An-Najah National University

Faculty of Graduate Studies

CHEMICAL DERIVATIZATION OF ZIDOVUDINE BASED ON CLICK REACTION FOR NOVEL ANALYTICAL METHOD DEVELOPMENT

By

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This Thesis Dissertation was Defended Successfully on 19/ 3/ 2023 and approved by

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Dr. Asma Hussien Internal Examiner	Signature

Dedication

I dedicate this thesis to God Almighty, my source of inspiration, wisdom, and knowledge. This thesis is also dedicated to my husband, Bashar, and daughter, Zaha, who has been my pillars of support and encouragement during the hurdles and obstacles through graduate school and life. My parents, Dawood and Bahia, deserve much of the credit for my success, and I thank them for being the kind of people who encouraged me to follow my dreams and work hard.

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Declaration

I, the undersigned, declare that I submitted the thesis entitled:

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I declare that the work provided in this thesis, unless otherwise referenced, is the researcher's own work, and has not been submitted elsewhere for any other degree or qualification.

Student's Name: _____

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ABSTRACT

Zidovudine is a drug used for the treatment of patients that are infected with the human immunodeficiency virus. It inhibits HIV-reverse transcriptase. Zidovudine has significant dose-limiting toxicities drug-specific, resulting in a small therapeutic window between the minimum and the maximum effective and tolerated doses, respectively. Serious adverse effects among them include hepatic abnormalities, myopathy, and bone marrow toxicity. Hence, a demand exists for analytical technologies that enable efficient and accurate measurements of Zidovudine. The adapted analytical techniques must have sufficient sensitivity, selectivity, or both in order to have more manageable and accurate measurements of various chemical procedures. Converting functional groups within a molecule is one of the adaptive techniques used in chemical derivatization. In this study, the development of a new analytical method for the chemical derivatization of Zidovudine was proposed. To that end, a click reaction with azide-conjugated alkyne was employed, resulting in a five-membered heterocycle (1,2,3-triazole) and an extended conjugation. The aim is to develop a sensitive and selective analytical method. The proposed analytical method has been developed using HPLC with UV/Vis detector and validated according to the International Conference of Harmonization and the Food and Drug Administration guidelines and with the use of the parameters such as accuracy, linearity, range, precision, limit of detection, and limit of quantitation. The developed method adapted λ_{max}=260 nm for the measurement of the derivatized Zidovudine. The method used HPLC using mobile phase Water-ACN 70:30 v/v. The eluted peak of the derivatized Zidovudine was separated from other used derivatization reagents. The analytical method was then validated, and validation parameters were found to be within the

accepted limits. The developed method was found to be linear (R^2 = 0.994), precise (RSD = 0.59), and accurate (% recovery = 101.17). Moreover, the developed method was sensitive to LOD ($4*10^{-8}$ mg/ml) and LOQ ($4*10^{-7}$ mg/ml). Therefore, the developed method is simple and feasible with high sensitivity and selectivity. Zidovudine may be analyzed in a variety of dosage forms and raw materials, including active pharmaceutical components. This line of research may be pursued further in the future, and the technique that has been established may be adopted in the testing of Zidovudine in biological systems.

Keywords: Zidovudine; Click Reaction; HPLC; Derivatization.

Chapter One

Introduction

Developing an antiviral and effective therapeutical treatment for persons infected with the human immunodeficiency virus (HIV) is a challenge. Challenges include chronicity of viral replication, virus-host cell dependence, high mutation-rate of the HIV RT, and virus integration into host cell DNA. These challenges make the development of antiretroviral therapies that are effective and specific a complex problem [1-4]. Currently exist, several RT inhibitors that were approved for the treatment of the HIV disease include 2',3'- dideoxynucleosides such as (1) stavudine (ZeritTM); (2) lamivudine (EpivarTM); (3) Zidovudine (RetrovirTM); (4) didanosine (VidexTM); and (5) Zalcitabine (HividTM) [5-7]. AZT is the first RT inhibitor to be approved for clinical use and is considered one of the most efficient anti-HIV drugs on the market. In HIV patients at advanced stages, Zidovudine (AZT) treatment was found to slow down the disease progression, thus increasing their chances of survival. Furthermore, neuropsychological functioning in adults with HIV-1 infection was shown to improve with treatment by AZT, while also decreasing the risk of HIV-1 transmission to newborns from their mothers' thanks to its pharmacokinetics, pharmacodynamics, and therapeutic efficacy [8]. However, problems associated with AZT chemotherapy include bone marrow toxicity and suppression, low therapeutic index owing to an inhibition of cellular polymerases, low localization in the brain, and a short half-life in the blood, necessitating AZT administration frequently in order to sustain a therapeutic drug concentration [9]. While AZT is effective in preventing HIV-1 replication, it has been established that its positive effects fade with time. In addition, it has no impact on the generation of the virus by chronically infected cells or on the transfer of infection to uninfected cells through syncytium formation. Because to the considerable dose-limiting toxicities generated by AZT, the therapeutic window between the lowest effective dose and the maximum dose that may be tolerated is constricted. Hence, a demand for analytical technologies that enable efficient and accurate validation and would enhance the AZT sensitivity, selectivity, or both by transforming into more manageable forms is a desirable consequence of various chemical procedures. Analytical chemistry is a dynamic and ever-changing field. Analytical instrument users are utilizing their instrument applications to measure and investigate many aspects of

what is referred to as the "Measurement of Chemical Systems." Pharmaceutical industries develop and monitor drugs in various dosage forms, the average time of drug manufacturing, the stability of drugs across various formulations, and the measurement of a drug's impurity level. The rise of pharmaceutical industries increased the demand for analytical technologies that enable efficient and accurate validation [10]. Enhancing a compound's sensitivity, selectivity, or both by transformation into more manageable forms is a desirable consequence of various chemical procedures. It is preferable to convert functional groups within a molecule to one adaptive to the technique used [11]. Derivatization is the process used to do this.

According to the research conducted, it is clear that the primary focus of the suggested methods that have been developed over the past two decades has been the development of analytical HPLC procedures that are rapid, accurate, precise, selective, and sensitive and that employ UV detection in order to perform direct assessments of ZDV [12-18]. To ensure the determination of the lower limit of quantification (LLOQ), specificity, linearity, recovery, accuracy, and precision, as well as compliance with the current recommendations for the validation of bioanalytical techniques that have been established by the Food and Drug Administration (FDA), all of these things were measured [19]. The reviewed methods, each approaching the problem differently, have shown to abide by the guidelines specifications and report their methods to be linear over a specified range [12-18]. However, most methods require elaborate setups, costly instrumentation, additional preparations, heating and multiple sequential reactions, and additional materials such as fluorescence. While proving their applicability in terms of performance, they are restrictive, prone to errors, and not scalable.

In this thesis, a novel analytical method was developed for the chemical derivatization of AZT. To that end, we employed a click reaction with Azide conjugated alkyne, resulting in a five-membered heterocycle (1,2,3-triazole) and an extended conjugation. As we show in the results, the developed analytical method is sensitive and selective. In contrast to the literature, our proposed novel development is more flexible, cost-effective, and scalable, all the while producing comparable results in terms of accuracy, precision, selectivity, sensitivity, and abiding by the guidelines [19].

1.1 Derivatization (chemical structure modification)

Derivatization improves the target analytes' detectability as well as their chromatographic performance, it is utilized in a significant amount of liquid chromatography-based bioanalysis procedures. Derivatization refers to the analytical procedures that are used in the process of bringing about the intended reaction between the reagent and the target molecules. This usually involves the functional groups hydroxyl, thiol, amino, carbonyl, and carboxyl [10]. In order to improve sensitivity or selectivity, this tactic is used in tandem with a high-wavelength detection technique, such as fluorescence or absorption in visible light. Examples of such technologies include when it comes to the analysis of the trace of complex biological components, this proves to be an exceptionally beneficial tool. Derivatization may also be the end consequence of organic or electrochemical processes, including oxidation and reduction, as well as the displacement and addition reactions. The reactivity of the substrate is the only factor that plays a role in determining whether a displacement occurs as a result of an electrophilic or nucleophilic attack. Nevertheless, given their lack of specificity, the processes of reduction and oxidation are not nearly as well-suited as other options, an essential aspect to be aware off. Methods such as gas chromatography, high-performance liquid chromatography (HPLC), and UV-VIS spectroscopy are examples of those that may benefit from derivatization [20, 21].

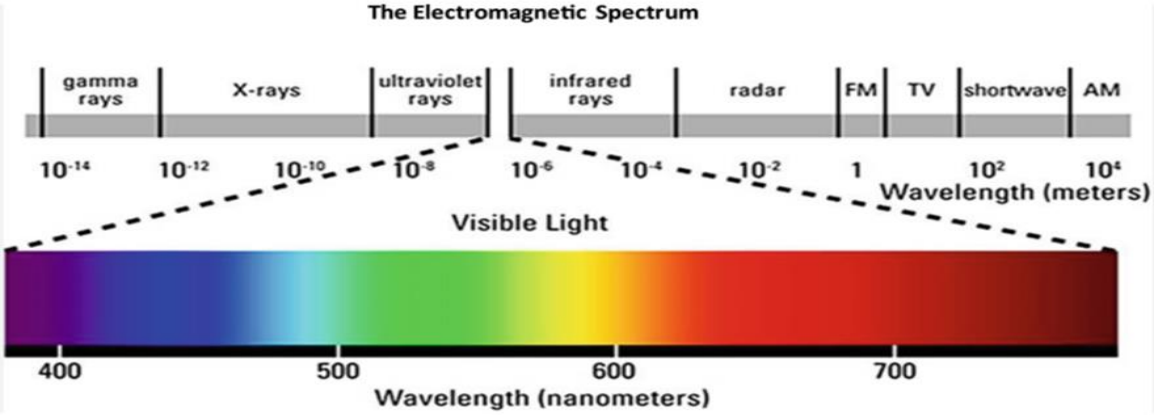
1.2 Ultraviolet-visible spectroscopy (UV–Vis)

Spectroscopy is performed by either absorbing or reflecting light in the near-infrared, ultraviolet, and visible spectrums [21, 22]. Spectroscopy of radiation that falls between the visible and ultraviolet (UV-Vis) spectrum, as shown in scheme 1.1. Because of the ways in which chemical compounds absorb or reflect light in the visual spectrum, these chemicals may be "seen." Electronic transitions are triggered in this portion of the electromagnetic spectrum by both atoms and molecules. For instance, fluorescence spectroscopy measures the transition from the excited state to the ground state, while absorption spectroscopy measures the transfer in the other direction from the ground state to the excited state.

The process of absorbing visible and ultraviolet (UV) light is caused by the movement of electrons inside the molecule to a higher energy state. This is the mechanism that is responsible for the absorption of light. This procedure is shown in the Scheme (1.2). It is possible for transitions to take place between the most fundamental vibrational state of the ground electronic state and any one of the many vibrational levels of the excited states. The most stable configuration for the molecule is one in which it is in its electrical ground state. The width of the ultraviolet spectrum is determined by the energy that is transferred from a single ground state to one of the multiple possible excited states. The pace at which energy may be transferred from the ground state to one of many excited states determines the extent to which ultraviolet spectra can cover a range of wavelengths. A representation of an ultraviolet spectrum may be seen in the Scheme (1.2), and it has multiple bands that correspond to discrete transitions between V_o and V_n. Even though there is significant overlap between the bands, it is still possible to make out the intricate vibrational structure. The breadth of the vibrational bands is affected by rotational transitions that take place at energy levels that are intermediate between each vibrational transition. It is essential to keep in mind that the vibrational behavior of the vast majority of molecules is complicated and that the overlap between the various energies of the transitions between vibrational states is much too wide for the fine vibrational structure to be detected [23]. For example, the energy required for rotational transitions is one hundred times that of vibrational transitions but only one hundredth of the energy required for electronic changes.

Scheme 1. 1

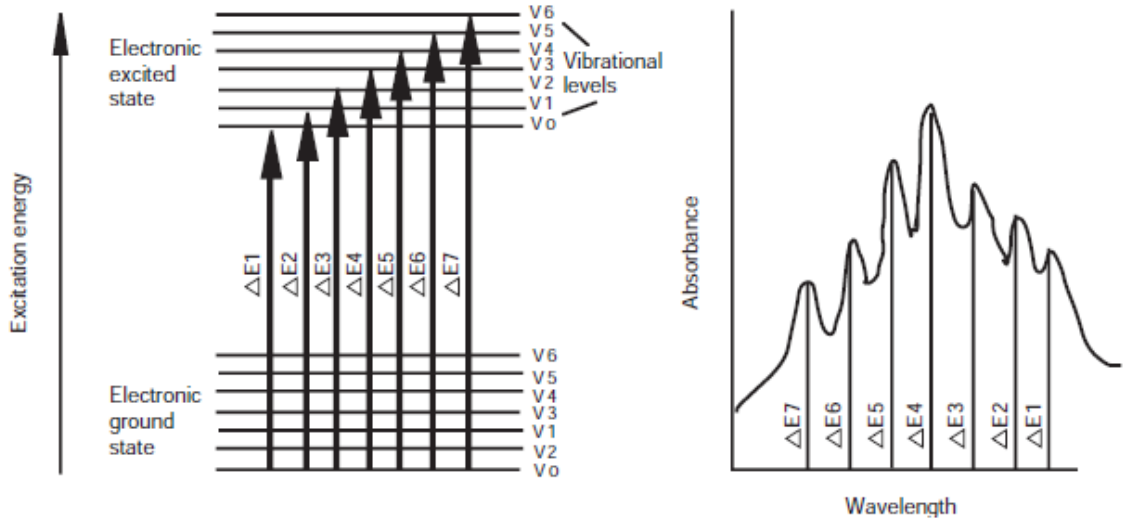
UV-Vis range



Adopted from "UV–Vis spectroscopy for food analysis," by Haque, F., et al. (2021), Techniques to Measure Food Safety and Quality: Microbial, Chemical, and Sensory, 169-193. Copyright 2021 Springer Nature Switzerland AG.

Scheme 1. 2

Electron excitation



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1.3 Liquid phase chromatography (HPLC)

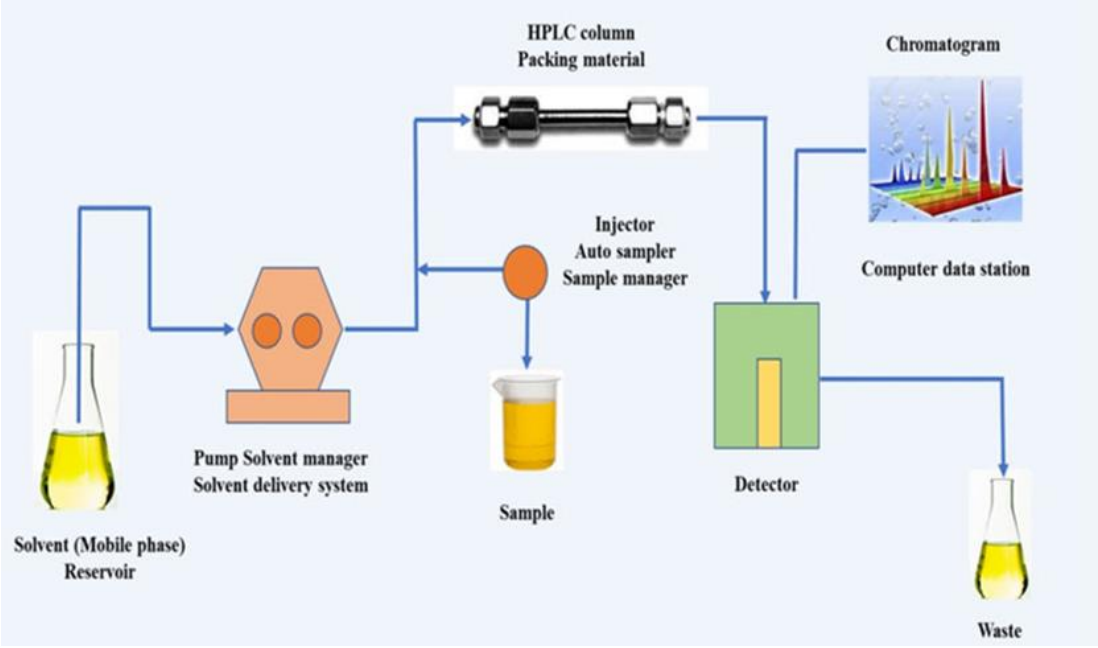
The most common and well-established analytical method is High-performance Liquid Chromatography (HPLC) and is also the predominant separation method employed. The HPLC method is used in quantitative and qualitative bioanalytical applications [23], as

it offers a diverse selection of possibilities for determining how to measure analytes or the derivatives of those analytes. Over the past four decades and more, it has been put to use in laboratories worldwide for pharmaceutical sciences, clinical chemistry, food and environmental investigations, and synthetic chemistry [24]. HPLC's rise to prominence can be mainly attributed to (1) reliability, provided by its pressure-driven liquid support; and (2) by its adaptability, the possibility of adjusting the composition of both mobile and stationary phases.

