An-Najah National University

Faculty of Graduate Studies

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

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This Thesis Dissertation was Defended Successfully on 25/ 6/ 2023 and approved by

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Dedication

I dedicate this thesis to God Almighty, my source of inspiration, wisdom, and knowledge. This thesis is also dedicated to my husband, Abdulrahman who has been my rock of support and inspiration during the hurdles and obstacles through graduate school and life. My parents, Amjad and Naheda, 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:

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

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: _____

Signature: _____

Date: _____

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Abstract

Norethindrone is a synthetic progestin, which mimics female natural progesterone's effects but with a greater potency. Norethindrone-only progestin pill is mainly used as birth control pills by increasing uterine lining thinning and cervical mucus thickening. It is also used as hormonal replacement therapy alongside other steroidal hormones in treating conditions caused by abnormal hormonal levels. Norethindrone has significant dose-limiting properties, resulting in a small therapeutic window. Consequently, there is a need for analytical methods that allow for effective and reliable measurements of norethindrone. The adapted analytical methods must have enough sensitivity, selectivity, or both in order to evaluate different chemical processes more easily and accurately. One of the adaptive strategies utilized in chemical derivatization involves converting functional groups within a molecule. In this thesis, we made a proposal to create a novel analytical technique for norethindrone's chemical derivatization. This was accomplished by using a click reaction with an azide-conjugated alkyne, which produced a five-membered heterocycle ring (1,2,3-triazole) and an extended conjugation. Our goal was to create a sensitive and selective analytical method. The analytical method has been created using HPLC with a UV/Vis detector and validated using parameters like accuracy, linearity, range, precision, Limit of detection, and Limit of quantitation in accordance with the ICH and the FDA's guidelines. The developed method adapted λ_{max}= 245 nm for the measurement of the derivatized norethindrone. The method used HPLC using mobile phase MeOH-Water 60:40 v/v. The eluted peak of the derivatized norethindrone was separated from other used derivatization reagents. The analytical method was then validated, and validation parameters were found to be within the permitted limits. The developed method proved to be linear (R^2 = 0.9995),

precise (RSD = 1.07), and accurate (% recovery = 106.5%). Moreover, the developed method was sensitive to LOD ($2*10^{-6}$ mg/ml) and LOQ ($2*10^{-4}$ mg/ml). Therefore, the developed method is simple and practical and has excellent sensitivity and selectivity. Norethindrone may be analyzed in a variety of dosage forms and raw materials, including active pharmaceutical components. The good sensitivity values of our developed method suggest that it may be applied to analyze norethindrone in biological systems.

Keywords: click chemistry, chemical derivatization, norethindrone, analytical method development, high-performance liquid chromatography.

Chapter One

Introduction

1.1 Analytical method development of pharmaceuticals

The development and validation of analytical methods are crucial when it comes to the discovery, creation, and production of medicines. As more pharmaceuticals reach the market each year, it is imperative to create innovative testing techniques for these drugs. The process of developing a methodology is what establishes the viability of such analytical method [1].

The key objective of analytical chemistry is the qualitative and quantitative identification of the constituent parts of substances, samples, and mixtures. In a qualitative analysis, the components or analytes of a mixture or sample are identified. Whereas, quantitative analysis determines the quantity of the mixture's or sample's constituents or analytes [2]. An analytical method uses a specific methodology and step-by-step instructions to analyze a sample qualitatively, quantitatively, or structurally for one or more analytes [3]. The two basic categories of analytical procedures are: traditional and instrumental techniques. The traditional approach is one where the signal is inversely correlated with the absolute concentration of the analyte. The three primary categories of traditional approaches are: analyte separation, qualitative analysis, and quantitative analysis. Extraction, distillation, precipitation, and filtering are all processes used for separation of analytes. Boiling point, freezing point, color, refractive index, and other physical attributes, are some of the qualities that may be analyzed qualitatively. Both gravimetric and volumetric analysis are the main attributes that could be used for a quantitative measure. On the other hand, the instrumental approach uses the relationship between the signal and the concentration of the analytes which can be attained using different spectroscopic, electrochemical, and chromatographic methods [4].

Infrared spectroscopy (IR), ultraviolet-visible spectroscopy (UV-visible), X-ray spectroscopy are examples of spectroscopic methods. Whereas, the most used chromatographic methods for analysis are: High performance liquid chromatography (HPLC), column chromatography, paper chromatography, thin layer chromatography (TLC), and gas chromatography (GC).

The examination of how electromagnetic radiation affects matter is known as spectroscopy. Radiation (energy) is absorbed and emitted by the matter as a result of these interactions. UV-visible spectroscopy is a popular example of an absorption spectroscopy which studies the spectra produced when electromagnetic energy is absorbed by a material and is also considered a well-mentioned example of molecular spectroscopy which studies the method of how molecules are affected by electromagnetic radiation which leads to the consequent modifications on energy that take place at the molecular level. Fluorometry is an example of emission spectroscopy which refers to the study of the spectra produced by electromagnetic radiation that a sample emits [5].

The amount of light caught at each wavelength in the UV and visible sections is counted during UV-visible spectroscopy. The visible (VIS), 400-800 nm, and ultraviolet (UV), 200-400 nm, respectively, are the areas used in this absorption spectroscopy, which utilizes electromagnetic radiations in this range [6]. The fundamental idea behind UV-visible spectroscopy is that various spectra are produced when ultraviolet or visible light is absorbed by a sample or chemical entity. When a molecule absorbs UV light, the electrons inside of it are excited, which causes them to change in electrical energy level from one that is lower to one that is higher. The reverse sort of transition results in the ultraviolet emission spectrum. The investigation of functional groups, conjugation, and the identification of geometric isomers are all applications of UV spectroscopy [5].

Chromatography is a physically and chemically based technique for separating mixtures of substances. In chromatography, a stationary phase and a mobile phase are used to separate a mixture of substances into their distinct components [7]. The most precise chromatographic analytical technique is High Performance Liquid Chromatography (HPLC), which is frequently used for both quantitative and qualitative medicinal product analysis [8]. HPLC is able to separate, recognize, and measure any part of the sample that is able to be dissolved in liquid [9]. Adsorption is the primary operating principle in this technique. In this type of chromatography, the mobile phase is liquid. Tested compound sample is dissolved to form a liquid solution. A liquid phase (mobile phase) and a porous substance (stationary phase) make up a column into which a sample is inserted. The sample moves across the column as the mobile phase is delivered at high pressure by a pump. The movement of sample elements depends on their affinity

for the stationary phase. The component moves more slowly if it has a stronger attraction for the stationary phase. The element travels much faster when it is less drawn to the stationary phase. The elements are isolated from one another [10]. Efficiency, retention factor, selectivity, and resolution are basic chromatographic characteristics [11].

There are many followed steps that should be taken into account when developing an HPLC analytical method: assessing the physiochemical characteristics of the tested drug molecule, choosing the appropriate chromatographic settings, developing the analytical strategy, method optimization, appropriate sample preparation, and method validation.

