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High Performance Liquid Chromatography Method for Determination of Aspirin after Derivatization Reaction and Kinetic Study

A Thesis

Submitted to the Council of the College of Science/University of Kerbala as a Partial Fulfillment of the Requirements for the Degree of Master of Science in Chemistry

By

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2025 AD

1446 AH

بِسْمِ اللَّهِ الرَّحْمَنِ الرَّحِيمِ

﴿يَرْفَعِ اللَّهُ الَّذِينَ آمَنُوا مِنكُمْ وَالَّذِينَ أُوتُوا الْعِلْمَ دَرَجَاتٍ وَاللَّهُ بِمَا تَعْمَلُونَ خَبِيرٌ﴾

صدق الله العلي العظيم

(سورة المجادلة ، الآية ١١)

Dedication

This work is dedicated with reverence to:

Imam Hussein (peace be upon him), who inspired me,

The Imam of our time (may God Almighty hasten his appearance),

My beloved mother, for her constant support and prayers, and my father,

My dear sisters …. My support (Noor, Tabarak),

My dear brother ….. My strength in life (Tholfakar),

To all my friends, who stood my side and supported me.

Finally, to my supervisors, with special appreciation.

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Finally, I also thank the Chemistry Department, College of Science, and the Head of the Department for their support in conducting this project.

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Report of the Head of Chemistry Department Chairman of Postgraduate Studies Committee

According to the recommendation presented by the Chairman of the Postgraduate Studies Committee, I forward this thesis "**High Performance Liquid Chromatography Method for Determination of Aspirin after Derivatization Reaction and Kinetic Study.**" for examination.

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We, the examining committee, certify that we have read this thesis and examined the student (**Rusull Lateef Naama**) in its contents and that in our opinion. It is adequate as a thesis for the degree of Master of Science in chemistry.

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Abstract

This study involves the quantification of Aspirin (Acetyl salicylic acid) using Reversed Phase High-Performance Liquid- Chromatography (RP-HPLC). A key element of this method was the synthesis and characterization of a novel Azo compound called (E)-2-acetoxy-5-((4-hydroxyphenyl) diazenyl) benzoic acid (AHPDBA). AHPDBA was synthesized through an Azo coupling reaction of Acetyl salicylic acid and a diazonium salt derived from 4-amino phenol. The resulting AHPDBA compound was subsequently characterized using UV-Visible spectroscopy, FT-IR spectroscopy, and gas chromatography-mass spectrometry (GC-MS).

Reversed-phase high-performance liquid chromatography (RP-HPLC) was employed for method optimization. The effects of mobile phase composition, pH, buffer, flow rate, injection volume, and temperature were carefully studied. Aspirin Calibration curve was created using concentrations ranging from (1 - 100 $\mu g.mL^{-1}$). By using RP-HPLC the kinetic study of the alkaline and oxidative degradation process of AHPDBA, the order rate constants, half-lives, and activation energies were calculated. Method validation of the proposed method included the determination of Linearity coefficient, precision, accuracy, Limit of Detection (LOD), and Limit of Quantification (LOQ). The method exhibited good linearity with the (1-100 $\mu g.mL^{-1}$) concentration ranges, with a correlation coefficient (r^2) of 0.9999. Excellent precision and accuracy were demonstrated with Relative Standard Deviation (RSD %) values below 0.291 % and recoveries ranging from 99.871-100.532 %. The LOD was 0.930$\mu g.mL^{-1}$, and the LOQ was 2.820$\mu g.mL^{-1}$. This study demonstrated that the proposed method was applied successfully for quantification of Aspirin in a pharmaceutical preparation.

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List of Abbreviation

Symbol	The Meaning
ASP	Aspirin.
D.W	Distal water.
ACN	Acetonitrile.
HPLC	High performance liquid chromatography.
NP-HPLC	Normal phase-High performance liquid chromatography.

RP-HPLC	Reversed phase-High performance liquid chromatography.
IEC	Ion exchange chromatography.
SEC	Size exclusion chromatography.
t_R	Retention time.
V_R	Retention volume.
F	Flow rate.
N	Number of theoretical plate.
W	Width of the peak.
L	Length of column.
H	Height of column.
R_S	Resolution of separation.
A_S	Asymmetric factor.
T_f	Tailing factor.
S	Slope of calibration.
FT-IR	Fourier Transformation Infrared.
UV-Vis.	Ultra violet visible.
GC/MS	Gas chromatography/ Mass
T_K	Temperature in kelvin.
T%	Transmission.
RSD%	Relative Standard Deviation.
LOD	Limit of Detection.
LOQ	Limit of Quantification.
Rec%	Recovery%.
S.D	Standard deviation.
λ_{max}	Maximum Wavelength in nm.
E%	Relative error.
Abs	Absorption.
r^2	Correlation Coefficient.

E_a	Activation energy
K_{obs}	Degradation rate constants
$t_{1/2}$	Half time
C_t	concentration remaining at time t
C_0	Initial concentration

Chapter one

Introduction

1. Introduction

1.1 Chromatography:

The term “chromatography” refers to chroma which means “color”, and graphein which means “to write”. Chromatography is the method used to separate, identify, and determine the chemical components found in complicated mixtures. This technique is widely used, similar to spectroscopy, and is a highly efficient instrument for both preparatory and analytical methods. Chromatography can be simply defined as follows: “It is the technique in which the components of a mixture are separated based upon the rates at which they are carried or moved through a stationary phase (column) by a gaseous or liquid mobile phase” (1). Chromatography separates molecules in a mixture based on their differential affinity for the stationary phase (solid or liquid) while being carried by a mobile phase. Effective factors for this separation method include adsorption (liquid-solid), distribution (liquid-solid), and molecular characteristics like variations in affinity and molecular weight. Because of these variations, some mixture components remain in the stationary phase longer and flow through the chromatographic system more slowly, while others migrate faster to the mobile phase and leave the system faster (2).

1.2 Classification of Chromatography

In all forms of chromatography, the components of the sample are separated through column material partitioning or adsorption. Partitioning separates a substance into two liquid phases, while adsorption binds a component to the surface of a solid phase (3). Solutes with different levels of affinity for the stationary phase are carried by a liquid phase when it passes over it in the normal mode chromatography (4). Fig. (1-1) displays the common chromatography classifications.

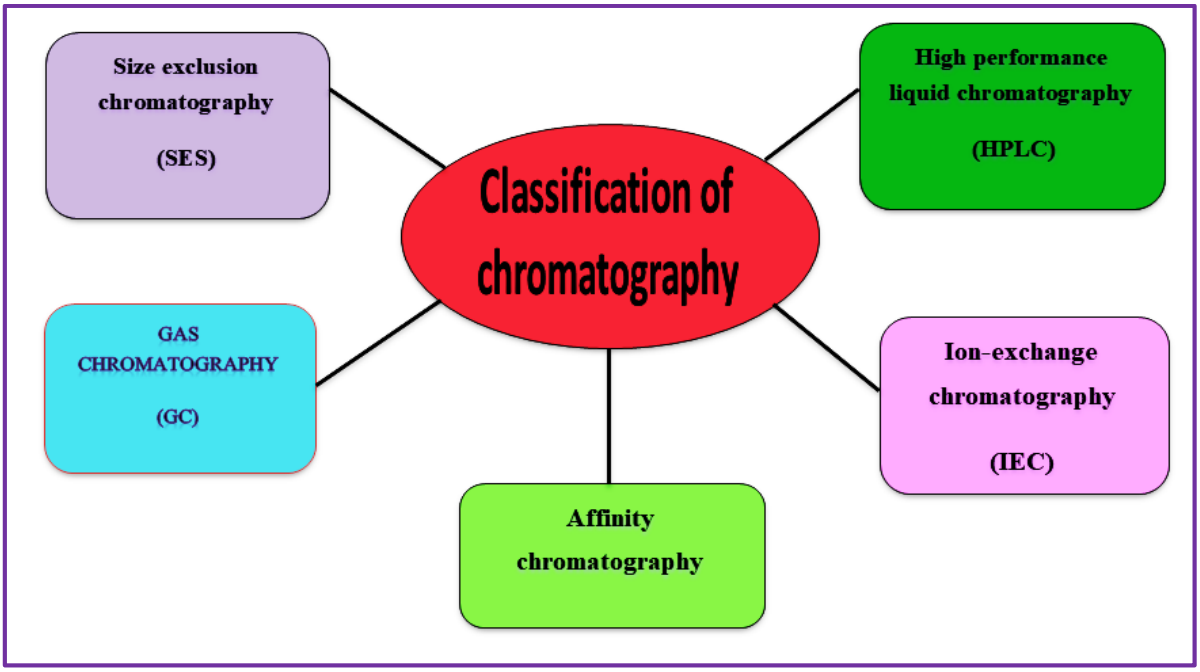


Figure (1-1): Schematic representation of some types of chromatography.

1.2.1 High-Performance Liquid Chromatography (HPLC)

HPLC developed in the late 1960s and early 1970 s, High-Performance Liquid Chromatography is now widely used for separations and purifications in a variety of areas including food industries, pharmaceuticals, biotechnology, and polymer chemistry (5). HPLC separates molecules by pumping a mobile phase through a column containing packing material (stationary phase). A detector measures the retention time of each molecule, which is determined by the interaction between the stationary phase, the molecule to be analyzed, and the solvent used. In chromatography, the sample is added to the mobile phase and separated based on interactions with the stationary phase (6). Gradient elution changes the composition of the mobile phase during the analysis. A gradient separates analyte mixtures according to its ability to it bind to the mobile phase. HPLC is the most frequently used for target compound analysis, where the compounds in a mixture are known, as reference standards can then be used to determine retention times (7). The HPLC method aims to separate and quantify the main drug substance, reaction impurities, synthetic intermediates, and degradants. High-Performance Liquid Chromatography is a powerful analytical chemistry tool. It is a

widely used analytical method for quantitative and qualitative analysis of drug products, including drug product stability determination (8).

1.3 HPLC Separation Techniques

1.3.1 Normal -Phase Chromatography (NP-HPLC)

Normal-phase chromatography (NP-HPLC) separates compounds based on their affinity for a polar stationary surface, such as silica. The strength of the interaction between the substance being analyzed and the surface that absorbs it is especially influenced by polar interactions such as dipole- dipole forces or hydrogen bonds (9). NP-HPLC effectively separates compounds that are easily soluble in non-polar solvents. It uses non-polar, non-aqueous mobile phase such as octane or chloroform. More polar compounds are retained longer and elute later because they interact more strongly with the polar stationary phase. Therefore, the least polar component elutes first (10).

1.3.2 Reversed Phase HPLC (RP-HPLC)

RP-HPLC consist of a non-polar stationary phase and a moderately polar aqueous mobile phase, typically surface-modified silica using RMe_2SiCl (R is a straight- chain alkyl group like $C_{18}H_{37}$ as well as C_8H_{17}). The elution of polar compounds is faster, while non-polar compounds are retained longer. Raise the water level in the mobile phase enhances retention of hydrophobic substances by increasing their attachments on hydrophobic materials stationary phase (10). Conversely, retention time can be reduced through adding more organic solvent content within the eluent. In reversed-phase chromatography, polar molecules elute earlier, while less polar molecules are retained longer. Increasing the water content of the mobile phase enhances the interaction between hydrophobic analytes and the stationary phase, thus lengthening retention times (11).

1.3.3 Hydrophilic Interaction Chromatography (HILIC)

HILIC, is a reversed- phase method, uses a polar stationary phase (e.g., amino, diol bonded phases, or unmodified silica) and the mobile phase with >70% organic solvent, typically acetonitrile, plus small amount of buffer, water, with other polar solvent. Analytes partition into aqueous-rich sub-layer formed by polar solvent adsorption on the stationary phase (12). HILIC retention mechanisms are complex, involving electrostatic and hydrogen-bonding interactions alongside hydrophilic partitioning. These mechanisms lead to an elution order that is exactly the opposite of the reversed- phase order. In HILIC, separation selectivity depends mainly on the organic modifier/aqueous ratio, However, the choice of stationary phase should enhance the functional groups of analyte (13).

1.4 Mobile Phase

In HPLC, the mobile phase, a liquid solvent or solvent mixture, flows through the column, influencing separation based on its composition. Reversed-phase HPLC typically employs water and a polar organic solvent, while normal-phase HPLC uses non-polar solvents (8). Pure inorganic salts and solvents are critical for mobile phase preparation. Typical solvents for the mobile phase are methanol, acetonitrile, water, and their combinations. Using progressive elution, in which the composition varies over time, is one way to increase separation by varying the composition mobile phase (14).

1.5 Stationary Phase

The stationary phase, a crucial element in chromatography, separates analytes based on their size, charge, polarity, and affinity through interaction as they pass. It can be a solid, liquid, or gas can be retained over a supporting medium (15). Therefore, analytes interact differently with stationary phase, leading to unique elution patterns and retention times. The choice of stationary phases in HPLC, whether polar or non-polar, is based on the desired separation type. Common phases include silica gel, reverse phase

$C_{18,}$ ion-exchange resins, and chiral phases. Key factors for selection are the polarity of the sample components and the intended separation method (8).

1.6 The HPLC parameters

Several factors serve as standards for a substance to ensure accurate analysis. Pump pressure, internal diameter, pore size, and particle size are common measurements that can be adjusted. For different compounds, the parameters can be changed according to their nature and chemical properties (16).

1.6.1 Retention time (t_R): The retention time is the difference in time between the injection point and the moment the peak maximum appears on the chromatogram. It is indicating how long a component takes to elute from the column. Several factors influence retention time like: stationary phase, column temperature, and mobile phase composition, properties of the compound such as size and polarity (17). Retention time reflects the interactions between the sample components (carried by the mobile phase), and the stationary phase within the column. These interactions cause different compounds to elute at different times, resulting in separated peaks on the chromatogram. The flow rate of the mobile phase and the length of the column also influence retention time and separation efficiency. Optimizing these factors enhances peak resolution and reduces the overlap of peaks (co-elution). Managing retention time is crucial for the reliable HPLC methods and for the effective separation and accurate identification of compounds in various applications (18).

$$t_R = V_R / F \qquad (1\text{-}1)$$

V_R is the Retention volume, F flow rate of mobile phase (ml/min) (19).

1.6.2 Void time or hold-up time (t_o), (t_M)

Hold-up time refers to the duration a particle remains within a packed column, specifically while interacting with the stationary phase. This represent the time the solute is retained within the system due to its interaction with stationary phase during a

chromatographic process (20). Hold-up time can be calculation using the following formula:

$$t_o = \frac{V_o}{F} \qquad (1\text{-}2)$$

Void volume (V_o) represents the mobile phase volume within the column. In chromatography, this volume is known by several names: hold-up volume, dead volume. (F) Is the flow rate of mobile phase (ml/min) (19).

1.6.3 Retention Volume (V_R)

Retention volume is the total volume of mobile phase needed to elute the analyte from the column. This volume includes the mobile phase volume associated with the hold-up time of solute and the volume related to the solute interactions with the stationary phase (10). The interaction is influenced by several factors, including column temperature, stationary phase polarity, and mobile phase properties. It can be calculated using the retention time (t_R) and the flow rate (F) (13):

$$V_R = t_R \times F \qquad (1\text{-}3)$$

1.6.4 Capacity factor (k)

Capacity factor also known as retention factor and it is a measure to interaction of the analyte with the stationary phase. It is calculate as the ratio of the time the compound spends in stationary phase to the time it spends in the mobile phase (21).

$$k = \frac{V_R - V_.}{V_.} = \frac{V`_R}{V_.} = \frac{t_R - t_.}{t_.} \qquad (1\text{-}4)$$

The retention time (t_R) of an analyte is the time it takes for it pass through and exit the chromatography column. Dead volume (t_o) is the time it takes for a non-retained compound to pass through the column. The capacity factor provides important information about the efficiency and selectivity of the chromatographic separation. A

larger k can be lead to better separation, but it may also increase analysis time because the analyte is retained longer on the stationary phase, Table (1-1) presents a general categorization of typical k values (22).

Table (1-1): Show general categorization of typical value of k

k Value	Stationary phase	The resolution	Peak shape
k< 1 (poor retention)	the stationary phase is not effective at retaining the analyte	low resolution	broadly peaks
1≤ k < 2 (Moderate retention)	the stationary phase is beginning to interact with the analytes	resolution starts to improve	Peaks get sharper.
٢ ≤ k < ١٠ (Good retention)	this range is frequently thought to be ideal for successful separations	good resolution	Peak shapes are supplied by the well retained analytes.
١٠ ≤ k < ٢٠ (High retention)	high level of interaction with the stationary phase	possibility of separation,	Very high values could result in broader peaks and excess extended analysis time.
k > ٢٠ (Very high retention)	strong interaction with the stationary phase	Typically unfavorable	Increases possibility of peak tailing or distortion and increases run times excessively.

The capacity factor is affected by factor such as particle size, column length, the chemical properties of the stationary phase, the properties of the mobile phase, and the structure of the molecule being analyzed (23).

1.6.5 Selectivity factor (α)

The selectivity is ability of the chromatographic system to distinguish between the sample components. It is calculated as the ratio of the retention factors (k) of two chromatographic peaks, effectively representing the relative distance between their centers.

$$\alpha = \frac{k_٢}{k1} = \frac{t_{R2} - t_o}{t_{R1} - t_o} \qquad (1\text{-}5)$$

Where: k_2 is the retention factor of peak 2, k_1 is the retention factor of peak 1.

t_{R2} is the retention time of peak 2, t_{R1} is the retention time of peak 1.

t_o is the void time (or dead time) (24).

While the (α) value doesn't directly indicate resolution, a high (α) value suggests good separation between peak centers. Selectivity is always greater than one. An (α) value of one means the two components elute together at same time (co-elution), meaning they have identical retention factors. The selectivity is influenced by chemical properties of the analyte, the mobile phase, and the stationary phase. Optimizing these factors helps to maximize selectivity in HPLC separations (19).

1.6.6 Efficiency (N)

In chromatography, efficiency is shown by the shape of the peak. Ideally, peaks should be sharp and narrow. However, dispersion causes peaks to spread out, creating a Gaussian shape. The plate number (N) measures this dispersion and assesses the column performance, similar to fractional distillation. A column with more theoretical plates improves separation and equilibrium. Column efficiency can easily assess by calculating the peak width at half height ($W_{1/2}$) and its retention time (shown in Fig.1-2) and using the following formula:

$$N = ١٦(\frac{t_R}{W})^2 \qquad (1\text{-}6)$$

Where: N is the theoretical plate count (25).

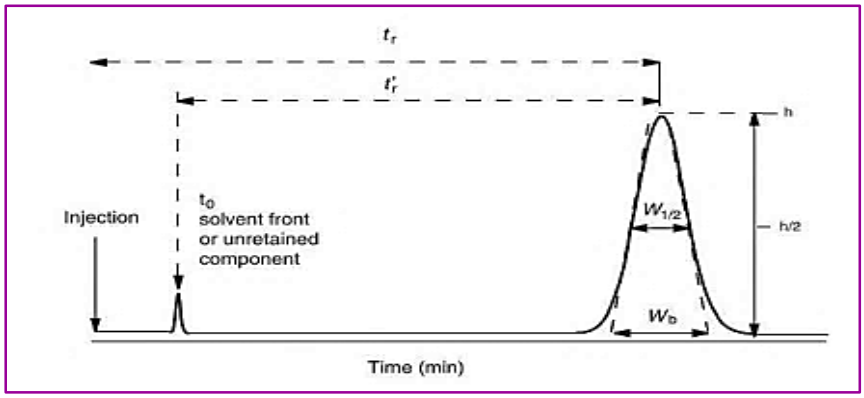


Figure (1-٢): displays a chromatographic peak at retention time t_R (25).

Higher N values mean the column has better efficiency. Also, peaks that appear later in the chromatograms are wider and contain more plates than earlier peaks. As N increase, the distribution of carbon numbers becomes narrower, resulting in smaller peaks and improved HPLC efficiency. In a fractionating column of length (L), a higher N value leads to a lower plate height (H), which allows for better separation. Plate height, also known as Height Equivalent to Theoretical Plate (HETP), is calculated using this formula (23):

$$H = \frac{L}{N} \qquad (1\text{-}7)$$

This equation gives a more accurate measure of column efficiency, particularly for analytes that elute quickly. Column performance, also known as column efficacy is determined using the theoretical plate count. If this count drops below a specific limit, the column may need to be replaced (10). Several factors within the chromatography system cause peak broadening as the analyte moves through it, which reduces efficiency. The main factors relating to the column are particle size, column dimensions, packing quality, and the presence of any voids. Longer columns improve peak sharpness and enhance efficiency and separation quality, but they also make the analysis time take longer. Increasing the column length (from 2.5 to 25 cm) improves peak efficiency significantly. A well-packed column that is 15 cm long and 0.46 cm in diameter, and uses 5 μm particles, typically produces 10,000-20,000 plates. Other factors like injection volume, dead volume, and flow rate also effect column performance and must be taken into account (26).

1.6.7 Resolution (R_S)

Resolution measures the ability of the chromatography system to separate two adjacent peaks. Higher resolution is crucial identifying and measuring the amount of each compound accurately, especially in complex mixtures. If the resolution is too low, peaks will overlap, making it difficult to determine concentrations (27). The resolution value

of 1.5 or higher is generally required for accurate peak quantification. Resolution (R_S) can be calculated using the following formula:

$$Rs = \frac{(t_{R2}-t_{R1})}{(W_{b1}+W_{b2})/٢} = \frac{٢\,(t_{R2}-t_{R1})}{(W_{b1}+W_{b2})} \qquad (1\text{-}8)$$

Where: t_{R1} and t_{R2} are the retention times of the two peaks.

W_{b1} and W_{b2} are the peak widths at the base of the two peaks (18).

For peaks that are very close together, the width of the second peak can be used as an approximation for the average peak width. The peak width at the Base is determined by tangents to the sides of the peak at the inflection points. Increasing resolution generally means longer separation times. The fundamental Resolution Equation identifies three key factors that influence resolutions are: efficiency, selectivity (separation factor), retention (capacity factor) (18).

$$R_S = {}^{١}/_{٤}\sqrt{N} \times \frac{\alpha-١}{\alpha} \times \frac{k}{١+k} \qquad (1\text{-}9)$$

Efficiency **selectivity** **Retention**

Column efficiency (N) indicated by number of theoretical plates (N), reflecting its performance. Selectivity, the ability of the stationary phase to distinguish compounds, is influenced by the choice of stationary and mobile phases, Fig. (1-3) shows the HPLC resolution as function of height equivalent to a theoretical plate. Greater differences in retention times enhance accuracy, while narrower peak widths improve resolution (28).

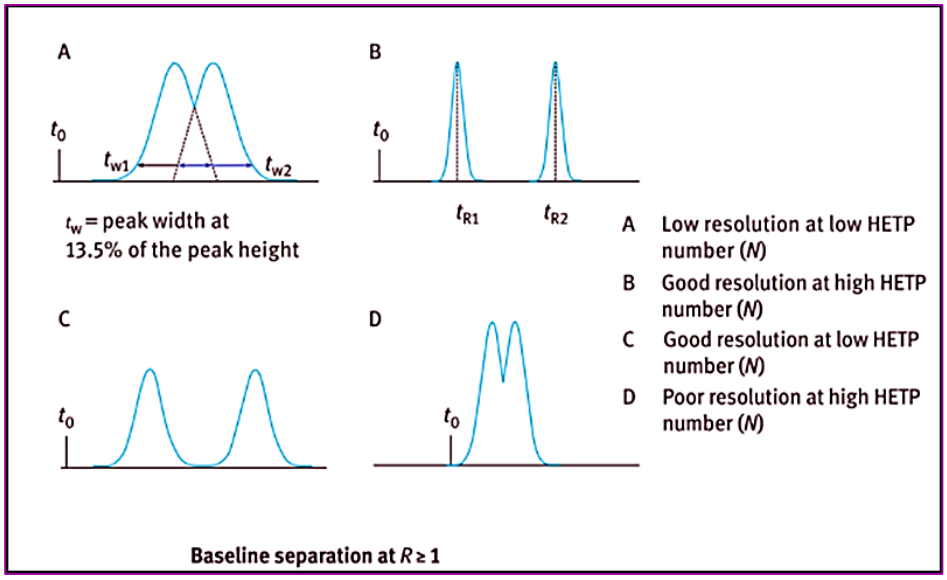


Figure (1-3): HPLC signal resolution depends on HETP, which represents the height of a theoretical plate (28).