Instrumentation: A pump, an injector, a column, and a detector, coupled with a data-handling device, are the essential components of a standard high-performance liquid chromatography system (Scheme 1.3).

Scheme 1. 3

High-Performance Liquid Chromatography (HPLC) [25]



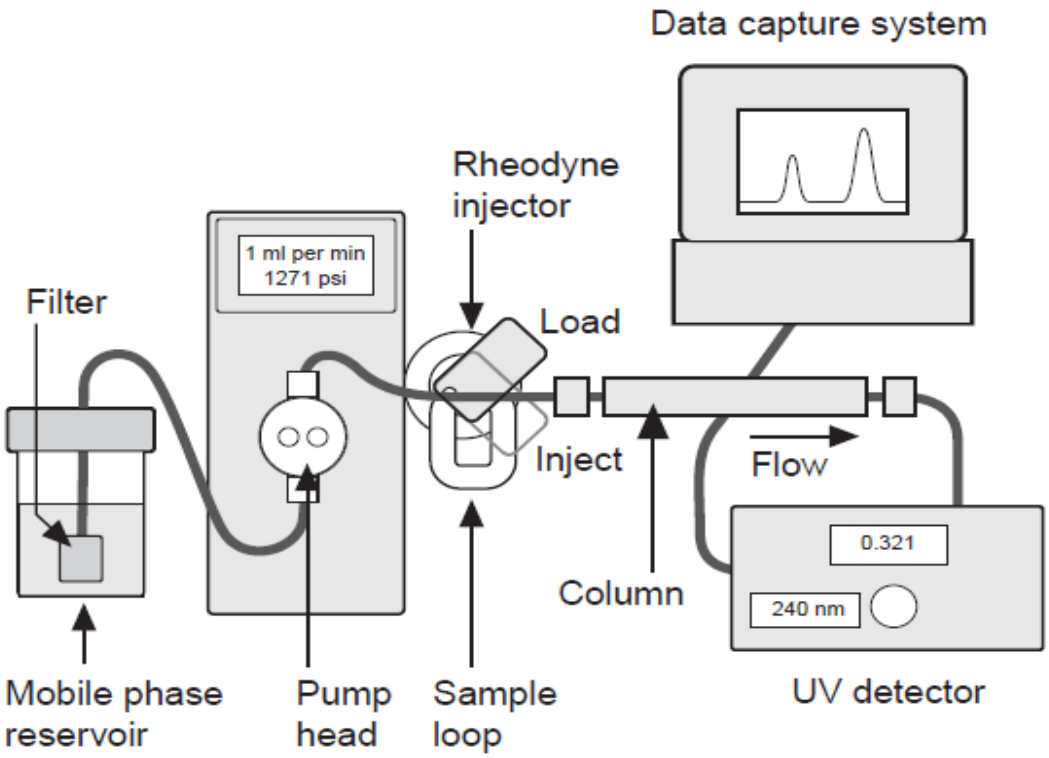
Adopted from "High Performance Liquid Chromatography: Recent Patents and Advancement," by Bhati, C., et al. (2020), Biomedical and Pharmacology Journal, 15(2), 729-746. Copyright 2023 Biomedical and Pharmacology Journal.

A typical isocratic elution HPLC instrument have the following basic components: (I) a solvent reservoir; (ii) a pump capable of pushing solvent at pressures of up to 4000 psi and flows of up to 10 ml/min; (iii) a loop injector with a fixed-volume loop ranging from 1 to 200 ml (20 ml is generally used as a standard); and (iv) a column, which is normally a High-performance liquid chromatography (HPLC) is a method for

measuring analytes and their derivatives that provides a broad range of options. UV-Vis absorption, fluorescence, evaporative light scattering, different electrochemical detection, and chemiluminescence are some of the numerous detection techniques utilized in HPLC. These are only a few of the various detecting techniques available. As a result, high-performance liquid chromatography, or HPLC, is widely regarded as the most effective approach for detecting inorganic and organic molecules in a wide range of matrices [27]. Yet, a considerable percentage of the compounds of interest lack the chromophoric, fluorophoric, or redox groups required for HPLC identification. To get around this issue, researchers are investigating induced derivatization methods to determine whether they can add the essential functional group (or groups) to the molecule [26]. These reactions must produce stable derivatives quickly and may be carried out in a number of ways, including the following:

Scheme 1. 4

A standard instrumental system for isocratic elution



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- **Pre-column mode**: The reaction is performed manually in vials before HPLC injection. This mode allows more flexible working conditions, for example, reaction time flexibility, excess reagent elimination, and more. We note that the derivative must be stable for the separation of the molecules to be successful after its modification through derivatization [26].

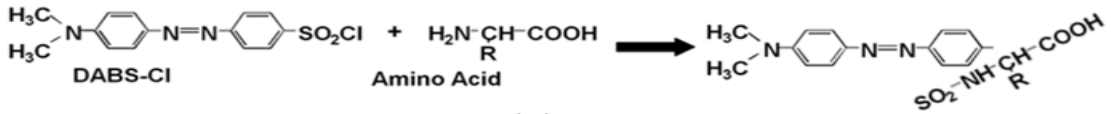
- **Post-column mode:** The reaction is performed by adding a derivatization reagent after separation has occurred; this is done before detection, utilizing a secondary HPLC pump. Although heavier equipment is needed in this approach, the number of manipulations can be considerably reduced by automation [20, 26].

Using derivatization analytes from a variety of classes has the potential to increase UV absorbance and fluorescence, as well as ionization efficiency and, most importantly, HPLC separation performance [21]. The term "derivatization" refers to the process of introducing a chemical change into a target material by combining an existing chemical reaction with additional processes such as heating, irradiation, or an electric field. In high-performance liquid chromatography, ultraviolet (UV) detection is regarded as the industry standard. In terms of sensitivity and selectivity, it often falls short when compared to other ways of monitoring and evaluating compounds, such as medicines. The process of chemical derivatization may be used to produce compounds with high UV absorption from molecules that have a low capacity for absorbing UV light. Derivatization reagents may be divided into five groups: (1) Non-fluorescent reagents are generally used in UV-VIS (benzoyl chlorides and sulfonyl benzene). (2) Fluorogenic reagents are generally non-fluorescent but react with target compounds to form conjugated fluorescent cyclic molecules (fluorescamine). (3) Fluorescent reagents have a highly fluorescent aromatic group (fluorophore) and a reactive group. (4) Reagents with redox properties, used in electrochemistry. (5) Reagent added to achieve enantiomeric separation. Chemical derivatization in can be divided into three classes: (1) Spectrophotometric (UV-VIS Detection); (2) Fluorescence detection; and (3) Derivatization for the resolution of chiral compounds. Next, we summarize each class and give derivatization examples.

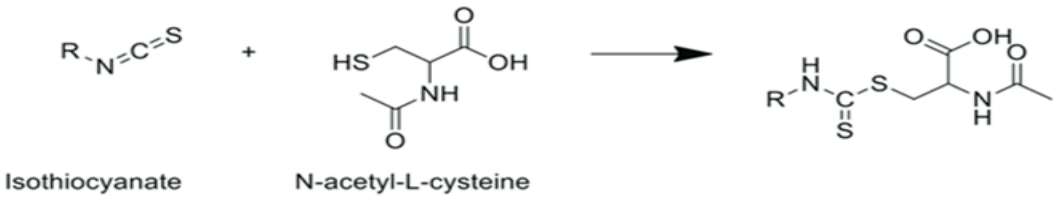
Spectrophotometric (UV-VIS Detection): UV detection is the usual approach in high-performance liquid chromatography (HPLC), despite the fact that it lacks the sensitivity and selectivity required for trace analysis. Using a chemical derivatization process, substances with a low UV absorption rate may be transformed into substances with a high UV absorption rate. In addition to being present in amino acids, amines may be found in almost every pharmaceutical medication (antibiotics, proteins, peptides, toxins, and environmental pollutants). Thus, the capacity to quantify them properly is crucial.

Figure 1. 1

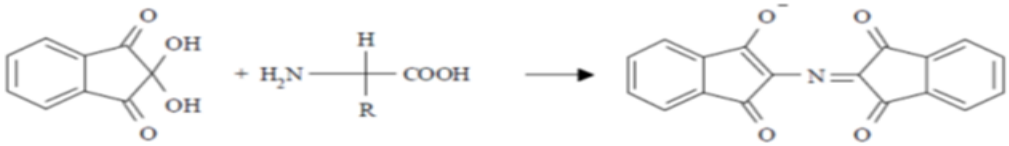
Reactions



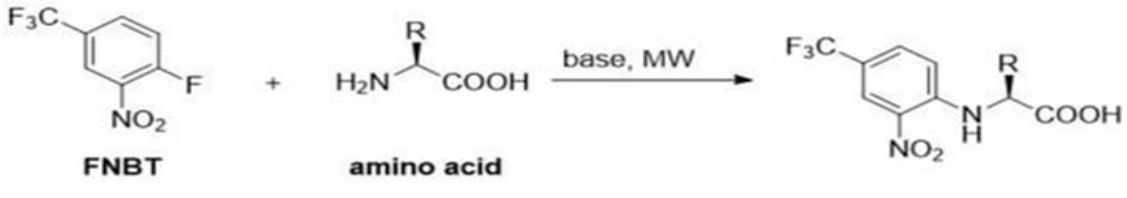
(a)



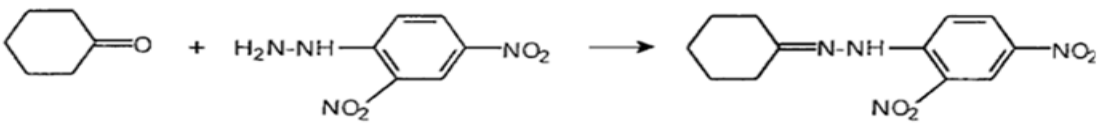
(b)



(c)



(d)



(e)

1.4 Derivatization in liquid chromatography

1.4.1 UV Detection

Available is a broad-range of reagents that allows introducing absorption group with high-UV. Furthermore, reagents are commonly aromatic compounds. This requires a high molar absorption coefficient of the chromophore group to be high. Examples are given in Figure (1), where, (a) DABS-Cl reacts with α-amino groups [27]; (b) Reaction of isothiocyanates with N-acetyl-L-cysteine [28]; (c) Ninhydrin is used for the derivatization of aminoglycosides such as streptomycin [29]; (d) Reaction of amino acid with FNBT [25]; and (e) Derivatization of CHN with 2,4-dinitrophenylhydrazine [30].

Acyl chlorides: To yield esters and amides, reagents such as aromatic acylation are to be reacted with hydroxy compounds, amines, and amino acids, respectively. Benzoyl chloride, 2-fuoryl chloride, m-toluoyl chloride, 3,5-dinitrobenzoyl chloride, and p-nitrobenzoyl chloride are all examples of aromatic acid chlorides often used as reagents [31].

$$\mathrm{R-CO-Cl+P-H\rightarrow R-CO-P+HCl} \tag{1.1}$$

Arylsulfonyl chlorides: Reacting with primary and secondary amines as the acyl chlorides at alkaline pH [31] are arylsulfonyl chlorides. While reacts slowly with phenol, it reacts with alcohols is even slower.

$$\mathrm{R-SO_2Cl+P-H\rightarrow R-SO_2-P+HCl} \tag{1.2}$$

Isocyanates and isothiocyanates: In N,N'-disubstituted ureas and N,N'-disubstituted thioureas, aryl isocyanates and aryl isothiocyanates may be used to convert primary and secondary amines. In the presence of aryl isocyanates [31], given by

$$\mathrm{R-NCO+R'NH_2\rightarrow R-NHCO-NHR'} \tag{1.3}$$

and aryl isothiocyanates,

$$\mathrm{R-NCS+R'NH_2\rightarrow R-NHCS-NHR'} \tag{1.4}$$

Some hydroxyl molecules, including alcohols or sugars, may combine with arylisocyanates to produce aryl urethane.

Other UV derivatizing agents

Post-column derivatization of amino acids with ninhydrin was often utilized, especially in commercial automated amino acid analyzers. High-resolution ion exchange or ion-pair chromatography was used to separate the amino acids, and then a post-column process involving ninhydrin made it possible to identify the amino acids.

$$NH_2 - CHR - COOH + 2C_9H_6O_4 \rightarrow CO_2 + C_{18}H_9NO_4 + RCHO + 3H_2O \qquad (1.5)$$

Amino acids and other amino compounds are often derivatized using 1-fluoro-2,4-dinitrobenzene (FDNB), popularly known as Sanger's reagent. The derivatization of aminoglycosides in body fluids was a common application. However, the DNS-Cl has already surpassed its usage in polypeptide sequencing (where FDNB was previously used) due to its greater accuracy.

$$FDNB + RNH_2 \rightarrow RNH - DNB + HF \qquad (1.6)$$

Phenacyl bromide: Carboxylic acids are esterified using aromatic halides in an aprotic solvent. Phenacyl bromide and naphthacyl bromide are the most often used reagents. These reagents are important for carboxylic acid groups in fatty acids, organic acids, and pharmaceutical compounds because to the high yield they provide [31].

$$C_6H_4CO - CH_2Br + R - COOH + R'_3N \rightarrow C_6H_4CO - CH_2 - OCOR + R'_3NH + Br - \qquad (1.7)$$

2,4-Phenylhydrazones are formed when DNPH combines with carbonyl-containing chemical molecules (such as ketones and aldehydes).

$$RR'C = O + C_6H_3(NO_2)_2NHNH_2 \rightarrow C_6H_3(NO_2)_2NHN = CRR' + H_2O \qquad (1.8)$$

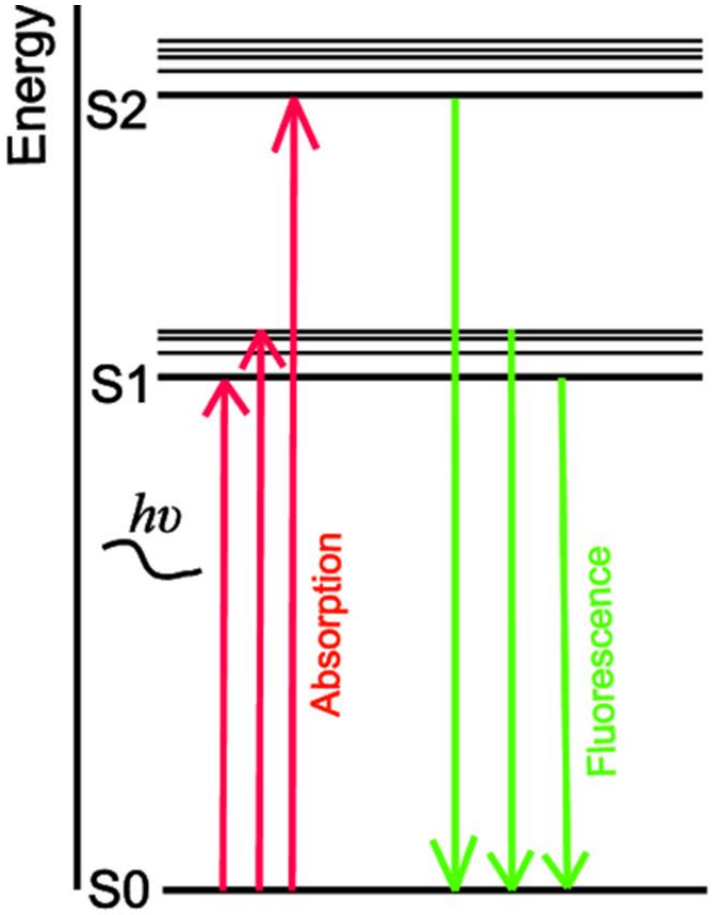
1.4.2 Fluorescence detection

In the ultraviolet range, fluorescence occurs when a molecule absorbs a photon, transitioning to an excited state; in the visible range, the molecule returns to the ground state by releasing a second photon at a higher wavelength, as shown in Scheme (1.5).

Fluorescence detection has a major benefit over UV detection technologies because of its much better sensitivity. Trace analysis relies heavily on fluorescence detection, and since fluorescent molecules are uncommon, they may be picked out of a muddled batch using just their fluorescent signature.