1.1.1 Synthetic steroidal hormones pharmacopeial analytical methods

Steroids are physiologically active chemicals that may be found in a variety of natural sources and they are manufactured for therapeutic and medicinal uses. Steroid hormones (sex hormones, mineralo- and glucocorticoids, and anabolic steroids) and cholesterol are the different types of naturally occurring steroids. The 17 carbon atoms that make up the four-fused ring core of all steroids are the same, and the differences between them come from the functional groups that are bonded to the core or side chains [12]. In particular, natural steroids have a significant impact on metabolism, immunity, and reproduction. However, several synthetic steroids that imitate the effects of natural steroids have been created and are now approved for the treatment of a wide range of illnesses [13].

The evolution of steroid hormone drug analysis over the past 15 years may be summarized by the fact that HPLC unquestionably gained the greatest regularly employed technology and that the significance of chromatographic-spectroscopic methods is growing. In European Pharmacopiea, UV spectrophotometry is the most popular assay test technique at approximately 240 nm for steroids with powerfully absorbing 4-ene-3-oxo or 1,4-diene-3-oxo groups and at roughly 280 nm for estrogens with a phenoltype ring A and its faint but distinctive spectra. Since the vast majority of impurities are tested with the primary component, this approach is inherently non-specific. Highly specific reversed phase HPLC is the main procedure in the US Pharmacopoeia. Porous octadecylsilica support is employed for the RP in the vast majority of instances. The UV detector's most common wavelength setting is 254 nm,

which is not the wavelength at which 4-ene-3-oxo- or 1,4-diene-3-oxosteroids have their full spectrum [14].

1.2 Norethindrone

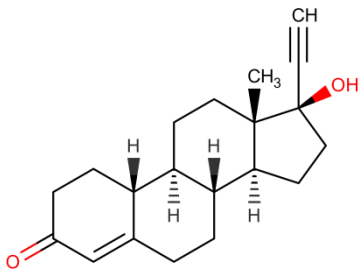
Norethindrone (NE), commonly known as norethisterone, is a synthetic second-generation progestin that imitates the actions of natural progesterone but with a greater achievable potency [15, 16]. Norethindrone exerts its pharmacological effect through its binding to the progesterone intracellular receptors in the reproductive system, which in turn activates the complex leading to its interaction with specific DNA sites, leading to an alteration of protein synthesis and as a result suppression of LH liberation [17]. Norethindrone tablet formulations have been used in oral contraceptive regimens. It is also used in hormonal replacement therapy as it helps in the prevention of endometrial hyperplasia. It is also proven to treat many cases such as endometriosis, secondary amenorrhea, and uterine hemorrhage brought on by abnormal hormonal levels [18].

Norethisterone inhibits ovulation when administered in amounts between 5-10 mg via acting on the pituitary. Additionally, endogenous oestrogen and progesterone synthesis is reduced, and the ectopic endometrium is transformed into a decidua resembled that of pregnancy. Norethisterone may affect tumor deposition directly or indirectly in cancer by inhibiting the pituitary [19].

Norethindrone has a chemical formula of $C_{20}H_{26}O_2$ and an IUPAC name of (8*R*,9*S*,10*R*,13*S*,14*S*,17*R*)-17-ethynyl-17-hydroxy-13-methyl-1,2,6,7,8,9,10,11,12,14,15 16-dodecahydrocyclopenta(a)phenanthren-3-one [15]. It belongs to the 19-progesterone-derived class of progestins [20]. Its molecular weight is 298.4 g/mol [21]. Figure 1.1 shows the norethindrone chemical structure [22].

Figure 1.1

Chemical structure of norethindrone



Norethindrone has an oral bioavailability of about 60% with a C_{max} ranging from 5.39 to 7.36 ng/mL and a T_{max} of 1-3 hours [22, 23]. A mean of 7.6 hours characterizes norethindrone's elimination half-life, which ranges from 5 to 12 hours [19]. Norethindrone is extensively biotransformed, with reduction being the most common pathway, followed by sulfate and glucuronide conjugation [24]. The given dosage is eliminated as metabolites in the urine and feces in amounts of around 60% [19]. The chromophores in the norethindrone structure absorb light at wavelengths greater than 290nm [24]. It has an attainable UV max absorption of 240nm in ethanol solvent [24]. Norethindrone has a water solubility of 7.04mg/L at 25℃ [24]. It is considered practically insoluble in water, soluble in chloroform and dioxane, and slightly soluble in ether [24]. It has a partition coefficient (log P) value of 2.97 [24]. Norethindrone has pKa values of -1.7 and 17.59 for its strongest acidic and strongest basic forms, respectively [22].

1.2.1 Literature ultraviolet-spectrophotometric determination of norethindrone/ norethindrone acetate

In 1970's, an ultraviolet curve of norethindrone was done using the U.S.P. reference standard material; its E (l%, lcm) at 240 nm was 575 [14, 25].

Reddy A., et al. 2010 developed a simple, accurate and validated UV-spectrophotometric method for the determination of norethindrone in commercially available tablets (primolut-N (Zota), Noritis (Cadila)) using Systronics – 2201 UV/Visible double beam spectrophotometer [26]. Via screening diluted solutions of norethindrone, a λ_{max} of 270 nm was found to be the optimum wavelength at which maximum absorbance is attained at minimum screened strength of the substance. A 0.5 µg/ml standard solution of norethindrone was prepared by using methanol as a solvent.

A calibration curve was then constructed through preparation of a series of standard solutions of norethindrone of appropriate aliquots (1.0-6.0 ml) from the originally prepared standard solution to give solutions with concentrations of 0.5-3.0 µg/ml after addition volume of methanol to the mark of 10 ml in a volumetric flask. Absorbance of each concentration was then measured at λ_{max} 270 nm against blank. The estimation of norethindrone in tablets were done via taking the average weight of twenty commercial pills of the norethindrone and then approximate of 50 mg was taken and further dilutions with methanol was made to attain a sonicated and filtered final solution of 1 µg/ml. Also, norethindrone's reference standard was prepared in the same way with same concentration level and absorbance was also measured on 270 nm against blank. Table 1.1 shows the amount found in tablet formulations (mg/tablet) vs. the label claim (mg/tablet) and the %recovery. The calibration plots' linear regression analysis results revealed a strong linear association with r2 = 0.999 in the measured concentration range of 0.5-3.0 g/ml. Norethisterone's LOD and LOQ were determined to be 19.87 mg/ml and 60.24 mg/ml, respectively.

Table 1.1

Norethindrone estimation in commercially available tablets

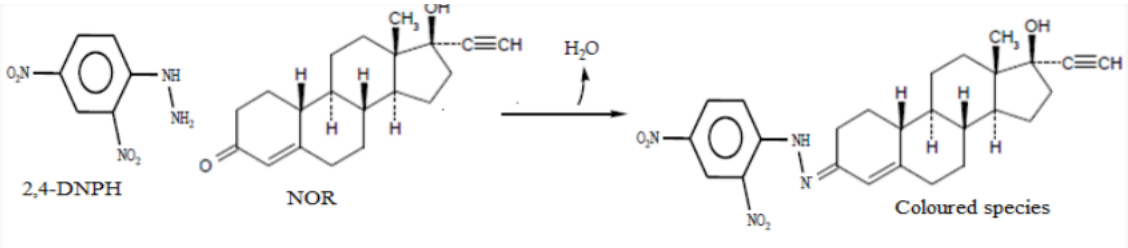
Brand	Label claim (mg/tablet)	Amount found (mg/tablet)	% Recovery
Primolut-N	5	4.907	98.15%
Noritis	5	4.965	99.3%