1.6.8 Peak Symmetry: Asymmetry factor (As) and Tailing factor (T_f)

Ideally, chromatographic peaks should be symmetrical and have a Gaussian shape. However, in practice, most peaks are asymmetrical and exhibit either fronting or tailing (shown in Fig.1-4). The Asymmetry factor (As) determined at 10% of the peak height, provides a quantitative measure of peak symmetry.

$$\text{A symmetry factor } As = \frac{b}{a} \qquad (1\text{-}10)$$

Where: a = Distance from the peak maximum to the front (leading edge)

b = Distance from the peak maximum to the back (tailing edge) (9).

When: As =1 indicates a perfectly symmetrical peak.

As < 1 indicates peak tailing, this means the tailing side of the peak is longer than the leading side. This can be caused by sample overload, issues with the column packing, or problems with the connection to the stationary phase.

As > 1 indicates peak fronting. This can be caused by column overload or excessive sample concentration. Optimal peak symmetry is important for accurate quantification,

repeatability, and consistent analyte detection. It ensures the area under the peak accurately represents the quantity of the measured compound (29).

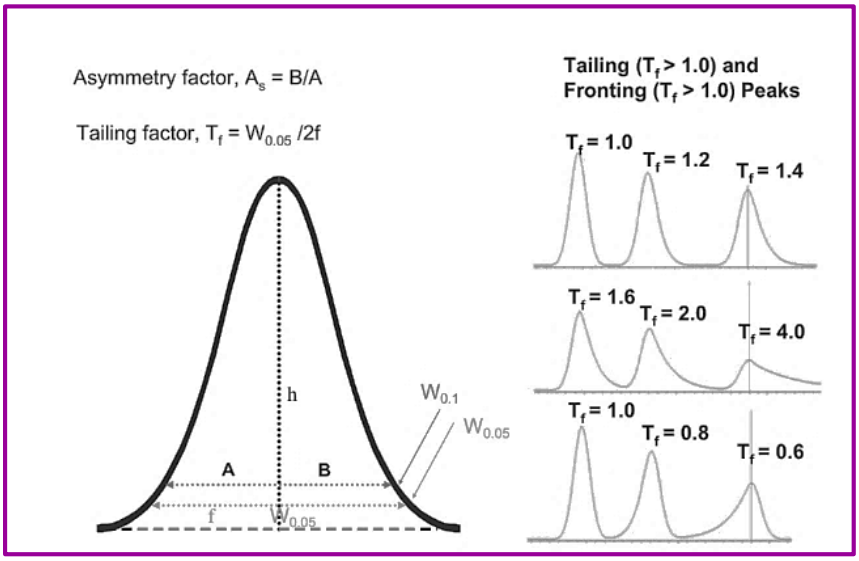


Figure (1-4): Calculation of asymmetry factor tailing factor (30).

Tailing factor (T_f) is calculated using the peak width at 5% of the peak height. Calculating the tailing factor is required for most pharmaceutical methods. A perfectly symmetrical peak has a tailing factor 1. A value above 1 indicates tailing, $T_f > 2$ indicates a tailing peak is not acceptable. The peak tailing typically results from analyte adsorption to the column material or extra column band broadening. Peak fronting, is usually caused by column overload or analyte reaction during chromatography (30).

1.7 HPLC Instruments

In High-Performance Liquid Chromatography (HPLC), a small liquid sample is injected into a column packed with tiny stationary phase particles (3 to 5 μm). A high-pressure pump forces a mobile phase through the column, separating the component of sample based on their ability to interact with the stationary phase. A detector measures these separated components, generating an output that is called “chromatogram” (Figure 1-5 illustrates the components of the HPLC system). Both Liquid Chromatography (LC) and HPLC use the same separation principles. However, HPLC offer advantages such

as faster separation, higher efficiency, more sensitivity, and easier operation. While HPLC is mainly used for analysis, traditional liquid chromatography is still employed for preparatory purposes (23).

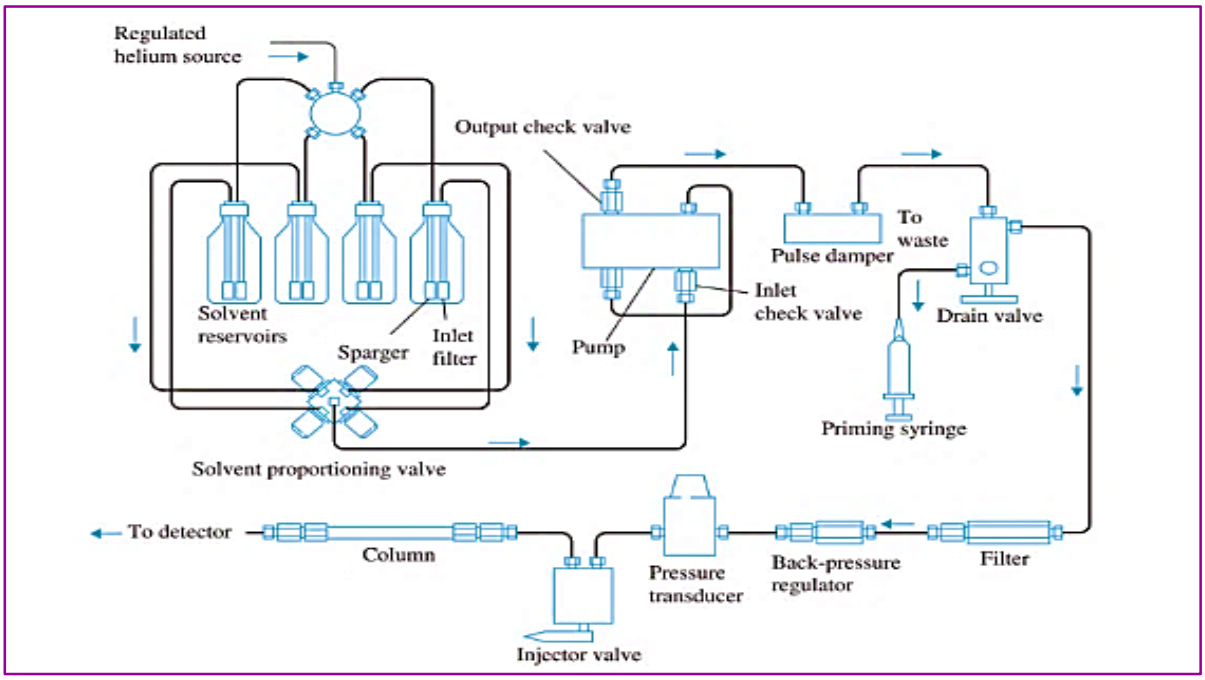


Figure (1-5): Component of HPLC (23).

An HPLC instrument consists of a solvent reservoir, pump, injector, column, detector, and data acquisition/display system (see figure 1-5). The column is the key component where separation occurs.

1.7.1 Solvent reservoir

The mobile phase, a mixture of polar and non-polar liquids, is carefully chosen to match the composition of sample. This mobile phase is stored in a glass reservoir. The separation of components depends on the type and composition of the mobile phase. We use high quality solvents specifically designed for HPLC. The choice of solvent varies depending on the method; reverse-phase HPLC typically employs a mixture of water and a polar organic solvent, whereas normal-phase HPLC generally uses non-polar solvents (31). The purity of the solvent and inorganic salts is essential in preparing the mobile phase. The simplest solvent reservoir consists of glass bottles connected to the

pump inlet with tubing. Degassers remove dissolved gases from the mobile phase to prevent column clogging and damage to the pumping/ injection systems. These gasses are removed via Vacuum pumping systems, Distillation units, heating and stirring devices, Sparking systems that remove dissolved gases from solutions using insoluble inert gas bubbles (32). Degassers and filters use to prevent column clogging and damage to pumping/injection systems from particulate matter from the solvents. Isocratic elution uses single solvent or constant mixture, unlike gradient elution which varies solvent composition (24).

1.7.2 Pump

An HPLC pump, like human heart, must continuously delivers a small volume of mobile phase at a constant pressure. Changes in pressure can lead to errors, so the pump must maintain consistent performance. The acceptable pressure range depends on the type of analysis. Standard HPLC procedures typically operate between 2000 to 5000 psi, while UHPLC applications can reach 15,000 to 18,000 psi (33). All HPLC systems include a pump to force the mobile phase through the system. This can generate injector pressures that may reach to 20,000 kPa (200 bars), depending on the particle size of the column, the viscosity of the mobile phase, and the flow rate. To minimize pressure pulsations, HPLC pumps are designed for consistent low flow rates, often using two pistons that work alternately to maintain a stable flow even when the mobile phase composition changes (8). In chromatography, a pump is used to move the mobile phase from the solvent reservoir, through the column and finally to the detector. The pump can deliver the mobile phase in a constant composition (isocratic) or changing composition (gradient). Inconsistent in the flow rate can lead to inaccurate results and effect on elution times. Pumps are designed to maintain a stable flow of the mobile phase flow at a constant pressure (34).

Constant pressure pumps, Syringe-type pumps, and Reciprocating piston pumps are the three kinds of pumps that are most commonly utilized.

Constant pressure pumps: These pumps use gas pressure from a gas cylinder to deliver a continuous, low flow rate across the column. Their valve design allows for rapid refilling of the solvent chamber.

Syringe type pumps: These pumps are suitable for columns with small diameters. They deliver a continuous, low-flow rate using a motorized screw system. The flow rate is adjusted by changing voltage of motor, which controls the amount of solvent delivered.

Reciprocating piston pumps: These pumps use a piston within a hydraulic chamber to distribute solvent, the piston draws solvent into chamber during its backward stroke and pushes the solvent out during its forward stroke (13).

1.7.3 Sample injector

The injector is a crucial component of HPLC systems. It injects liquid samples into the system at high pressures (up to 4000 psi) with consistent volumes, typically ranging from 0.1 to 100 μL. Injectors may be either automated or manual. Auto samplers are used for processing large sample volumes or when manual injector isn't feasible. Injectors are designed to deliver constant sample volumes injection to ensure accuracy, requiring inert materials and reproducible performance. In HPLC, injecting a precise sample volume rapidly onto the column is essential for maintaining the stability of mobile phase (13). The valve operates in two stages:

• Loading: The pump connects to the column. A syringe is used to load the sample into a fixed loop at atmospheric pressure.

• Injection: The valve rotates 60 degrees, introducing the sample from the loop into the mobile phase. The loop ensures consistent sample volume is delivered into the HPLC system. Syringes alone cannot overcome the high back pressure required for accurate injection into the moving mobile phase (35). The injector type is:

• Manual injection:

These systems use a 6-port rotary valve to inject samples. In the LOAD position, the sample loop is filled at atmospheric Pressure, but the sample bypassed the column.

When switched to the INJECT position, the valve directs the sample to the column, flushing excess sample to waste to remove air bubbles and clean the loop.

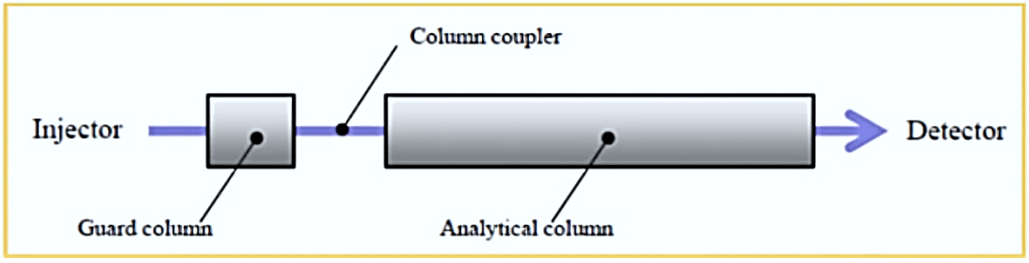
• **Automatic injection*:***

Automatic injection reduces human error and improves lab productivity. Modern HPLC systems use auto-samplers and injectors. The software controls the auto sampler to transfer samples to the column and manage the injection sequence (34).

1.7.4 Columns

HPLC Columns are typically made of polished stainless steel, with lengths ranging from 50 to 300 mm and internal diameters from 2 to 5 mm. The stationary phase particles inside the column are usually between three and ten µm. Micro bore columns have internal diameters less than 2 mm. Maintaining a consistent temperature for both the column and mobile phase is crucial for optimal analysis. The stationary phase separates the sample components but creates high back pressure at low speeds, requiring a powerful pump force. Following the supplier requirements is essential to ensuring accurate results and separation in each analysis (23). Columns types which include:

a- Guard columns: These columns protect analytical columns by filtering out impurities and particles from solvents and samples that can bind to the stationary phase (Fig. 1-6 illustrates HPLC guard column). They also reduce solvent loss by saturating the mobile phase with the stationary phase. Guard column usually contain larger particles and a similar composition to the analytical column. When contaminated, the guard column can be repacked or replaced with a new one(36).



Figure(1-6): illustration of HPLC guard column and analytical column connection (37).

b- Analytical columns: These are the key components of high-performance liquid chromatography (HPLC). Analytical columns are typically 10 to 30 cm long. Common particle sizes range from 5 or 10 mm, with internal diameters from 4 to 10 mm. A typically column has 4.6 mm diameter, 25 cm long with 5 mm particles, providing 40,000 to 60,000 theoretical plates per meter (Fig. 1-7 shown Schematic illustration of a standard HPLC column). Most columns are made of smooth-bore stainless steel, but glass and polymer tubing like polyether ketone are also used (38).

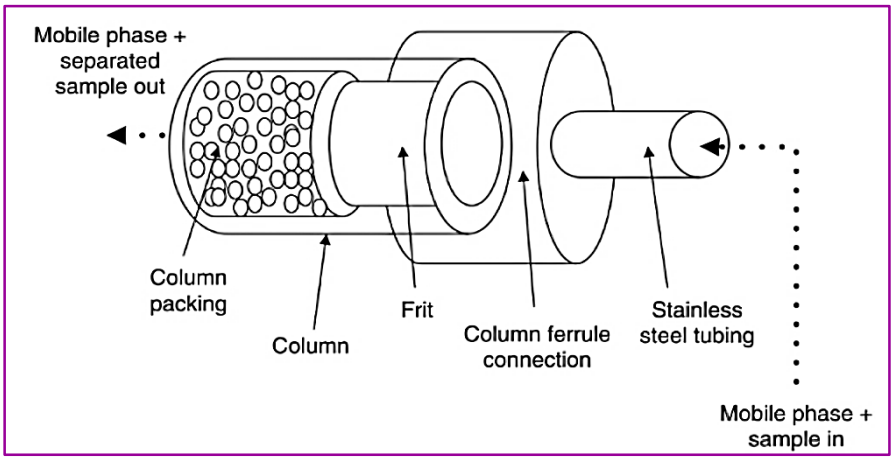


Figure (1-7): A schematic diagram of a typical HPLC column(39).

Maintain a consistent column temperature is crucial for reliable chromatography results, although it's not always needed if the application works well at room temperature. Ovens or heaters can help stabilize the temperature. Furthermore, modern technologies might require higher temperatures to adhere to specific regulations(40).

1.7.5 Detector

After analytes exit the HPLC column, they are quantified using detection methods such as UV spectroscopy and mass spectrometry. The resulting liquid chromatogram shows the detectors response as each component elutes independent of the mobile phase composition (41), (Fig. 1-8 depiction of different kinds of detectors. The optimal detector properties include:

1- Sensitivity is appropriate to the work at task.

2- Reproducibility and stability are good.

3- The broad linear dynamic response range.

4- Fast response time that is not influenced by a low rate.

5- Not affected by variations in temperature, flow rate, or solvent composition.

6- Cell design that prevents the split bands from remixing.

7- High reliability and ease of access for maintenance.

8- Non- destructive analysis of the sample (34).

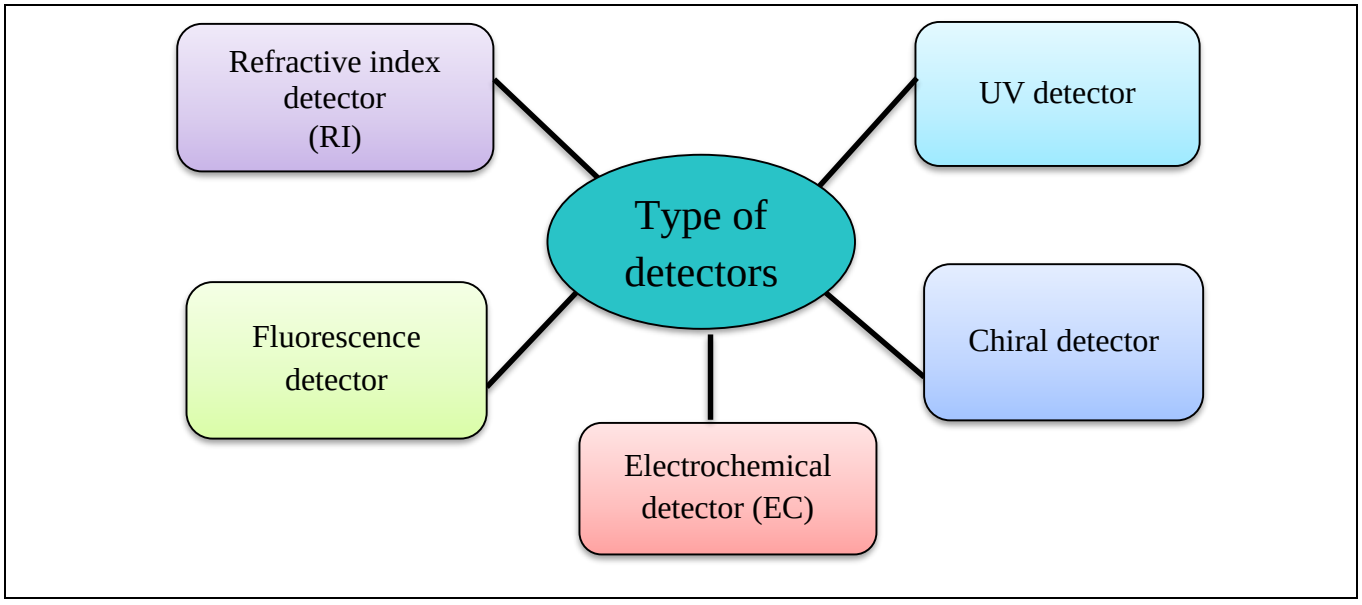


Figure (1-8): Schematic representation of some types of detector.

1.7.5.1 Refractive index detector (RI)

Refractive index detector Measure the change in refractive index of the mobile phase as analytes elute. RI Useful for compounds that do not absorb UV light, such as carbohydrates, lipids, and polymers. The Limitations of RI are less sensitive than UV-Vis or fluorescence detectors (42).

1.7.5.2 Fluorescence detector

Fluorescence detector measure the fluorescence emitted by analytes after excitation with UV or visible light. Fluorescence detector is highly sensitive for compounds that naturally fluoresce or can be derivative to fluoresce (43).

1.7.5.3 Electrochemical detector (EC)

Electrochemical detector (EC) measure changes in electrical current or potential, often used for electrochemically active compounds (44).

1.7.5.4 Chiral detector

A chiral detector in HPLC is a specialized detector used to identify and quantify enantiomers, which are chiral molecules that are mirror images of each other. These detectors are crucial in pharmaceutical and other fields where the specific biological activity of a drug or compound is linked to its chiral form (٤٥) .

1.7.5.5 UV detector

UV detectors are a common type of detector used in liquid chromatography (LC). They are widely used in various industries, including medicines and environmental testing, to analyze different compounds. The detector measure the amount of light that passes through the sample and this measurement is used to determine the concentration of the sample. This concentration is reported as absorbance (46). The UV absorbance is based on electronic transitions within molecules. Many UV detectors use a mercury lamp that operates at a fixed wavelength of 254 nm, which excites electrons in the ($\pi \rightarrow \pi^*$), ($n \rightarrow \pi^*$), ($n \rightarrow \sigma^*$) molecular orbital. To achieve the optimal UV sensitivity and linear response, it is crucial to choose the right mobile phase solvent and buffer. The UV cutoffs the solvent, which is the wavelength at which the solvent starts to absorbs light, is particularly important, especially when working at low wavelengths(47). UV detectors can be divided into these types: variable wavelength, photodiode arrays detectors, fixed-wavelength detector, and detectors that use multiple wavelengths from a broad spectrum light source. Fixed wavelength detectors were common in older HPLC systems but are now rarely used. Low-pressure mercury lamps are still frequently used in photodiode array and variable wavelength detector because they emit light at 254 nm. Variable wavelength detectors can be adjusted to improve selectivity or to match the absorbance peak of analyte. They can also switch wavelengths during a

chromatographic run to detect different analytes. These detectors use a tungsten lamp for visible light and deuterium lamp for UV light. The light passes through a slit and then to a diffraction grating, which separates the light into different wavelengths. By rotating the grating, a specific wavelength is selected and directed to a photodiode through the detector cell (8). Figure (1-9) shows a diagram of a variable wavelength detector.

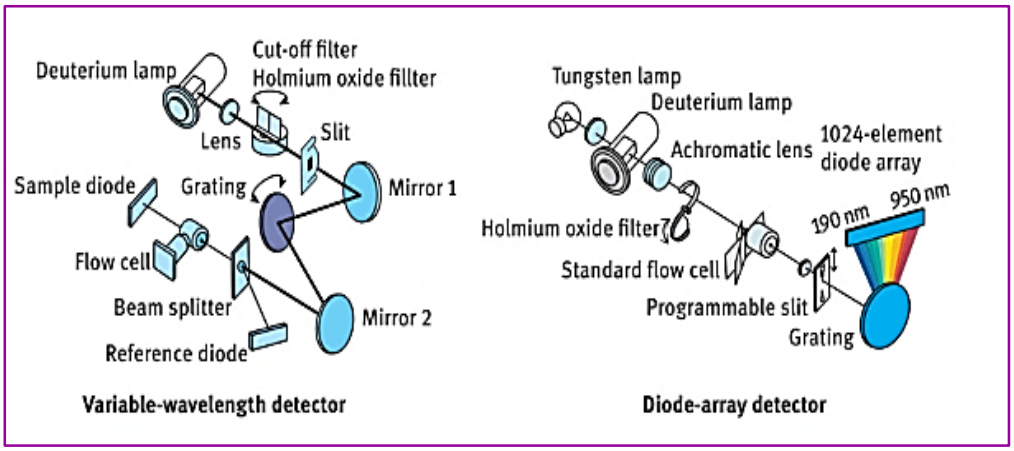
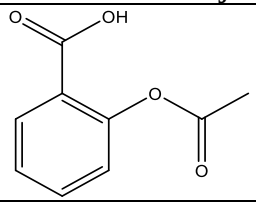


Figure (1-9): UV-visible absorption detector schemes for HPLC systems include: Right diode-array detector, left variable-wavelength detector (28).

1.8 Aspirin drug (ASP)

Acetylsalicylic acid (ASA), commonly known as aspirin, is an important nonsteroidal drug (NSAID) with cardio protective and anti-inflammatory effects. Initially reported by Charles Frederic Gerhardt in 1853, aspirin was developed from salicylic acid, a traditional medicine made from the bark of willow. Aspirin is Bayer AG's first notable product, efficiently synthesized by esterifying acetic anhydride and salicylic acid using a catalytic amount of sulfuric or, less commonly, phosphoric acid, Table (1-2) shown the Physical and Chemical properties of Aspirin. Today, Aspirin is one of the most widely used medications for various treatments and illnesses (48).