Scheme 1. 5

Electron energy states and fluorescence



Adopted from "Single-molecule fluorescence microscopy review: shedding new light on old problems," by Shashkova, Sviatlana, and Mark C. Leake (2017), Bioscience reports 37, no. 4. Copyright 2022 Portland Press.

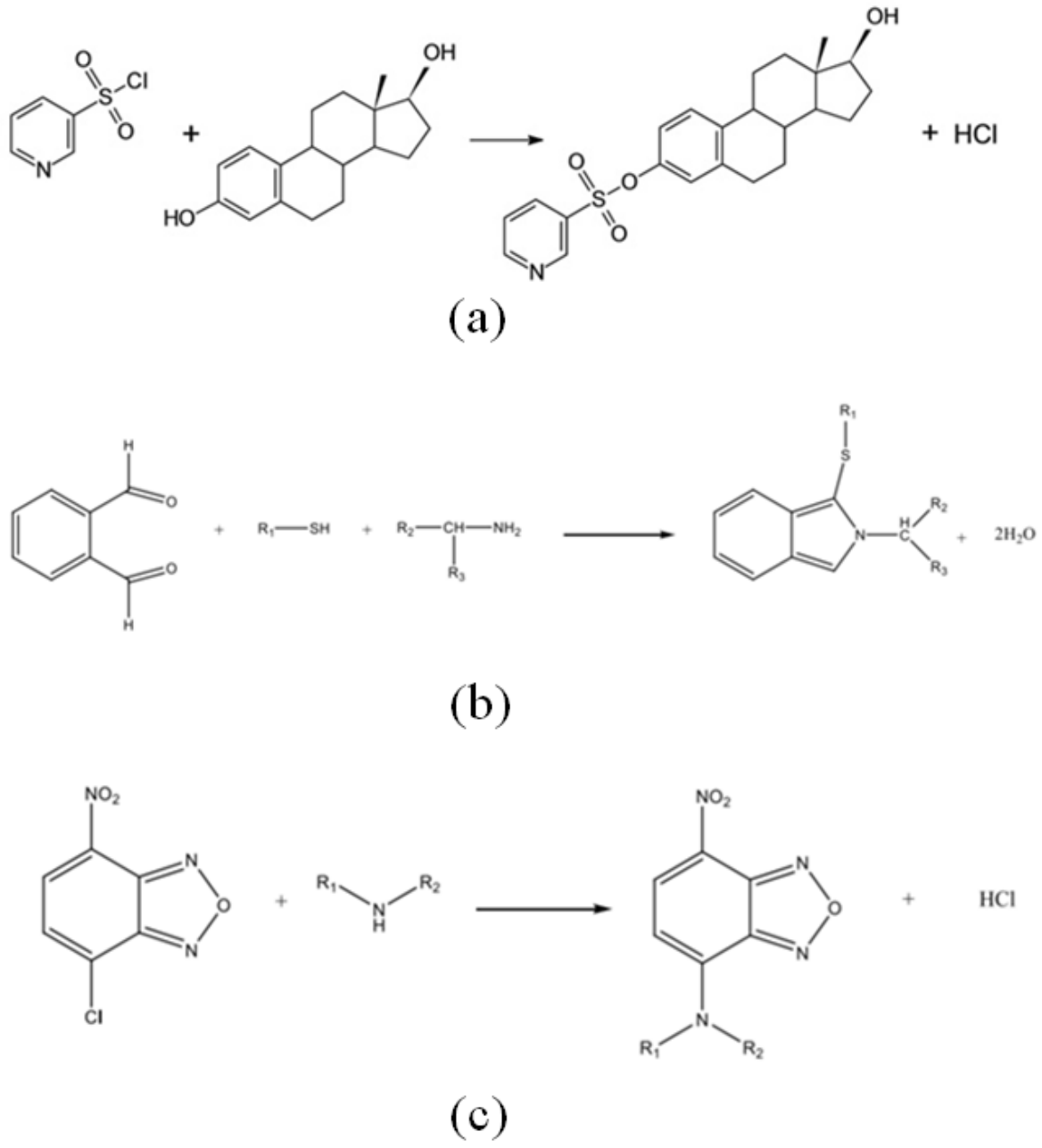
Sulfonyl chlorides: One of the most used fluorescent derivatization reagents for amino acids is dansyl chloride, also known as 5-dimethylaminonaphthalene-1-sulfonyl chloride, which interacts with amines at a slightly alkaline pH. DNS-reaction Cl's with phenols and alcohols is slower at higher pH. Consequently, DNS-Cl may, depending on the conditions, produce a wide variety of N-dansylated, O-dansylated, or didansylated tyrosine derivatives (c.f., Figure 1.2a, [32]). DNS-Cl, being an aromatic sulfonyl chloride, undergoes reactions in basic environments. Solvent used and the DNS-

derivative structure dictate the fluorescent efficiency of DNS-derivatives. Reversed-phase chromatography with fluorometric or UV detection is used to distinguish DNS-amino acids [31].

$$C_{12}H_{12}ClNO_2S + R_2NH \rightarrow C_{12}H_{12}ClNO_2SNHR_2 + HCl \quad (1.9)$$

Figure 1.2

Derivatization reactions



Chloroformate derivatives: For peptide synthesis, the 9-fluorenylmethyl chloroformate (FMOC-Cl) protecting group is often used. FMOC-Cl, like DNS-Cl, may be detected using ultraviolet light. FMOC-Cl is reactive toward a wide variety of organic compounds, including amines (both primary and secondary), amino acids, and alcohols. Within buffer environments, FMOC-Cl interacts with amino groups to produce carbamates (urethanes).

Other fluorescence derivatizing agents: The aldehydes and ketones react with dansylhydrazine (DNS-H) to produce the respective hydrazones. These analogs share the DNS-fluorescence amide's properties. The range of 525-540 nm is used to determine the intensity of a fluorescent light's emission. Both 360-370 nm and 240 nm may be used for excitation.

- *O-phthaldialdehyde (OPA):* When it comes to post-column derivatization, OPA is now preferred over ninhydrin (Figure 1.2b, [30]). Before separation in a biological fluid, OPA may be used for amino acid derivatization in the presence of mercaptoethanol. The derivatization of secondary amines is not appropriate for OPA [31]. The approach is suitable for regular measurement of picomolar or even femtomolar amounts of amino acids, especially when combined with automated sample preparation.

- *Halogenonitrobenzofurazans:* When reacted with aliphatic amines, amino acids, peptides, and proteins, the non-fluorescent compound 4-chloro-7-nitro-2,1,3-benzoxadiazole (NBD-Cl) produces extremely fluorescent derivatives (Figure 1.2c, [33]). In alkaline condition, NBD-Cl also interacts with thiols or phenol to generate derivatives that are fluorescently dim or nonfluorescent altogether. Numerous investigations have been conducted using these fluorogenic dyes to identify amine functional groups in an effort to create techniques for measuring amines and amino acids in pharmaceutical and biological materials. The fluorescence emission of NDB-amides is measured at 512 nm, whereas the excitation wavelength is 464 nm.

- *Fluorescamine:* To create fluorescent derivatives, fluorescamine interacts with molecules containing nucleophilic functional groups such as primary and secondary amines, alcohols, and water. The derivatization of fluorescamine might occur either pre- or post-column. The formation of a derivative is very sensitive to the pH value.

1.5 Validation methods

The new technique was verified according to ICH standards for its specificity, linearity, accuracy, precision, sensitivity, robustness, and solution stability [19, 34].

1.5.1 Precision

The precision of an analytical procedure is defined as the degree to which a series of measurements obtained from multiple samplings of the same homogenous sample carried out under the same conditions are in close agreement with one another. Repeatability, intermediate precision, and reproducibility are three subcategories derived from it. The standard deviation, variance, or coefficient of variation of a data sequence is the standard way in which one can express the degree of precision that can be achieved by an analytical method [35]. The circumstances in which the procedure is intended to be used directly affect the immediate precision that can be achieved. The accuracy of the analytical procedure can be affected by several random events, including the particular day, the analyst performing the work, and the equipment. It is not thought to be necessary to investigate each of these effects independently. It is strongly recommended that a controlled experiment be carried out.

1.5.1.1 Repeatability

The precision attained under identical operating conditions and within a short period is termed repeatability. Repeatability is synonymous with intra-assay precision. The essay would probably be repeated by the same individual using the same apparatus. A separation between the sample preparation method and the instrument performance can be reached within repeatability. Hence, assuming that the dilution steps and sample extraction are more susceptible to variation than the instrumental analysis, a well-maintained instrument precision would be superior to its whole repeatability [36].

1.5.1.2 Intermediate precision

Intermediate precision describes the variance in accuracy within a laboratory when various analysts perform the analysis using different equipment and on different days. Hence, equipment standardization and data handling procedures while also ensuring the entire lab analysts are held to the same standards, a lab could reduce the likelihood that such deviations would be significant [35].

1.5.1.3 Reproducibility

The accuracy between laboratories is expressed by reproducibility. A round-robin trial, in which numerous laboratories are invited to carry out qualitative and quantitative analysis of a sample whose composition is only known to those organizing the trial, is a popular approach for reviewing the performance of a method for new methods. When transferring a process from one corporation area to another, such a trial would be conducted. The data produced via such technique transfer is not often included in the marketing dossier required to get a product license [35].

1.5.2 Accuracy

It has already been shown that procedures may be precise without concurrently being accurate. Doing an accurate assessment when analyzing raw medicinal components is an easy operation. Comparing the test chemical in question to a quantity for which the accuracy is previously established is the easiest procedure. The drug's reference standard is a formulation that has been exhaustively studied, investigated, and assessed in terms of its chemical composition. Assessing the precision of commercial drug screening tests is a difficult task. Evaluating a drug or placebo formulation that has been manufactured with a known amount of material injected into it may be accomplished with more precision if the analysis is conducted on a small scale as compared to a large scale when the same procedure is performed. A comparison to a well-established reference procedure, such as an approach based on a pharmacopoeial handbook, may also be used to determine the validity of the methodology. The degree of accuracy should be expressed as a percentage of the known amount of analyte added to the sample vs. the amount determined by analysis [36].

1.5.3 Linearity

One way to determine whether or not a procedure is linear is to examine the degree of straightness in a calibration plot of response vs concentration. The accuracy of a procedure may be determined by examining its linearity. For the purpose of determining linearity, a single series of measurements at a variety of analyte concentrations may be sufficient. After that, the data are evaluated using a linear regression using the least squares. The figure that was produced offers the required information about linearity by means of its slope, intercept, and correlation coefficient [36]. Linearity can be modeled

by an equation of a straight line that takes the known form: y = ax + b, where the parameter *a* is the slope of the line and the parameter *b* is the line intercept with the y-axis [23].

1.5.4 Robustness

Peak retention, resolution, and efficiency are standard criteria used to assess the applicability of chromatographic procedures to systems. The following experimental elements are often changed while testing the robustness of a method entails: (I) the age of the standards and sample preparations; (II) the time it takes to run the sample analysis; (III) variations in the pH of the mobile phase; (IV) variations in the composition of the mobile phase; (V) the temperature at which the analysis is run; (VI) the flow rate; (VII) the column manufacturer; and (VIII) the type of filters used to prevent centrifugation. The use of the statistical design of experiments in robustness research is very desired since it allows for data-driven method control [35].

1.5.5 System Suitability

At one point in time, the pharmaceutical industry was under the impression that system suitability testing of pharmaceuticals was the best way to determine whether or not a particular chromatographic system was suitable for routine use in pharmaceutical laboratories, which is where the quality of the results is considered to be of the utmost importance. The following is a list of the parameters that were utilized in the system suitability tests (SST) report: Number of theoretical plates, also known as Capacity factor Separation (K), Efficiency (N), also known as Relative retention, Resolution (Rs), Tailing factor (T), and Relative Standard Deviation (Rs) are the variables that are measured (RSD) [37].

1.5.5.1 Number of theoretical plates/Efficiency (N)

In the context of a column, "efficiency" is defined as the degree of peak dispersion, and it should possess the properties of the column [37]. The number of theoretical plates is often used in order to provide a graphical depiction of efficiency. As an example, the formula that may be used in order to find N is shown below.

$$N = 5.54 \left(\frac{V_e}{w_{\frac{1}{2}}} \right)^2 \tag{1.10}$$

1.5.5.2 Capacity ratio or Capacity factor (k)

Termed retention factor has no dimension and is independent of the mobile phase's flow rate and column dimensions, which measure the extent of retention relating to an analyte relative to an un-retained peak [37]. Where t_R implies the sample peak's retention time and an un-retained peak's retention time is t_M.

$$k' = \frac{t_R - t_M}{t_M} \tag{1.11}$$

k' = 0 means no compound is left in the column. Generally, a value of k' greater than 2 is an acceptable value.

1.5.5.3 Resolution

Increasing the temperature, increasing the length of the column, decreasing the particle size of the particles, and modifying either the eluent or the stationary phase are all effective strategies [37] for increasing the resolution of the column, which is defined as the ability of the column to separate two medications into two distinct peaks or chromatographic zones. It is possible to represent it as a ratio of the angular distance between the average tangential widths of two peaks. Here is one way it may be stated. The following is the formula that was used in the process of determining the resolution.

$$R_s = 2\frac{t_{R_2} - t_{R_1}}{t_{w_1} + t_{w_2}} \tag{1.12}$$

1.5.5.4 Tailing factor or Asymmetry factor

When analyzing the chromatographic peak, the Gaussian distribution is used as an assumption [39]. In contrast, in the actual world, there is almost always at least one outlier, which demonstrates that migration and distribution are not completely consistent with one another. As a direct consequence of this, regulatory agencies such as USP and EP now consider this to be a proposed factor for determining the acceptability of a system. In most cases, the asymmetry factor and the tailing factor will be quite near to one another; nonetheless, they will seldom be correct and equal to one another. In general, you want your values to fall between 1.0 and 1.5, and anything that is higher than 2.0 is considered improper. Using this information, we should be able to compute the greatest asymmetry.

$$A_s = \frac{B}{A} \tag{1.13}$$

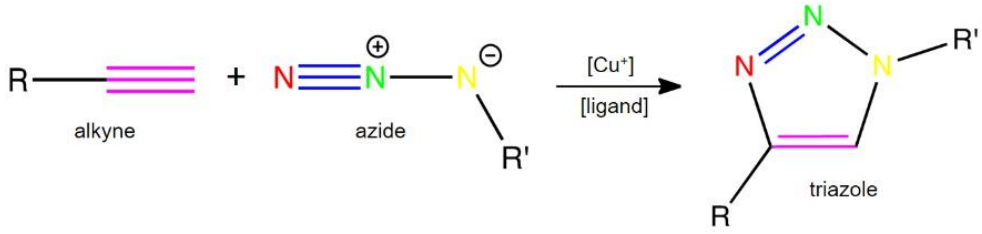
where A_s is the peak asymmetry factor; and B is the distance from the point at peak midpoint to the trailing edge; and A is the distance from the leading edge of peak to the midpoint (measured at 10 % of peak height).

1.6 Click Chemistry

CuAAC-Cu catalyzed alkyne azide cycloaddition is a reaction between azide and alkyne that produces a covalent product - 1,5-disubstituted 1,2,3-triazole, as illustrated in the Scheme (1.6). Copper catalysis is the foundation of Click Chemistry. A method that is widely used, dependable, and straightforward for making covalent connections between building blocks that each contain a different set of functional groups. Applications in organic synthesis, medicinal chemistry, surface and polymer chemistry, and bioconjugation have all been used at some point [38].

Scheme 1. 6

Copper-catalyzed azide-alkyne cycloaddition reaction



The catalyst is frequently presented as a Cu-TBTA complex. The requirements of the experiment will typically determine which catalyst option is available, and in most cases, a variety of combinations will yield the desired results. Several distinct copper (I) sources may be incorporated into the reaction. Salts of copper(I) such as iodide, bromide, chloride, and acetate, as well as coordination complexes of copper(I) such as [Cu(CH3CN)4]PF6 and [Cu(CH3CN)4]PF6, can be used to synthesize copper(I). OTf has been utilized quite frequently in recent years [38].

The decision was made to use Click Chemistry as a conjugation chemical reaction as opposed to any of the hundreds of other possible organic reactions. Due to the multiple advantages that Click Chemistry provides, the choice was taken. It is fully unilateral. To perform the Click Chemistry technique, just the azide and alkyne components are

required [39]. This procedure has no effect on the majority of other chemical groups present in tagged DNA and proteins, including amino and carboxyl groups. Azides and alkynes are not present in biomolecules found in nature. In the synthesis process, azide-activated ester and alkyne-activated ester may be used to synthesize azide-labeled and alkyne-labeled proteins, respectively.