Lakshmi R., and R. Swamy, 2020 published a research paper similar to the later for the estimation of norethindrone in pure and commercially marketed formulation using UV-spectrophotometric analysis method while taking into account the advantage of the presence of keto group in norethindrone structure which allowed the development of an orange-colored derivatized compound of norethindrone through the condensation reaction with the chromogenic reagent 2,4-dinitrophenyl hydrazine (2,4-DNPH) with the presence of sodium hydroxide (NaOH) and potassium iodate (KIO_3) (scheme 1.1). Stock solution of 1mg/ml of norethindrone was prepared and then a standard solution of 100 µg/ml was made via a step wise dilution of the stock solution with distilled water. A series of 10 ml volumetric flasks were filled with aliquots of a (0.2 to 1.2 ml, 100 g/ml) Norethindrone working standard solution followed by the addition of 2,4-DNPH,

potassium iodate and sodium hydroxide to each of the aliquot's solutions, then stirred until the appearance orange color of the derivatized. With distilled water, the mixture was diluted up to 10 ml. The spectrum of absorption was captured, and the absorbance of the remaining solutions was assessed at 475 nm. The constructed calibration curve of the plot ranges showed a good linear relationship with r^2 = 0.998. Assay of norethindrone in the tested tablet gave a % recovery of 97.8% and the method was validated according to ICH guidelines. The precision (%RSD) was 1.418. LOD and LOQ for norethisterone was found to be 0.290 µg/ml and 0.890 µg/ml, respectively.

Scheme 1.1

The condensation reaction between norethindrone and 2,4-DNPH



In 2018, a UV-spectrophotometric method for the estimation of norethindrone acetate in bulk pharmaceutical products was developed and validated. The optimum λ_{max} was investigated via scanning of a standard stock solution of norethindrone acetate on a uv-spectroscopic range of (190-400 nm). The spectrum was obtained using methanol and water as the blanks in a 50:50 ratio, and the wavelength with the highest absorption was 256 nm. The calibration curve was constructed by preparing and scanning a range of solutions of different concentrations from norethindrone (25- 150 µg/ml). Accuracy percentage recovered was calculated to be 100.2%, from experiments on linearity, the correlation coefficient R2 was determined to be 0.9979, %RSD was determined to be 0.22 and 0.88, respectively, from investigations on precision and intermediate precision. LOD and LOQ were found to be 0.9 µg/ml and 3 µg/ml respectively. Premoult-Nor's commercial formulation was used for the assay, and the results showed that the purity level was 101.91% [27].

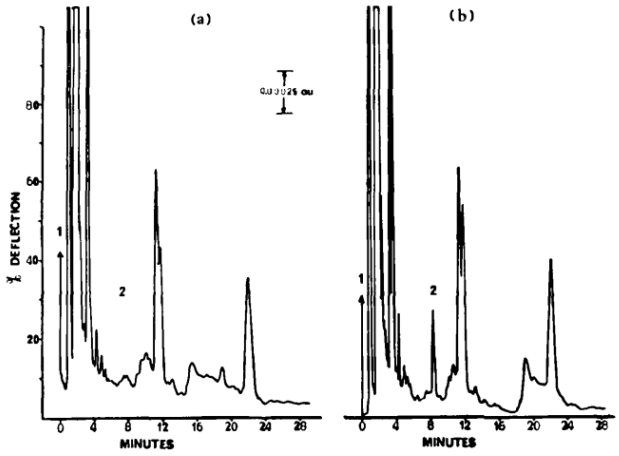
1.2.2 Literature HPLC analysis of norethindrone/norethindrone acetate alone and/or with other steroidal hormones

Currently, High Performance Liquid Chromatography is among the most effective instruments in analytical chemistry.

In accordance with the tremendous advancements in high-performance liquid chromatography (HPLC), many articles on the separation and analysis of steroids have been published in the late 1970s. The measurement of norethindrone in plasma is presented using a sensitive, precise, high-performance liquid chromatographic method configured with a UV detector using a wavelength of 254 nm to chromatograph the plasma organic solvent extraction on a reversed-phase column; quantification from plasma samples containing 2 ng/ml norethindrone were reported. Figure 1.2 shows a) a chromatogram shows the analysis of an injected sample with norethindrone at a concentration of 5 ng/ml and b) a chromatogram made from plasma that was collected from 8 human donors [28].

Figure 1.2

HPLC chromatogram of norethindrone from a prepared sample of known concentration and spiked human plasma sample, 1= injection, 2= norethindrone



In July. 1987, a chemist in OSHA analytical laboratories named Duane Lee was asked to do an HPLC analysis for norethindrone from air samples using FWS-B filters. Norethindrone was supplied by sigma Aldrich (CAS#: 68—22-4). Conditions and parts used in HPLC analysis: Alltech C18 10-µm, mobile phase ACN/H2O 70:30, at a flow rate of 1.0 ml/min, using Ultraviolet detector (Waters 440) Wavelength = 254 nm,

injection volume of 20 µl/injection. The chromatogram of norethindrone gave a peak with good resolution and symmetry on retention time of 6.4min and a calibration curve was constructed by plotting detector response vs. standard concentrations. A linear regression relationship was attained and the concentration of norethindrone in air samples were then measured through the application of linear equation of the calibration curve.

Several other papers focused primarily on the separation of progesterone and estrogens. Oral contraceptives (OCs), which combine the progesterone of 19-norethindrone (NE) or levonorgestrel (LN) with the exceptionally strong synthetic estrogen ethinyl estradiol (EE), are frequently used by women to prevent pregnancy [29]. Despite the fact that these OC medications are often used in the developed world, many women cease using contraceptive tablets for reasons related to tolerability [30]. As a result, the effective dosage for OC has been further decreased to typically 150–1500 µg/tablet/day for NE (or LN) and 10–50 µg/tablet/day for EE [31, 32]. (Kay R. Bagon and E. W. Hammond,1978) [33] published a paper that discussed the HPLC assay of norethindrone and ethinyl estradiol in oral contraceptive pills. However, their conducted study showed inefficient separation of the two steroids according to the HPLC conditions used; as EE and NE had a selectivity factor of 1.0 (comparable K' values). As a result, efforts have been made to overcome two fundamental issues in the simultaneous HPLC analysis of EE and NE: the lower EE concentration in the capsule in comparison to the concentration of NE (35 µg vs. 500 πg, respectively) and the problem that EE has a low UV molar absorptivity at 254 nm (one of the most widely utilized and accessible wavelengths in UV detectors). Even though the UV molar absorptivity of EE is significantly higher at lower wavelengths (20000 cm/mol at 210nm); there are the usual additional issues when choosing lower wavelengths, such as the need for a more expensive variable wavelength detector and an inherently noisy baseline. Moreover, NE still absorbs significantly at 210 nm, which may require the changing of attenuation before the EE is eluted [34]. Correspondingly, an optimized, accurate, and quick separation of EE and NE from oral capsules was attained on 280 nm with the usage of optimum mobile phases, columns, and a suitable internal standard [14, 34]. Two reasons contributed to the choice of 280nm as an optimum wavelength which allowed the simultaneous possible analysis for both steroids utilizing the same optical

density: the low dosage EE has a maximum sensitivity (λ_{max}) at 280 nm, while the highly concentrated NE has a substantially lower absorption (450 cm/mol at 280 nm vs. 14000 cm/mol at 244 nm).