Table (1-2): Physical and chemical properties (49):

Chemical name	2- Acetoxy benzoic acid, Acetylsalicylic acid
Molecular structure	$C_9H_8O_4$
Molecular weight	180.159 g/mol
Solubility	Slightly soluble in cold water; soluble in ether, alcohol, and hot water.
Appearance	Colorless needles, crystalline
Pka	3.5
Density	1.40 g/cm^3
Melting point	159^o C
Uses	Aspirin inhibits cyclooxygenase, preventing arterial and venous thrombosis. It treats headaches and reduces inflammation. It may lower cancer risk and is crucial for heart attack patients, also serving as first-line treatment for acute rheumatic fever symptoms like fever and joint pain.
Structural form	

1.8.1 Uses of Aspirin

1. Pain Relief: For headaches, muscle pain, toothaches, and minor arthritis.

2. Anti-Inflammatory: Reduces swelling and inflammation.

3. Fever Reduction: Helps lower high body temperature.

4. Heart Health (Low-Dose Aspirin): Prevents blood clots, Reduces risk of heart attack and stroke (usually in people with cardiovascular disease or high risk) (50).

1.8.2 Functional Groups in Aspirin

1. Carboxylic acid (-COOH): Contributes acidity and reactivity

2. Ester (-COO-): Formed from salicylic acid and acetic anhydride

3. Aromatic ring: Stabilizes the molecule and affects its physical properties (51).

1.8.3 Reactivity of Aspirin

1. Sensitive to moisture and heat.

2. Reacts with strong bases (alkaline hydrolysis).

3. Can undergo esterification or hydrolysis (52).

1.9 Azo compound

Azo dyes are produced through a simple diazotization and coupling process, with various methods used to optimize color, yield, and dispersibility. Over 60% of all dyes are azo dyes, that constitute about 70% of industrial dye usage (53). These dyes are distinguished by the azo functional group (-N=N-) linking symmetrical or asymmetrical alkyl or aryl radicals (54). Azo dyes are the primary synthetic colorants used in textiles and printing. Azo dye waste urgently requires treatment or safe conversion due to its harmful effects on human and aquatic life. Colorants are compounds that impart color to materials and are widely used across industries like textiles, paints, and plastics (55). Fig. (1-10) shows the general structure of Azo dyes.

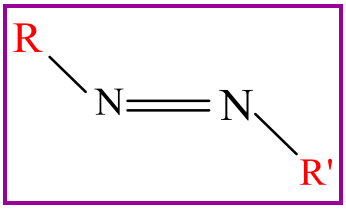


Figure (1-10): The general structure of Azo dyes.

1.10 Synthesis of Azo dyes

Di Azo compounds and diazonium salts are important intermediates in organic chemistry, known for their versatility. Azo compounds feature an Azo group (-N=N-) linked to sp^2 hybridized carbon atoms. Their synthesis involves two steps: diazotization of an aromatic amine and Azo coupling. Azo groups usually connect to substituted aromatic or hetero aromatic rings(56).

1.10.1 Diazotization

Diazotization involves converting an aromatic or hetero aromatic primary amine into a diazonium ion, which then couples with an electron-rich nucleophile in acidic aqueous solution using nitrous acid (HNO_2) at low temperatures (0-5) °C. The benzene ring's high electron density stabilizes the N-N group; however, the diazonium ion becomes highly unstable when formed by combining sodium nitrite ($NaNO_2$) with a mineral acid like hydrochloric acid. Fig. (1-11) shows the mechanism of diazotization Aromatic amine (17).

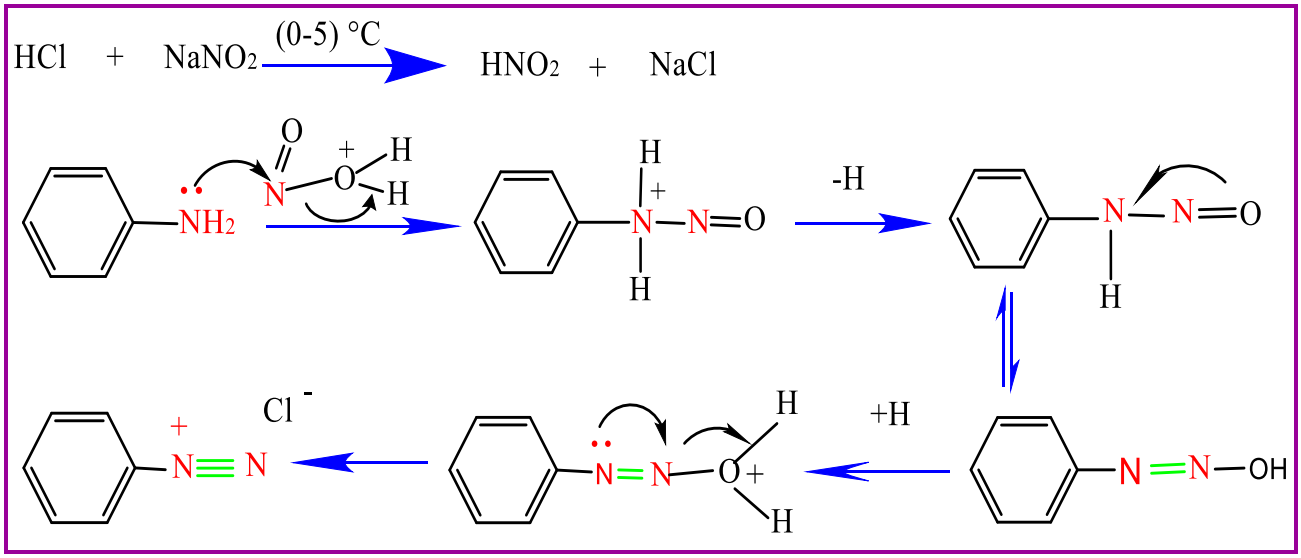


Figure (1-11): Aromatic amine diazotization mechanism.

1.10.2 Azo coupling reaction

The coupling process involves adding a coupling substance, like phenols or aromatic amines, to the unstable diazonium salt in a cold aqueous solution. Fig.(1-12) shows the Azo coupling reaction (57).

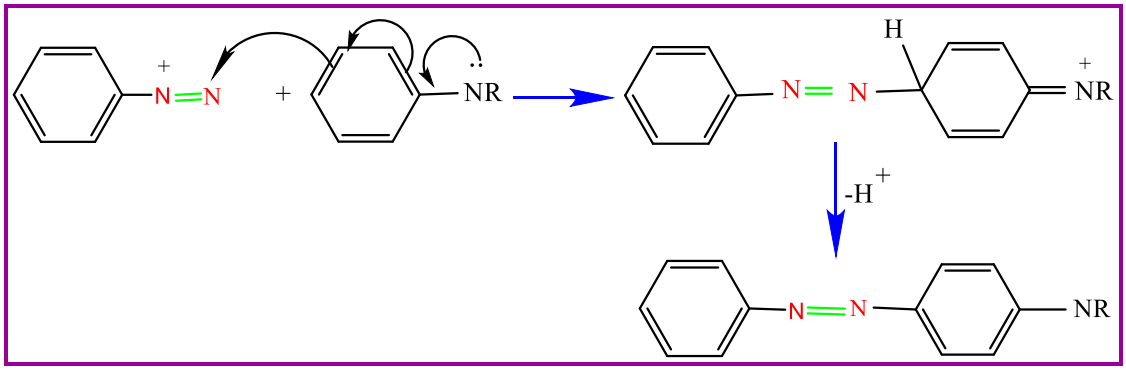


Figure (1-12): Azo coupling reaction

1.11 Physical characteristics, structure, and connections

Aryl azo compounds exhibit bright colors, especially red, orange, and yellow, due to π-delocalization. An example is Disperse Orange 1. Certain azo compounds, like methyl orange, serve as acid-base indicators (58). Additionally, blue Azo dye is commonly used as the recording layer in most Digital Versatile Disc Recorder (DVD-R/+R) and some CD-R discs (59). Azo dyes are primarily solid salts, with the anion often serving as the colored component; however, cationic Azo dyes also exist. Most of these dyes are anionic due to the presence of 1-3 sulfonic acid groups, which are fully ionized at the pH of the dyed material:

$$RSO_3H \rightarrow RSO_3^- + H^+$$

Anionic dyes bind to leather and wool via electrostatic forces due to protein's positive charge. Cationic Azo dyes, containing quaternary ammonium, dye leather and wool through ion exchange (60).

1.12 Coupling reaction of Azo compound

Azo dye coupling process occurs when a diazonium salt react with an aromatic coupling agent. As an electrophile, the diazonium salt engages the electron-rich benzene ring of the coupling agent (61). When an azo compound is added to a cold solution, a brightly colored solid form and separates out. Due to its intense color properties, this solid is frequently utilized as a colorant. The coupling agent typically reacts at two or four

positions on its benzene ring, with a functional group taking one spot. The choice of coupling agent with the diazonium salt determines the color of the Azo compound produced, as its chromophoric properties are influenced by agent (62).

1. Coupling with phenols

The reaction of diazonium salt with a phenol compound produces yellow or orange Azo compound, with the particular structure of the phenol influencing the observed color. Fig. (1-13) shows the Reaction between phenols and diazonium salt (63).

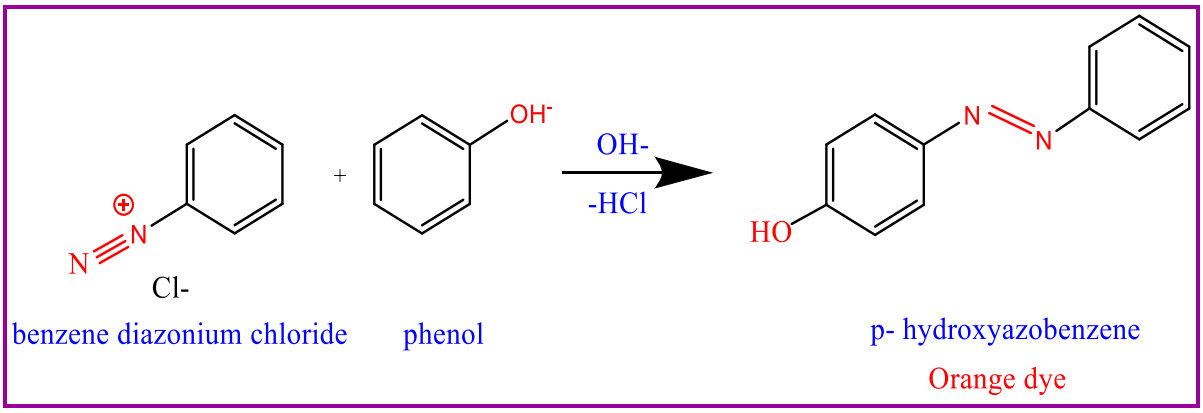


Figure (1-13): Reaction between phenols and diazonium salt

2. **Coupling with Amines**

Aryl amine reactions with diazonium salts typically produce a yellow color due to the coupling of the two, forming Azo compounds distinguished by Azo group (-N=N-) (64). Fig. (1-14) Presents the reaction of aryl amines with diazonium.

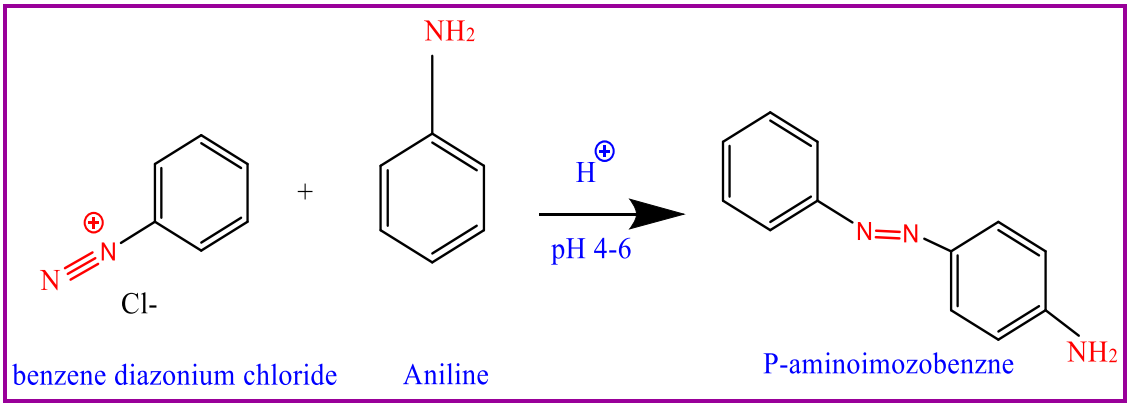


Figure (1-14): Reaction of Aryl Amines with diazonium.

1.13 Application of Azo compounds

Azo compounds are widely used in research, technology, and medicine. Aryl Azo groups find applications in pharmaceuticals, and many Azo compounds are key in dye and cosmetic production (65). This chemical is used in various industries. In analytical chemistry, Azo compounds and their metal complexes act as spectral reagents due to their color properties (62). Heterocyclic Azo compounds are important for dyeing textiles, especially using thiazolyl Azo ligands as dye agents (66). Photography and adsorption studies have both used Azo compounds(67). They also have number of medical applications; the initial Azo dye, prontosil, was used as an antibacterial agent, representing a key milestone (68), Fig. (1-21) illustrates the application of azo compounds.

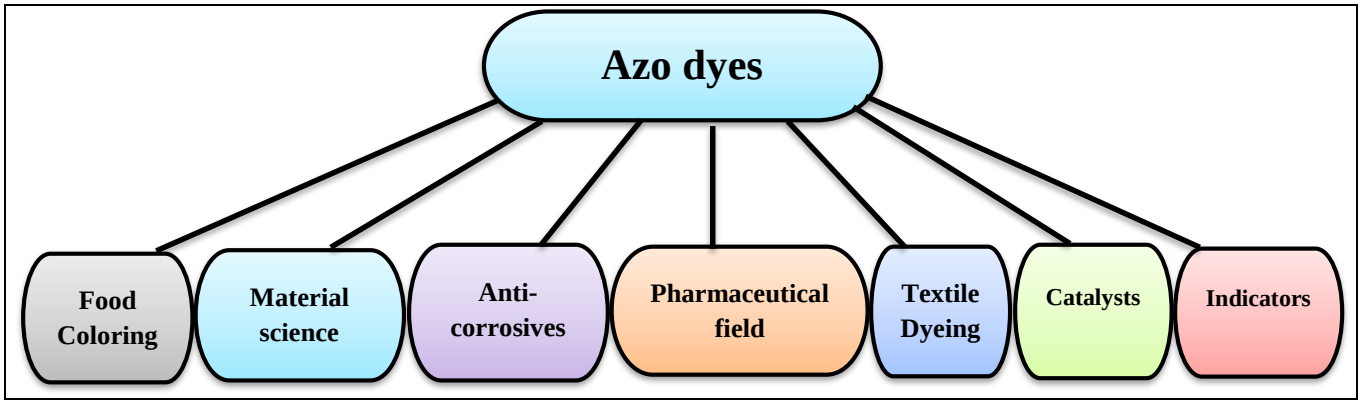


Figure (1-15): Application of Azo compounds.

1.14 Literature Review

(HPLC) has become a preferred analytical technique for aspirin determination because of its high sensitivity, specificity, and reproducibility. Many studies have focused on determining the concentration of aspirin. This study involved synthesizing a new Azo dye compound, and determining the concentration of aspirin using HPLC. This resulted in a new, accurate method with a low (LOD) and (LOQ). In the Table (1-3), different studies for aspirin determination will be discussed.

Table (1-3): Aspirin determination studies by HPLC method

Method and Compound	Flow rate (ml/min)	Retention time(min)	Recovery %	RSD %	LOD (µg.mL^{-1}), LOQ (µg.mL^{-1})	Linearity	Condition	Ref.
Lansoprazole and aspirin	1	4.3	100.49 %	0.933	LOD= 2.56, LOQ= 7.78	65-390	The Kromasil C_{18} column (250× 4.6mm, 5µm), uses a mobile phase of 10mM phosphate buffer pH3 :ACN 55:45 and UV detection at 284nm.	(69)
Ramipril, Aspirin and Simvastatin	1	2.7	99.76-99.90	1.1	LOD= 0.1, LOQ= 10	50-150	A mobile phase of 0.5% Ortho phosphoric acid: ACN: Methanol (20:10:70 v/v) and UV detection at 226nm was used with the SHISHE-DO C_{18} column, which was 250×4.6 nm and 5µm particle size.	(70)
Aspirin and Omeprazole	1	3.076	100.83 ± 0.448	0.921	LOD =1.564, LOQ =4.693.	10-100	C_{18} column (15cm ×4.6 mm, 5 µm particle size) with UV detection at 240 nm and mobile phase (acetonitrile: water 60:40 v/v).	(71)
Aspirin and pravastatin	1.5	3.654	99.58 ± 0.386	0.385	LOD= 0.204, LOQ= 0.680.	10-30	The Phenomx C_{18} (200mm × 4.6mm, 5 µm) column, a detector set at 260 nm and mobile phase consisting of water, acetonitrile, and acetic acid (40:59::0.1, v/v/v)	(72)
Atorvastatin, Aspirin, Ramipril, and Metaprolol	1	4.497	99.6 ± 0.436	0.4443	LOD= 2.6739, LOQ= 8.100	22.5 - 67.59	The column (250*4.6mm,) C_{18} column with a 5µm particle size. The mobile phase, was a 90:10 (v/v) mixture of phosphate buffer (pH 4) an acetonitrile, the volume of 20 µl of the solution	(73)

							was injected into column, UV detection 210 nm.	
Aspirin and Omeprazole by RP-HPLC method implementing AQbD approach	1.15	2.94	98 - 102%	0.577	LOD =0.014, LOQ= 0.044.	10-60	C18 column (250 × 4.6 mm, 5 µm), and mobile phase consisted of methanol: Disodium hydrogen phosphate buffer (68:32 v/v), pH adjusted to 4.5 with phosphoric acid, at a detection wavelength of 280 nm.	(74)
Methocarbamol and Aspirin	1	5.81	100.98 ± 1.52	1.65	LOD=1.3, LOQ= 4	25-450	C_{18} column, diluted acetic acid (pH 3.2): acetonitrile 79: 21 v/v, flow rate =1 mL min, at λ_{max} = 273 nm.	(75)
Omeprazole and Aspirin	0.6	1.720	99.59	0.09	LOD =1.85, LOQ =5.26.	10-50	The column is C_{18} (150 mm × 4.6 mm, 5 µm), the mobile phase is acetonitrile-methanol (20:80 v/v) injection volume was 20 µL, recorded at 233 nm	(76)
Aspirin and Omeprazole	1	4.248 ±0.02	100.62 ± 1.55	1.54	LOD=5.209, LOQ=17.19	20-400	Ultra C_{18} (4.6×250 mm, 5 µm particle size) column, The mobile phase was 0.05 M disodium hydrogen phosphate buffer pH 3 and acetonitrile in the ratio 60:40 (by volume), recorded at 235nm.	(77)
Green Chromatographic Approaches for Simultaneous Determination of Aspirin, Rosuvastatin and Clopidogrel in their	1	3.1	100.74 ± 1.202	1.083	LOD= 3, LOQ= 10	10.00 – 200.00	The column was Prontosil Hyperchom C_{18} (250 × 4.6 mm, 5 µm), the mobile phase was methanol: water with 1% TEA (80: 20, v/v, adjusting pH to 3.65 ± 0.05 using glacial acetic acid),and the injection volume was 20 µL, recorded at 260 nm	(78)

Tertiary Mixture								
Aspirin after derivatization reaction	1	1.65	100.123	0.291	LOD= 0.930, LOQ= 2.820	1.00-100	The column ODS-C_{18}, The mobile phase is acetonitrile-water (80:20), column temperature of 40 °C, at pH=3,	Present work

An azo dye was synthesized through a diazotization reaction, followed by coupling with a suitable aromatic compound, yielding a stable, colored Azo dye compound. The interaction between the dye and aspirin was optimized, and the resulting complex was separated and quantified using HPLC with UV detection. Key parameters of the method:

1. Detection Wavelength: Optimized to achieve maximum absorbance of the azo dye
2. Limit of Detection (LOD): Low, indicative of high sensitivity.
3. Limit of Quantification (LOQ): Low, enabling accurate quantification even at low concentrations.
4. Retention Time: Sharp, well-defined peaks confirmed the method's selectivity.

The newly developed HPLC method using a synthesized Azo dye provides a reliable, sensitive, and specific approach for determining aspirin concentration. Compared to conventional techniques, this method exhibits significantly lower LOD and LOQ values, making it suitable for pharmaceutical quality control and potential clinical applications

1.15 Kinetic Study

Kinetic studies explore the speed of chemical reactions and the elements that influence them, such as temperature, concentration, and catalysts. By analyzing reaction progress over time, kinetics elucidates reaction pathways and mechanisms. Understanding reaction kinetics is crucial for predicting outcomes and optimizing conditions for

diverse applications such as environmental science, drug discovery and development, and industrial chemistry (79).

1.16 Essential Elements of kinetic Studies

1.16.1 Rate of reaction: The rate of reaction quantifies how quickly a chemical reaction proceeds. It is generally determined by measuring the change in concentration of a reactant (consumed during the reaction) or a product (formed by the reaction) over a specific time interval. This measurement provides a numerical value representing the reaction's speed. Factors such as temperature, the presence of a catalyst, and reactant concentrations significantly influence the rate. A higher rate indicates a faster reaction, while a lower rate means the reaction is slower (80).

Reaction rate units can also be expressed as changes in:

Mass per unit time ($g.s^{-1}$), Volume per unit time ($dm^{-3}.s^{-1}$), and Moles per unit time (mol. S^{-1}) (81). it is typically measured in $mol.dm^{-1}.s^{-1}$.

1.16.2 Factors Influencing reaction rates

1. Concentration: reaction rates generally increase with higher reactant concentrations (82).

2. Temperature: higher temperatures increase kinetic energy, generally accelerating reaction rates (82).

3. Catalysts: catalysts accelerate reactions without being consumed (82).

4. Surface area: increased surface area accelerates solid-phase reactions (82).

5. Presence of light: most reactions accelerate in the presence of light or radiation(83).

6. Nature of reactants: reactant bond strengths and characteristics affect product formation rates (83).

1.16.3 Rate Laws: rate laws express the relationship between reactant concentrations and reaction rate. For A+B→ C, a typical rate low is:

$$\textbf{Rate} = k[A]^m[B]^n \qquad (1\text{-}11)$$

Where; m and n represent the order of the reaction with regard to reactants A and B, k is the rate constant (84).

1.16.4 Rate constant of reaction: the rate constant, often denoted as k, quantifies the speed of a chemical reaction. Specific to a given reaction at a particular temperature, it is influenced by factors like temperature, catalysts, and reactant concentration (83). The Arrhenius equation explains the relationship between temperature and the rate constant:

$$k = Ae^{-Ea/RT} \qquad (1\text{-}12)$$

Where: A= pre-exponential factor, E_a= activation energy, R= universal gas constant, T= temperature in kelvin (84).

1.16.5 Order of Reaction

The order of reaction refers to the power to which the concentration of a reactant is raised in the rate law of a chemical reaction. It indicates how the rate of reaction is affected by the concentration of reactants (85).