It is possible to employ aqueous DMSO, DMF, acetonitrile, alcohols, buffers, and water in its purest form. There is an immediate response that can be seen and quantified. In order to ease the creation of conjugate nano molecules in dilute solutions, "Click Chemistry" may be applied. At any pH level, the reaction will occur.

In contrast to NHS ester reactions with amines and other chemical processes involving conjugation, it is not essential to adjust the pH of the reaction mixture. As Click Chemistry is effective throughout a broad pH range from 4 to 11 [39], no buffer, acid, or base is required. Click Chemistry has thus grown into a technique that may be used for fluorescence labeling, conjugate synthesis, and the universal modification of DNA and proteins. This includes the antiviral drug zidovudine, which will be used in our research. Zidovudine derivatives would be produced through a click reaction, which the proposed research would use to help avoid these limitations.

1.7 Zidovudine

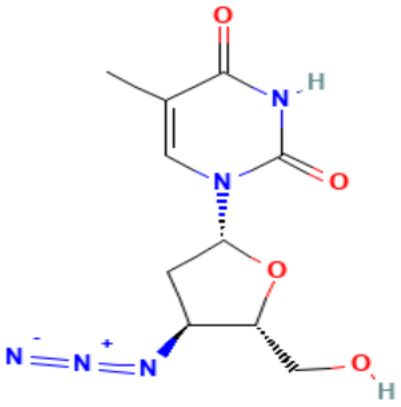
It is the first FDA-approved drug for HIV infection, it is a reverse transcriptase inhibitor and is commonly used in combination with other antiretroviral agents to give synergistic treatment (Scheme 1.7). Zidovudine is a nucleoside reverse transcriptase inhibitor. It is a synthetic analog of thymidine, a nucleoside (NRTI). Zidovudine is an antiviral medication that works by substituting itself for thymidine in newly produced viral DNA and acting as a chain terminator for viral DNA. This process allows it to perform its antiviral function. Because of this, the HIV-1 reverse transcriptase enzyme cannot produce viral DNA from the RNA template, which disrupts the HIV-1 life cycle. (Scheme 1.8) [40]. In order to be transformed into an active triphosphate, AZT requires an intracellular conversion by host cell kinases [41]. Zidovudine cannot exit the cell because it is converted intracellularly to the triphosphate form, despite its half-life in plasma being just around an hour. As a result of Zidovudine intracellular trapping and around seven hours half-life, it allows for a more suitable frequency dosage.

Zidovudine is considered to be effective against HTLV-1, which is a retrovirus that causes adult T cell leukemia-lymphoma [42]. In the same manner that it is effective against HIV-1, zidovudine may also be useful against HTLV-1. Since zidovudine has a relatively low bioavailability (only 64 percent), taking medication with food will just delay the absorption process. Zidovudine is eliminated almost entirely in the form of zidovudine 5'-glucuronide, with the parent compound accounting for just about 14% of the drug's clearance. Zidovudine is eliminated from the body mostly via the kidneys' functions. In addition, the digestion of it is a collaborative effort across many microsomal routes [43]. Since this medication has the potential to induce deadly adverse effects, such as lactic acidosis and myelosuppression, the dosage must be administered with the utmost care. Hence, it is feasible to get an appropriate dose with a limited number of adverse effects by making use of procedures that are sensitive and selective [17].

Despite the potential and benefits such a method may have, we only could find a few works in the literature that performed derivatization of Zidovudine which we will describe in the next sub-section. In this study, we aim to develop facile chemical derivatization of Zidovudine based on click reaction. The method will increase the **UV** detection range, which facilitates the development of a unique analytical detection method.

Scheme 1. 7

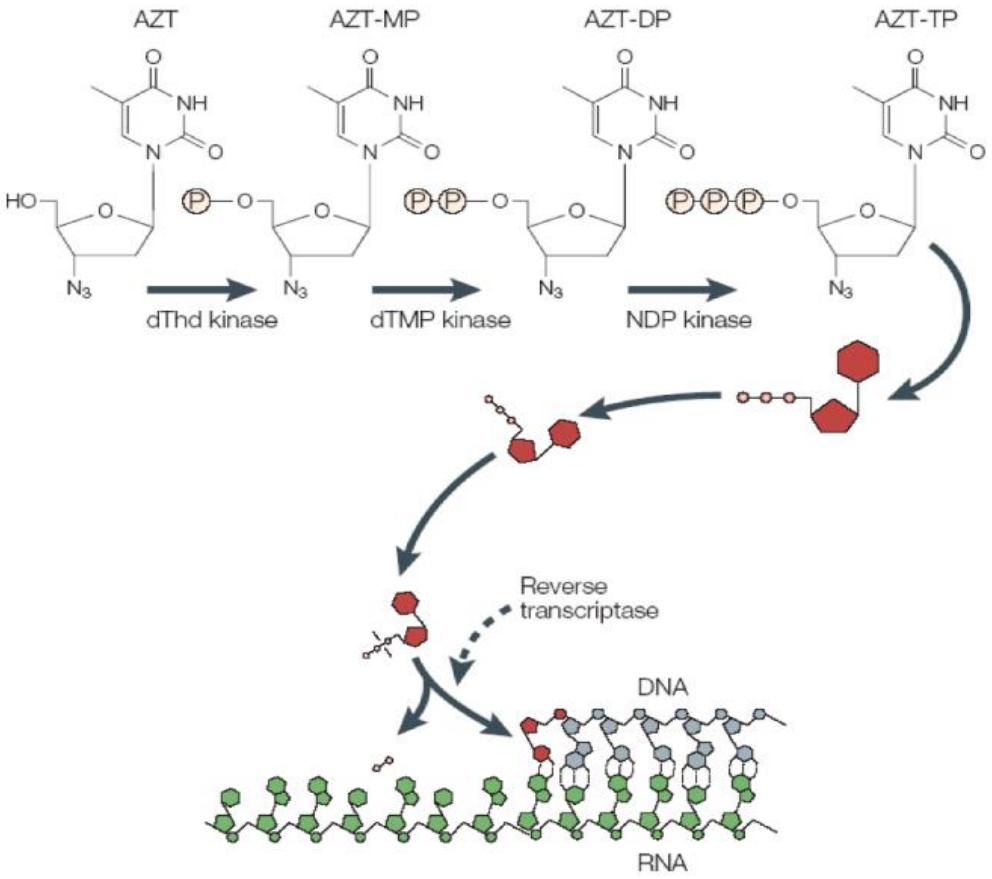
Zidovudine structure



Adopted from "Antiviral Strategies," by De Clercq, E. and Neyts, J., (2009). Handbook of Experimental Pharmacology, vol 189. Springer, Berlin, Heidelberg. Copyright 2022 Portland Press. Copyright 2009 Springer-Verlag Berlin Heidelberg.

Scheme 1. 8

Mechanism of antiviral action of Zidovudine



Adopted from "Antiviral Strategies," by De Clercq, E. and Neyts, J., (2009). Handbook of Experimental Pharmacology, vol 189. Springer, Berlin, Heidelberg. Copyright 2022 Portland Press. Copyright 2009 Springer-Verlag Berlin Heidelberg.

1.8 Literature Review

Kusuma et al. (2011) [16], proposed an HPLC test technique developed and validated to indicate zidovudine tablet stability as part of the suggested strategy. The goal of the paper was to provide an HPLC approach with UV detection used to directly assess Zidovudine in pharmaceutical dosage forms, raw materials, spiking human blood, and drug dissolution studies, eliminating the need for laborious extraction or evaporation procedures. Samples taken during expedited stability tests might be analyzed using the new technology to help determine when drugs would expire, as suggested by the scientists. This means that chromatographic conditions Using a room-temperature Zodiac C18 column. The mobile phase comprises a combination of methanol and acetonitrile, a flow rate of 1 ml/min. The mobile phase was degassed and filtered using nylon 0.45µm– 47mm membrane filters before use. The elution was tracked using a UV

detector set to 270nm with an injection volume of 20µL. Optimizing chromatographic conditions were studied, and this included looking at the effects of mobile phase, pH, flow rate, and solvent ratio. Capacity factor, asymmetry factor, resolution, and column efficiency were among the chromatographic characteristics calculated from the resulting chromatograms. The condition with the best estimate also had the greatest resolution, symmetry, and capacity factor.

- **Standard preparation of standard solutions (100µg/ml):** To prepare a Zidovudine standard solution of 1000 µg/mL, 25 mg was moved to a 25 mL volumetric flask after being weighted, dissolved, and diluted with methanol until the desired concentration was reached. After further diluting with the mobile phase, this solution (1ml) served as a 100 µg/ml working standard stock solution for the RP-HPLC method.

In compliance with FDA bioanalytical method validation requirements. Validations were done by evaluating its specificity, LLOQ, recovery, linearity, and precision. Methanol: acetonitrile (40:60v/v/v) was the mobile phase of choice. Using a Zodiac100-5 C18) 250×4.6mm, 20 µL\samples were injected. The samples were detected at 270 nm. The linearity (r = 0.995) was observed throughout a concentration range of 0.1-0.6 µgmL-1; the slope was 188680, the intercept was 4018.33, and the limit of detection was 0.062 µg mL-1. It was put to good use in analyzing pharmaceutical formulations free of interfering excipients and endogenous substances. Similarly, Zidovudine concentrations for in vitro dissolution studies might be determined using this method.

Balamuralikrishna and Syamasundar (2011) [44] proposed the simultaneous assessment of abacavir, lamivudine, and zidovudine in tablet dosage forms has been made possible as a result of the development of a straightforward, dependable, and readily repeatable RP-HPLC technique. While performing chromatography on a Luna C18 column with a flow rate of 1.2 mL/min, the mobile phase consisted of 70 percent acetonitrile, 20 percent methanol, and 10 percent 0.05M ammonium dihydrogen phosphate. The mobile phase included methanol, acetonitrile, and ammonium dihydrogen phosphate as its constituents. The measurement was taken at a wavelength of 266 nm when it was carried out. The levels of abacavir, lamivudine, and zidovudine that were discovered in the body were found to be retained for 2.6, 6.1, and 8.9 minutes, respectively. The linearity of the calibration curves for abacavir was seen across the range of 50-150

g/mL, the linearity of the curves for lamivudine was observed over the range of 20-60 g/mL, and the linearity of the calibration curves for zidovudine was observed over the range of 30-90 g/mL. After its validation in accordance with the requirements provided by ICH and USP, it was determined that the technique under consideration was appropriate for the routine quality control analysis of tablets containing a variety of pharmacological dose forms. The author developed a speedy, accurate, and exact RP-HPLC method for the simultaneous evaluation of abacavir sulfate, lamivudine, and zidovudine in both tablet and bulk medication dose forms. With the help of this procedure, the chromatographic conditions were determined. The separation was carried out with the assistance of a Shimadzu HPLC system that was equipped with a Luna C18 column that measured 250 mm in length and 4.6 mm in diameter and had a resolution of 5. At a flow rate of 1.2 mL per minute, a mobile phase that was composed of 70 parts acetonitrile, 20 parts methanol, and 10 parts 0.05M ammonium dihydrogen phosphate was moved down the column using a PU 2080 isocratic pump. In order to lower the pH of the mobile phase down to 4.5, O-phosphoric acid was used. The sample was injected by using a Rheodyne 7725 injection valve in conjunction with a 20 L Hamilton syringe. A program named spin chrome was used in order to collect the necessary data. Using a Loba ultrasonic bath sonicator, the mobile phase and the other solutions were degassed so that the experiment could proceed. Using an Elico UV-Visible spectrophotometer allowed for the collection of the UV spectra of each of the numerous medications.

In accordance with the current guidelines provided by the Food and Drug Administration (FDA) of the United States for the validation of bioanalytical techniques, we conducted experiments to establish the method's accuracy, linearity, recovery, specificity, precision, LLOQ, and stability. The proposed RP -HPLC method was found to be rapid, precise and accurate. This indicates that it can be used for the routine quality control analysis for the simultaneous determination of the Abacavir sulphate, Lamivudine and Zidovudine in their tablet dosage forms.

Nandi et al. (2013) [18] determined Lamivudine (LMV), Zidovudine (ZDV), and Nevirapine (NVR) concentrations in human plasma using the UV-HPLC technique designed and validated in accordance with FDA criteria (Scheme 1.9). Liquid-liquid extraction was used to prepare the sample, and while using a chromatographic separation on a Hypersil BDS. This allowed within a short run time of 10 minutes

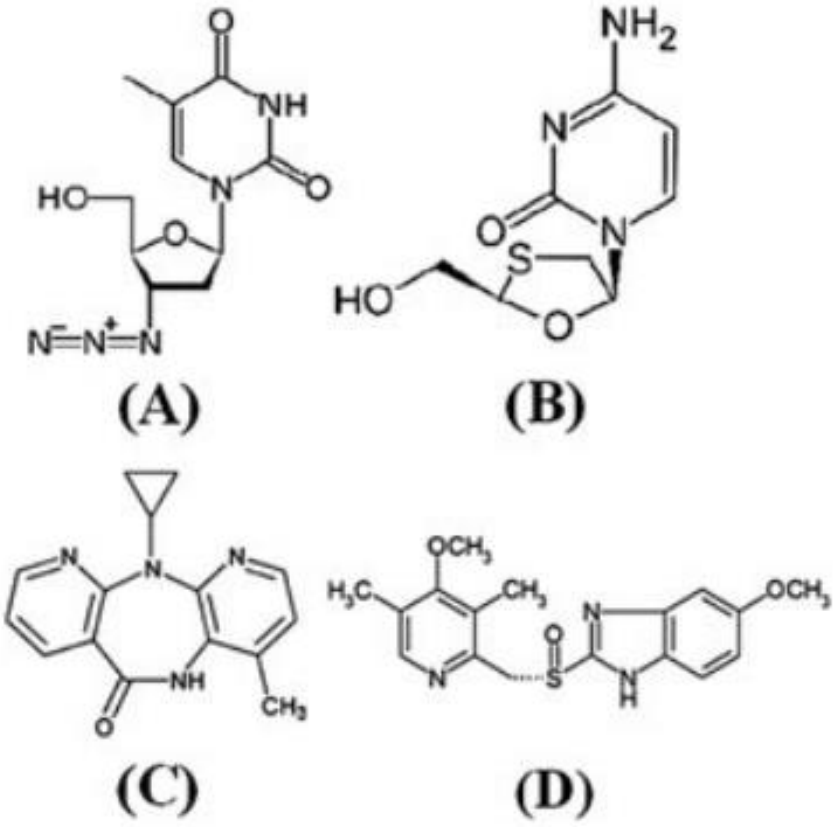
simultaneously estimate the three analytes in the plasma. Isocratic chromatographic separation at room temperature was achieved by using a Hypersil BDS, with a guard column, C18. The mobile phase consisted of methanol (40:60, v/v), a buffer 0.1 M ammonium acetate in 0.5% acetic acid, v/v, and with a flow rate of 0.85 ml/min, a 50 ml sample injected into the column.

- **Sample and Standard solutions preparation:** Appropriate aliquots of the working solution of each analyte were spiked into plasma, and the seven calibration standards (CC) were produced. These standards had concentrations in the range of 50-2000 ng/ml for LMV; 50-3000 ng/ml for ZDV; and 10-3000 ng/ml for NVR. These concentrations were the maximum amount of IS that could be found in the plasma of the blood was 300 ng/ml. Using the same method as described above, quality control (QC) samples were prepared for LMV , ZDV, and NVR. The concentrations of these samples were as follows: 150, 150, and 30 ng/ml (LQC), 50, 50, and 10 ng/ml (LLOQ), 1500, 900, and 1500 ng/ml (MQC), and 2500, 1750, and 2500. A temperature of 20 degrees Celsius was maintained for the polypropylene vials that contained the aliquots of the quality control samples that had been transferred there.

Industrial guidelines for validating bioanalytical methods were used to ensure this approach was robust enough for use in the field. The results reveal that the method is linear, 50 to 2000, from 50 to 3000, and 10 to 3000 ng ml-1, with a LLOQ of 50, 50, and 10 ng ml-1 for LMV, ZDV, and NVR, respectively. Above 90.16 percent of the analytes were recovered during the extraction process while working with plasma samples.

Scheme 1. 9

Chemical structures of (A) Zidovudine; (B) Lamivudine; (C) Nevirapine; and (D) Esomeprazole



Adopted from "Development and validation of an HPLC-UV method for simultaneous determination of zidovudine, lamivudine, and nevirapine in human plasma and its application to pharmacokinetic study in human volunteers," by Nandi et al. (2013). Drug testing and analysis, 5(6), 485-491. Copyright 2012 John Wiley & Sons, Ltd.