A more recent research paper in 2015 was published by Koneru A., et al. discussed the estimation of NEA and EE in pharmaceutical formulation via analysis on a reversed-phase HPLC (RP-HPLC) using C18 column, a mobile phase of 84:16 v/v methanol: phosphate buffer, a 1 ml/min flow rate, and at 220 nm as the selected λ_{max} for detection. With a resolution of 4.17 min, ethinyl estradiol and norethindrone acetate eluted at 3.5 and 4.7 min, respectively. The peaks obtained had theoretical plates more than 2000 and were symmetrical with tailing factors less than 1.5. All parameter findings were determined to be within acceptable ranges after the devised technique was verified in accordance with ICH criteria. In the concentration range of 5-1000 g/mL, the technique was determined to be linear, and %RSD of the precision study resulted in a value of around 2.0%. The assay findings for EE and NEA were 102.94 (2.01) and 105.37 (1.94%) with mean% Recovery of 106.30 and 95.45, respectively.

In literature, many other chromatographic methods have been developed for the analysis of norethindrone acetate [29, 35-39]. However, most of these chromatographic analytical methods have one of these two drawbacks: long time of analysis and/or the usage of expensive detectors [40].

Hashem , H., S. Abd EL-Hay, and T. Jira [40] developed an inexpensive, rapid, and fully validated analytical procedure for the determination of norethisterone acetate (NEA) in bulk powder, tablets, and plasma using a stability-indicating HPLC technique to overcome previously analytical drawbacks. A simple model of HPLC equipped with a diode array detector (DAD) system, a 220 volts UV-lamp, and an analytical reversed-phase c-18 column 150 x 3.0 mm with an isocratic mobile binary phase of ACN/H2O at a flow rate of 1ml/min were used. After tablet examination containing norethindrone acetate NEA alone, the peak that was produced is discovered to be homogenous, and the absence of co-eluting peaks indicates that the procedure is specific. The excipients in the formulation did not obstruct the determination of NEA, according to a contrast between the chromatogram of the raw NEA and that of extracted NEA from tablets. It is discovered that NEA's elution time is 2.946 0.01 minutes. figure 1.3/a shows the HPLC

chromatogram of norethindrone acetate NEA from bulk powder and from tablets. Figure 1.3/b demonstrates how the approach nailed to separate NEA when Norethisterone, Levonorgestrel, or Estradiol are also present. The combination of the steroids was eluted when the concentration of ACN% rose to more than 55%. Separation of norethindrone acetate from plasma was attained. The chromatogram of plasma sample with and without norethindrone acetate NEA is displayed in figure 1.3/c. The technique was examined for the following criteria: system suitability, linearity, accuracy, limits of detection and quantitation, specificity, and robustness. Results of the validation criteria are shown in table 1.2.

Figure 1.3

a) NA chromatogram from bulk powder and from tablets. b) separation of NA from other steroids in same formulation. 1- norethindrone 2- levonorgestrel 3- estradiol 4- norethindrone acetate. c) separation of NA from plasma

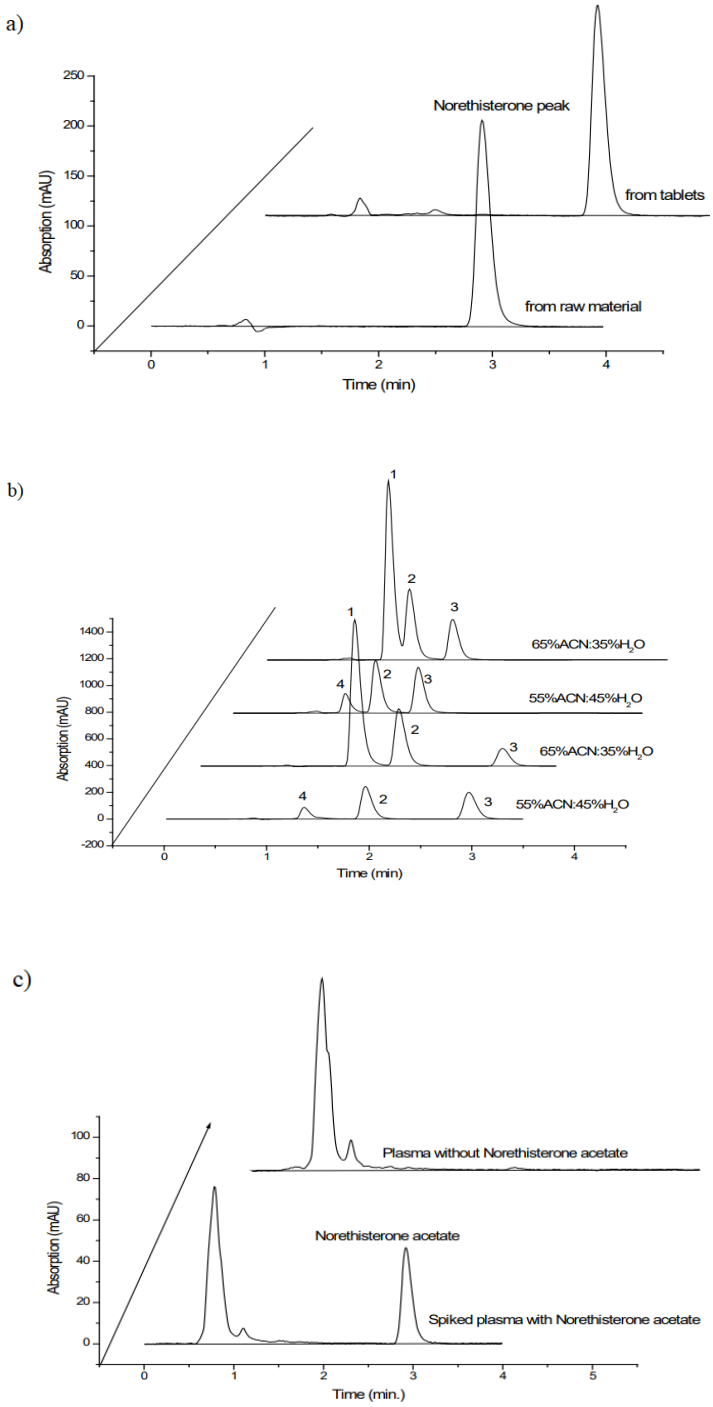


Table 1.2

Values of the tested validation parameters

System suitability	
Theoretical plates (N)	17078 (pro meter)
Retention factor (K')	1.964
Linearity and regression data	
Linearity range (µg/ml)	0.125-50
Detection limit (µg/ml)	0.0625
Quantitation limit (µg/ml)	0.125
Slope	0.220
Intercept	2.661
Coefficient of determination (R^2)	0.9998

Note. (Hashem et al., 2015).

1.3 Chemical derivatization

The sample preparation procedure and the primary analytical methodology are two especially difficult aspects of the bioanalytical process that are typically emphasized in two separate directions [41, 42]. Making a sample appropriate for a particular analytical technique and/or optimizing analytical efficiency (precision, accuracy, detection limit, stability, chromatographic retention, selectivity, etc.) are two of the many objectives of sample preparation. [43].