1.16.5.1 Zero Order: the rate of reaction is constant and independent of the concentration of reactants (86).

$$Rate = k \qquad (1\text{-}13)$$

The unit of zero order reaction: [M $Time^{-1}$]

$$t_{1/2} = {}^{a}/_{٢k.} \qquad (1\text{-}14)$$

1.16.5.2 First Order: the rate is directly proportional to the concentration of one reactant (86).

$$Rate = k\,[A] \qquad (1\text{-}15)$$

$$K_1 = \frac{٢.٣٠٣}{t} \log \frac{C}{C.} \qquad (1\text{-}16)$$

Where: C the concentration of a reactant at time (t) , C_0 is the initial concentration of the reactant, The unit of First order is: [$Time^{-1}$] (87).

$$t_{1/2} = {}^{0.693}/_{K_1} \qquad (1\text{-}17)\ (87).$$

1.16.5.3 Second Order: A second-order reaction is a chemical reaction where the rate of reaction is proportional to the square of the concentration of a single reactant, or the product of the concentrations of two reactants. This means the overall reaction order is two reactants (86).

$$Rate = k\,[A]^2 \qquad (1\text{-}18)$$

$$K_2 = \frac{1}{a\,t}\,\frac{X}{(a-X)} \qquad (1\text{-}19)\ (88)$$

$$Or \qquad Rate = k\,[A]\,[B] \qquad (1\text{-}20)$$

$$K_2 = \frac{2.303}{(a-b)}\log\frac{b(a-x)}{a(b-x)} \qquad (1\text{-}21)\ (88).$$

The unit of second order is: [M Time]$^{-1}$ (83).

$$t_{1/2} = \frac{1}{k_2.a} \qquad (1\text{-}22)\ (89).$$

1.16.5.4 Third order reaction: A third-order reaction is a chemical reaction where the rate of reaction is dependent on the concentration of three reactants or the cube of the concentration of a single reactant. The rate law for such reactions can be expressed as Rate = k[A]^3 or Rate = k[A][B][C], where k is the rate constant and [A], [B], and [C] are the concentrations of the reactants (86).

$$K_3 = \frac{1}{2\square}\left[\frac{x(2\square-x)}{a^2(a-x)^2}\right] \qquad (1\text{-}23)\ (90).$$

The unit of Third order reaction is: [M^{-2} Time $^{-1}$] (90).

$$t_{1/2} = \frac{3}{2K_3} \times \frac{1}{a^2} \qquad (1\text{-}24)\ (91).$$

1.16.6 Calculating Kinetics:

1. Experimental methods: like spectrophotometry, manometry, and chromatography are commonly used to monitor concentration changes over time (69).

2. Graphical analysis: can determine reaction order; for example, a plot of ln [A] vs. time yielding a linear relationship a first-order reaction (80).

1.17 Application of kinetic study

Kinetic studies are fundamental in understanding reaction rates and mechanisms, and they have wide-ranging applications in various fields. They help optimize industrial processes, design more efficient chemical reactors, and improve drug formulations. Furthermore, kinetics plays a crucial role in environmental science, food chemistry, and even our understanding of biological processes (92).

1.17.1 Industrial Processes

Kinetics helps optimize production rates and yields in chemical manufacturing (93).

1.17.2 Designing Chemical Reactors

Kinetic studies are instrumental in the design and optimization of chemical reactors, facilitating efficient and cost-effective production by informing reactor sizing and operating condition decisions. (94).

1.17.3 Pharmaceutical Development

1. Drug Stability: Kinetic studies are essential for predicting drug degradation over time, a critical factor in establishing the shelf life and stability of pharmaceutical formulations. (95).

2. Drug Absorption and Metabolism: A thorough understanding of drug absorption, distribution, metabolism, and elimination (ADME) is critical for the development of efficacious and safe pharmaceutical products (96).

3. Drug Interactions: Kinetics helps predict drug-drug interactions, leading to safer and more effective treatments (97).

1.17.4 Environmental Science

Kinetics helps determine the rate at which pollutants are broken down in the environment, aiding in the development of strategies for pollution control (98).

1.17.5 Wastewater Treatment

Kinetics can be used to optimize wastewater treatment processes to remove harmful contaminants (99).

1.17.6 Food Chemistry

Knowledge of reaction kinetics is crucial for food preservation, flavor enhancement, and safety measures against spoilage. (e.g., refrigeration, canning, drying).

1.17.7 Materials Science

Kinetics plays a role in understanding the behavior of materials at different temperatures and pressures, which is important for developing new materials with desired properties (100).

1.17.8 Understanding Biological Processes

Enzymes and other biological catalysts follow kinetic principles, and understanding these principles is crucial for understanding various biological processes (101).

1.17. 9 Developing New Technologies

Kinetic studies are essential for developing new technologies in various fields, such as nanotechnology and energy storage (102).

1.18 Aims

1. Developed simple, fast and accurate method for quantification of Aspirin (Acetylsalicylic acid).

2. Synthesis of a new Azo compound from Azo derivative, and Characterize the Azo compound using different techniques.

3. Validate the developed method to obtain the best optimal conditions to determine the aspirin.

4. Applying the proposed method to determine Aspirin in pharmaceutical products.

5. Study the kinetics of Aspirin by using the RP-HPLC for alkaline and oxidative degradation, determining rate constants, half-lives, and activation energies.

Chapter two

Experimental part

2.1 Chemicals

In the table below are lists all chemicals used in this work and their suppliers; no further purification was required.

Table (2-1): List of chemical materials

Seq.	Chemicals	Chemical formula	Company supplied	purity
1	Standard aspirin	$C_9H_8O_4$	Sigma Aldrich	≥99.0%
2	Standard Amiloride	$C_6H_8ClN_7O$	Sigma Aldrich	≥99.0%
3	Standard Furosemide	$C_{12}H_{11}ClN_2O_5S$	Sigma Aldrich	99.0%
3	4-Amino phenol	C_6H_7NO	LOBA CHEMI PVT. LTD., India	99.0%
4	Sodium nitrate	$NaNO_2$	E.M.darmstad	99%
5	Sodium Hydroxide	NaOH	B.D.H	99 %
6	Hydrochloric acid	HCl	J.K Baker Netherlands	37 % (w/w)
7	Ethanol grade	C_2H_5OH	Fluka	99 %
8	Acetonitrile, HPLC grade	C_2H_3N	HIMEDIA	99.7 %
9	Methanol, HPLC grade	CH_3OH	HIMEDIA	99.7 %
10	Water, HPLC grade	H_2O	HIMEDIA	
١١	Potassium dihydrogen phosphate	KH_2PO_4	Thomas Baker	98 %

12	Sulfuric acid	H_2SO_4	G.C.C	99 %
13	Glacial acetic acid	CH3COOH	Sharlu	<98 %
14	Phosphate buffered saline (PBS)	$Cl_2H_3K_2Na_3O_8P_2$	HIMEDIA	99 %
15	Sodium acetate	CH_3COONa	HIMEDIA	99 %
16	Ammonium sulfate	$(NH_4)_2SO_4$	HIMEDIA	99 %
17	Phosphoric acid	H_3PO_4	Fluka	80 %
18	Disodium hydrogen phosphate	Na_2HPO_4 .12 H_2O	HIMEDIA	99 %
19	Dipotassium hydrogen phosphate	K_2HPO_4	HIMEDIA	99 %
20	Potassium hydroxide	KOH	Fluka	98 %

2.2 Instruments

The instruments used in this study are listed in the table below.

Table (2-2): Instruments used

NO.	Instrument name	Type origin	place
1.	High performance liquid chromatography (HPLC)	UFLC-20A, Shimadzu, Japan.	Kerbala university College of science.
2.	Double beam UV-visible spectrophotometer.	AA- 1800, Shimadzu, Japan.	Kerbala university College of science.
3.	Fourier-transform infrared spectroscopy	8400 S, Shimadzu, (Japan).	Kerbala university College of science.

4.	Shaker.	GEMMYORBIT VRN480 England.	Kerbala university College of science.
5.	Ultrasonic	DAIHAN, Scientific, Korea.	Kerbala university College of science.
6.	MS spectrophotometer	Shimadzu GCMS-QP210 PLUS	Samara university College of science.
7.	Hotplate Magnetic stirrer	Heido - MrHei- standard, Germany.	Kerbala university College of science.
8	pH meter	HANNA, ITALY	Kerbala university College of science.

2.3 Methodologies

2.3.1 Preparation of chemical solution

A number of solutions were prepared including:

2.3.1.1 Preparation of aspirin standard solution (1000 µg.mL^{-1})

Standard solution of aspirin (1000 µg.mL^{-1}) was prepared by exactly weighting 0.100 g of standard aspirin (M.wt 180 g/moL) and transferred it into a 100 mL volumetric flask and completes the volume with mobile phase. The solution was sonicated for 15 min or until the reference standard dissolved completely.

2.3.1.2 Preparation of Reagent solution (4- aminophenol) (1000 µg.mL^{-1})

A reagent was made by dissolving 0.100 g of 4-amino phenol in small amount of methanol, M.Wt. is (109.13 g/moL), then complete the volume with 100 mL of methanol to the mark of volumetric flask.

2.3.1.3 Preparation of sodium hydroxide solution (10%)

A 2.50 gm of sodium hydroxide was dissolved in 25 mL of distilled water to prepared 10% (w/v) of NaOH.

2.3.1.4 Preparation of hydrochloric acid solution (1M)

A 4.17 mL of concentrated hydrochloric acid 37 % (w/w) was dissolved in 50 mL D

2.3.1.5 Preparation of sodium hydroxide solution 0.1 M

0.100 g of sodium hydroxide was diluting in 25 mL distilled water.

2.3.1.6 Preparation of hydrochloric acid solution 0.1M

This solution prepared by dissolving about 0.42 mL of concentrated hydrochloric acid in 50 mL distilled water.

2.3.1.7 Preparation of Phosphate buffer solution pH =2

The solution was prepared by dissolving 0.429 g of disodium hydrogen phosphate and 0.17 g potassium dihydrogen phosphate in 50 mL D.W. the pH adjusted to 2 by addition diluted phosphoric acid.

2.3.1.8 Preparation of Sulfate buffer solution pH =2

This solution was prepared by dissolving 13.21 g of Ammonium sulfate in 50 mL of D.W (solution A). A 0.14 mL of Sulfuric acid solution was dissolved carefully with cooling in 50 mL D.W (solution B). Equal volumes of solutions A and B were mixed.

2.3.1.9 Preparation of Sodium acetate buffer solution (0.1 M) pH =4

Exact 0.822 g of sodium acetate was dissolved in 100 mL of D.W (solution A). A 1.44 mL from glacial acetic acid was dissolved in 250 mL of D.W (solution B). 100 mL of solution B was titrated using about 20 mL of solution A.

2.3.1.10 Preparation of Phosphate Buffered Saline (PBS)

The solution was prepared by dissolving 1.079 g of PBS in 100 mL of D.W the pH=7.2 by using buffer.

2.3.1.11 Preparation of Phosphate buffer solution (0.1 M) pH =8

This solution was prepared by dissolving 0.026 g of potassium dihydrogen phosphate and 0.8365 g of dipotassium hydrogen phosphate in 50 mL of D.W with the same solvent.

2.3.1.12 Preparation of Phosphate buffer solution (1 M) pH =8

This solution was prepared by dissolving 6.805 g of potassium dihydrogen phosphate in 50 mL of D.W. The pH was adjusted to 8 by addition a drops of 1 M sodium hydroxide.

2.3.1.13 Preparation of buffer phosphate solution pH =9

Accurately weighed 1.74 g of potassium dihydrogen phosphate in 80 mL of D.W, and the solution was adjusted pH to 9 with potassium hydroxide 1 M, and diluted with D.W to the mark.

2.3.2 Preliminary investigations

2.3.2.1 Preliminary investigations of 4- amino phenol and aspirin interactions

A group of standard drugs (furosemide, aspirin, Amiloride) were prepared at 1000 $\mu g.mL^{-1}$. The reagent 4-amino phenol was prepared at 1000 $\mu g.mL^{-1}$. In a test tube, 1 mL of each drug stock solution was placed. Sequentially, drop by drop, 2 mL of the reagent was added to the test tube with shacking, colored precipitate (Brown precipitate) began to appear at interaction between the aspirin drug and the reagent, while no precipitate appeared at the interactions between the other drugs and the reagent. Drops of Hydrochloric acid 0.1 M and Sodium hydroxide 0.1 M were added to two different parts of this mixture to observe the impact of the acid function. The objective was to test the effect of the acid and base on color creation. Under basic conditions, a significant color change indicated a favorable interaction. Conversely, the absence of color change in acidic conditions suggested no interaction. This investigation found that aspirin and the reagent 4-aminophenol show a significant color response in basic conditions, while no color change is observed in acidic conditions.

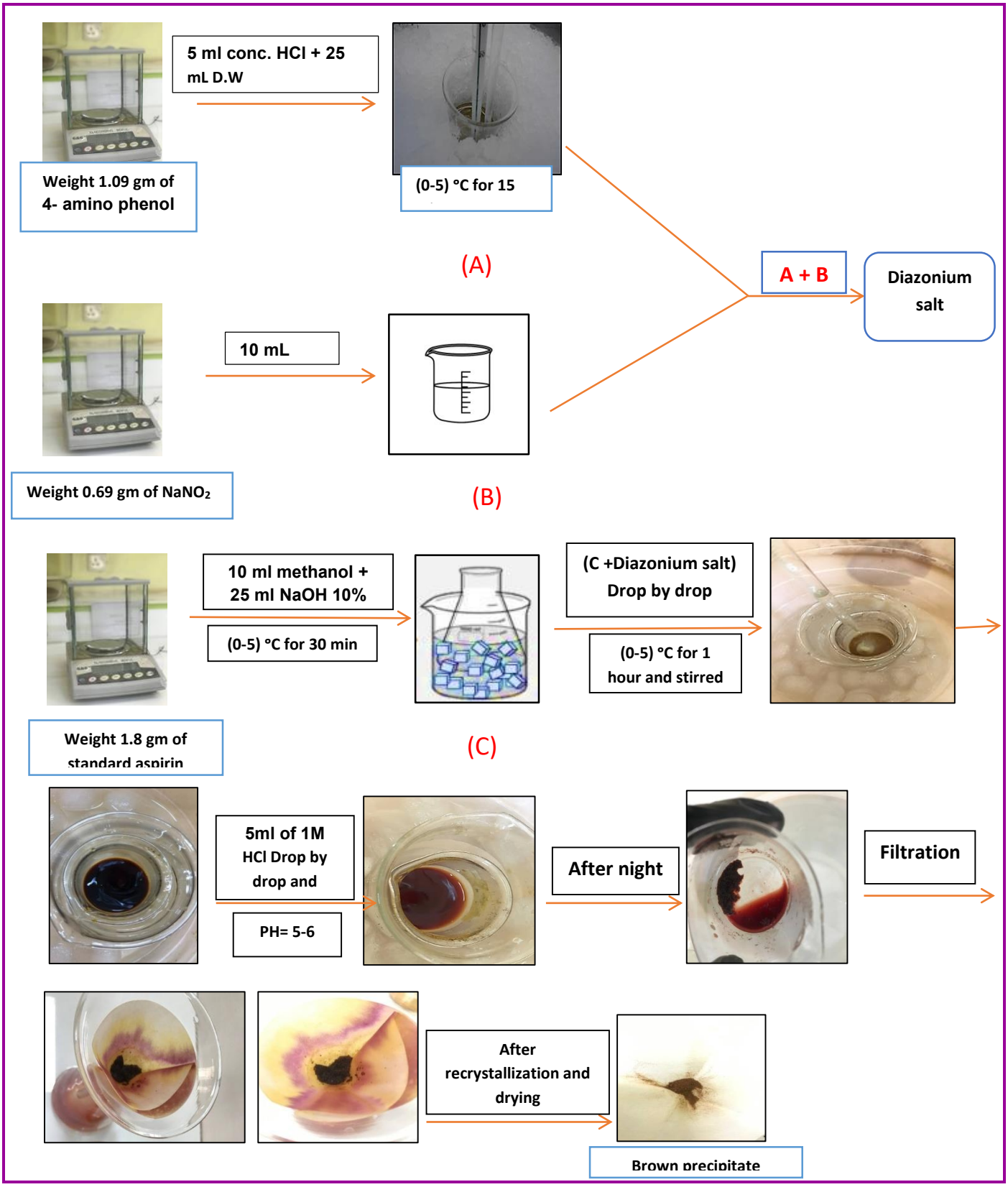
2.3.2.2 Preliminary investigations of standard aspirin by HPLC

To obtain a mobile phase that would be suitable for the quantification of aspirin**,** different mobile phases were investigated. The mobile phases that were employed included various ratios of organic modifiers (water, acetonitrile, and methanol) are some of the solvents were evaluated, using an isocratic mode. A mixture of water: acetonitrile at varying ratios (10:90, 20:80, 30:70, 40:60, and 50:50) and methanol: acetonitrile at different ratios (90:10, 80:20, 70:30, 60:40, 50:50) was employed as the mobile phase to increase the proportion of organic solvent.

2.3.3 Synthesis of ((E)-2-acetoxy-5-((4-hydroxyphenyl) diazenyl) benzoic acid) (AHPDBA)

Method for synthesis of diazoninm salts: An aromatic amine solution (4-amino phenol) (1.090 g, 10 mmol) was dissolved in a mixture of 25 mL of distilled water and 5 mL of concentrated HCl. This solution was used to form diazonium salt pairs for Imidazole liquor synthesis. After cooling the mixture to 0-5 °C (this degree remains constant by adding sodium chloride to the ice bath), a solution of sodium nitrite (0.690 g, 10 mmol) in 10 mL distilled water was added, the nitrogenation was completed by stirring and cooling to 0-5°C the solution for 15 minutes for the next step.

Method for synthesis of azo compounds ((E)-2-acetoxy-5-((4-hydroxyphenyl) diazenyl) benzoic acid) (coupling reaction): a (1.8 g, 10 mmol) of standard aspirin was dissolved in 10 mL methanol and 25 mL of 10% NaOH, the solution should be cooled to between (0-5) °C and returned to sit for a half hour. The salt solution was cooled between (0-5) °C and gradually infused with this imidazole derivative solution. After being given a deep red color, the mixture was stirred at 0-5 °C for 1 hour. Gradually added 5ml of 1M diluted HCl until a brown precipitate forms, achieving an acidity pH of 5-6. After separation, the resulting solid was filtered, washed thoroughly with distilled water to remove any sodium sulfate, dried, and recrystallized using methanol (103) and the yield was 42%. The procedure illustrated in Fig. (2-1).



Scheme (2-1): Schematic diagram of synthesis of AHPDBA

2.4 UV-Visible measurement

The UV-Visible spectrum for aspirin at 25µg.mL^{-1} was recorded from 200 - 800 nm using a dual-beam UV-Visible spectrophotometer; the measurements were carried out using quartz cells with a thickness of 1 cm. The maximum absorbance λ_{max} of aspirin was found to be 277nm. The UV-Visible scan was done for 4-amino phenol to determined λ_{max} , which was determined to be equivalent to 234nm. The UV-Visible scan for AHPDBA at concentration 25 µg.mL^{-1}, the peak absorbance for AHPDBA was observed at λ_{max} = 304nm.

2.5 Infrared spectra (FT-IR)

The FT-IR spectra were obtained as (KBr disc) for the reagent 4-amino phenol and aspirin and AHPDBA, to compare to standard spectra to confirm their purity.

2.6 Mass spectrometry (MS)

MS was applied to study the fragment of the synthesized azo dye compound and to study the m/z value by comparing it with the theoretical value.

2.7 Analysis of AHPDBA by using HPLC method

2.7.1 Mobile phase composition: water, acetonitrile, and methanol are some of the solvents that had been evaluated applying the isocratic mode with a C_{18} column and various organic modifications studies. Acetonitrile was selected instead of methanol because of it strong dipole moment and limited hydrogen bonding. The ideal mobile phases for HPLC analysis consist of acetonitrile: water in the ratio 80:20 (v/v).

2.7.2 Effect of pH

The effect of changing the pH Mobile phases on the peak shape and retention times of the test solution was investigated using pH values between 3 and 8 under optimal conditions.

2.7.3 Effect of pH Buffer

The effect of different pH buffer (2, 4, 7.2, 8, and 9) on retention time and the shape peak was investigated.

2.7.4 Effect of flow rate

The effect of different flow rates by injection AHPDBA at (0.3, 0.5, 0.8, and 1 ml/min) under optimal condition was examined.

2.7.5 Effect of volume injection

The effect of injection volume for AHPDBA with (10, 20, 30, 40, and 50 µL) was investigated under ideal conditions.

2.7.6 Effect of temperature

The effect of column temperature on resolution in reversed-phase high-performance liquid chromatography (RP-HPLC) was studied at optimal conditions between 30 and 50℃.

2.7.7 Calibration curve

The calibration curve of AHPDBA was done by preparing different concentrations of AHPDBA ranging from (1.00-100 $\mu g.mL^{-1}$) in the mobile phase, the peak area of each concentration was plotted against the concentration to calculate the calibration equation and correlation coefficient.

٢.٨ Validation of the method

The following parameters were used to validate the improved HPLC method:

2.8.1 Linearity and range

Linearity of the method was assessed by injecting AHPDBA prepared in the mobile phase in the range of (1.00-100 μg.mL^{-1}). The peak areas were plotted against the corresponding concentration to obtain the calibration curve.

2.8.2 Precision

The precision of method was confirmed by repeatability (104), repeatability were tested using three concentrations (10, 50, 100 μg/mL) of aspirin drug.

2.8.3 Accuracy

The accuracy of method is defined by the proximity of the measured value to the true sample value, while the precision refers to the consistency of repeated measurements under the same conditions (105). Recovery studies in pharmaceutical research assess the precision and accuracy of analytical methods. This process includes adding a known analyte amount to a sample matrix and measuring the recovered amount. Researchers validate techniques by comparing observed recovery rates to expected values.

2.8.4 Limit of detection and limit of quantification (LOD and LOQ)

The sensitivity of the suggested method was assessed using the Limit of detection (LOD) and limit of Quantitation (LOQ), defined as LOD =3.3SD/S and LOQ= 10 LOQ (106).

2.9 Kinetic investigation of AHPDBA by HPLC

2.9.1 Kinetic investigation for alkaline

To investigate alkaline degradation kinetics, a 1000 μg /mL stock solution of AHPDBA was prepared by dissolving 50 mg of AHPDBA in 50 mL of methanol. One mL of the

stock solution were transferred to a series of test tubes, followed by addition of 1 mL of 1 M NaOH solution. The test tubes were sealed and incubated in a thermostatic water bath at controlled temperatures of 50, 60, and 70 ℃. From time zero until 1.25 hours, the contents of each tube were neutralized to pH 7 every 15 min by adding 1 mL of 1M HCl. The tube contents were transferred to 10 mL volumetric flask and completing the volume with the mobile phase, filtered, and analyzed by HPLC. Residual AHPDBA concentrations were calculated for each temperature and time point. The logarithm of percentage of remaining AHPDBA concentration was plotting against the corresponding time interval (in hours) for each temperature, and the regression equations were calculated.

2.9.2 Kinetic investigation for oxidative degradation

For this study, 50 mg of AHPDBA was dissolved in 50 mL of methanol; 1 mL of solution has been placed into different test tubes, following by additional 2 mL of 3 % methanolic hydrogen peroxide solution. The test tubes were stoppered and placed in thermostatic water bath at different temperature, 60, 70, and 80 ℃. From zero time until 1.25 hours, the contents of each tube were neutralized to pH 7 every 15 min by adding 1 mL of 1M HCl. Tube contents were transferred to 10 mL volumetric flask and completing the volume with the mobile phase, filtered, and chromatographed. Residual of AHPDBA concentrations were calculated for each temperature and time point. The logarithm of percentage of remaining AHPDBA concentration was plotting against the corresponding time interval in hours for each temperature, and the regression equations were calculated.