Haneef et al., (2013) [15] proposed a reverse-phase validation of Zidovudine in its pure tablet dosage forms may be performed rapidly and accurately using an HPLC technique described by [15]. For this purpose, chromatography was performed at a flow rate of 1.0ml/min on a symmetric C18 (4.6 x 150mm, 5m) column using a mobile phase consisting of a 65:35 v/v methanol:phosphate buffer (v/v) combination, with detection set to 264nm. The retention time was 2.55±0.02 minutes. Waters HPLC equipped with an autosampler and UV detector and a C18 (4.6 x 150 mm, 5 m) column were used as the chromatographic conditions of note. The sample was injected using a 20 µL Rheodyne injection syringe. To prepare the mobile phase, we utilized HPLC-grade methanol and phosphate buffer. The mobile phase consisted of a freshly mixed 65:35 (v/v) solution of methanol and 0.05M potassium dihydrogen phosphate buffer (pH-2.8).

Before being used, the solvents were sonicated and filtered using a 0.45 μ membrane filter. The column temperature was preserved at room temperature, and the mobile phase flow rate was held constant at 1 mL/min.

- **Preparation of phosphate buffer:** To make phosphate buffer, put 7.0 grams of potassium dihydrogen phosphate in a beaker and dissolve it with HPLC water until you have 1000 milliliters. After then, the pH was lowered using orthophosphoric acid to around 2.8.

- **Preparation of mobile phase:** Mobile phase was prepared by mixing 350 mL (35% concentration) of the aforementioned buffer with 650 mL (65% concentration) of Methanol HPLC in an ultrasonic water bath for 5 minutes to remove air bubbles. Filter using a 0.45-micron filter in a vacuum. It's important to remember that Mobile phase was employed as filler.

- **Sample and Standard solutions preparation:** When making up the sample solutions, we weighed 5 zidovudine pills and took the mean. Weigh out 10mg of Zidovudine and carefully transfer it to a 10ml volumetric flask. Sonicate 10 ml of diluent to ensure full dissolution. Harmonize the ingredients and strain them through a 0.45-micron filter. Pipette 0.4 ml of the aforementioned stock solution into a 10 ml volumetric flask and then filled the flask with diluent until it reaches the appropriate mark. Sonicate it in roughly 10 ml of diluent until it dissolves, and then use that solvent to get the volume up to the required amount. In addition, transfer 0.4 ml of the aforementioned stock solution by micropipette into a 10 ml volumetric flask, and dilute to the appropriate concentration with diluent. Combine and strain through a 0.45 m membrane.

The approach produces linear responses between 20 and 80 mg/ml of Zidovudine. The procedure was useful for ensuring the purity of both raw materials and medicinal formulations. The developed RP-HPLC method was also checked for both daily and weekly variation. On the same day, six separate injections of the identical Zidovudine (40 mcg/ml) solution yielded a relative standard deviation (RSD) in a peak area of 0.16 percent. Over the course of six days and six injections, it was also determined that the daily variance was 0.27%. Results showed that the results for % RSD were all within a range of 2, suggesting that the approach was reliable. Since the new HPLC approach

was shown to be rapid, sensitive, specific, and reproducible, the authors concluded that it was effective in the determination of Zidovudine.

Serife Evrim Kepekci Tekkeli (2013) [45] identified that the presence of substances similar to Zidovudine, requires use of spectrophotometric methods that are uncomplicated, selective, and sensitive. UV detection cannot be performed due to the absence of a groups within their structure, namely, chromophore. Under these circumstances, the creation of ion pair complexes makes it possible to detect both ultraviolet (UV) and visible light. Studies that were published on drug analysis made use of a variety of hues. The intricate nature of the drug served as inspiration for the color choices that were made. One that was designed specifically for ZVD; nevertheless, in order to work, it requires a greater number of ingredients and a more involved reaction procedure. In this research we propose the development of a spectrophotometric method for LVD and ZVD that makes use of bromocresol purple (BCP) and has the potential to be used in regular laboratories without the requirement for costly equipment and trained personnel. One example of this would be high-performance liquid chromatography (HPLC). The conventional technique for determining LVD and ZVD in bulk and pharmaceutical forms is uncomplicated, quick, accurate, exact, and sensitive.

Measurements were acquired by Model U-2900 Hitachi spectro-fluorimeter with a Xenon lamp. pH measurements were carried out with WTW pH 526 digital pH meters, calibrated with buffer solution (pH 7.0).

- **Reagents and Solutions:** The analysis used only chemicals and reagents of the highest possible quality in every step of the process. Both LVD and ZVD, as well as GlaxoSmithKline Pharmaceutical Industry's formulations Zeffix (containing 100 mg of LVD per tablet) and Retrovir (containing 250 mg of ZVD per capsule), were generously provided by the company. GlaxoSmithKline Pharmaceutical Industry is based in Istanbul, Turkey. Merck, located in Darmstadt, Germany, contributed to the BCP. The success of the entire operation relied on water that had been twice distilled. After adding 10% ethanol to water, the resulting solution contained 0.05% BCP.The LVD and ZVD stock solutions (1 mg mL1) were both prepared using water as the solvent.

- **Procedure:** After transferring LVD and ZVD stock solutions in different quantities to sealed glass tubes, the final volumes of the LVD and ZVD solutions were brought up to 1 mL and 0.5 mL, respectively, by the addition of water. This brought the total volume of the LVD and ZVD solutions to 1 mL. Each tube received an addition of two milliliters of a phthalate buffer solution with a pH of 2.0 for LVD and 2.5 for ZVD, as well as two milliliters of a BCP solution with a concentration of 0.05%. Using a vortex mixer and giving it 2 minutes of time, each 5 mL reaction mixture was extracted with 5 mL of chloroform. After the completion of the two phases' separation process. By adding chloroform to the volumetric flasks, the total volume of the organic layers was raised up to 5 milliliters.

Assessment of the feasibility for the analysis of the proposed procedure of ZVD and LVD in both pure and formulations by applying it to the samples analysis by recommended techniques. The method was tested by applying it to the examination of six drug duplicates, and the outcomes were compared to the values that were to be anticipated in accordance with Beer's law in order to validate its accuracy and precision. The comparatively tiny RSD values give rise to the possibility of good precision and repetition being achieved. The relative standard deviation (RSD) values for the results that were reproduced were low. In order to determine whether or not the process was successful, recovery experiments using the more traditional addition strategy were carried out. For each medication, a pure sample solution was combined with a standard drug solution of varied concentrations in order to conduct the tests. Average percentage recoveries for LVD and ZVD were somewhere in the range of 100.04 to 101.28 respectively.

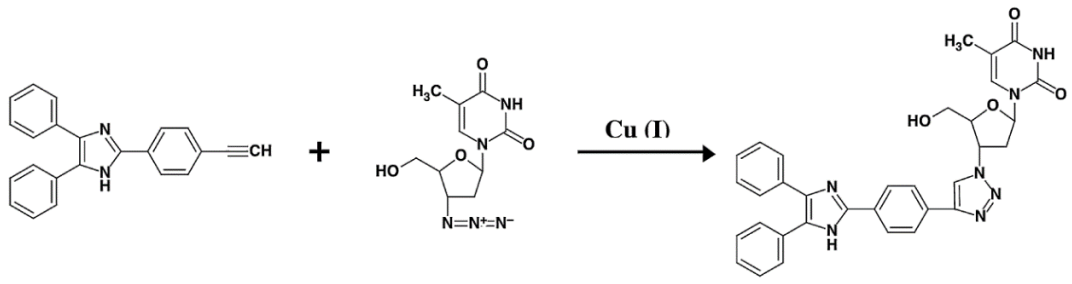
A significant advantage of utilizing this method is that it enables one to utilize an extraction spectrophotometric method to identify chemicals that are contained within a complicated mixture. In comparison to the complex and expensive methods of gas chromatography and high-performance liquid chromatography, the apparatus is simple and very inexpensive. Due to the method's sensitivity, it was found to be well suited for determining ZVD and LVD in dosage and even in pure forms. The suggested method makes use of a reagent that is both less expensive and simpler to get, and it does not call for any reaction conditions that are mission-critical or any sample preparation processes that are time-consuming. The outcomes of the study were not impacted in any way by

common additions. They consist of lactose, starch, magnesium stearate, magnesium trisilicate, and carboxymethylcellulose. When compared to previous methodologies that have been published, the one that has been offered is not only simple to put into action, but also quick to carry out, and it yields very accurate results. The suggested approach can be used to conduct analyses of these pharmaceutical chemicals in a variety of laboratory settings, including those dedicated to quality control and research as well as more traditional settings.

Maeda et al., (2014) [17] aiming to lessen Zidovudine's negative effects, such as lactic acidosis and myelosuppression, devised a more sensitive and selective analytical technique for determining the drug's concentration. Accordingly, the scientists used a Huisgen reaction-based pre-column fluorescence derivatization technique for chromatographic analysis. Specifically, an alkyne and an azide undergo a cycloaddition process. A fluorescent alkyne, DIB-ET, was also developed and synthesized by the authors for use as a reagent; DIB-ET is built around a lophine skeleton that contains an alkyne that functions as both a reactive center and a fluorophore. Solvent A: acetonitrile-5 mM Tris-HCl buffer and Solvent B: water was used for the chromatographic separation utilizing a Cosmosil5C18-AR-II column and a gradient elution protocol (acetonitrile). The gradient schedule looked like this: 0% B (from 0 to 9.5 minutes), linear progression from 0% to 100% B (from 9.5 to 10.0 minutes), and 100% B (10.0 to the end of the session) (10.0–19.0 min). At room temperature, a flow rate of 1.0 mL min-1 was used. We used 310 nm and 400 nm for the excitation and emission wavelengths. Two processes were carried out: (1) the synthesis of DIB-ET, and (2) a fluorescent derivatization technique for regular Zidovudine. Each is described in detail below.

Scheme 1. 10

Fluorescence derivatization reaction between Zidovudine and DIB-ET



DIB-ET **Zidovudine** **DIB-zidovudine**

Adopted from "Fluorescence derivatization method for sensitive chromatographic determination of zidovudine based on the Huisgen reaction," by Maeda et al. (2014). Journal of Chromatography A 1355: 206-210. Copyright 2014 Published by Elsevier B.V. All rights reserved.

Using copper (II) sulfate and L-ascorbic acid as catalysts, Zidovudine was discovered to react with DIB-ET. The suggested approach enabled Zidovudine detecting and determination of DIB-zidovudine by fluorescence detection, and the authors verified that Zidovudine might be transformed to DIB-zidovudine following interaction with DIB-ET (Scheme 1.10). Zidovudine in rat plasma was successfully measured using the described technique after injection with no interference from biological components.

The heat of more than 40 degrees Celsius for more than 5 minutes. It was discovered that this produced a reactivity level very close to that at the higher temperature. However, the reaction was somewhat less vigorous than before heating. This behavior was seen at ambient temperatures. Consequently, 40 degrees Celsius was chosen as the reaction temperature and five minutes as the reaction time since these values provided the highest and most stable reactivity.

Atlas and Nalanda (2016) [12] proposed an isocratic, simple, precise, and accurate HPLC-UV-based method. The authors reports that this is so since there is a need to look into a novel analytical approach for quantifying zidovudine in human plasma mostly because no officially recognized bioanalytical methods have been shown to be effective. Effective treatment for HIV infection is available in the form of AZT, which has a greater affinity for the HIV reverse transcriptase than it does for human DNA polymerases. In the 1990s, the discovery of AZT as an effective treatment for AIDS transformed the prognoses given to patients with the disease and helped shatter the

misconception that HIV/AIDS was an unavoidable death sentence. Zidovudine, on the other hand, is typically administered in conjunction with a number of other antiretroviral medications 4. A metabolic conjugation to the glucuronide is responsible for the rapid elimination of approximately 75% of an AZT dose, while the excretion of the unmodified medicine in the urine is responsible for the rapid elimination of the remaining 20%. It is widely held that zidovudine triphosphate can only be created by the chemical synthesis of the inactive drug itself. The fact that the hematological toxicity of AZT is both dose-dependent and reversible is a major cause for concern. Because of this, determining the concentration of AZT in plasma could one day be utilized to individualize the dosage of medication for each patient5. The application of nanotechnology to the development of new drug delivery systems has shown promise as an effective strategy for targeting the delivery of therapeutic molecules to certain locations, thereby paving the way for the development of innovative treatment alternatives for HIV/AIDS. Several different in vitro and in vivo assays have been utilized in order to investigate the efficiency of this delivery mechanism. Novel antiviral drug delivery systems have also been investigated in animal models to determine whether or not they are safe. Because of the parallel increase in the number of preclinical studies, it is now more vital than ever to develop and validate analytical methods for measuring the quantities of antiretroviral medications in physiological fluids.

Many methods for AZT determination have been recorded, with chromatography emerging as the most common from a survey of the relevant published information. Ten PBMC samples were collected from HIV-positive patients using ZDV medication, and their intracellular ZDV and anabolite levels were analyzed using High Performance Liquid Chromatography (HPLC) and Radioimmunoassay (RIA). With the help of click chemistry and fluorescence-based detection, commonly known as radiometric analysis, zidovudine-incorporated DNA may now be observed. The AZT concentration was determined using a combination of analytical techniques, including fluorescence derivatization, micellar liquid chromatography, and absorptive stripping voltammetry. Zidovudine in conjunction with other anti-retroviral medications in bodily fluids has been determined using a variety of extraction methods, including solid-liquid extraction, liquid-liquid extraction, column switching, deproteination, FT-IR, and UV. The findings have been shared with the public. A new method has been devised using surface-

enhanced Raman scattering (SERS) and density-functional theory (DFT) that relies on the molecular electrostatic potential (MEP) and the selective Raman bands enhancement. High performance thin layer chromatography (HPTLC), liquid chromatography (LC), and electrospray ionization mass spectrometry (MS/MS) have been proposed as a means of simultaneously estimating AZT concentrations in biological fluids and pharmaceutical goods. Methods for the identification of AZT and its active forms in aquatic environments and plasma include anion exchange solid phase extraction, liquid chromatography-tandem mass spectrometry, and LC-APCI-MS-MS. The substance's chemical make-up can be determined using these techniques. Neither a simple nor a cheap method for verifying AZT levels in human plasma is discussed in any of the available literature. The current study aimed to develop a validated HPLC-UV method for measuring AZT in solution and human plasma to facilitate routine pharmacokinetic and bioequivalence studies of AZT and related nanotechnology studies. For the chromatographic analysis, a Phenomenex C18 column was utilized, and the temperature was kept at room temperature. The mobile phase was created by mixing methanol and orthophosphoric acid at a ratio of 40:60% volume/volume at a flow rate of 1.0 milliliters per minute. The wavelength of 265 nm was decided to be ideal for the detector. The total amount of time spent running was ten minutes, and the volume of the injection was twenty liters.

In sum, this study was conducted with the intention of developing and validating method that makes use of nevirapine as an internal standard for quantifying Zidovudine in human plasma. The method was to make use of HPLC-UV technology. The extracted samples were chromatographed on a Phenomenex C18 column at room temperature using a mobile phase consisting of sixty percent orthophosphoric acid (v/v), one milliliter (mL) per minute (mL/min), forty percent methanol, and ultraviolet light with a wavelength of two hundred sixty-five nanometers (nm). The SCL-10AVP system controller is responsible for overseeing and managing the daily operations of the chromatographic equipment as a whole. For the purpose of the data gathering, LC solutions version 1.23 Service Pack 1 was used. During the chromatographic analysis, a Phenomenex C18 column was used, and the temperature was kept at room temperature. The ratio of methanol to orthophosphoric acid in the mobile phase was 40:60% (v/v), and the flow rate was 1.0 milliliters per minute. The wavelength of 265 nm was decided

to be ideal for the detector. The total time of the run was ten minutes, and the amount of the injection was 20 µL.

- **Sample and Standard solutions preparation:** Produced in diluent and kept at a temperature lower than 10 degrees Celsius, the drug stock standard solution had a concentration of 1.15 mg/mL, while the internal standard (IS) stock standard solution had a concentration of 1 mg/mL. The solutions were appropriately diluted in the medium of choice before to each and every application. We stored all of the solutions in the refrigerator at a temperature of four degrees Celsius and out of the light. After adding 25 L of working solutions to 475 L of drug-free human plasma, the resulting concentrations of AZT and IS (50 µg/mL) were 81.70, 163.4, 488.5, 1029.83, 2059.65, 3089.48, 4119.30, and 5263.5 µg/mL respectively. A comparable number of samples for quality control (QC) were prepared individually and pooled at low, medium, and high concentrations (245.10, 2631.78, and 3718.81 µg/mL, respectively). The samples were stored in a freezer at a temperature of -20 degrees Celsius until they were analyzed.