High performance liquid chromatography (HPLC), which offers a variety of separation processes and detections serves an essential method in quantitative and qualitative bioanalysis [44]. The vast range of detection methods used in HPLC provides several numbers of options for detecting the analytes, including UV-Visible absorption, fluorescence, refractive index (RI), different electrochemical detection methods, evaporative light scattering (ELS), etc.

Most of the pharmaceutical medications lack the proper chromophores that would allow for analysis at wavelengths other than the non-specific UV region of the electromagnetic spectrum (EM). Therefore, derivatization procedures are performed to change these medications into easily measurable molecules whose characteristics and concentrations may be linked to the original compound [45]. Chemical derivatization has grown in importance for quantification and confirmation in chromatography and enables analysts to identify substances in quantities that would otherwise be challenging

or unattainable to be detected by direct chromatographic methods. So, instrumental analysis can be way better improved via the application of chemical derivatization methods on specific compounds [46]. Chemical derivatization is a familiar followed technique in which a molecule is chemically altered to improve its suitability for a certain analytical method [47].

Derivatization is frequently employed in liquid chromatography-based bioanalysis to improve the target analytes' chromatographic outputs or make them more detectable. The derivatization process is explained in terms of the analytical approach utilized to yield chemical alterations to the target molecules either through the interaction between a chemical reagent, which depends on the kind of analyte which can be either an electrophile or a nucleophile, and the target molecules (an analyte containing hydroxyl, thiol, amino, carbonyl, and carboxyl which act as the principal functional groups engaged in derivatization) and/or subjecting these target molecules to physical variables (i.e., the use of heat, radiation, or an electric field) resulting in the formation of a new compound (the reaction's derivate) with improved chemical and physical properties for analysis [48, 49].

The goal of the analysis and the proposed technique of detection should be taken into account before choosing the derivatization reaction. Common derivatizations try to introduce analyte moieties comprising chromophores, fluorophores, or groups into the molecule to increase their detectability. As a result, several classes of analytes are known to benefit from chemical derivatization in terms of UV absorbance, fluorescence, ionization efficiency, HPLC separation performance, and chemical stability [50, 51].

Derivatization may be described in terms of the application type, the quantity of samples, the type of reagent employed (if it is a commonly used compound or a newly created reagent), also the functional group selectivity for which the structural change is operating [52].

The reaction parameters (reagent quantity, reaction time, temperature, and so on) are tuned to produce the preferred derivative and the highest practical reaction yield. Additional sample cleaning processes can be designed to remove undesired byproducts and superfluous reagents, resulting in fewer analyte inferences during analysis [49, 53]. To avoid derivatization at many places in the target molecule, metabolites, or

endogenous components, reagents must be selective (target one particular site of the molecule) [54]. For instance, acid chlorides (R-COCl) or anhydrides (RC(=O)OC(=O)R) need to be avoided when derivatizing a compound containing both hydroxyl and amino functional groups as they will derivatize both functional groups [49]. On the other hand, dansyl chloride ($C_{12}H_{12}ClNO_2S$) is a superior derivatization reagent for amino and phenol functional groups due to the fact that it is inert towards aliphatic alcohols [55]. Other factors to consider in reagent selection are commercial availability, purity, and cost. Reagents are often inexpensive and hence do not pose a barrier to their usage [56]. Chemical derivatization processes that use optimum conditions are often robust enough to be used for pharmaceutical bioanalysis and fulfill regulatory standards. This is often proved via a thorough validation procedure in which numerous criteria, including but not limited to accuracy, precision, selectivity, matrix effect, and so on, are evaluated [57, 58]. Using an internal standard (ISTD) is essential to account for any analyte loss that can occur during the various sample processing stages and bioanalysis, maintaining the assay's robustness [59-61]. When feasible, employ deuterium or ^{13}C stable internal standard; otherwise, use an analog with comparable reactivity, recovery, and chromatographic characteristics [49].

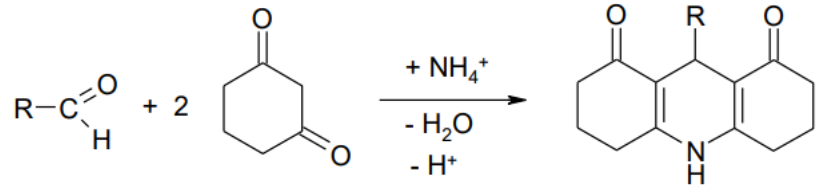
1.3.1 Chemical derivatization techniques in HPLC

There are several derivatization approaches for the bioanalysis of analytes in HPLC. In situ derivatization is considered the easiest method for high-throughput bioanalysis in which chemical derivatization is carried out by directly adding reagent(s) to the liquid sample. If the reagent is water soluble, it can be dissolved in both an organic solvent that is likewise soluble in water as well as an aqueous or partly aqueous solvent. An example, biomatrices containing low molecular weight aldehydes (LMWA) don't absorb UV light, however after being derivatized with cyclohexane-1,3-dione in an ammonia-induced condensation process that results in dihydrolutine derivatives, they may be detected by a UV detector (Scheme 1.2). An aqueous solution with a high concentration of acetic acid (AA) and ammonium acetate ($C_2H_7NO_2$) is used to dissolve the derivatization reagent. Tridecanal and heptadecanal, which are produced frequently in a variety of biochemical processes like peroxisomal oxidation of long-chain 3-methyl-branched and 2-hydroxy fatty acids, as well as microsomal degradation of

phosphorylated sphingoid bases, have both been successfully determined using this derivatization [62].

Scheme 1.2

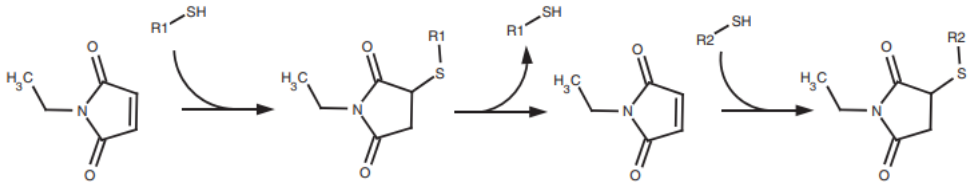
in-situ derivatization: derivatization of aldehydes in biomatrices



Derivatization is commonly used to enhance compound chromatographic characteristics in such a way of lowering the polarity of the derivatized compound and thus inhibiting their bonding with the hydrogen bonding sites in the stationary phase such as in the context of analyzing via the liquid chromatography [47]. So, structural alterations of the analyte can also affect its affinity towards the stationary phase used for HPLC separation. In RP-HPLC, the hydrophobicity of the solutes—which is often defined by the decimal logarithm of octanol-water partition constant, log K_{ow}—is used to assess the affinity for hydrophobic stationary phase [63, 64]. In general, the value of log K_{ow} affects both the retention in the HPLC process as well as the extraction in an organic solvent. Based on the building block derivatized and linked to the analyte's structure, K_{ow} value can be either enhanced or diminished. An approach called hydrophobicity-tailored derivatization. This derivatization strategy used for the alteration of the hydrophobic nature in which the ion-pairing mechanism is considered a substitute for the separation-based method for improving the interaction of polar analytes with the hydrophobic surface of the reversed-phase RP stationary phase [65]. An example from the literature, the free thiols in a recombinant monoclonal antibody may be easily quantified by RP-HPLC with hydrophobicity-tailored thiol derivatization. The authors did this by using compounds based on the maleimide thiol group that have distinct levels of hydrophobicity depending on how the thiol group reacts with the C=C bond in the derivatization reagent (Scheme 1.3) [66]. When N-ethylmaleimide (NEM) is used, the derivatives' log K_{ow} value rises by around 1.38, which was enough to ensure retention in RP-HPLC and to guarantee chromatographic selectivity.