2.10 Analysis of a marketed formulation

For the analysis of aspirin in pharmaceutical dosage form, 10 tablets (Aspirin 100 mg) were accurately weighed, finally powdered, an accurate 100 mg was weight of the

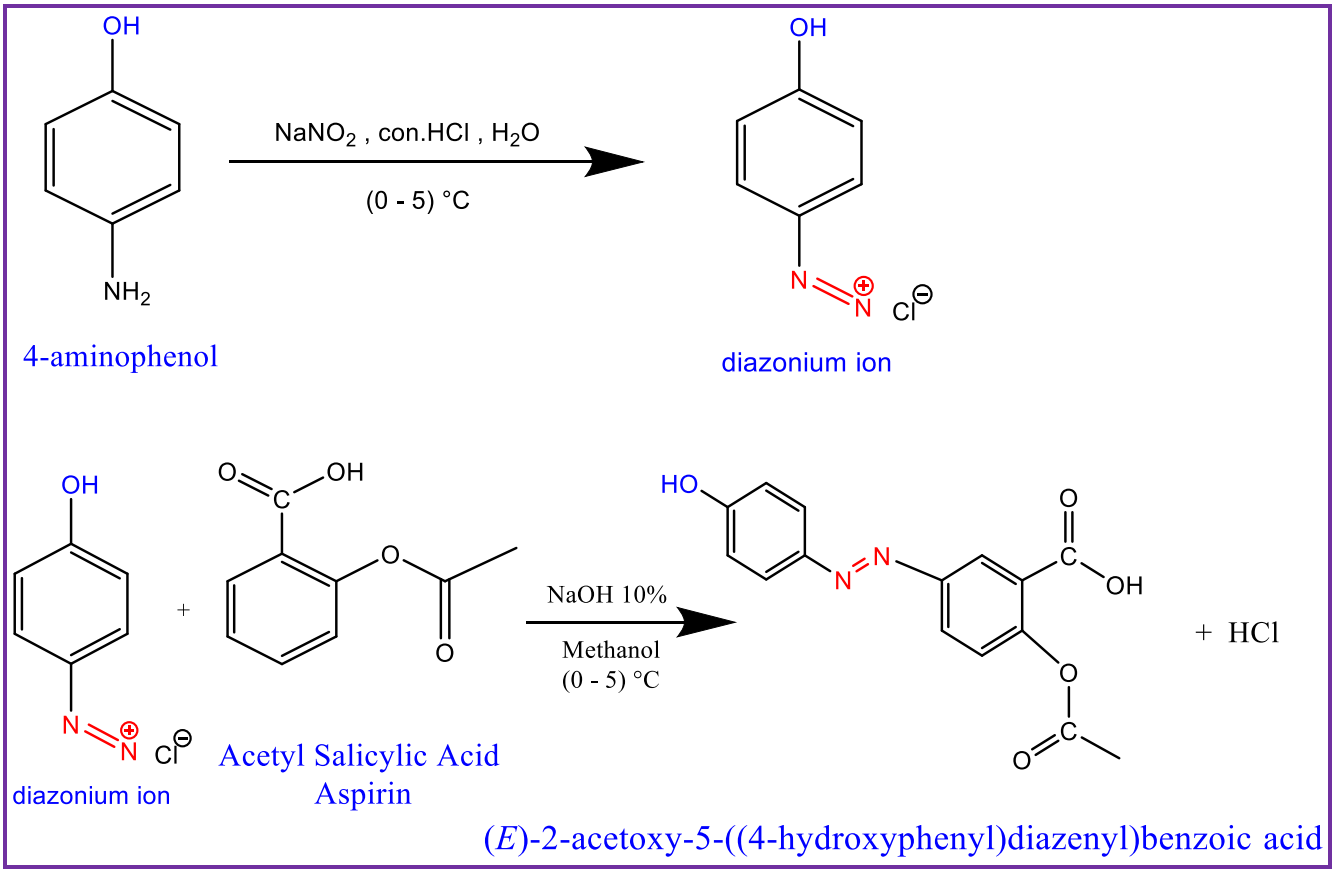
powder containing of acetyl salicylic acid and transferred to a 100 mL volumetric flask, the solution was sonicated to dissolve the powder in 50 mL of mobile phase composition acetonitrile: water (80:20 v/v) after the diluted up to mark with the mobile phase adjusted with 0.1 M HCl to pH 3. To prepared solution was filtered through a 0.45 µm nylon filter, then the appropriate volume was transferred into the vial. The solution flowed at a flow rate of 1 ml/min for 5 minutes and the column temperature was 40℃. The injection volume was 30 µL were injected into HPLC column.

Chapter three

Result and discussion

3.1 Synthesis of (E)-2-acetoxy-5-((4-hydroxyphenyl) diazenyl) benzoic acid (AHPDBA)

The reaction scheme of Azo dye compound from 4-amino phenol is shown in scheme (3-1). The first step in this mechanism includes formation of diazonium salt at 4-amino phenol, and the second step includes coupling of diazonium salt with aspirin drug (Acetyl salicylic acid).



Scheme (3-1): Synthesis of AHPDBA.

The solubility of (AHPDBA) in various solvent is shown in table (3-1).

Table (3-1): Solubility of (AHPDBA) in different solvents

compounds	Ethanol	methanol	Acetonitrile	Distal water	DMSO	chloroform
AHPDBA	++	++	+++	-	+	+

3.2 Characterization of aspirin, 4-amino phenol, AHPDBA

3.2.1 UV- Visible measurement of 4- amino phenol, standard Aspirin, and AHPDBA

The UV-Visible spectroscopy was used to determine the λ_{max} for the reagent 4-amino phenol which was found to be 234nm, and the maximum absorbance wavelength (λ_{max}) of standard aspirin was determined to be 277 nm. The UV-Visible study of the Azo dye compound (AHPDBA) showed a peak in the visible region, with a maximum absorbance at 304 nm, the azo group can donate electrons, resulting in an electronic transition where electrons move from non-bonding (n) orbitals to anti-bonding (π^*) orbitals. This type of transition, known as ($n \rightarrow \pi^*$) electronic transition, is characteristic of molecules containing heteroatoms with lone pairs of electrons, such as nitrogen atoms in the Azo group (-N=N-) (107). This is shown in Fig. (3-1).

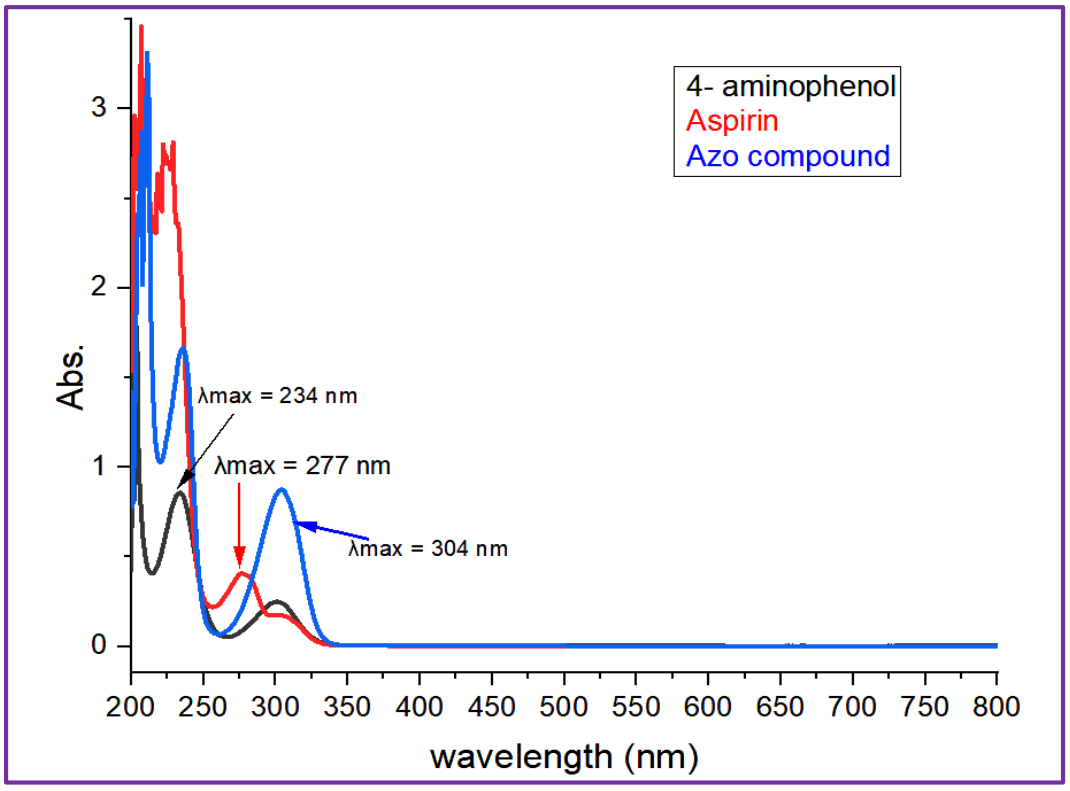


Figure (3-1): The UV-Visible scan of 4-amino phenol (Black), standard Aspirin (Red), Azo compound (AHPDBA) (Blue).

3.2.2 FT-IR Spectra

3.2.2.1 FT-IR spectra for 4-aminophenol

The infrared spectrum (FT-IR) of 4-amino phenol is displayed in Fig. (3-2), for primary amine observe strong peaks at 3340 cm^{-1} for (N-H stretching amine) due to NH_2 group in 4-amino phenol. The hydroxyl O-H group for phenolic compound appears at (3282 cm^{-1}), and peaks appear at (3028 cm^{-1}) for aromatic C-H stretching, as well as peaks at (2920 cm^{-1}) for aliphatic C-H stretches, in the region of (1512-1473 cm^{-1}) sharp peaks corresponding to the stretching of C=C bands in the aromatic ring. The peak at (1234 cm^{-1}) for C-N stretching of the amine group, peaks at 1616 cm^{-1} for bending vibrations related to the amine group, a Peak at 1091 cm^{-1} for C-O stretching due to the alcohol group, and a single sharp peak at 821 cm^{-1} due to para- substitution in the aromatic ring (108).

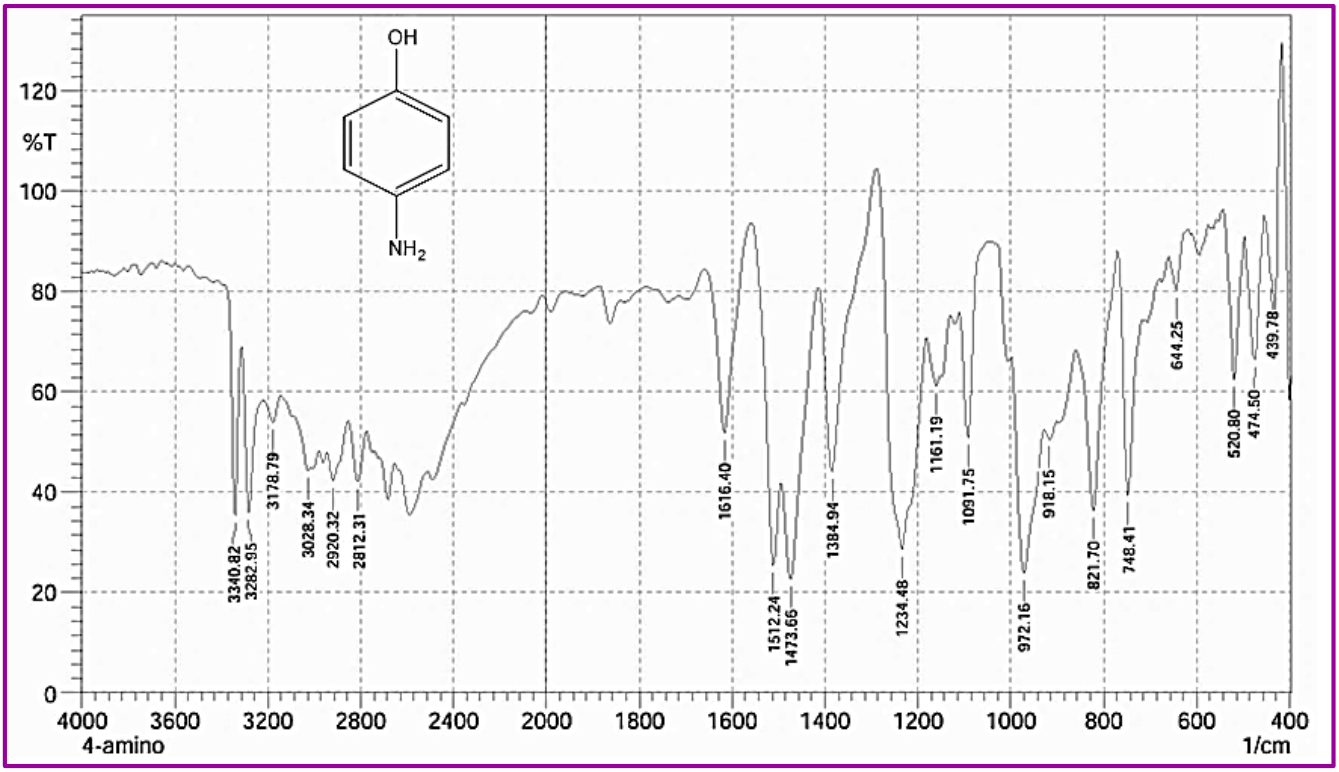


Figure (3-2): The FT-IR spectrum of 4- amino phenol**.**

3.2.2.2 FT-IR spectra for Aspirin

The FT-IR spectrum of acetyl salicylic acid (aspirin) can be obtained in Fig. (3-3) which shows several characteristic peaks that corresponds to its functional groups. A broad peak appears at 2870 cm^{-1} due to the hydroxyl (O-H) group, and a peak at 2997 cm^{-1} for the aromatic C-H stretching vibrations. The sharp peak appears at 1759 cm^{-1} due to the carbonyl (C=O) stretch of the ester group and carboxylic acid. As well as peaks in (1519-1604 cm^{-1}) correspond to the C=C stretching vibrations for the aromatic ring. While C-O stretching for the ester and carboxylic group appears at 1219 cm^{-1}, a single and strong absorption band at 756 cm^{-1} for Ortho- substitution aromatic ring (109).

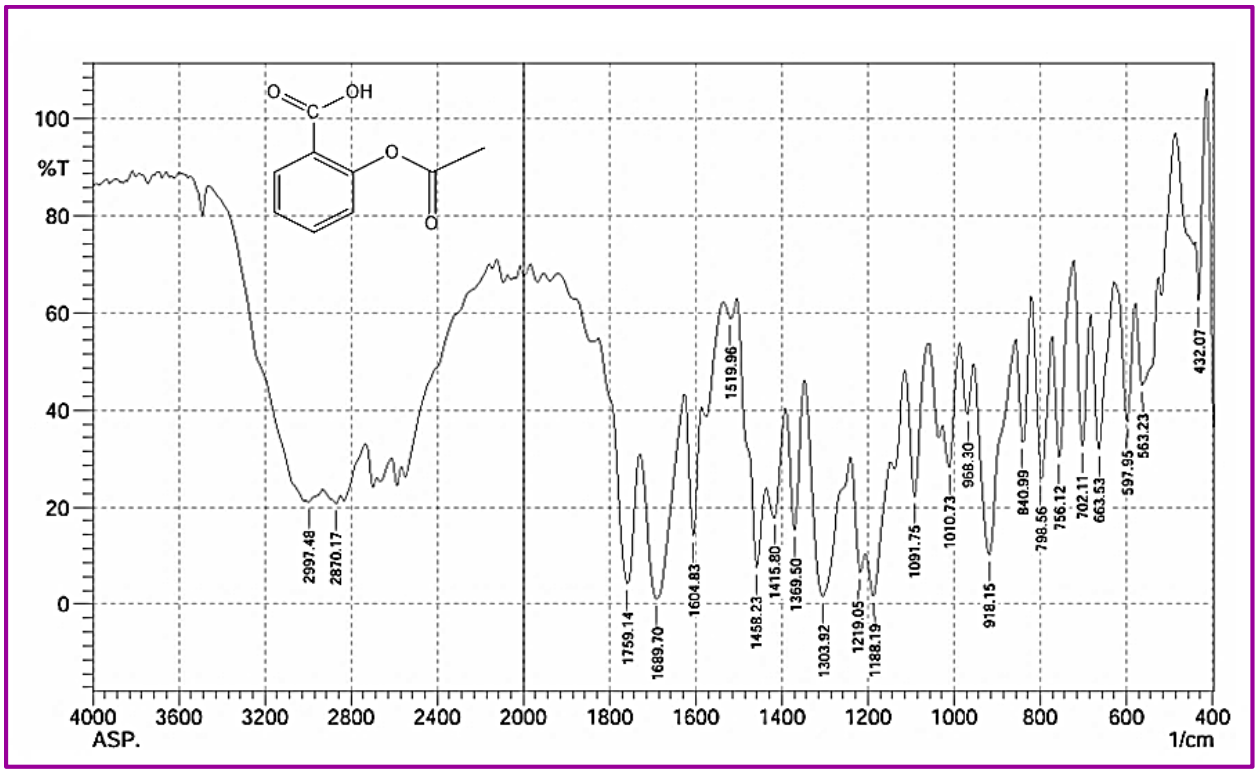


Figure (3-3): The FT-IR spectrum of aspirin.

3.2.2.3 FT-IR spectra of AHPDBA

The FT-IR spectrum of (AHPDBA) exhibits several characteristic peaks that correspond to different molecular vibrations as in Fig. (3-4). Based on observed peaks, there is a broad peak at 3236 cm^{-1} indicating hydroxyl groups (OH), while new bands are observed for the aromatic C-H (3012 cm^{-1}) stretches, and absorption band for C-H aliphatic at (2858cm^{-1}), the absorption band for the carbonyl group C=O appears at (1612 cm^{-1}), and the NH_2 absorption band at (3100-3400 cm^{-1}) is absent after the reaction, while the recognizable peak is frequently seen at 1573 cm^{-1} due to the stretching vibrations of the azo group (-N=N-), and show strong absorption bands at 1207 cm^{-1} for (C-O phenolic). The C=C for the aromatic compound appears at (1612cm^{-1}), Depending on the particular chemical and its substituents, this peak may vary slightly (110).

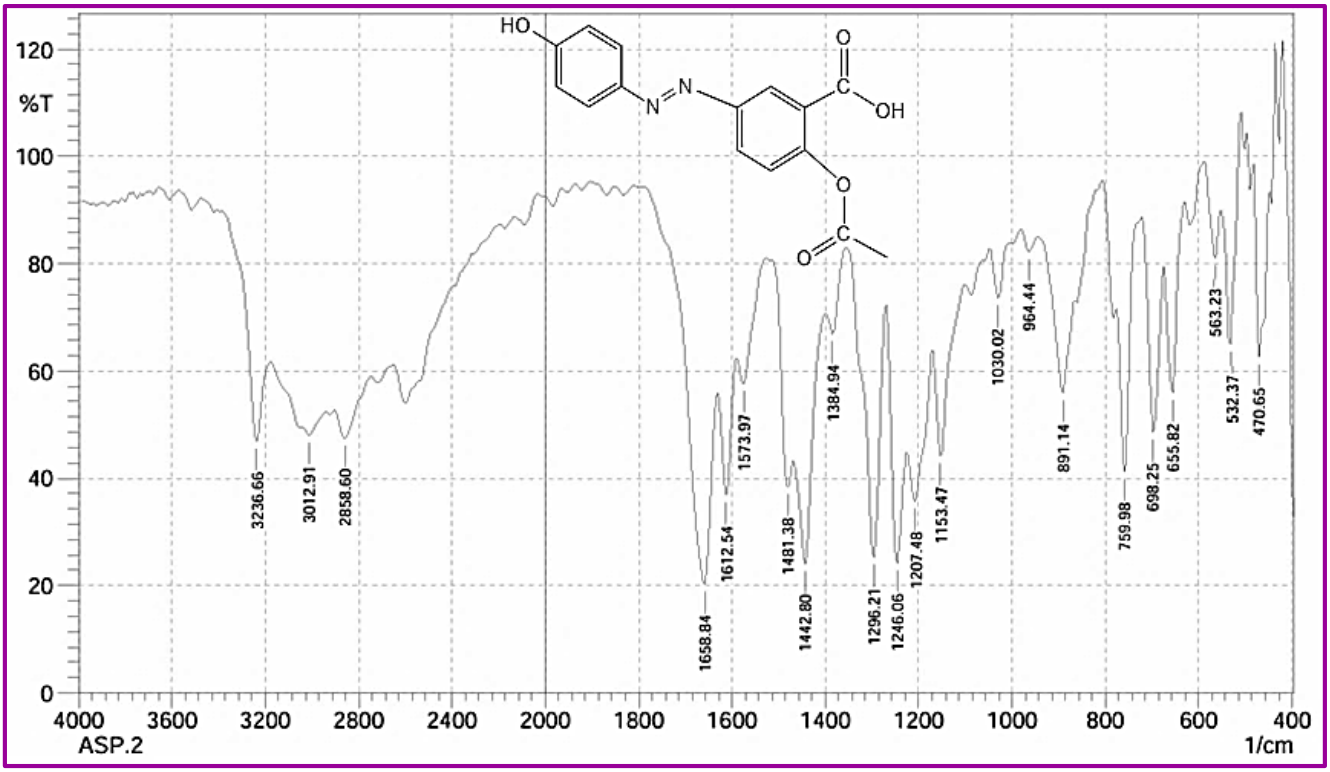


Figure (3-4): The FT-IR spectrum of AHPDBA.

Table (3-2): show the main data of FT-IR spectrums of new Azo dye with the source material.

Type of bond	Wave number (cm^{-1}) of 4-amino phenol	Wave number (cm^{-1}) of Aspirin	Wave number (cm^{-1}) of AHPDBA
St. N-H	3340	------	-----
N-H bending	1616	------	-----
(C-H) aromatic	3028	2977	3012
(C-H) aliphatic	2920	2870	2858
(C=C) aromatic	1512,1473	1519,1458	1612
N=N	------	------	1573
C=O	------	1759	1658
C-N	1091	------	1030
St. (C-O) phenolic	1234	1219	1207

3.2.3 Mass for AHPDBA

The Mass (MS) spectrum of AHPDBA is shown in Fig. (3-5). The molecular weight from the experiment is 300 g/mol closely matches the calculated molecular weights 300 g/mol. This agreement confirms the accurate the proposed method.

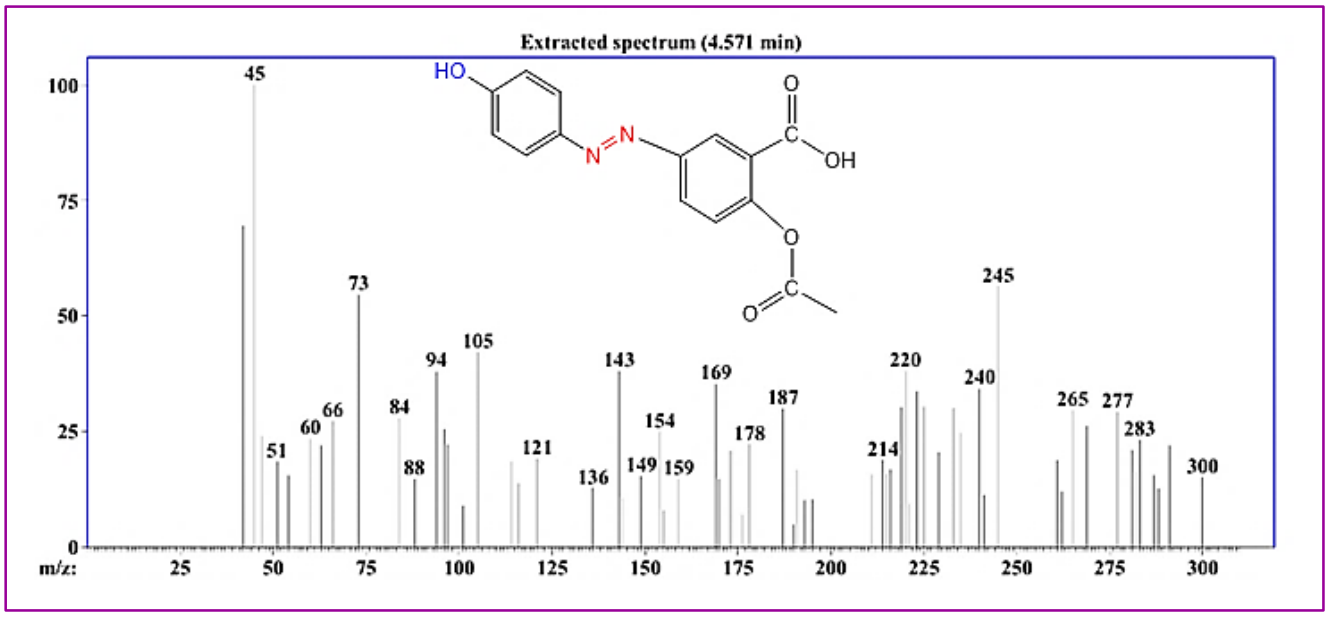
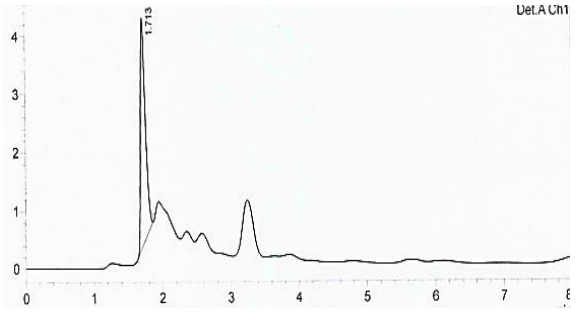


Figure (3-5): MS spectrum of AHPDBA.