- **Treatment of Plasma Samples:** In preparation for the analysis, the frozen plasma samples were warmed back to room temperature. The samples were centrifuged at 5000 revolutions per minute for ten minutes in order to separate the plasma. An aliquot measuring 1.0 mL was pipetted into a 2.0 mL Eppendorf microcentrifuge tube, then 1 mL of methyl-t-butyl ether was added, and the mixture was vortexed for a total of two minutes. After that, the mixture went through another round of centrifugation at 5000 rpm for five full minutes. The residual powder was reconstituted by adding 300 µL of mobile phase and being aggressively vortexed after the supernatant layer was moved to a new tube and evaporated to dryness at 40 degrees Celsius in a nitrogen atmosphere. The HPLC system was given an injection of 20 µL.

In accordance with the current guidelines provided by the Food and Drug Administration (FDA) of the United States for the validation of bio analytical techniques, we conducted experiments to establish the method's accuracy, linearity, recovery, specificity, precision, LLOQ, and stability. The literature indicates that the upgraded HPLC-UV approach is sensitive, specific, and trustworthy. According to what was reported by the authors, linearity was also detected throughout a large range. This

method is appropriate for use in HIV/AIDS clinical therapeutic drug monitoring programs owing to the fact that it is simple to use and requires just a minimal amount of plasma sample preparation in order to accomplish rapid liquid-liquid extraction free of contamination. The authors come to the conclusion that the novel method is suitable for the regular assessment of Zidovudine in physiological fluids and is pertinent to studies pertaining toxicokinetics, Pharmacokinetics, bioavailability, and bioequivalence.

The blank plasma collected from different donors was evaluated to determine the selectivity of the suggested approach. Six separate batches of blank plasma were analyzed by chromatography to identify any endogenous components that might potentially interact with Zidovudine and the internal standard (nevirapine). Three independent elutions were performed on each sample, and the findings indicated that neither the medication nor the internal standard coeluted with any of the samples within their retention time range. Zidovudine's peak and the internal standard's peak were easily distinguishable from one another (Figures 1.3 and 1.4).

Figure 1.3

Chromatogram of Blank plasma

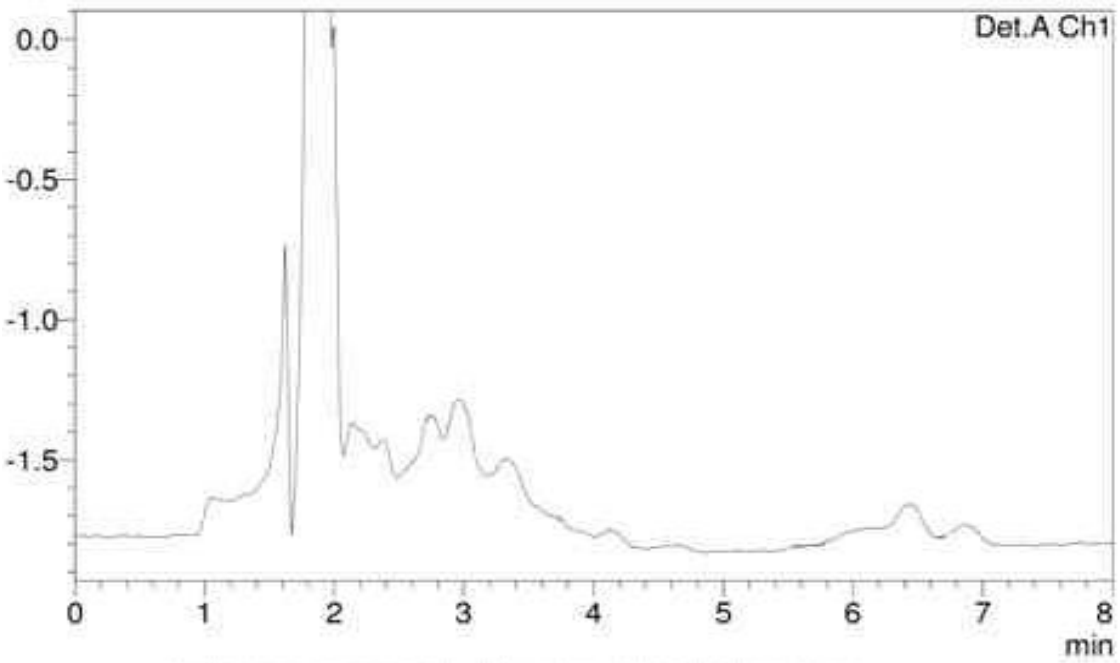


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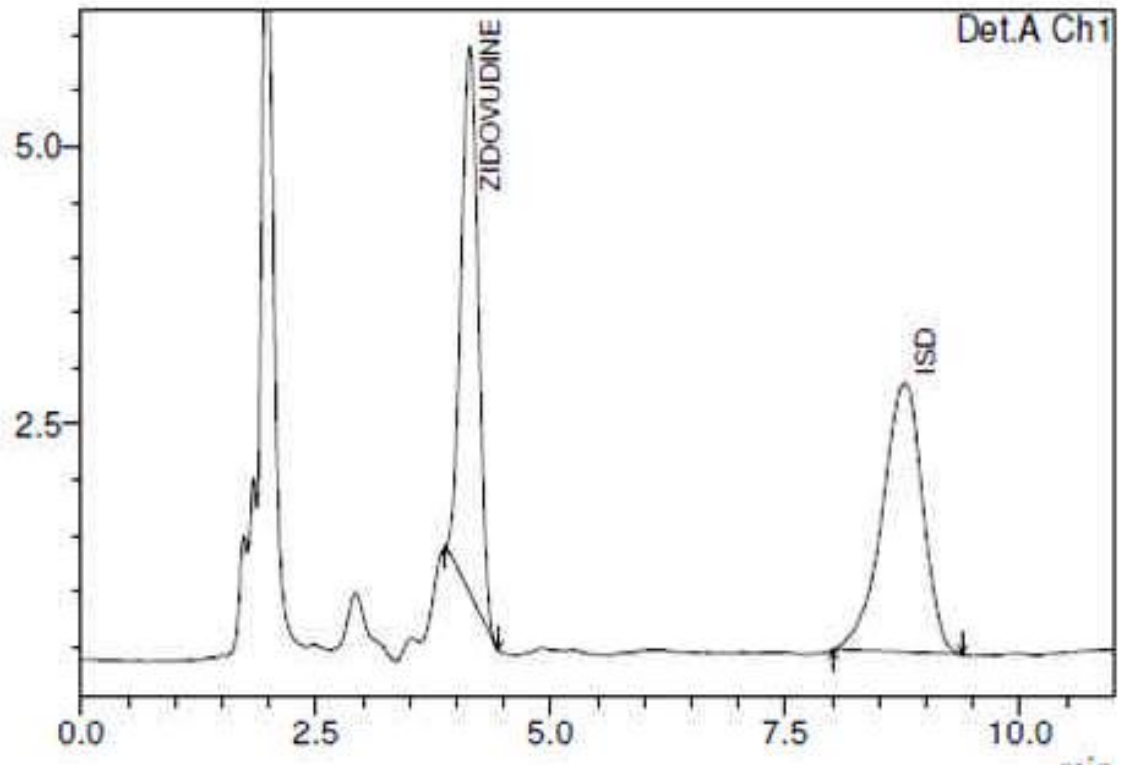
Figure 1.4

Chromatogram of Plasma spiked samples with Zidovudine and internal standard



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There was no interference between the drug and internal standard peak using this approach, and it was shown to be AZT specific. In addition, the technique was shown to have excellent linearity across the concentration ranges of 81.70 and 5263.55 µg/mL, with a r2 of 0.9974. Day-to-day fluctuations ranged from 0.375% to 4.955%. Overall, AZT had a 55.48% success rate in terms of patient recovery. The medicines were shown to be stable in plasma under all of the circumstances tested. The authors stated that the suggested technique was suitable for AZT therapeutic medication monitoring programs in clinical settings and would be helpful in evaluating pharmacokinetic profile and bioequivalence studies in HIV research.

Dayaramani et al. (2020) [13] provided a validated RP HPLC technique for quantifying Zidovudine Bulk that is straightforward, selective, precise, accurate, and economical. Chromatographic settings included a phenomenex® C18 (250 X 4.6 mm, 5 µm)

stationary phase, a mobile phase of water and methanol (60:40 v/v), a flow rate of 1.0 mL/min, and a constant column temperature of room temperature. The PDA detector was able to pick up Zidovudine at 266 nm.

- **Sample and Standard solutions preparation:** Using a volumetric flask with a 100 mL capacity, we opened 20 capsules, measured out 10 mg of Zidovudine, dissolved it in 60 mL of methanol, and sonicated the mixture for 30 minutes. The filtered solution was then rinsed with methanol to remove any remaining solids from Whatman filter paper No. 41. Methanol was used to make the solution the correct concentration. When making up a batch of the typical solution, the authors to make a standard solution with a concentration of 100 µg/mL, 10 mg of Zidovudine was accurately weighed, transported to a 100 mL volumetric flask, dissolved in 50 mL methanol, and diluted up to mark using methanol.

- **Analysis of Zidovudine in Capsule formulation**: The concentration in a range of 0.01 to10.0 µg/mL for Zidovudine was determined by diluting three separate aliquots of the sample solution with methanol. Finally, solutions were produced and evaluated by chromatography. Zidovudine concentration in the sample was calculated by plugging the area response into the regression equation, including terms for both medications.

The new technique was verified according to ICH standards for its specificity, linearity, accuracy, precision, sensitivity, robustness, and solution stability [19]. Zidovudine in bulk and capsule form may be routinely analyzed using the suggested approach. 20 µL of fluid was injected. Zidovudine was eluted at about 4.5 min during the chromatographic technique, whereas any possible interference peaks were detected after an analytical duration of 6 min. Zidovudine's purity peak plot at a purity index of 0.99974.

Dezani et al. (2021) [14] described, developed, and validated a selective and simple reversed-phase high-performance liquid chromatography (RP-HPLC) method for the simultaneous measurement of Zidovudine (AZT), stavudine (d4T), and lamivudine (3TC) in samples taken from Single-Pass Intestinal Perfusion experiments (Figure 1.5). For each and every test, a high-performance liquid chromatography (HPLC) system (Merck-Hitachi LaChrom®, USA) was used. The L-7100 pump, L-7200 autosampler,

L-7300 column oven, L-7400 UV detector, and L-7500 vacuum degasser made comprised this unique system (L-7612). An interface known as D-7000 allowed for all of the components of the system to speak with the software that was responsible for data collection and processing. This software had the product number 810866201, and it was designed to be used in conjunction with a chromatographic station. The program was known by its full title, which was HPLC System Manager Version 4.1, and it remained in operation from the years 1994 to 2001. The diameter of the Gemini C18 Phenomenex column that was employed as the stationary phase was 150 millimeters, while the thickness measured 4.6 millimeters and 5 micrometers. As a shield, this column used a C18 guard column with dimensions of 4 millimeters in thickness and a minimum diameter of 3.0 millimeters. The isocratic mobile phase was made up of a mixture of a 20mM phosphate buffer, acetonitrile, and methanol in a ratio of 90:7:3%. In order to get the pH down to 4.5µ, orthophosphoric acid was used. Before being used, the mobile phase was put through a filtration process using a PVDF membrane that had a pore size of 0.45 microns. Chromatographic analysis was carried out in an isocratic environment at a constant flow rate of 0.7 mL/min, while the UV detector signal was calibrated to a wavelength of 270 nm. The volume of the injection was set at 30 µL, and the temperature in the column oven was maintained at 35 degrees Celsius.

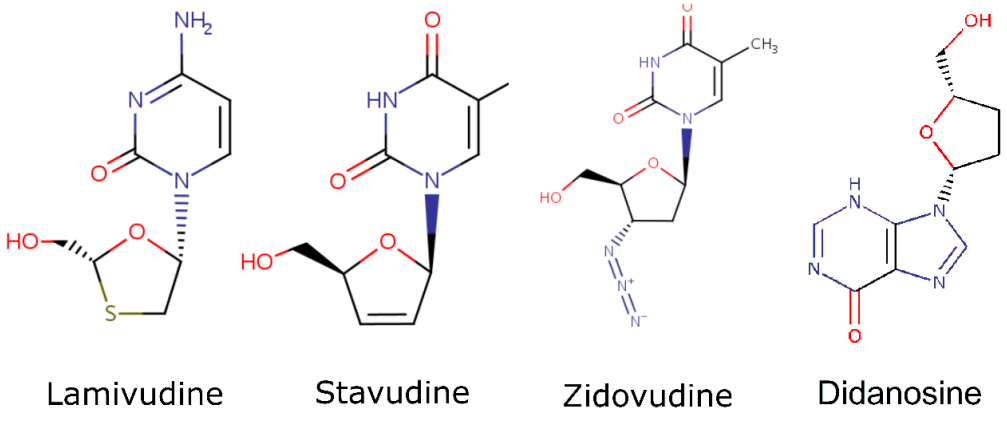
- **Sample preparation:** The perfusate samples were ready after being centrifuged at room temperature (3500rpm for 10min.) Mobile phase: 20 mM phosphate buffer, acetonitrile, and methanol, 90:7:3, v/v, %; sample: 150 µL; IS didanosine solution: 150 µL; total volume: 1200 µL. Prior to HPLC analysis, the mixtures were vortexed.

- **Standard solutions preparation:** Concentrations of 0.5–50 µg/mL, 10–200 µg/mL, and 10–200 µg/mL for d4T, 3TC, and AZT, respectively, were obtained by diluting the stock standard solution in a water and methanol combination with a ratio of 80:20%. The HPLC technique was developed using all standard operational solutions. The same steps were used to obtain the calibration curve during the validation process; only the perfusion solution was used instead of the water and methanol solution. Following the necessary procedures, all solutions were sonicated for 30 minutes before being used. After all that, an IS with a concentration of 125 µg/mL Didanosine was created by mixing water and methanol in a ratio of 80:20.

In the chromatographic study that was carried out using a Gemini C18 column, didanosine served as the reference standard that was measured against (IS). During the validation process, we took into consideration a variety of factors, including the linearity, selectivity, accuracy, and precision of the system. We were able to determine concentrations of 3TC as low as 0.32 ng/mL, d4T as low as 0.11 ng/mL, and AZT as low as 0.45 ng/mL. The upper limit for the quantification of 3TC was 1.06 g/mL, the lower limit for d4T was 0.38 g/mL, and the upper limit for AZT was 1.51 g/mL. Both repeatability and intermediate accuracy, both of which were expressed as a percentage of relative standard deviation, ranged anywhere from 1.05 to 1.31 and 1.50 to 1.87, respectively (RSD). Based on these findings, it is reasonable to conclude that the validated RP-HPLC method might be used for the simultaneous detection of 3TC, d4T, and AZT in perfusate samples. It is essential to have a technique that is both easy to use and accurate enough to test for many antiretroviral medicines in a single sample. This is because antiretroviral medications are often given in conjunction with one another while treating HIV/AIDS patients. The findings of this study might also serve as a basis for developing processes that can be used in settings that are analogous to the one that was under investigation.

Figure 1.5

Chemical structures of the antiretroviral drugs used in this study



Adopted from "Development and validation of RP-HPLC method for simultaneous determination of lamivudine, stavudine, and zidovudine in perfusate samples: Application to the Single-Pass Intestinal Perfusion (SPIP) studies," by Dezani, T.M., et al. (2021). Brazilian Journal of Pharmaceutical Sciences, 57. Copyright 2021 Brazilian Journal of Pharmaceutical Sciences.

1.9 Literature summary

Chromatography was found to be the method that is used to detect AZT the most frequently after researchers reviewed the existing literature and discovered that it has been described as one of the many AZT detection techniques. Chromatography was found to be the most effective method for detecting AZT. These results further highlight the need of creating detection systems that are not only straightforward but also selective, reasonably priced, sensitive, and scalable. Unfortunately, due to the fact that their structure does not include any chromophore groups, it is not possible to do direct UV detection with them. The majority of techniques call on the formation of ion-pair complexes as a prerequisite for detection in the visible and ultraviolet spectrums. Studies that were published on drug analysis made use of a variety of hues. The intricate nature of the drug served as inspiration for the color choices that were used. Certain procedures call for extra reactions, heating, and expensive equipment, which limits their applicability to particular fields of endeavor and renders them unfit for wide-scale implementation. As a direct consequence of this, they are only suitable for a select few applications.