Scheme 1.3

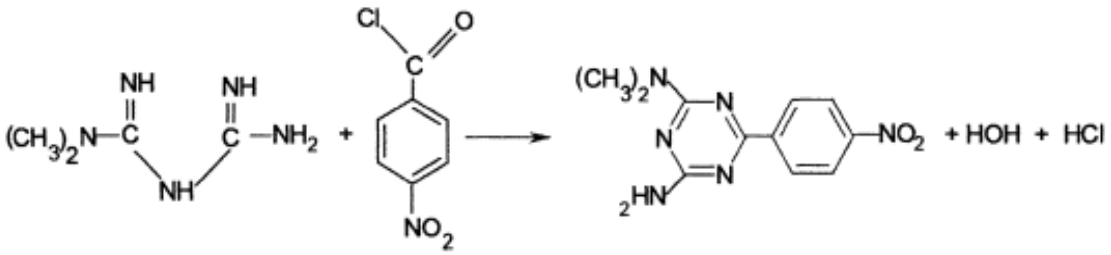
Hydrophobicity-tailored derivatization: addition of thiols to maleimides



Liquid-liquid extraction (LLE) is a typical technique for preparing samples for bioanalysis. It serves two main purposes: HPLC analysis sample enrichment and analyte isolation. Derivatization and LLE can be coupled in a variety of experimental techniques [65]. A two-phase liquid-liquid system (dichloromethane/plasma sample) was used to derivatize the very hydrophilic drug metformin (MTF), an anti-diabetic biguanidine. (Scheme 1.4) [67]. The derivatization process transforms the analyte, which is very hydrophilic, into a less hydrophilic chemical before it gets inserted into the organic layer. However, excess p-nitrobenzoyl chloride (C7H4ClNO3), a derivatization reagent, is hydrolyzed to a salt and moved into the aqueous phase. By eliminating the large excess of the derivatization reagent in this manner, the column overload that was causing issues with the chromatographic separation is also eliminated.

Scheme 1.4

LLE-derivatization technique: derivatization of hydrophilic metformin by p-nitrobenzoyl chloride



Solid-phase analytical derivatization, chiral derivatization, isotope-labeling derivatization, and post-column derivatization are also other regularly followed derivatization techniques that give high throughput yields for analytical determination of compounds.

1.3.2 Literature analysis methods of steroidal hormones via chromatographic methods using different derivatization techniques

Many derivatization techniques for estrogens and/or combination formulations of estrogens and norethindrone have been published as concerns over potential medication interactions and probable failure of contraception in women taking OC have increased since the introduction of low dosage combinations of these agents. One of the main proposed mechanisms of OC-drug interactions is the increased clearance of OC medications as a result of activating the enzymes responsible for drug metabolization, such as cytochrome P450 CYP3A4. Hence, it is crucial to investigate any possible interactions between novel medication candidates and low dosage OC during the drug development process to maintain the ideal OC exposure throughout concurrent therapy [68]. Reza Anari et al. 2002 [69] introduced a sensitive and precise technique to assess trace pictogram quantities of EE from tiny volumes of plasma (usually 50-100 L) from preclinical species (Rhesus monkey). A straightforward aqueous phase derivatization technique was performed in an attempt to modify the EE core structure via the incorporation of a highly ionizable moiety (dansyl chloride as derivatization reagent). The incorporation of a dansyl moiety is expected to dramatically boost the ionization of EE, according to calculations of the ionization capacity of a set of probable derivatives with the 3- or 17-hydroxyl functional groups. Little volumes of EE-dosed rhesus monkey plasma were used to establish a very sensitive and selective LC/MS/MS technique for the measurement of EE due to the powerful 3-dansyl-EE signal seen using positive turbo ion spray mass spectrometry. Scheme 1.5 illustrates the derivatization reaction between EE and dansyl chloride that achieved a high consistent yield in about 3 minutes. Using electrospray tandem mass spectrometry (ESI), it was possible to examine the ionization and fragmentation of the isolated dansyl derivative. According to expectations, under the influence of positive turbo ion spray, the 3-dansyl-EE formed a strong molecular ion peak at m/z 530 (figure 1.4/a). A strong fragment ion at m/z 171 that corresponds to the 5-(dimethylamino)-naphthalene moiety was formed by the collision-induced dissociation spectra of m/z 530 (figure 1.4/b). The unique fragment ion at m/z 171 caused by the dissociation of the C–S bond and consequent reorganization of one hydrogen atom in the direction of the naphthalene nucleus via a modified McLafferty rearrangement process, which was detected by electron ionization mass spectrometry. 20% of the strength of the ion at m/z 171 came from the product ion

at m/z 170, which is the consequence of straight C-S cleavage without hydrogen rearrangement. With ideal collision energy at 51 eV, the product ion's intensity at m/z 171 was 600% higher than the protonated parent ion's intensity at m/z 530 (figure 1.4). This is a substantial increase over the underivatized EE's fragmentation efficiency, which was 1.4% utilizing the m/z 295 f 145 transition under the influence of negative electrospray ionization.