3.3 Preliminary investigations of standard aspirin by HPLC

To evaluate standard Acetylsalicylic acid (Aspirin), different mobile phase ratios were tested. These included methanol: acetonitrile mixtures (90:10, 80:20, 70:30, 60:40, 50:50 v/v), as well as acetonitrile: water mixture (90:10, 80:20, 70:30, 60:40, 50:50 v/v), The result of this investigation is shown in table (3-3), (3-4).

Table (3-3): Initial study results for standard Aspirin using a mobile phase (ACN: Water).

Mobile phase	Composite (v/v)	Retention time (min)	Peak area	Height	Tailing factor	
ACN	100	1.713	19414	3928	1.695	

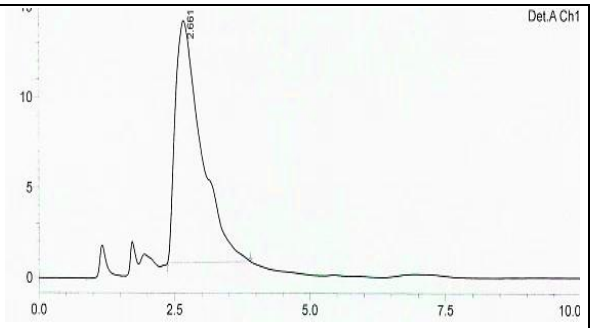
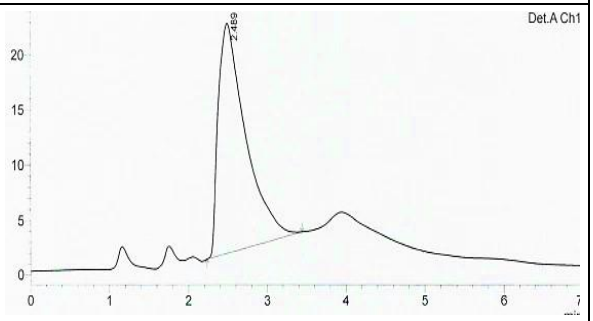
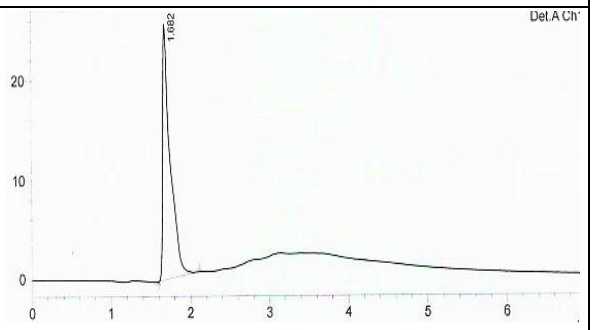
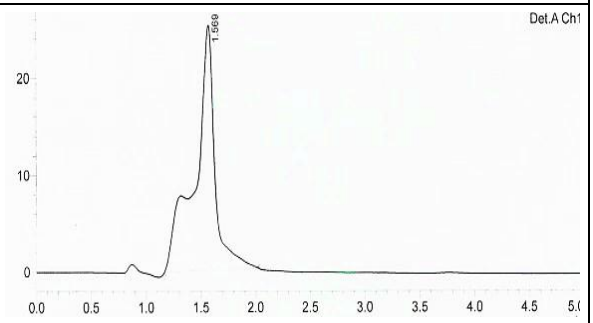
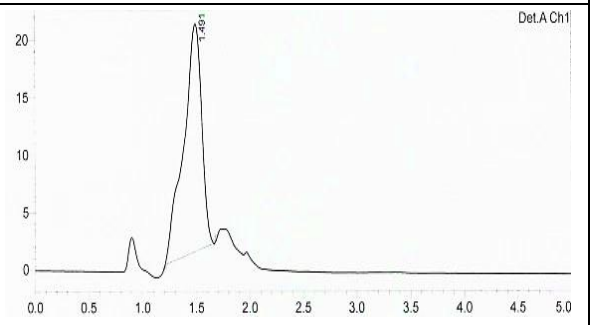
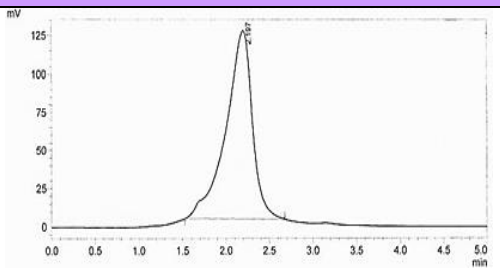
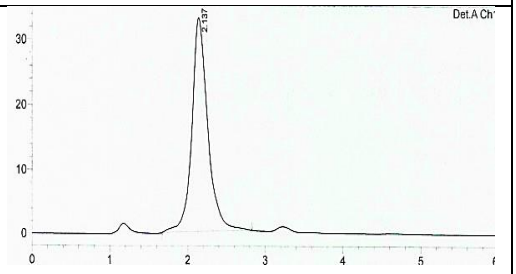
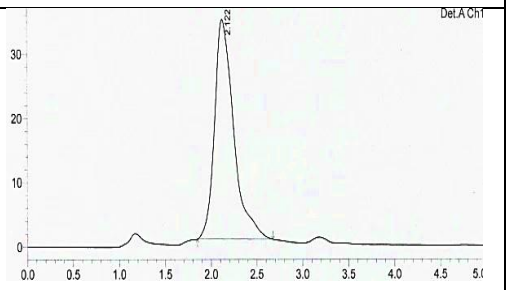
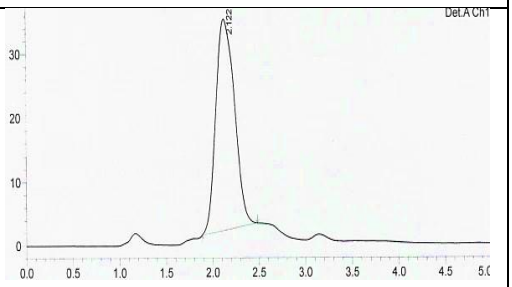
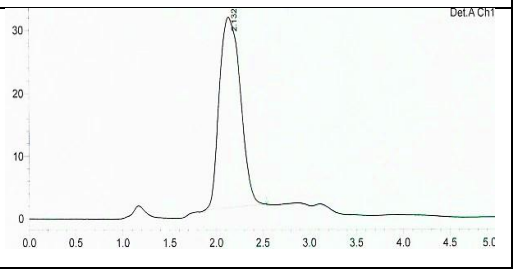
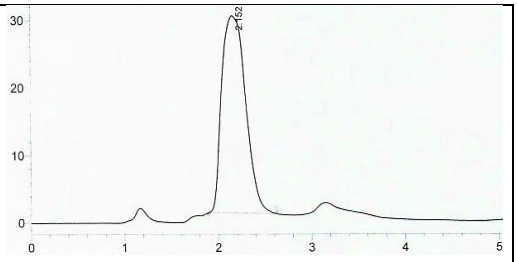
ACN :water	90:10	2.661	446302	13399	2.318	
ACN :water	80:20	2.489	507808	20908	2.312	
ACN :water	70:30	1.682	169035	25843	2.593	
ACN :water	60:40	1.569	322233	25314	0.864	
ACN :water	50:50	1.491	233819	19818	0.760	

Table (3-4): Initial study results for standard aspirin using a mobile phase (methanol: ACN)

Mobile phase	composite	Retention time	Peak area	Height	Tailing factor	
methanol	100	2.162	382883	29460	1.101	
methanol: ACN	90:10	2.137	478844	33224	1.195	
methanol: ACN	80:20	2.122	499960	34189	1.479	
methanol: ACN	70:30	2.122	465974	33123	1.198	
methanol: ACN	60:40	2.132	480539	30366	1.224	

methanol: ACN	50:50	2.152	519626	29213	1.302	

3.4 Analysis of AHPDBA by using HPLC method

3.4.1 Mobile phase composition

The AHPDBA analysis was initially tested with different mobile phase compositions to find the best eluent mixture. These tests examined the changing of the proportions of organic solvents such as water, acetonitrile, and methanol. The organic solvents were evaluated using a gradient elution. Specifically, a mixture of water and acetonitrile was tested at varying ratios, including (10:90, 20:80, 30:70, 40:60, and 50:50 v/v). As well as mobile phase consisting of methanol and acetonitrile mixtures was evaluated at different ratios, including (90:10, 80:20, 70:30, 60:40, and 50:50 v/v). The purpose of employing these different solvent ratios was to systematically increase the relative proportion of the organic solvent component within the mobile phase to investigate the effect of the mobile phase on the retention and separation. Through careful evaluation of the results obtained with each mobile phase composition, it was determined that the ratio (80:20 v/v) acetonitrile: water proved optimal for analysis. This determination was based on an assessment of suitability parameters, this parameter included retention time, tailing factor, and overall peak shape. Fig. (3-6) shows the optimal result.

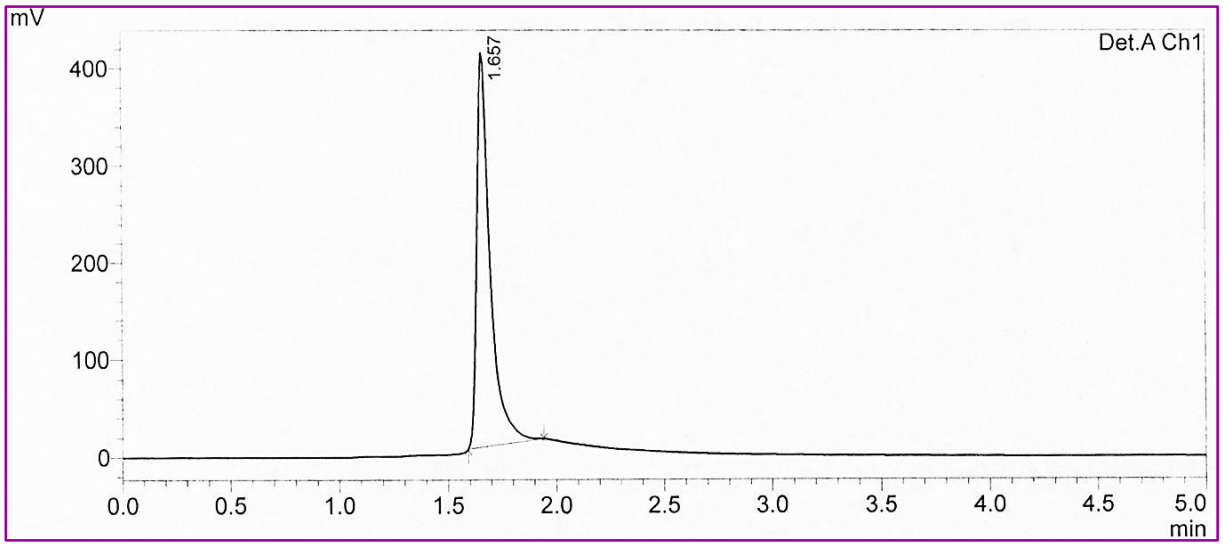
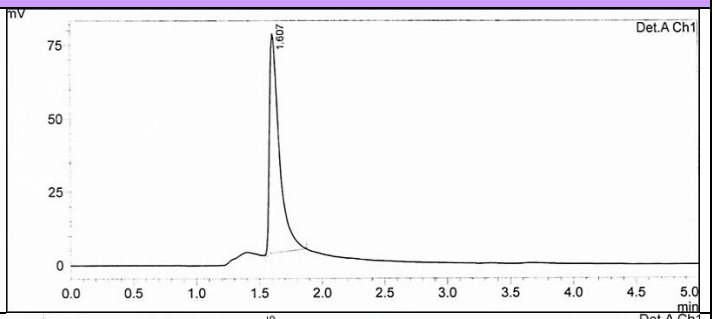
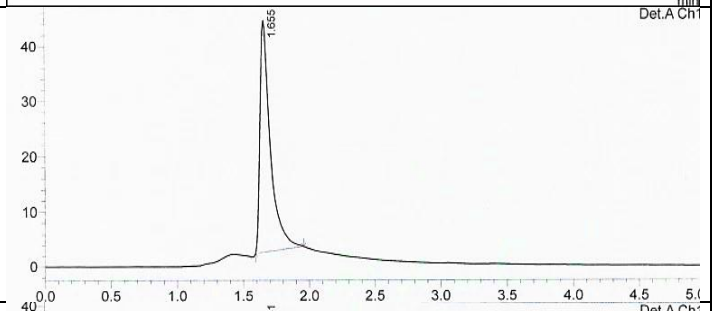
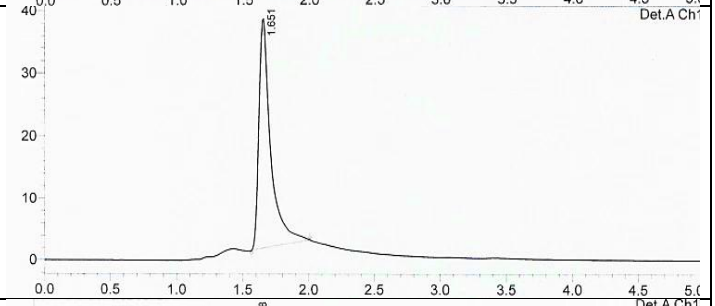
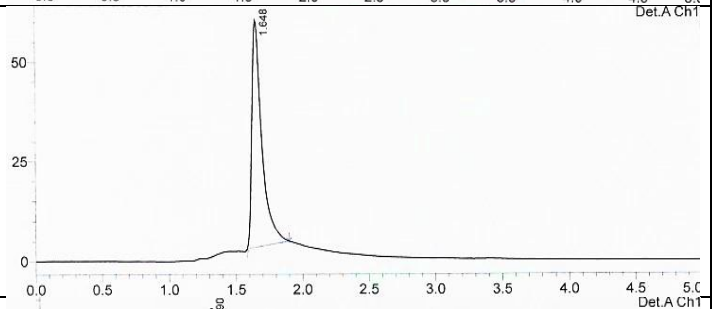
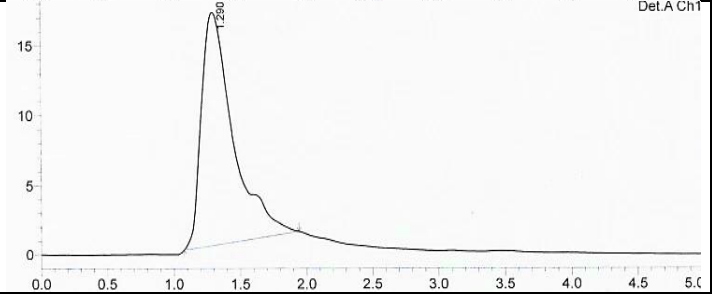


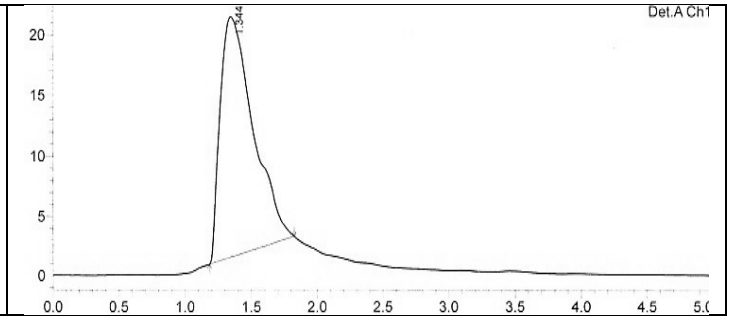
Figure (3-6): Chromatogram of AHPDBA using a mobile phase ACN: Water (80:20 v/v).

3.4.2 Effect of pH

The influence of pH was investigated for Aspirin, at pH levels ranging from 3-8. 1M HCl and 1M NaOH were employed to adjust the pH. The results of this investigation clearly indicated that a pH of 3 is the optimal pH for HPLC analysis of Aspirin because this pH keeps the compound stable and improves separation and detection. Additionally, at pH 3, there is less interference from other substances. Aspirin is also more stable at lower pH, which prevents the hydrolysis of aspirin to salicylic acid and acetic acid at higher pH levels, especially in alkaline conditions. Fig. (3-7) shows that pH 3 is the optimal pH for Aspirin.

Table (3-5): Effect of pH on analysis Aspirin

PH	t_R (min)	Area	Height	Tailing factor	
3	1.607	203618	55297	1.595	
4	1.655	232751	42015	2.218	
5	1.651	233608	36902	2.197	
6	1.648	299857	57199	2.082	
7	1.290	280516	16751	2.022	

8	1.344	346844	19957	2.006	

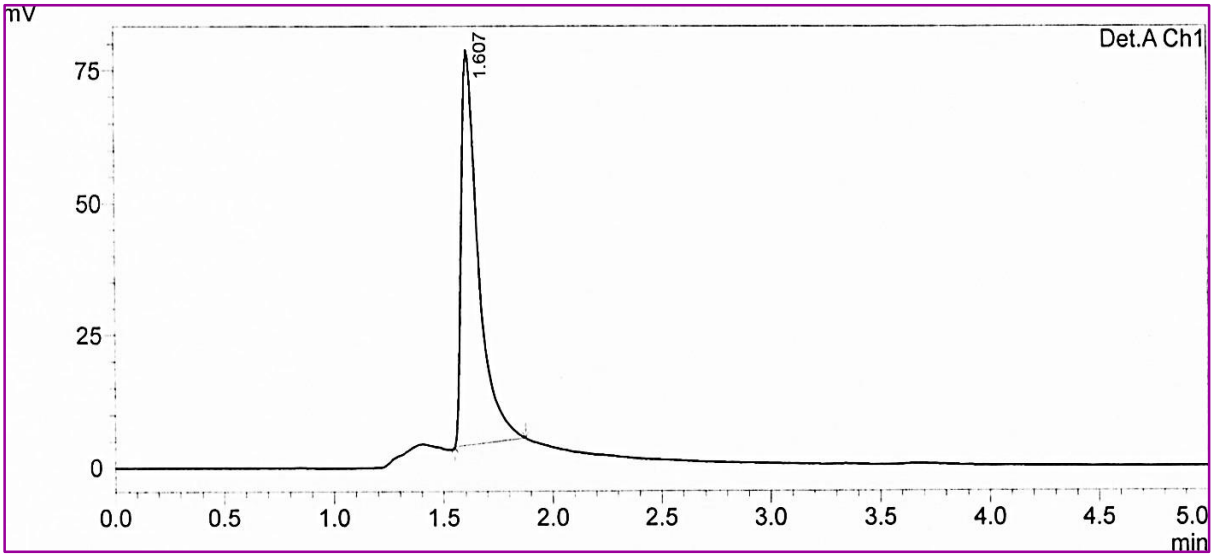


Figure (3-7): Chromatogram for AHPDBA at pH 3 (the optimal result).

The result in Figure (3-7) and table (3-5) showed the pH effect on retention time and tailing factor, and shape peak.

3.4.3 Effect of Buffer solution

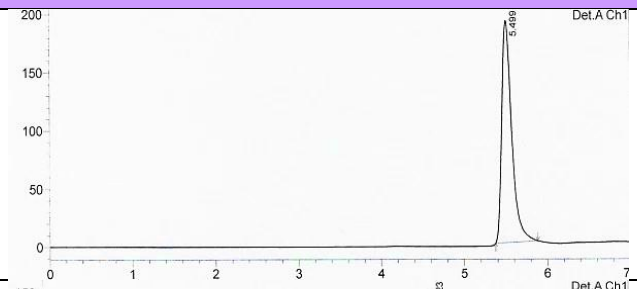
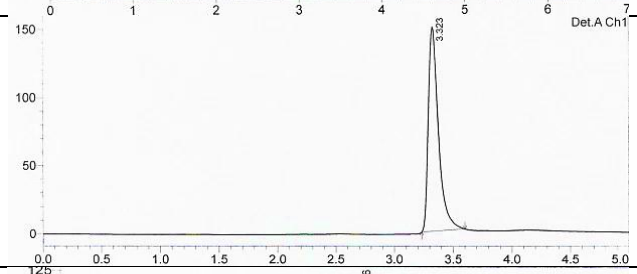
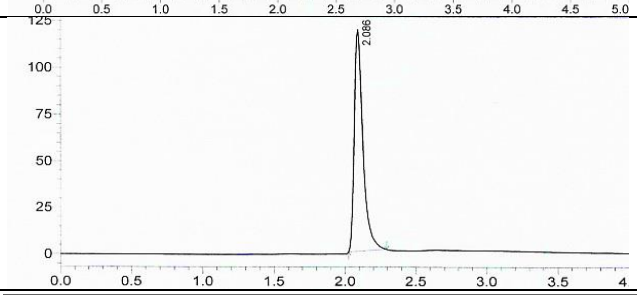
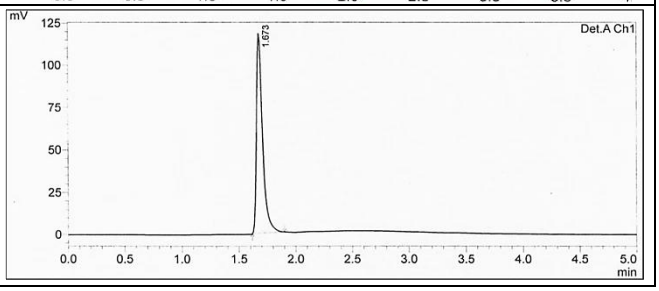
An investigation was conducted to study the effect of buffer solutions on Aspirin analysis. This study specifically focused on examining the impact of buffers at different pH levels. The pH values of the buffer solutions used were (2, 4, 7.2, 8, and 9). After careful observation and analysis of the results obtained during the study, it was determined that the effect of buffer solutions, at the pH levels tested, was negative.

3.4.4 Effect of flow rate

The optimal flow rate was determined by testing solutions at flow rates of (0.3, 0.5, 0.8, and 1 ml/min). Based on figure (3-8), the optimal flow rate for analysis aspirin solutions

is 1 ml/min. This flow rate, commonly used in HPLC for Aspirin analysis, provides fastest analysis time, and it allows enough interaction between analyte and the stationary phase to maximize separation, minimize peak broadening, and provide good column efficiency.

Table (3-6): Effect of flow rate on analysis of Aspirin.