1.10 Zidovudine analysis

In accordance with the recommendations of the international pharmacopoeia, zidovudine was subjected to analysis by infrared absorption spectrophotometry, standard comparisons, and liquid chromatography. Liquid chromatography was also used to establish zidovudine's level of purity. Zidovudine can be identified in pharmaceutical formulations through the use of ultraviolet absorption spectrophotometry, thin-layer chromatography, and liquid chromatography. Ultraviolet absorption spectrophotometry and liquid chromatography are both used to determine the concentration of zidovudine [50, 51]. It has been reported as a straightforward and fast method for determining the concentration of zidovudine in plasma to use high-performance liquid chromatography (HPLC) with a reversed-phase column and detection at 265 nm. The detection criteria for the drug in plasma and urine, respectively, were set at 20 and 200 ng/mL. It has been shown that zidovudine can be measured using isocratic high-performance liquid chromatography in serum, milk, and tissues. Zidovudine, much like other anti-human immunodeficiency virus (HIV) medications, is now put through a colorimetric test to determine whether or not it is

effective. There have been reports that zidovudine and its metabolites may be concurrently measured in serum and urine by employing HPLC using a technique that involves column swapping. In order to figure out how much zidovudine is present in the serum, researchers have turned to both the enzyme-linked immunosorbent test and the time-resolved fluoroimmunoassay. Paper and thin-layer chromatography were used in an investigation of zidovudine and many other nucleoside compounds [51].

1.11 Aim of the study

We aim in this thesis to develop a series of Zidovudine derivatives based on click reaction using adequate chromophores. Thus, the need to understand the Pharmacokinetics of drugs that can cause beneficial or adverse side effects, even in small amounts, is essential. Therefore, a sensitive analytical method is paramount when determining small amounts of drugs in biological samples. Due to its high sensitivity and selectivity, High-performance liquid chromatography (HPLC) detection is a powerful analytical technique for determining drugs. However, many chemicals of interest cannot be detected using HPLC because they lack the necessary chromophoric, fluorophoric, or redox groups required for detection. To that end, we develop a simple, chemical derivatization of Zidovudine based on the click reaction with Azide conjugated alkyne, resulting in an extended conjugation, five-membered heterocycle (1,2,3-triazole). This results in a shift in drug absorbance which in turn translate it into a more of a bathochromic and hyperchromic shift. As we will show, expands the UV detection range, facilitating the creation of a novel analytical detection method.

1.12 Objectives

Our objective in this thesis is to:

- Develop a series of derivatives of Zidovudine that are based on the click reaction. This while also using adequate chromophores and developing an accurate and sensitive HPLC method for their validation.

- To synthesize pure chemical derivatives of the Zidovudine and use it as standard in the method development.

- Validation with an HPLC method using standard metrics, such as the range, robustness/ruggedness, linearity accuracy, the limit of detection (LOD), the limit of quantification (LOQ), and precision.

Chapter Two

Methodology

2.1 Reagents and materials

Zidovudine (Cas. #: 30516-87-1), Ethynyltoluene (Cas. # 766-97-2), N, N-Diisopropylethy-lamine (CaS. #: 7087-68-5), were attained from Sigma-Aldrich Co., United States. The product was purified into column chromatography using silica gel (Pore size 60 Å, 40-63 µm particle size, 230-400 mesh particle size, Sigma Aldrich Co.). Thin layer chromatography (TLC) (DC-Fertigfolien Alugeram ® Sil 6 G/UV 254, Macherey Nagel Company, Germany) was used to examine the reaction. All materials and reagents are used with the highest grade of purity.

Dichloromethane (DCM), hexane (Hex), and Ethyl acetate 99.5% (EtOAc) were acquired from C.S. Company, Haifa. Copper(I) iodide (CuI) was purchased from Merck Millipore, USA and acetic acid was purchased from El Sahms Co, Palestine. Acetonitrile supragradient grade for chromatography (ACN) was purchased from SDFCL, India.

2.2 Instrumentations

Used a binary HPLC pump (Waters 1525, Singapore) and a photodiode array detector. The spectral data were acquired using a Bruker 500 MHz-Avance III NMR spectrometer in Switzerland. A Jenway/7315 ultraviolet (UV) spectrophotometer. Weighing scale with extreme precision (Adventurer ®, Ohaus Company, USA). Sonicator for a water bath (Elmasonic S 70 Hz, Elma, Germany). The rotary evaporator (Stuart ® RE400/MS, made in the UK) is in use. Shaker (MEMMERT, GMBH).

All synthetic and analytical procedures were performed at the Pharmacy Labs (An-Najah National University, Nablus), except for NMR was done at the University of Jordan.

2.3 Synthesis of Zidovudine derivative

2 mL of dichloromethane was put on a mixture of Zidovudine (100.9 mg, 0.37 mmol), CuI (14.4 mg, 0.074 mmol), DIPEA (17.8 mg 24µl, 0.148 mmol) acetic acid (1µL,0.0148 mmol) and Ethynyltoluene (47.45 mg, 51.8µl, 0.077mmole) which was

stirred for 2h at room temperature. The reaction was monitored by TLC using mobile phase (Ethyl acetate 3: Hexane 1) and when it showed completion of the reaction, the reaction was quenched. The organic layers were collected and evaporated in a rotary evaporator, once the reaction was handled with DCM and 2M HCl (x3). A solid white product was obtained with a yield of 58% after the remaining crude was refined by flash chromatography on silica gel eluting with (Hex: EtOAc 1:2). Rf is 0.76 (EtOAc:Hex 3:1). (EtOAc:Hex 3:1). H NMR (500 MHz, DMSO): δ 11.36 (s, 1H, NH), 8.72 (s, 1H, Ar), 7.84 (s, 1H, H-triazole), 7.75 (d, 2H, *J* = 7.8 Hz, Ar), 7.28 (d, 2H, *J* = 7.3 Hz, Ar, CH pyrimidine), 6.45 (t, 1H, *J* = 6.4 Hz, CHN), 5.41-5.38 (m, 1H, CH-triazole), 5.31-5.27 (m, 1H, CH ribose), 4.27 (bs, 1H, OH), 3.74-3.65 (m, 2H, $C\underline{H_2}OH$), 2.81-2.68 (m, 2H, CH_2 ribose), 2.33 (s, 3H, CH_3), 1.82 (s, 3H, CH_3).

2.4 HPLC Analytical Method Development

2.4.1 Analytical method development

In order to calculate the optimal value for the zidovudine derivative λ_{ma}, a working standard solution containing 0.2 mg/ml was prepared. This solution was then scanned in the ultraviolet wavelength range of 200-400 nm using MeOH as a blank. It was discovered that the medication exhibited maximum absorbance at 260 nm; hence, this wavelength was selected as the detection wavelength for the purpose of determining the zidovudine derivative concentration. To get appropriate and optimum HPLC conditions. It was found necessary to test several mobile and stationary phases. The preparation of the mobile phase contained three different compositions: (1) methanol and water in a ratio of 75:25v/v; (2) water and acetonitrile in a ratio of 50:50v/v; and (3) water and acetonitrile in a ratio of 70:30v/v. The mobile phase was operated at a flow rate of between 1 and 2 ml/min.

2.4.2 Analytical Method Validation

The validation was developed following the FDA and ICH guidelines [19, 34]. The validation parameters that were tested include the followings: linearity, range, accuracy, precision, robustness, and ruggedness [19, 46]. The system suitability test was checked for the HPLC-developed method, where the eluted peaks were tested for parameters such as symmetry, theoretical plates, and resolution.

2.4.3 Linearity and range

In order to evaluate the linearity and the range, five reaction mixtures of Zidovudine with concentrations of 2, 4, 6, 10, and 12 mg/ml were injected into the HPLC. A plot of the mean area under the curve (AUC) versus the concentration generated by the calibration curve. The generated calibration curve allows us to calculate the square correlation coefficient (R^2) and the regression equations.

2.4.4 Accuracy

The accuracy of a method is described by the closeness of the measurement from the regression line obtained from the AUC values given by the HPLC against the concentrations used (Sec., 2.4.2). The accuracy was calculated by preparing a reaction of Zidovudine with a concentration of 8 mg/ml. This was then evaluated by calculating the percentage of recovery given by the ratio between the AUC_{sample} and AUC_{std} as follows [36]:

$$Accuracy = \frac{AUC_{sample}}{AUC_{std}} \times 100\% \tag{2.1}$$

2.4.5 Precision

Several layers of precision work were accomplished. To begin, five injections of derivatized Zidovudine (4 mg/mL) were performed, and the percent relative standard deviation (RSD) of the chromatogram's produced peaks was determined to assess the instrument's precision. Concentrations of 2 and 4 mg/mL were used to investigate intraday accuracy, while a concentration of 10 mg/mL was used to evaluate inter-analyst precision. Later, the %RSD was calculated. If the RSD is less than 2, the established procedure is deemed precise.

2.4.6 Robustness

Subtle adjustments to the mobile phase composition, detecting wavelength, and mobile flow rate was used to test the suggested method's robustness[47]. The effects of the mobile phase flow-rate (0.9 & 1.1 mL/min), the detection wavelength (258 nm and 262 nm), and the mobile phase composition (41:59 and 39:61) were all examined.

2.4.7 Sensitivity

The sensitivity is measured in relation to these two detection thresholds. To determine the two values, we use within the HPLC baseline the signal-to-noise ratio (S/N). LOD & LOQ values of the tested compounds were resolved while the (S/N) is 3 to 1 and 10 to 1, respectively [21].

Chapter Three

Results and Discussion

3.1 Synthesis of Zidovudine derivative [48]

The click reaction, a targeted cycloaddition between an alkyne and an azide in the presence of Cu(I), has inspired a new pre-column derivatization technique for the chromatographic study of azide compounds. Click reaction is one of the most effective in creating a very pure product with a high yield; azide is conjugated to alkyne to form a five-membered heterocycle (1,2,3-triazole) with an extended conjugation, which changes the absorbance of the drug to a more hyperchromic and bathochromic shift. Since Zidovudine contains an azide and ethynyl toluene, an alkyne, we may employ these groups to catalyze the reaction between complementary chromophores, so increasing their UV detection in a novel fashion. Figure (3.1) depicts the reaction synthesis while the TLC was used to monitor and help determine the optimum time for which the reaction will be completed. The optimal response time was determined to be 2 hours after several experiments. 1H NMR analysis, as shown in Figure (3.2), was used to confirm the product's structure. At 8.72-6.5 ppm, the aromatic ring proton and pyrimidine proton were detected. The two methyl groups were detected at 2.33 and 1.82 ppm, while the ribose moiety's proton range was from 5.42 to 2.69 ppm.

Figure 3.1

Synthetic scheme Zidovudine derivative

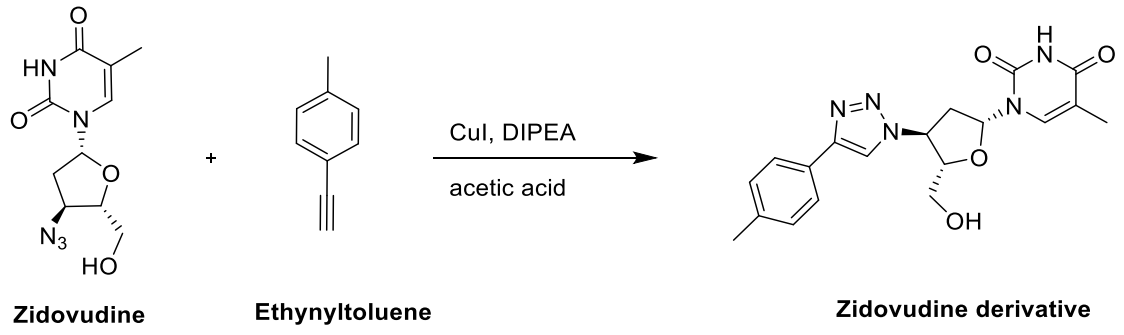
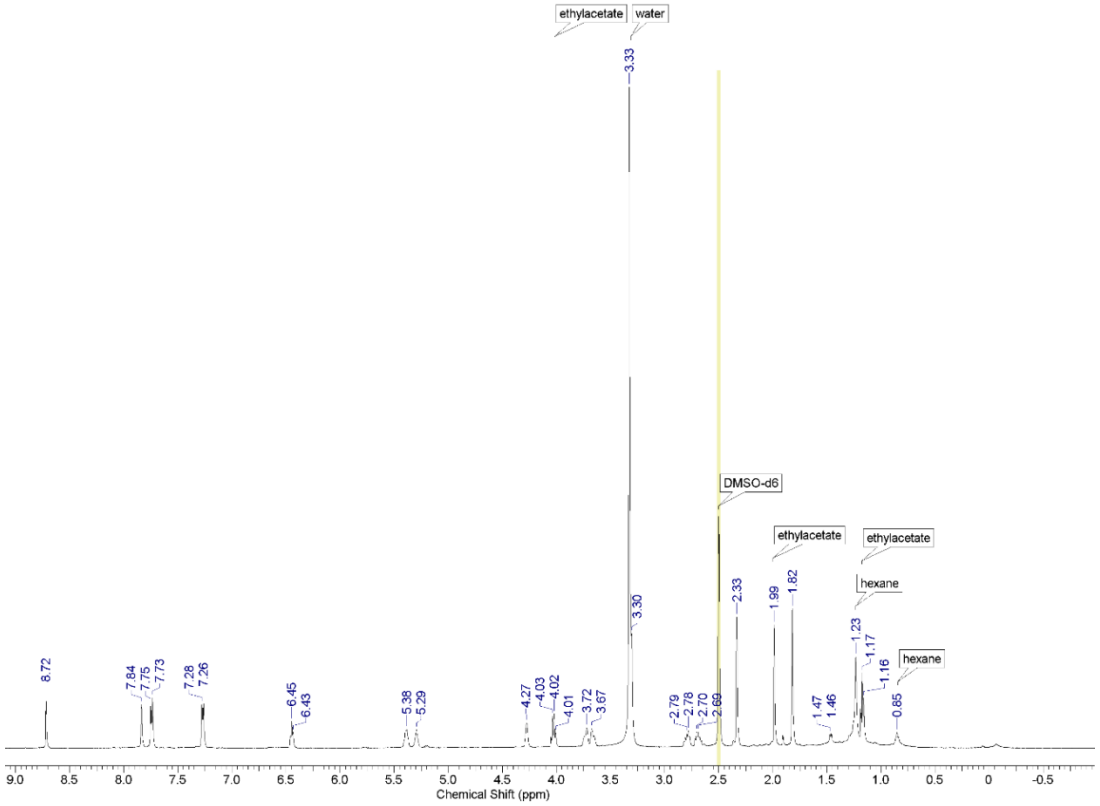


Figure 3.2

1H NMR spectrum of zidovudine derivative



3.2 HPLC analytical method development

3.2.1 Method development

A reverse-phase HPLC analytical method was developed and validated according to ICH guidelines [19]. Several trials were performed to attain the best separation by mainly modifying the ratios of the mobile phase mixture, the diluent used for the component mixture, and the HPLC column until the best well-separated peaks were obtained. Specifications for the final reverse-phase chromatographic HPLC system that was created. The XTERRA® MS system with a C18 column and a 260 nm detection technique was used. Table (3.1) summarizes the HPLC conditions. As can be seen in Figure 3.3, the eluted peaks were symmetrical with limited broadening and eluted at distinct retention durations, 2.650 min for Zidovudine, 3.255 min for the derivative, and 7.145 min for ethyl toluene (Figure 3.3).