Scheme 1.5

Chemical derivatization reaction between EE and dansyl chloride

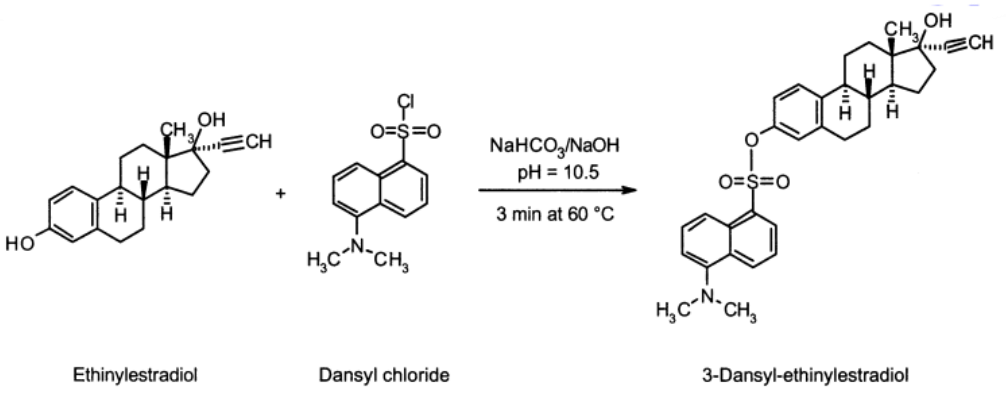
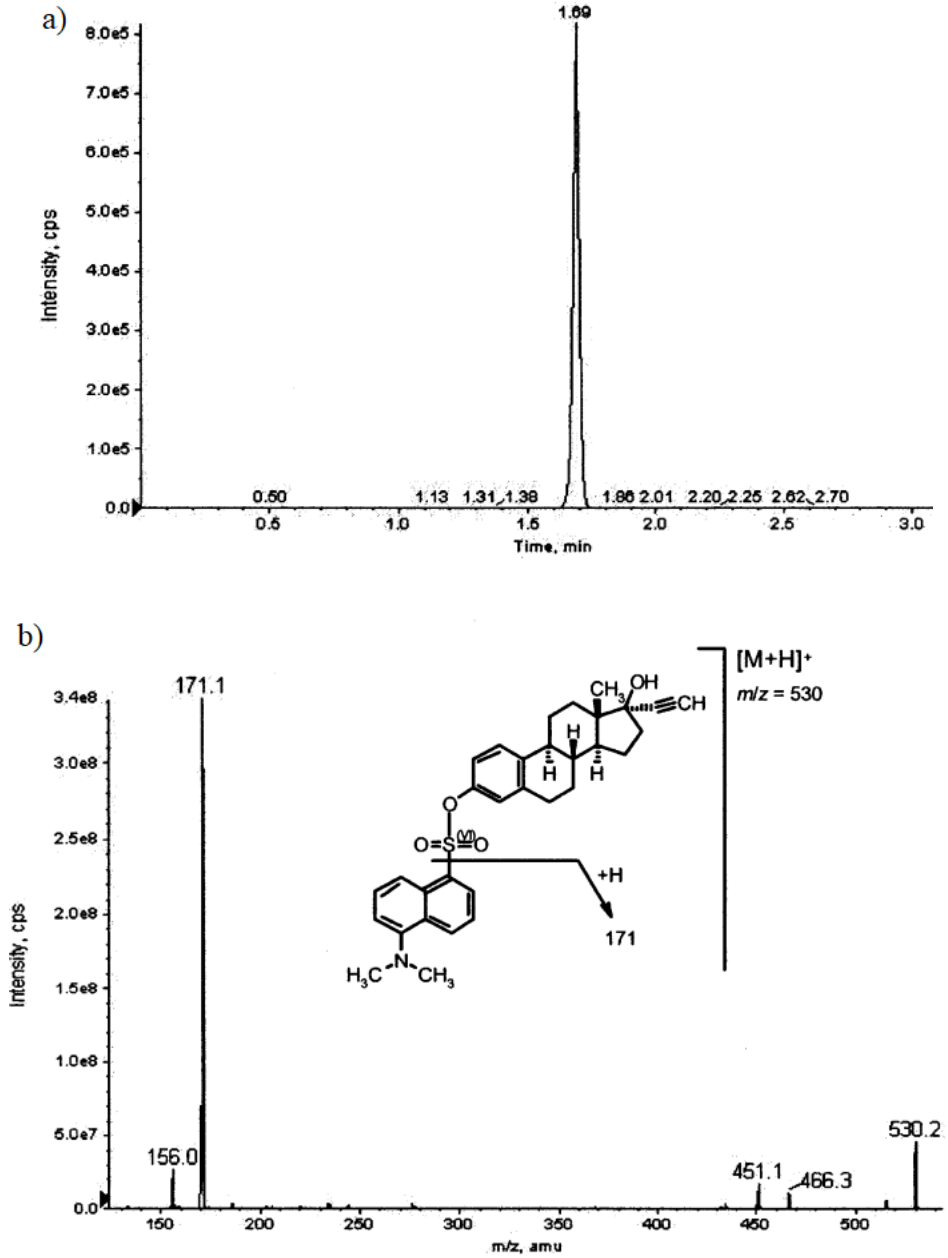


Figure 1.4

a) 10 µL of derivatized and extracted EE-spiked plasma samples were put onto the column after being spiked with EE at a concentration of 5 ng/mL. b) Product ion scan of the m/z 530 at collision energy of 51 eV under ideal source conditions

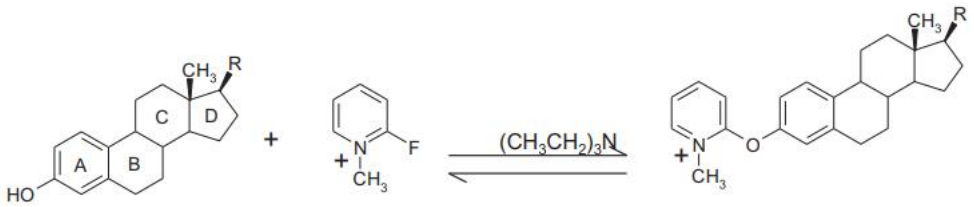


As analytical difficulties arise from the fact that postmenopausal women have estrogen circulation levels less than 20 pg/mL and at these low levels, immunoassay quantification is inaccurate. Liquid chromatography tandem mass spectrometry (LC-MS/MS) gives higher specificity and occasionally greater sensitivity. However, ionization of estrogens is insufficient. So, ionization may be enhanced by the addition of

charged molecules, but many of these estrogen derivatives produce non-specific product ions coming from the "reagent" group. Therefore, a method that would produce derivatives with product ions unique to each estrogen was developed. Andrew et al., 2016 [70] published a paper encompassed the application of derivatization of estrogens in which solid phase extraction was used to separate the estrogens from the human plasma and serum, and 2-fluoro-1-methylpyridinium-p-toluenesulfonate (FMP-TS) was used to derivatize the estrogens (scheme 1.6). After LC separation, "FMP" derivatives of estrogens were quantified by electrospray in positive mode. The technique was linear from 1-400 pg/sample, with detection and quantitation limits of 0.2 pg/sample. The variability, precision, and accuracy measurements within and across assays were within acceptable limits (< 20%).

Scheme 1.6

The derivatization of an estrogen with 2-fluoro-1-methylpyridiniumptoluenesulfonate (FMP-TS) in the presence of triethylamine (TEA), illustrated by the formation of methylpyridinium ether derivatives of phenolic estrogens



A comparable research was published before in 2003 [68], which stated the measurement of oral contraceptive ethinyl estradiol (EE) using (LC/MS/MS) technique on 1 mL of human plasma sample and then validated over the curve range of 2.5-500 pg/ml. Methyl t-butyl ether was used to extract EE and EE-d4 internal standard from the plasma matrix before it was derivatized with dansyl chloride and then re-extracted into hexane phase. The straightforwardly ionizable tertiary amine function group was introduced to EE using this derivatization process. The hexane phase was dried by evaporation, reconstituted, and added to the LC/MS/MS apparatus. A C18 column was used, an isocratic mobile phase of 20:80 water: acetonitrile (v/v) adjusted with 1% formic acid. By this derivatization technique, analysts were able to reach the targeted

lower limit of quantification at 2.5 pg/mL and significantly increase analyte sensitivity in electrospray ionization.

Similar research in 2005 was published that discussed the development of an (LC-MS/MS) analytical method for the simultaneous measurement of norethindrone and ethinyl estradiol in 0.5 ml of plasma sample and validated across the concentration ranges of 50–10,000 pg/ml and 2.5–500 pg/ml, correspondingly [71]. N-butyl chloride was used to extract NE, EE, and their internal standards NE13C2 and EE-d4 from human plasma matrix. The extract was derivatized with dansyl chloride after the organic solvent had evaporated, and the resulting mixture was then added to the LC-MS/MS apparatus. The derivatization-related potential interference peaks were separated from the analytes of interest using optimal chromatographic conditions: on a RP-18 column, the gradient chromatographic elution was accomplished using acetonitrile, water, and formic acid as the mobile phase, at whole runtime of 5.0 minutes, and at a flow rate of 1.0 ml/min. The inter-day precision (%RSD) of the quality control samples for NE and EE at three different concentration levels was 6.8% and 4.2%, respectively. Whereas, the accuracy (%RSD) values were recorded 4.4% for NE and 5.9% for EE. Excellent linearity in the prescribed concentration ranges (50–10,000 pg/ml for NE and 2.5–500 pg/ml for EE) was attained, with correlation values above 0.9988 for all validation batches. The LLOQs for NE and ethinyl EE in the presented research were 50 and 2.50 pg/ml, respectively.