Flow rate	t_R (min)	Area	Height	Tailing factor	
0.3	5.499	1592055	191481	1.599	
0.5	3.323	904373	150591	1.688	
0.8	2.086	507803	118990	1.749	
1	1.673	296387	100244	1.132	

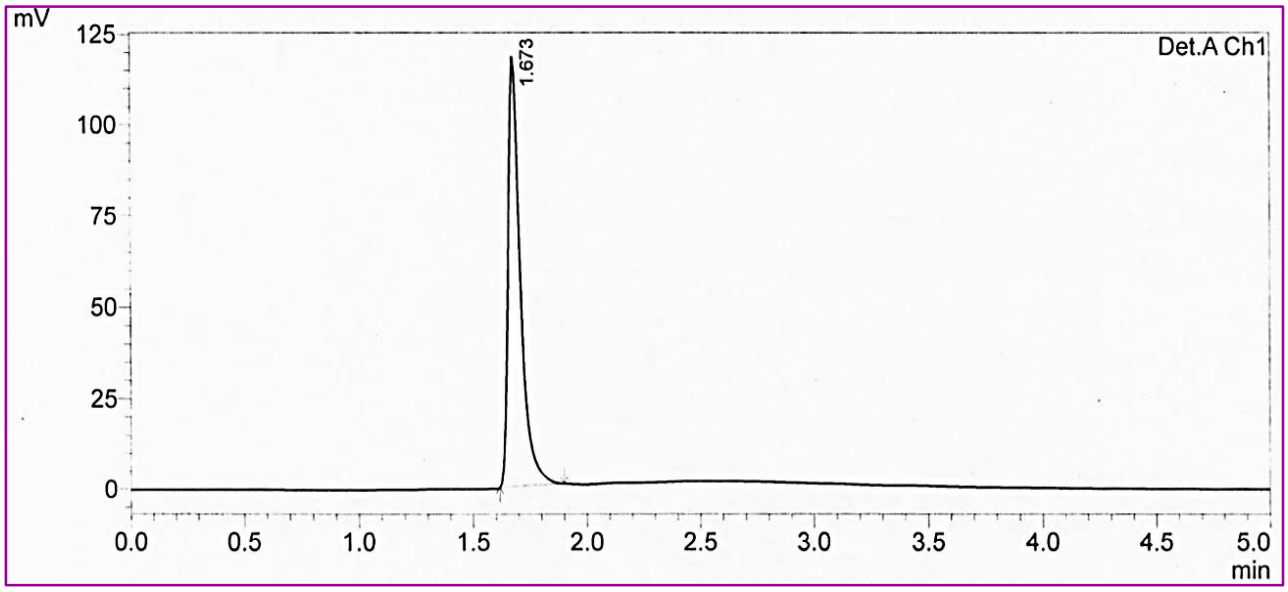


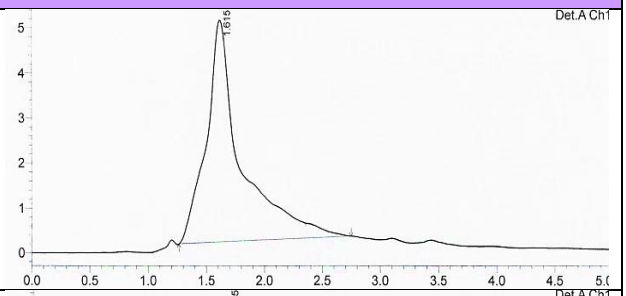
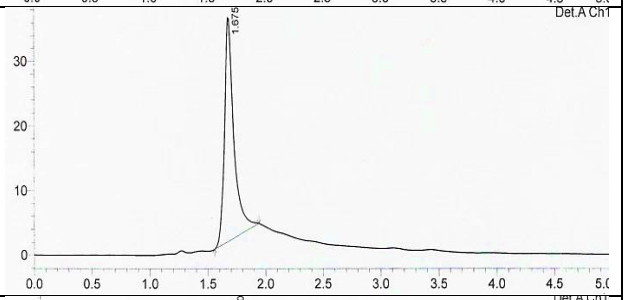
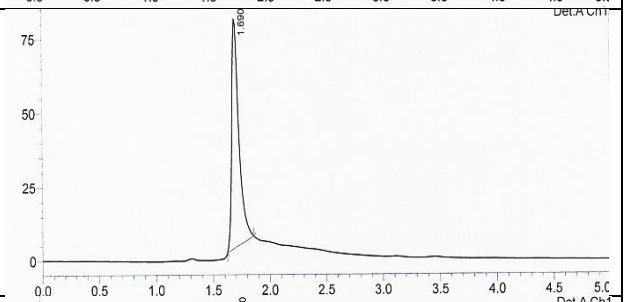
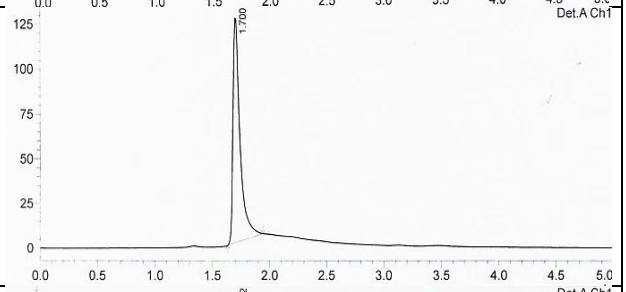
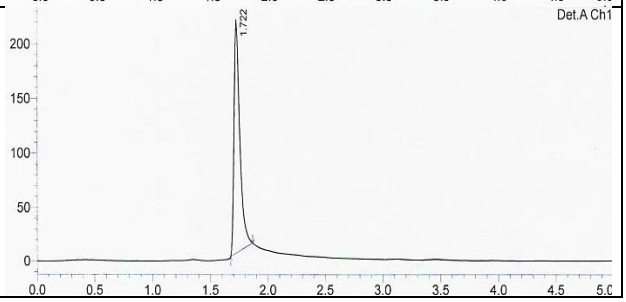
Figure (3-8): Chromatogram for AHPDBA at flowrate 1ml/min (the optimal result).

A flow rate of 1 mL/min was chosen for this investigation because it provided a short analysis time, good peak shape, and manageable pressure, and short retention time.

3.4.5 Effect of volume injection

Increasing the sample volume injection up to 30 µL improved the signal strength and achieved a stable baseline. Injection excessive volume can overload the column, resulting broad peak and increase tailing factor. Therefore 30 µL was chosen as the injection volume in HPLC analysis of aspirin. This volume provides a good balance between sensitivity, reproducibility, and a voiding column overload, while maintaining a good baseline. The optimal result is shown in fig. (3-9), and Table (3-7).

Table (3-7): Effect of volume injection (µL) on Aspirin analysis

Injection volume	t_R (min)	Area	Height	Tailing factor	
10	1.615	104123	4934	1.895	
20	1.675	198678	34728	1.591	
30	1.690	346027	77586	1.533	
40	1.700	545172	125751	2.226	
50	1.722	761283	215452	1.898	

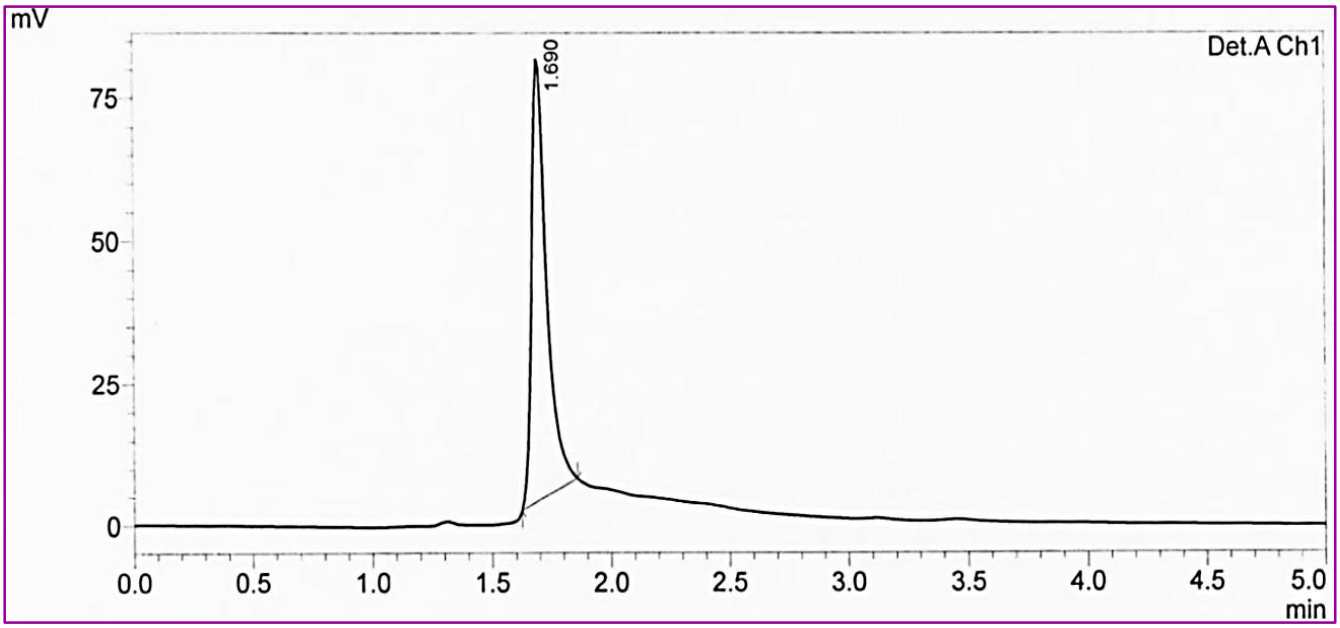
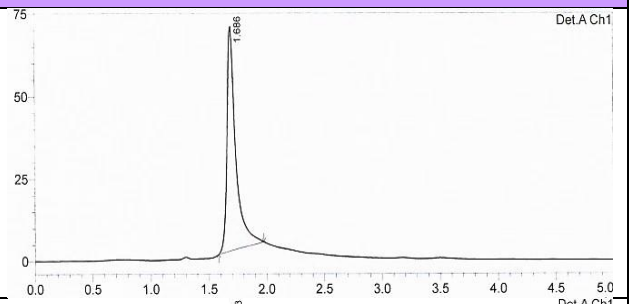
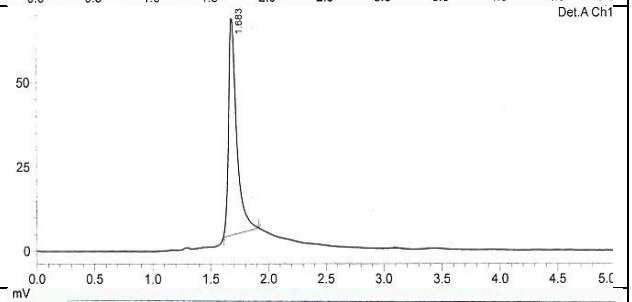
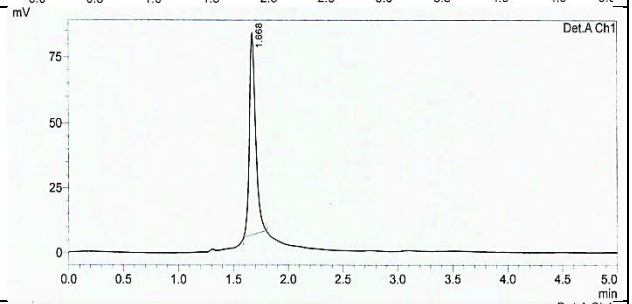
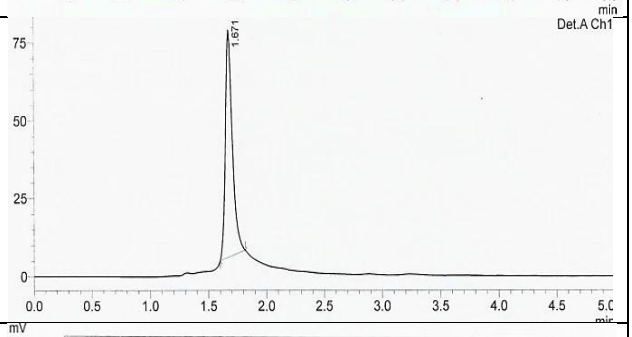
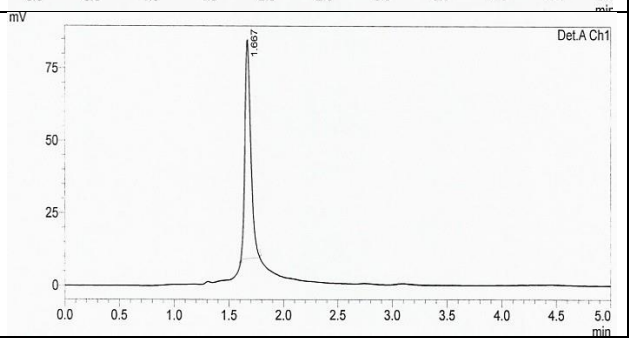


Figure (3- 9): Chromatogram for AHPDBA at volume injection 30 µL (the optimal result).

3.4.6 Effect of Temperature

The Column temperature was tested at (30, 35, 40, 45, and 50 ℃) using a heat oven. In RP-HPLC, increasing the column temperature usually reduces the viscosity of mobile phase, enabling faster flow rates without increasing back pressure. The temperature was raised to 50 ℃, but 40 ℃ was found to be optimal temperature. Because higher temperatures can improve peak shape and shorten retention times by reducing band broadening and improve diffusion within the mobile phase. The optimal temperature is shown in fig.(3-10).

Table (3-8): Effect of temperature (°C) on analysis Aspirin

Temperature (°C)	t_R (min)	Area	Height	Tailing factor	
30- at room temp.	1.686	362481	68100	1.885	
35	1.683	316203	64234	1.759	
40	1.668	310132	77401	1.246	
45	1.671	306639	72867	1.446	
50	1.667	285383	75688	1.411	

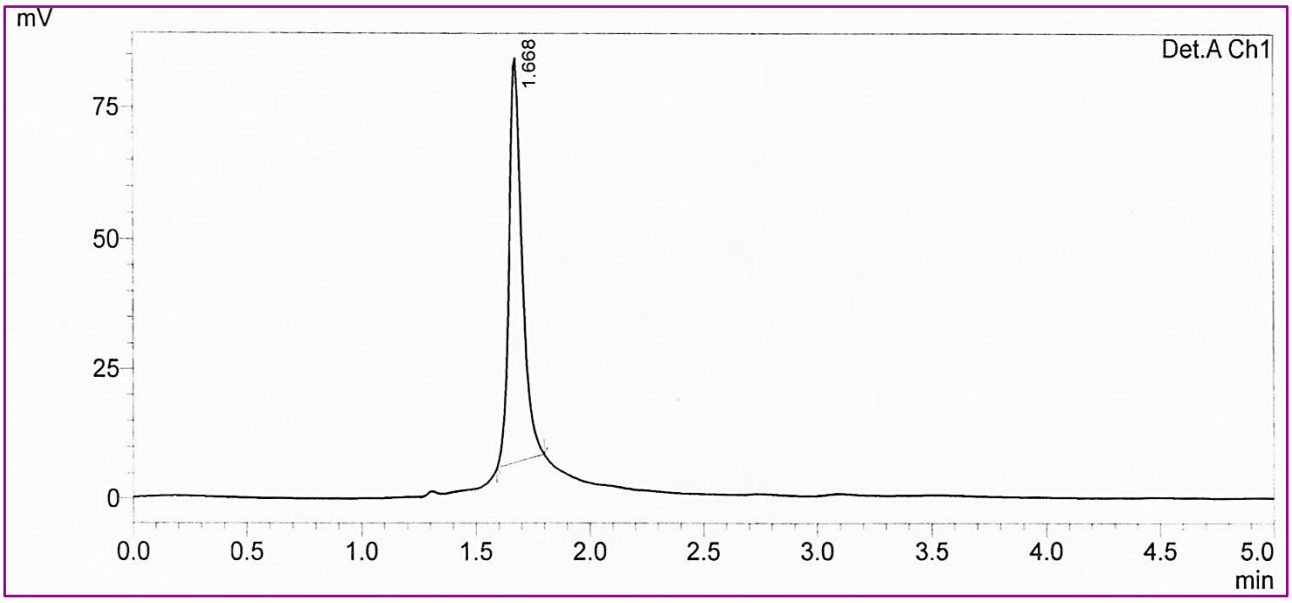


Figure (3-10): Chromatogram for AHPDBA at 40 ℃ (The optimal result).

3.5 Method Validation

The analytical method has been validated. Most parameters tested yielded results within acceptable limits. Table (3-11) summarizes the regression data, accuracy, precision, limit of detection (LOD), and limit of quantification (LOQ).

3.5.1 Calibration Curve and Linearity study

A Calibration Curve was created using various solutions of AHPDBA ranging of (1-100 $\mu g.mL^{-1}$) (Table 3-9). Fig.(3-11) shows a plot of peak area versus concentration, resulting in the regression equation Y= 2108x. This equation represents a strong linear correlation between the peak area and the concentration, supported by high r^2, indicating near-perfect correlation (That shown in table 3-10).

Table (3-9): Calibration curve of Aspirin

Conc. (µg.mL^{-1})	Peak area
5	10618
10	21470
20	43218
30	65106
40	84472
50	105820
60	127212
70	148505
80	168200
90	189614
100	209080

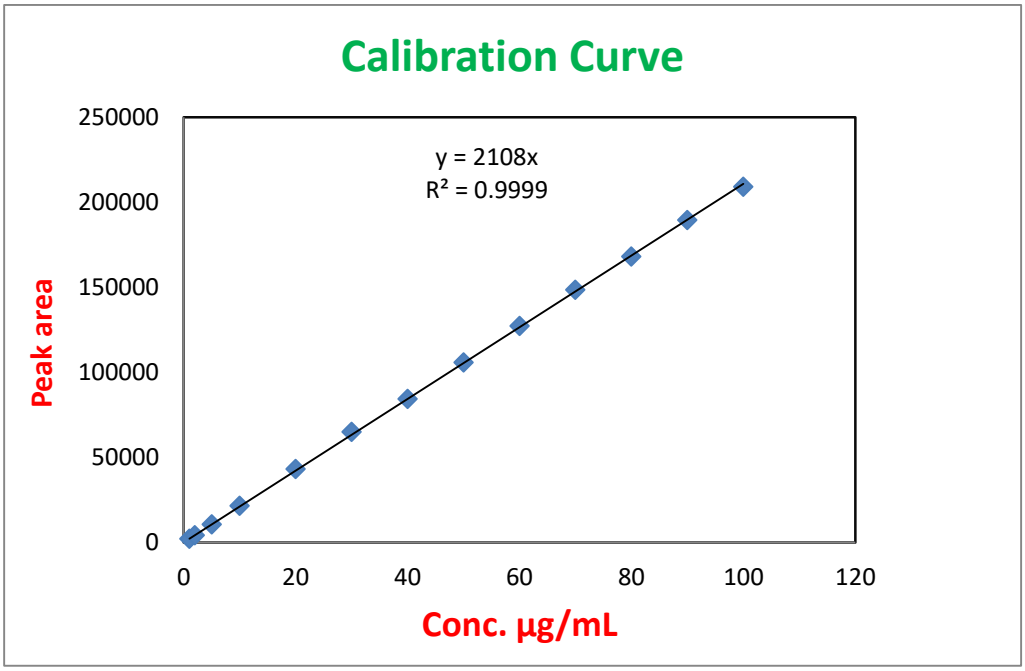


Figure (3-11): Calibration curve of Aspirin.

Table (3-10): Summery of Linearity study of Aspirin

Parameters	Value
Linearity range (µg.mL^{-1})	1-100
Regression equation	Y = 2108x
Intercept	0
Slope	2108
Correlation coefficient (r^2)	0.9999

3.5.2 Precision

The precision of the new method for aspirin detection, which refers to agreement between repeated measurements of the same material under controlled conditions (111), was evaluated. Accuracy and systematic errors were assessed by three samples known concentration of aspirin (10, 50, and 100 µg.mL^{-1}) prepared from a 1000 µg.mL^{-1} aspirin solution along with other additives. Table (3-11) shows high precision, indicated by low relative standard deviation (RSD %) values (≤ 0.291).

3.5.3 Accuracy

This study aimed to ensure the accuracy of analytical methods by comparing test results with known true value (111). The accuracy of method was determined by studying recovery at three different concentrations of aspirin drug (10, 50, and 100 µg.mL^{-1}) by HPLC method. Table (3-11) presents accuracy results, which are displayed at recovery percentage ranging (99.871-100.532%).

3.5.4 Limit of Detection and Limit of Quantification

The Limit of quantification (LOQ) indicates the sensitive of method. It is the lowest concentration of substance that can be reliably measured. It is determined through a series of dilutions (112). In this study, the Limit of Detection (LOD) and Limit of Quantification (LOQ) were calculated using the standard deviation of the response and

the slope of calibration curve. Specifically, the LOD was calculated as 3.3 times the standard deviation divided by the slope (3.3 σ/s), and the LOQ was 3.3 times the LOD. According to Table (3-11), the LOQ was 2.820 $\mu g.mL^{-1}$, and the LOD was 0.930 $\mu g.mL^{-1}$.

3.6 Application

Ten tablet of (Aspirin 100 mg) were weighed and ground into a fine powder. Exactly 100 mg of this powder, containing Acetyl salicylic acid, was weighed and transferred to a 100 ml volumetric flask. 50 ml of a mobile phase consisting of acetonitrile and water (80:20 v/v) was added to the flask, and the mixture was sonicated to dissolve the powder. The solution was then diluted to the 100 mL mark with the same mobile phase, adjusted to pH 3 with 0.1 M HCl. This solution was filtered through a 0.45 µm nylon filter, and an appropriate volume was transferred to a vial. The solution was then run at a flow rate of 1 ml/min for 5 minutes, with an injection volume of 30 µL, and column temperature of 40 °C, with λ_{max} 304 nm.

Table (3-11): The accuracy and precision in the quantification of aspirin within a pharmaceutical product

Asp. Drug($\mu g.mL^{-1}$) Taken	Asp. Drug($\mu g.mL^{-1}$) found	Recovery% 100.123± 0.291	E % n=3
10	9.987	99.871	-0.128
50	50.266	100.532	0.532
100	99.965	99.965	-0.034
Mean		100.123	
S.D		0.29178	
RSD%		0.29142	
LOD		0.930	
LOQ		2.820	

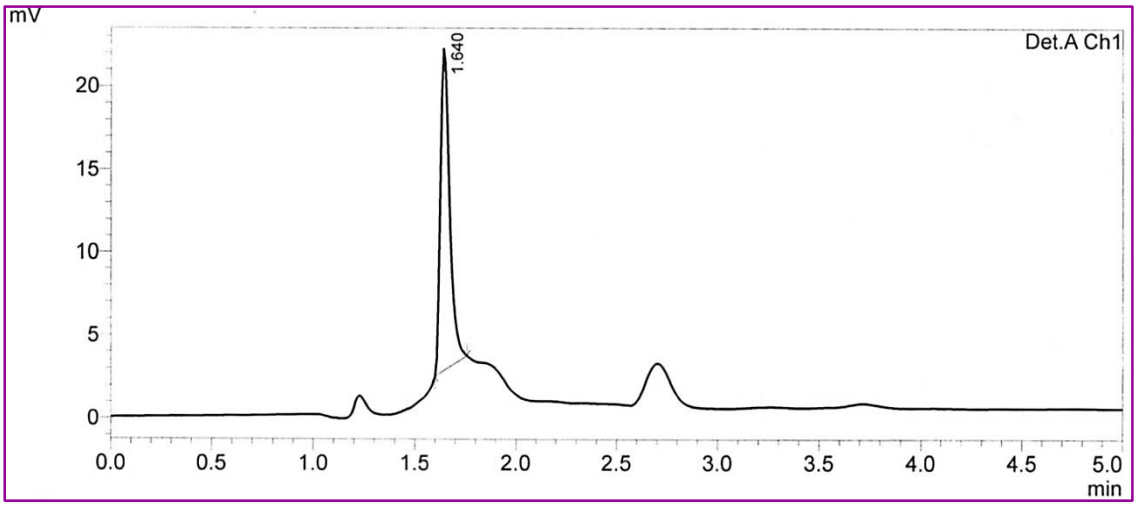


Figure (3-12): Chromatogram for the determination of aspirin drug at 10µg.mL^{-1}.