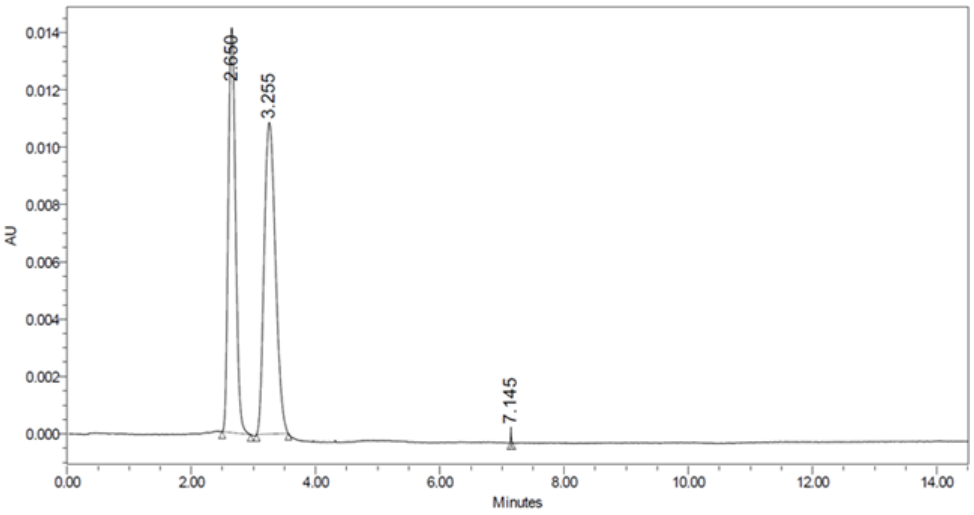
Table 3.1

The HPLC chromatographic conditions

HPLC Chromatographic conditions	
Mobile phase composition	Water-ACN 70:30 v/v
Flow rate	1mL/min
Wavelength (λ)	260 nm
Stationary phase	XTERRA® MS C18, 5µm, 4.6×250 mm cartridge
Column T	25°C
Injection V	10 mL
Run time	20 min

Figure 3.3

Component mixture of eluted peaks form the Chromatogram



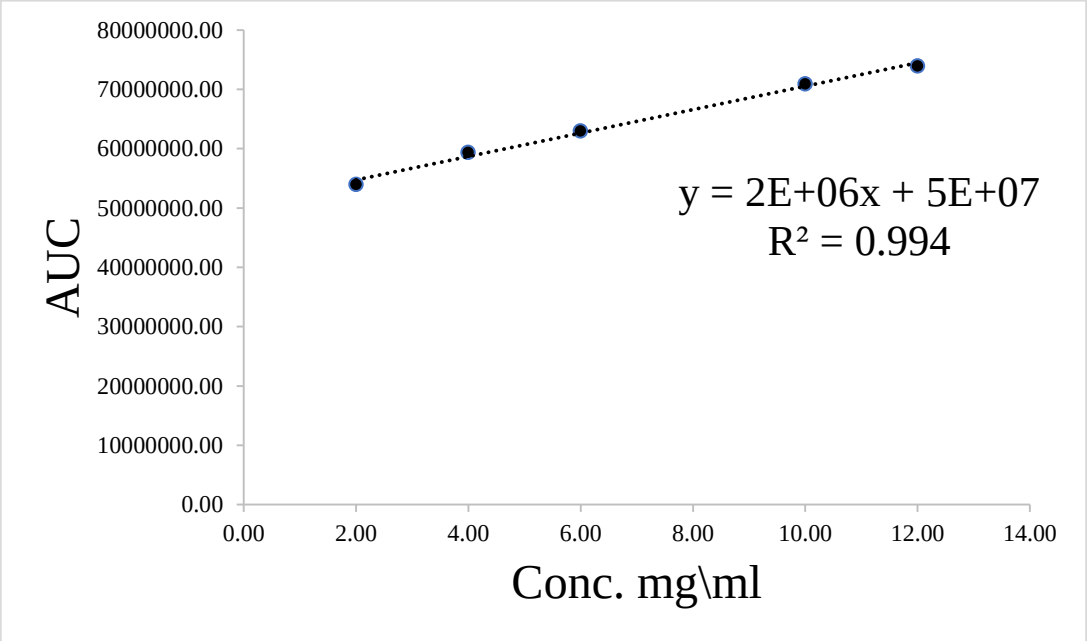
3.3 Method validation

3.3.1 Linearity and range

The area under the curve calculated from the drug's HPLC readings vs. concentration was used to evaluate the method's linearity. Figure (3.4) displays the slope of the regression line for the derivative. As shown by an R^2 value was 0.994, indicating that a linear relation between the measured peak area and sample concentration. This is within the accepted limits according to the ICH guidelines which needs to be above 0.970 [49].

Figure 3.4

Linearity curves for the product



3.3.2 Accuracy and selectivity

The accuracy of the method is computed using the AUC_{sample} (peak area of 8 mg of Zidovudine) and AUC_{std} (peak area of the synthesized standard) that resulted in 101.17%, a 1.17% difference between the measured and the value calculated by applying the regression line equation for the value of 8 mg/ml. These results demonstrate the quality of the fitted regression line parameters and the established linear relation between the sample concertation and measured AUC. According to the ICH guidelines [49], the criteria for acceptance with a range average recovery of 80-120% and 90-110% were met, with it being 101.17%.

3.3.3 Precision

Several distinct ranges were investigated for this parameter; the %RSD was determined to be 0.74 by syringing 4 mg/mL 5 times on HPLC, indicating the instrument's precision. For the intraday precision, calculations were performed by repetition of the measurements three times with two doses 2 and 4 mg/mL. All concentrations were tested in triplicate, and the findings revealed that the %RSD was less than 2.0. In addition, three sets of measurements were performed at 10 mg/mL to assess repeatability among analysts, yielding an %RSD of 0.36%. A breakdown of results is listed in Table (3.2). According to the ICH guidelines [49], our reportable result were found to be within the specified range. Additionally, the individual results were also found to agree with the predefined RSD, 2%, and are covered by the validation range.

Table 3.2

Results of the intermediate precision, instrument precision, and different analysts

Precision parameter	Conc.mg/ml	AUC	Average	%RSD
Intermediate precision	2	63142215	63376544	0.52
		63845203		
		63142215		
	4	60411534	60011319	0.77
		60254781		
		59367643		
Instrument precision	4	60411534	59800541	0.74
		60254781		
		59367643		
		59505041		
		59463705		
Different analysts	10	58318820	58469379	0.36
		58770496		
		58318820		

3.3.4 Detection and quantification limit (LOD &LOQ)

The detection limit, or LOD is the lowest amount of analyte in a sample that can be detected. It may be expressed as a concentration that gives a signal-to-noise ratio of approximately 3:1. The limit of quantification or LOQ is the lowest amount of analyte in a sample that can be determined with acceptable precision and accuracy with a signal-to-noise ratio of approximately 10:1. The LOD was calculated for the product. It was found to be $4*10^{-8}$ mg/ml. This while the calculated LOQ was $4*10^{-7}$ mg/ml. According to the ICH guidelines [49], with a ratio of 3:1 signal-to-noise. Estimating the

detection limit with such ratio is acceptable. As for the LOQ, a 10:1 ratio is the desirable one. For chromatographic procedures. The signal-to-noise ratio for a chromatographic procedure, are ought to be determined inside a predefined region. The best outcome will be if the ratio is situated in an equal manner around the peak of interest. Our results show that these were met.

3.3.5 Robustness

Analytical procedures may be judged by how well they withstand typical use and how little they change when subjected to small but purposeful changes in the technique parameters. For this experiment, we experimented with different wavelengths, flow rates, and mobile phase compositions. Tables (3.3), (3.4), and (3.5) display the data (4.5). Take note of the variation in an area that results from the varying amounts of sample injections. According to the ICH guidelines [49], the final reportable result is within the specifications and the individual results agree to a defined RSD, 2%, and are covered by the validation range.

Table 3.3

Results of the robustness validation of the wavelength of the maximal absorption parameter

The wavelength of maximal absorption (λ_{max})	AUC	Average	%RSD
262 nm	206174254	202154930	1.15
	200970567		
258 nm	200789556		
	200685346		

Table 3.4

Results of the robustness validation the flow rate parameter

Flow rate	AUC	Average	%RSD
Flow rate of 1.1 mL/min	98615015.6	99964898	0.96
	98615015.6		
	100874657		
Flow rate of 0.9 mL/min	100629378		
	100593529		
	100461798		

Table 3.5

Results of the robustness validation of the Mobile phase composition parameter

Mobile phase composition	AUC	Average	%RSD
61:39	60788341	59798039	1.6
	60237052		
	57942643		
59:41	59063583		
	60744347		
	60012272		

3.4 System suitability

Tests of a system's appropriateness are carried out in order to validate the precision of an analytical approach and the data that it relies on. Using the novel methodology, it was discovered that all of the conventional system suitability criteria, such as resolution, symmetry of peaks and theoretical plates, and retention factor (K), fell within the parameters of what are considered to be acceptable ranges. The depiction of these findings can be seen in Figure (3.5), and the relevant data can be found in Table (3.6). According to the requirements outlined in Section 2.5.5, a resolution is considered final if it meets or exceeds the 1.5 criterion. In this instance, we obtained a score of 2.24. Additionally, for the asymmetry or tailing factor, the ideal peak will have A_s =1, however, values in the range 0.9 –1.1 are also acceptable, with the tailing becoming apparent when the asymmetry factor equal to or exceeds 1.2. Here we reached the ideal one with A_s =1, yet another indication of the stability of the system and its robustness to changes. In the case of the theoretical plates, it should not fall below 2000; in our case, it was 2608. Finally, the retention factor, ideally, would be greater than 2.0; in our experiments, it was 1.99, which is ideal. In sum, in all System suitability metrics, we met the required conditions and reached the ideal case for the asymmetry, tailing, and retention factors.

Figure 3. 5

Chromatogram for system suitability

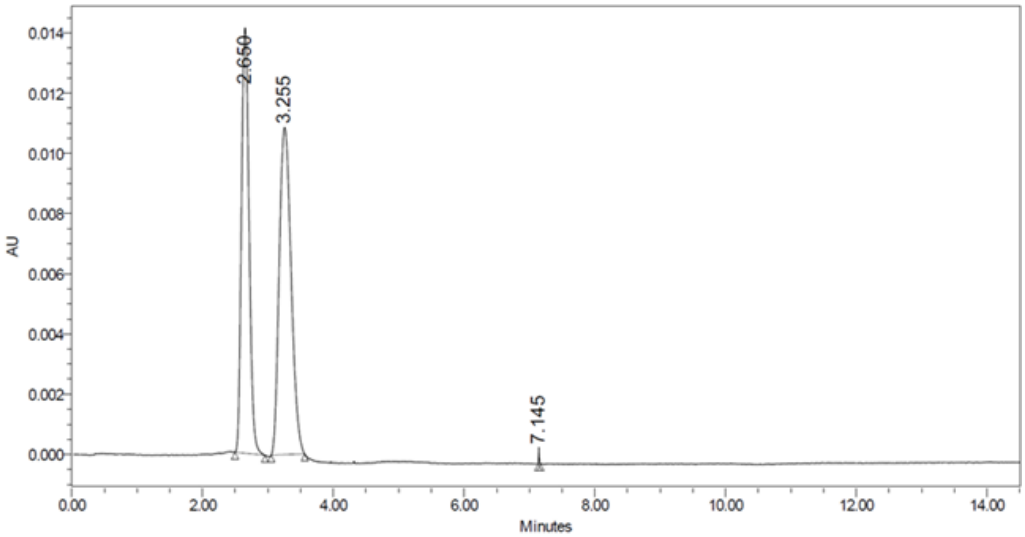


Table 3.6

System suitability

Zidovudine derivative	
Resolution (R) BP	2.24
Symmetry of peaks	1
Theoretical plates (N)	2608
Retention factor (K)	1.99

3.5 Comparison to state-of-the-art

Next, we compared our results with state-of-the-art methods by [16] and [15]. Results are listed in Table (3.7), and demonstrate we outperform [16] in all metrics while we outperform the method by [15] in terms of the LOD, LOQ, and tailing factor and comparable results in the other metrics. These results show the potential of using our proposed approach over other methods. Note that this is done while requiring fewer resources and through more simple and cost-effective procedures. The improvement we demonstrate by an order-of-magnitude in LOD and factor in LOQ values demonstrate that this method is much more sensitive, thus further improving detection. This, as we showed in the introduction and literature review is paramount for the derivatization of AZT. Additionally, the tailing factor metric also shows that our method is more selective than the other methods by sharpness of the peaks as we demonstrated through the results section.

Table 3.7

Comparison to other methods

Metric	Kusuma et al. (2011) [16]	Haneef et al. (2013) [15]	Ours
linearity (R2)	0.995	0.999	0.994
Accuracy [%]	102.5	100.65	101.17
Pre intraday (RSD%)	1.01	0.16	0.52
LOD [µg/ml]	0.154	0.27*10-3	0.41*10-4
LOQ [µg/ml]	0.825	0.83*10-3	0.37*10-3
Tailing factor [-]	1.16	1.3	1
Theoretical plate [-]	4044	2157	2608
robustness (RSD%)	NA	NA	1.24

Chapter Four

Conclusion

In this thesis, an HPLC analytical method has been developed and validated for the purpose of estimating Zidovudine within a formulation. The proposed method is accurate, simple, rapid, and specific. The simple nature of the proposed method opens the possibility to perform routine Zidovudine analysis in low-budget laboratories, ones that lack sophisticated analytical instruments. The proposed HPLC method with UV/Vis detector was found to meet the criteria of drug system suitability, resolution, and peak integrity. Additionally, we showed that the method is found to be sensitive by the limits achieved in quantification and detection. The analysis shows that it adapted derivatization of Zidovudine measured at λ_{max} = 260 nm, for which the Zidovudine derivatize eluted peak was separated from other reagents. Then validated and found to be within the accepted limits, linear (R^2 = 0.994), precise (RSD = 0.59), accurate (% recovery = 101.17), and sensitive to LOD ($4*10^{-8}$ mg/ml) and LOQ ($4*10^{-7}$ mg/ml).

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جـامعـــة النجاح الوطنية

كليـــة الدراســـات العليـــا

الاشتقاق الكيميائي للزيدوفودين بناءً على تفاعل كليك لتطوير طريقة تحليلية جديدة

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قدّمت هذه الرسالة استكمالاً لمتطلبات الحصول على درجة الماجستير في العلوم الصيدلانية، من كلية الدّراسات العليا، في جامعة النجاح الوطنية، نابلس– فلسطين.

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الملخص

الزيدوفيدين دواء يستخدم لعلاج المرضى المصابين بفيروس نقص المناعة البشرية (HIV). بعد تحويله بواسطة الإنزيمات الخلوية إلى زيدوفيدين تريوفوسفات، يثبط الزيدوفيدين النسخ العكسي لفيروس HIV يحتوي عقار الزيدوفيدين على سميات كبيرة خاصة بالأدوية ومحددة للجرعات تؤدي إلى نافذة علاجية صغيرة تتراوح بين الحد الأدنى من الجرعات الفعالة والحد الأقصى للجرعات التي يمكن تحملها. وتشمل أخطر هذه الآثار الضارة سمية نخاع العظام، والاعتلال العضلي، والتشوهات الكبدية. ومن ثم، يوجد طلب على التقنيات التحليلية التي تتيح قياسات فعالة ودقيقة لـ الزيدوفيدين، ويجب أن تتمتع التقنيات التحليلية المكيفة بحساسية كافية أو انتقائية أو كليهما من أجل الحصول على قياسات دقيقة أكثر قابلية للإدارة للإجراءات الكيميائية المختلفة. يعد تحويل المجموعات الوظيفية داخل الجزيء أحد الأساليب التكيفية المستخدمة في الاشتقاق الكيميائي. في هذه الرسالة، تم تطوير طريقة تحليلية جديدة للاشتقاق الكيميائي لعقار الزيدوفيدين تحقيقًا لهذه الغاية، استخدمنا تفاعل كليك مع أزيد ألكين المترافق، مما أدى إلى حلقه خماسية (1،2،3-تريازول) واقتران ممتد. في هذه الأطروحة، نهدف إلى تطوير طريقة تحليلية حساسة وانتقائية. تم تطوير الطريقة التحليلية المقترحة باستخدام HPLC مع كاشف UV / Vis وتم التحقق من صحتها وفقًا لإرشادات المؤتمر الدولي للتنسيق وإدارة الغذاء والدواء وباستخدام المقاييس مثل الخطي، المدى، الدقة، حد الكشف وحد التعيين الكمي. اعتمدنا بالطريقة المطورة $\lambda max = 260$ لقياس مشتق زيدوفودين. الطريقة المستخدمة هي HPLC باستخدام طور متحرك متكون من ماء وأسيتونتريل بنسبه

70:30 v\v. تم فصل ذروة التصفية منزيدوفودين المشتق عن كواشف الاشتقاق الأخرى المستخدمة. ثم تم التحقق من صحة الطريقة التحليلية، ووجدت معاملات التحقق ضمن الحدود المقبولة. تم العثور على الطريقة المطورة لتكون خطية (R2 = 0.994) ومضبوطة (RSD = 0.59) ودقيقة (نسبة الاسترداد = 101.17). علاوة على ذلك، كانت الطريقة المطورة حساسة لـ حد الكشف (4*10−8 ملغم\ملل) وحد التعيين الكمي (4*10−7 ملغم\ملل). لذلك، فإن الطريقة التي تم تطويرها بسيطة وممكنة وذات حساسية وانتقائية عالية. يمكن تطبيقها في تحليل زيدوفودين في أشكال الجرعات والمواد الخام المختلفة، بما في ذلك المكونات الصيدلانية الفعالة. يمكن أن يستمر هذا العمل البحثي في المستقبل، ويمكن استخدام الطريقة المطورة لاختبار زيدوفودين في النظم البيولوجية.

الكلمات المفتاحية: زيدوفودين، تفاعل كليك، HPLC، الاشتقاق الكيميائي.