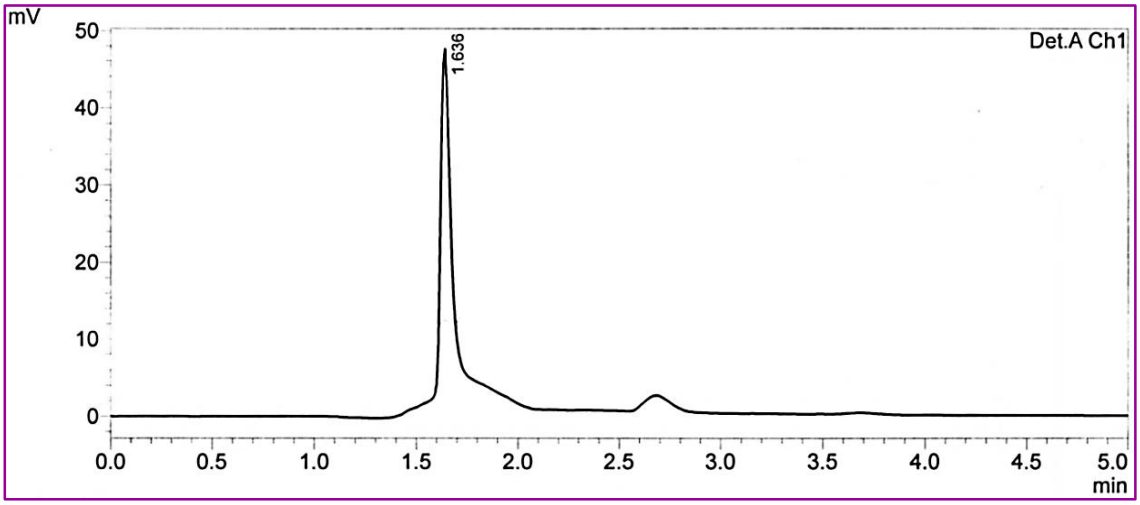


Figure (3-13): Chromatogram for the determination of aspirin drug at 50 µg.mL^{-1}.

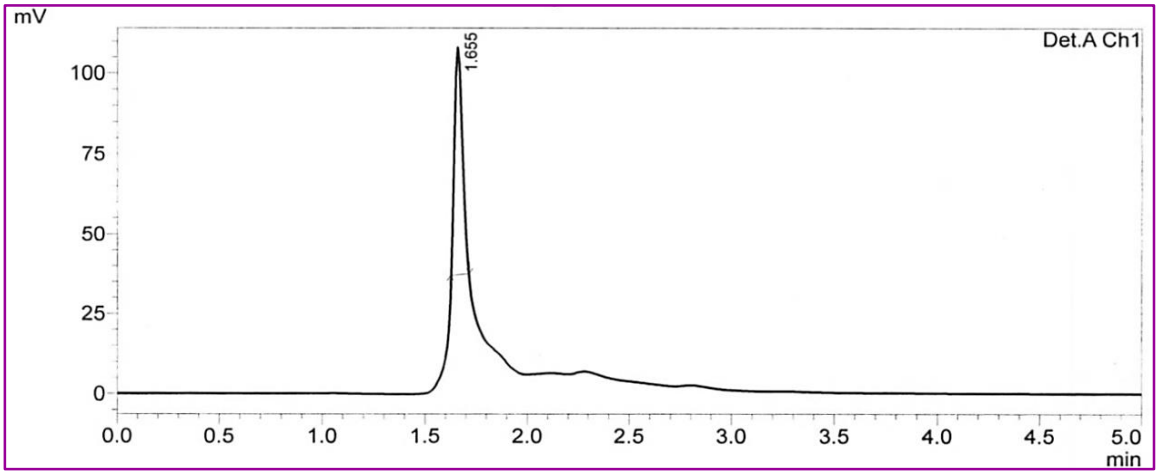


Figure (3-14): Chromatogram for the determination of aspirin drug at 100 µg.mL^{-1}.

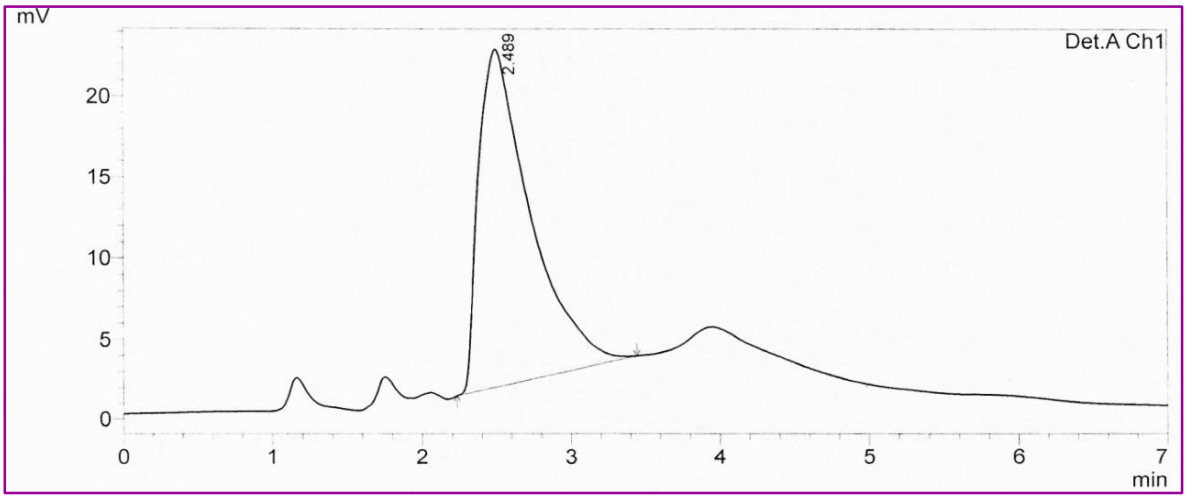


Figure (3-15): Chromatogram standard aspirin before derivatization reaction.

Figure (3-15) shows a standard aspirin sample before any derivatization was done. The sample was a pure, known as the standard of aspirin. The data was collected before any chemical modifications or derivatization procedure was performed on the sample to enhance its detectability or chromatographic properties.

3.7 Result of kinetic studies

The kinetic study of AHPDBA was studied under alkaline and oxidative conditions. Aspirin's structure contains an ester moiety susceptible to hydrolysis. This reaction, involving the cleavage of the ester bond, yields salicylic acid and acetic acid as products. The stability of aspirin is pH-dependent, with acidic or basic conditions accelerating the hydrolytic degradation process (113). The study was done using 1M NaOH and a 3% methanolic hydrogen peroxide. These conditions were selected to provide the degradation happened at rates that allowed for accurate kinetic data collection. An HPLC method was used to measure the degradation, and the result showed a consistent decrease in Aspirin concentration over time. Table (3-12), Table (3-13), Fig. (3-16) and (3-17) illustrate the temperature effects on degradation process. For each degradation process, the logarithm of percentage of remaining drug concentration was plotted against time (in hours) at various temperatures. These plots

yielded straight lines, and regression equations were determined for each line. Because sodium hydroxide (1M) and 3% methanolic hydrogen peroxide were in excess, the alkaline at (50, 60, and 70 °C) and oxidative at (60, 70, and 80 °C) degradation of AHPDBA conformed to first-order kinetics. This means the degradation rate depended on both the AHPDBA concentration and the temperature. Apparent first order degradation rate constant (K_{obs}) and half-life were calculated from the regression line slopes at each temperature in Table (3-14), (3-15) using the following equation:

$$\text{Log } (C_t/C_0) + 2 = -K_{obs}\, t \qquad (3\text{-}1),$$

$$t_{1/2} = 0.693/K_{obs} \qquad (3\text{-}2).$$

C_t =concentration remaining at time t, C_0 =Initial concentration, K_{obs} =Apparent rate constant, $t_{1/2}$ = half time (88). By graphing the logarithm of K_{obs} (log K_{obs}) against the inverse the absolute temperature (1/T), [refer to Fig. (3-18), (3-19)]. These plots show a linear relationship within the tested temperature ranges. The Arrhenius equation explains the reaction rates change with temperature. This relationship is based on the idea of activation energy (114). The activation energies were calculated using the following equation:

$$\text{Log } K_{obs} = \log A - E_a /2.303RT \qquad (3\text{-}3)$$

Where: R represents the universal gas constant, and T is represents the absolute temperature in Kelvin. A is the frequency factor, and E_a is the activation energy (83). The activation energy (E_a) was obtained (Table 3-16) for the alkaline degradation AHPDBA. For the oxidative degradation of AHPDBA, the activation energy (E_a) was obtained (Table 3-17).

Table (3-12): Kinetic Study of AHPDBA Degradation: Log (C_t/C_0 + 2) at Various Temperatures with 1M NaOH

time (h)	(C_t/C_0) at 50 °C	Log (C_t/C_0)+2 at 50 °C	(C_t/C_0) at 60 °C	Log (C_t/C_0)+2 at 60 °C	(C_t/C_0) at 70 °C	Log (C_t/C_0)+2 at 70 °C
0	0.724964	1.860316693	0.724964	1.860317	0.724964	1.860317
0.25	0.621678	1.793565314	0.522469	1.718061	0.406291	1.608837
0.5	0.540582	1.732861382	0.404722	1.607157	0.252743	1.402679
0.75	0.473947	1.675729514	0.315236	1.498635	0.164328	1.215711
1	0.41596	1.619052058	0.259127	1.413512	0.102986	1.012777
1.25	0.350137	1.544237734	0.207306	1.316613	0.070913	0.850728

Table (3-13): Kinetic Study of AHPDBA Degradation: Log (C_t/C_0 + 2) at Various Temperatures with 3% methanolic hydrogen peroxide.

time (h)	(C_t/C_0) at 60 °C	Log (C_t/C_0)+2 at 60 °C	(C_t/C_0) at 70 °C	Log (C_t/C_0)+2 at 70 °C	(C_t/C_0) at 80 °C	Log (C_t/C_0)+2 at 80 °C
0	0.739083	1.868693	0.739083	1.868693	0.739083	1.868693
0.25	0.622857	1.794388	0.522824	1.718355	0.436294	1.63978
0.5	0.513051	1.71016	0.40144	1.603621	0.282658	1.451261
0.75	0.428655	1.632108	0.311405	1.493325	0.183451	1.263521
1	0.36864	1.566602	0.239801	1.379851	0.125269	1.097843
1.25	0.310949	1.492689	0.191425	1.281999	0.085964	0.934315

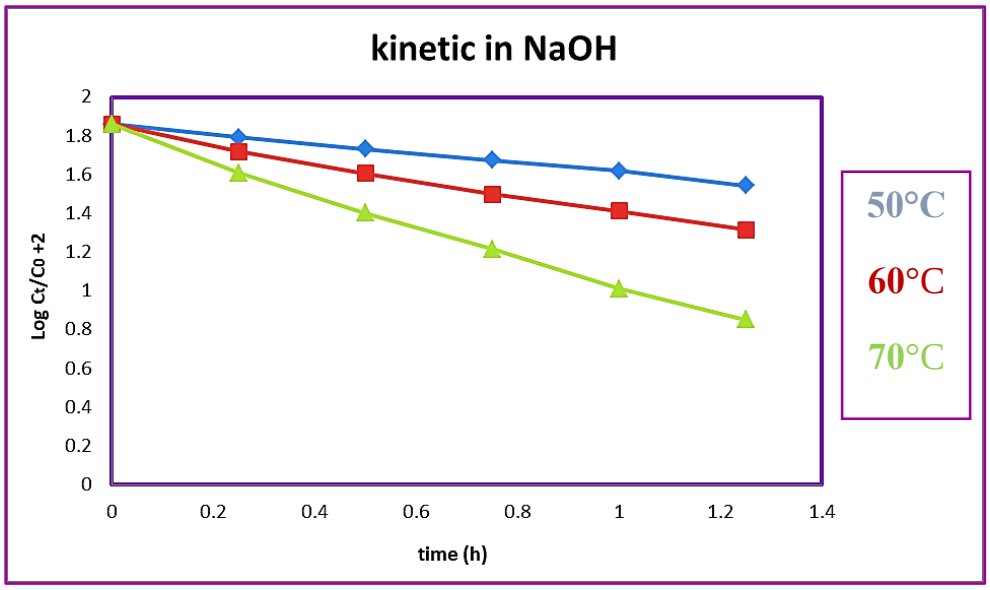


Figure (3-16): Kinetic plots depicting the degradation of AHPDBA with 1M NaOH.

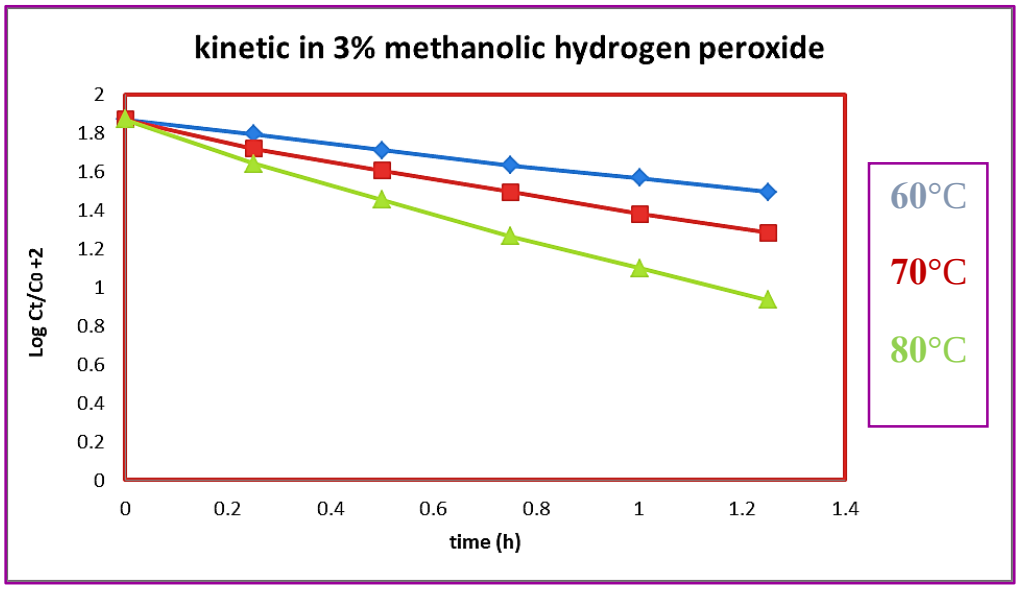


Figure (3-17): Kinetic plots depicting the degradation of AHPDBA with 3% methanolic hydrogen peroxide.

Table (3-14): Degradation rate constants K_{obs} and half-lives ($t_{1/2}$) for AHPDBA in 3% methanolic peroxide for proposed HPLC method

Temperature (°C)	K_{obs} (h^{-1})	Half-life ($t_{1/2}$)
60	0.3019	2.295462
70	0.4639	1.493856
80	0.7412	0.93497

Table (3-15): Degradation rate constants K_{obs} and half-lives ($t_{1/2}$) for AHPDBA in 1M NaOH for proposed HPLC method

Temperature (°C)	Kobs (h^{-1})	Half-life ($t_{1/2}$)
50	0.247	2.805668
60	0.4275	1.621053
70	0.8026	0.863444

Table (3-16): The activation energy (E_a) for AHPDBA in alkaline degradation

T (Kelvin)	Kobs (h^{-1})	Ea (kcal mol^{-1})
323.15	-0.607	17.369
333.15	-0.369	17.544
343.15	-0.095	17.641

Table (3-17): The activation energy for (E_a) for AHPDBA at oxidative degradation

T (Kelvin)	Kobs (h^{-1})	Ea (kcal mol^{-1})
333.15	-0.520	18.241
343.15	-0.333	17.956
٣٥٣.١٥	-0.130	18.705

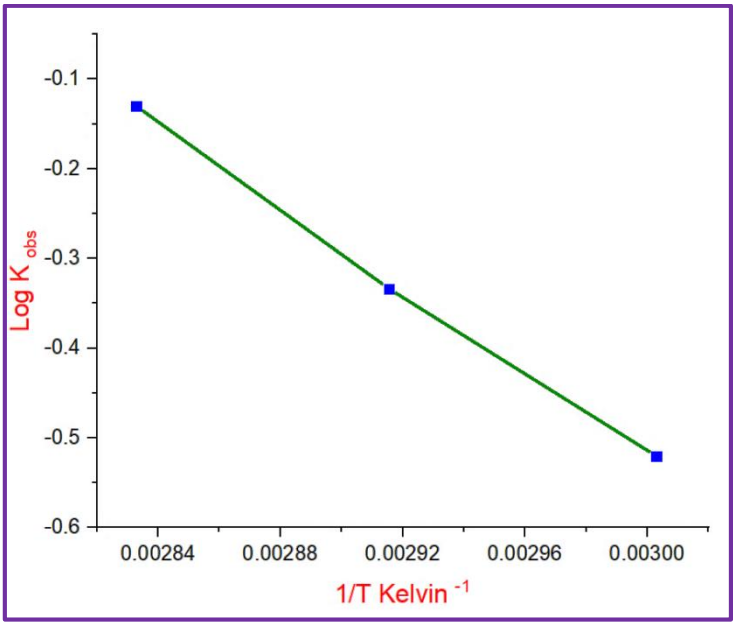


Figure (3-18): The relationship between the log K_{obs} and 1/T of oxidative degradation process.

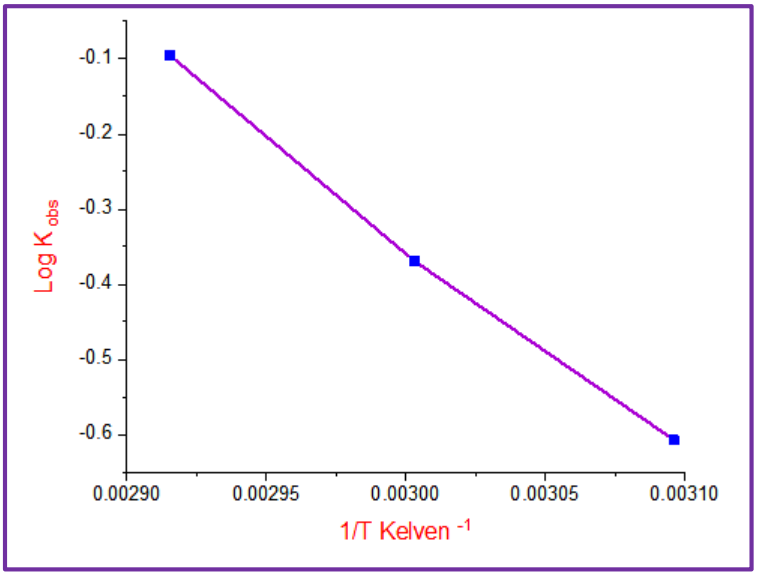


Figure (3-19): The relationship between the log K_{obs} and 1/T of alkaline degradation process.

As result from table [(3-16), (3-17)] Higher temperatures speed up the breakdown process by increasing the kinetic energy available for the reaction. The pH is also a critical factor; degradation typically proceeds more rapidly under acidic or alkaline conditions compared to neutral pH (79). High activation energy results in slower reaction rate because more energy is required for the reaction to reach the transition state. With a higher energy requirement, fewer molecules have enough energy to overcome the barrier at a specific temperature. This leads to fewer successful collisions and a slower rate of product creation. On the other hand, reactions with lower activation energies happen faster because more molecules have the energy needed to reach the transition state (115).

3.8 Conclusions

1. A reversed phase HPLC method using UV detection was developed and validated for the quantification of Acetylsalicylic acid. This method offers improved convenience and effectiveness for quality control analysis of the drug substance in pharmaceutical formulations.

2. This methodology is simple, economically efficient, and exhibits excellent accuracy. It provides good precision and has a low Limit of Detection and Limit of Quantification. With the rapid analysis time of below 5 minutes, and the ability to quantify at low concentrations of the drug, it is useful for many analytical applications, including the quality control and analysis of drug substances and drug formulations. The presented methodologies enabled direct analysis of the drug.

3. This approach reduced errors, reduced analysis time, and lowered overall cost. A simplified mobile phase employing isocratic elution and a low pH was utilized, the good recovery was observed in tablet dosage forms. Kinetic studies reveal that the drug in alkaline and oxidative degradation show that is degrades easily under these conditions. Therefore, using appropriate buffer agents, and selecting suitable solvents protect against oxidation. These precautions are essential during the manufacturing, formulation, packaging, and storage processes.

4. The relative standard deviation (RSD %) was below 0.291%, indicating the high precision of the method. Therefore, this method is suitable for the quantitative determination of the drug in tablet formulations. Consequently, this method is applicable for routine sample analysis in quality control laboratories.

3.9 Future work

1. Applying the proposed method by using another Azo compound.

2. Applying another technique rather than HPLC to determine the drug.

3. Investigating the interferences that might be conflict with Aspirin.

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الخلاصة

تهدف هذه الدراسة إلى تقدير كمية الأسبرين (حامض أسيتيل الساليسيليك) باستخدام تقنية كروماتوغرافيا السائل عالي الأداء ذات الطور المعكوس (RP-HPLC) وكان العنصر الأساسي في هذه الطريقة هو تخليق وتوظيف مركب آزو جديد، أُطلق عليه اسم-2-(E) أسيتوكسي-٥-((٤-هيدروكسي فينيل) ديازينيل) حامض البنزويك (AHPDBA) . تم تصنيع مركب AHPDBA عن طريق تفاعل اقتران آزو بين حامض أسيتيل الساليسيليك وملح ديازونيوم مشتق من ٤-أمينو الفينول. بعد ذلك، تم تشخيص مركب AHPDBA الناتج باستخدام طرق تحليلية مختلفة، وهي: مطيافية الأشعة فوق البنفسجية والمرئية، ومطيافية تحول فوريير للأشعة تحت الحمراء(FT-IR) ، وكروماتوغرافيا الغاز- قياس الطيف الكتلي(GC-MS) .

لتحسين كفاءة طريقةRP-HPLC ، تمت دراسة عوامل مختلفة بعناية، بما في ذلك: تركيبة الطور المتحرك، ودرجة الحموضة، والمحلول المنظم، ومعدل التدفق، وحجم الحقن، ودرجة الحرارة. تم إنشاء منحنى معايرة الأسبرين باستخدام تركيز تتراوح من ١ إلى ١٠٠ ميكروغرام/مل. وباستخدام تقنية-RP HPLC، تم حساب الحركية لعملية التحلل القاعدي والتأكسد لمركب AHPDBA ، وثوابت معدل التفاعل، وأنصاف الأعمار، وطاقات التنشيط. وللتحقق من صحة الطريقة المقترحة، تم تحديد: معامل الخطية، والدقة، والتوافق ، وحد الكشف (LOD) ، وحد الكمية (LOQ) أظهرت النتائج خطية جيدة ضمن نطاقات التركيز المستخدمة (١-١٠٠ ميكروغرام/مل)، مع معامل ارتباط (r^2) قدره ٠,٩٩٩٩. كما تم إثبات دقة وتوافق ممتازتين، حيث كانت قيم الانحراف المعياري النسبي (RSD) أقل من ٠,٢٩١٪، ونسبة الاسترداد النسبي تتراوح بين ٩٩,٨٧١٪ و ١٠٠,٥٣٢٪. وكان حد الكشف 0.930 (LOD) ميكروغرام/مل، في حين كان حد الكشف الكمي 2.820 (LOQ) ميكروغرام/مل. بشكل عام، أظهرت هذه الدراسة نجاح تطبيق الطريقة المقترحة لتقدير كمية الأسبرين في المستحضرات الصيدلانية.

جمهورية العراق

وزارة التعليم العالي والبحث العلمي

جامعة كربلاء - كلية العلوم - قسم الكيمياء

طريقة كروماتوغرافيا السائل عالي الأداء لتقديرالاسبرين بعد تفاعل الاشتقاق ودراسة الحركية

رسالة مقدمة الى

مجلس كلية العلوم – جامعة كربلاء

وهي جزء من متطلبات نيل درجة الماجستير في علوم الكيمياء

تقدمت بها

رسل لطيف نعمه

بكلوريوس علوم الكيمياء (٢٠١٦) – جامعة كربلاء

باشراف

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