Despite the widespread use of derivatization techniques, there is a significant and noticeable difference between instrumental analytical approaches and supportive derivatization: modern analytical tools are highly standardized and frequently automated. The processing and interpretation of measured data are frequently automated using sophisticated algorithms that are backed by extensive digital libraries. Scientists can achieve valid results even if they are unfamiliar with the actual measurement device. These approaches spread beyond the domain of skilled professionals to become instruments in regular laboratory work. On the other hand, using derivatization procedures often necessitates a considerably greater understanding of the instruments, the actual measurement methodology, the sample, and the chemistry involved. It is frequently far from routine [72]. Despite advances in contemporary technology, the requirement for inexpensive procedures for pharmaceutical analysis will keep

derivatization approaches important in the quality control of these substances, particularly in low resource economies [45].

1.4 Click chemistry

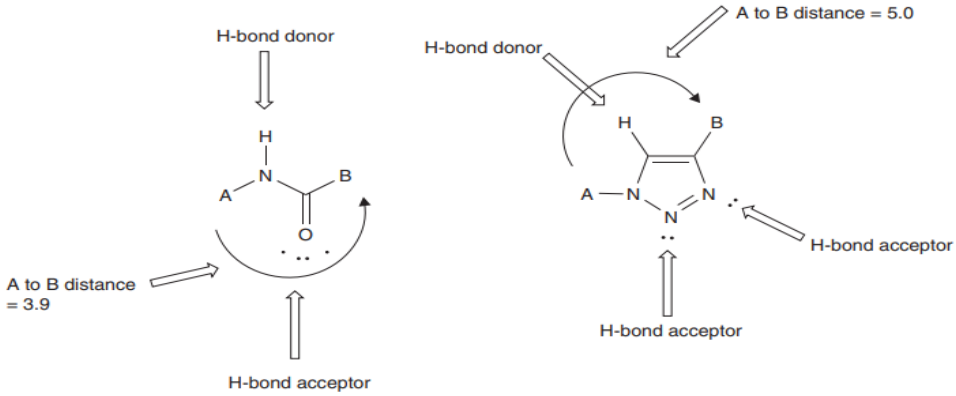
Nowadays, medicinal chemists and analysts are searching for analytical methods to synthesis substances or libraries of substances during the lead optimization or drug discovery procedures while ensuring highly quantified yield with the production of lesser harmless products as much as possible under mild conditions [73, 74]. As a result, the development of synthetic techniques that enable the assembly of a large number of physiologically active molecules, quick production of novel molecules, and the introduction of new molecular characteristics became the focus of all efforts [75]. Alongside being quick, efforts are poured toward searching for an ideal primary synthesis characteristics that fulfill good efficiency, adaptability, and selectivity [74]. An immense variety of activities in many domains have been driven or inspired by the persuasive concept that anybody may create large chemical entities with competent chemistry. Numerous fields of biorthogonal chemistry have worked together to create new, complex chemical reactions with the maximal level of mechanistic knowledge. This is because it is actually quite challenging to find methods for the precise and reliable establishment of bonds [75]. In 2001, Sharpless and colleagues firstly introduced click chemistry which grew out of a desire to use molecular assembly's capabilities for as many different types of applications as feasible. The "click" in "click chemistry" refers to the ease and satisfaction of snapping items together using a baggage strap connector. Whatever the components, as long as the buckle's two ends can connect, the linkage can be created [75]. The term "click chemistry" was originally used to refer to reactions that met a strict set of requirements which include: "The reaction must be modular, wide in scope, offer very high yields, only generate inoffensive by-products that can be eliminated by non-chromatographic means, and be stereospecific (but not necessarily enantioselective)". Click chemistry necessary process attributes include simple reaction circumstances, easily accessible starting materials and reagents, the use of no solvents or a solvent that is benign (like water) or can be quickly removed, and straightforward product isolation under simple reaction conditions [76]. Click reactions are wide and varied with the huisgen 1,3-dipolar cycloaddition of azides to alkyne-containing molecule affording the formation of 1,2,3-triazole moiety being the

chief model of all its available reactions. Although this reaction was discovered at the start of the 20th century, Huisgen et al. didn't fully understand its potential or its mechanism until the 1960s [77]. Since a number of substituents can be created by combining alkyne and azide components, this reaction has a very high potential [78]. However, because the directing impact of the substituents is typically negligible, this reaction has been plagued by a lack of selectivity for more than 40 years, producing a mixture of the 1,4- and 1,5-regioisomers [78]. Furthermore, this transformation necessitates heating and prolonged reaction periods to be completed and the two regioisomers can sometimes be difficult to separate using traditional chromatographic techniques. In 2002, Two independent studies conducted found that copper (I) salts could speed up this reaction by up to 10^7 million times [79, 80]. More crucially, only one of the two regioisomers, specifically the 1,4-regioisomer, is produced by the copper catalyst, at room temperature or with relatively modest heating as it can be carried out across a broad range of temperatures (0-160°C) which normally does not require temperature elevation, in a number of solvents (including water), and over a broad spectrum of pH values (from 5 to 12). When compared to the uncatalyzed variant, it can move up to 10^7 times more quickly, and product filtering is the main form of purification [79-84]. The conditions detailed in these publications also met the click chemistry criteria listed above, turning this reaction to be well used and applied [76, 78]. Furthermore, steric variables have little impact on it. This transition is easily carried out by varyingly substituted primary, secondary, tertiary, and aromatic azides. The acetylene component has great tolerance for changes [79]. Under normal circumstances, azides and terminal alkynes are remarkably stable and simply installable, which are two further factors contributing to the popularity of this cycloaddition [85, 86]. They can both withstand oxygen, water, regular organic synthesis requirements, biological molecules, a broad variety of solvents and pHs, as well as the circumstances in which living systems respond (reducing environments, hydrolysis, etc.) [81, 86, 87]. Aliphatic azides are thermodynamically more likely to decompose, but a kinetic barrier prevents this from happening under the conditions mentioned above [81]. Until a dipolarophile, such as an alkyne, gets in touch, they will practically stay to be "invisible" in the solution [81].

The produced triazoles that are 1,4-disubstituted are not brand-new in medicinal chemistry. In fact, more than 7,000 1-H-1,2,3-triazole molecules with a 1,4-disubstituted ring have been discovered prior to learning about the copper-catalyzed click reaction. In the domain of medicinal chemistry, triazoles have been found to exhibit a number of favorable characteristics. For instance, triazoles are resistant to both reductive and oxidative conditions, also to acid and basic hydrolysis, demonstrating a strong aromatic stability. This heterocycle can possibly potentially play an active role in the production of hydrogen bonds as well as in interactions between dipoles and p stacking because it has a large dipole moment (approximately 5 D) and the moiety is also comparatively robust to metabolic breakdown [88]. Tazobactam, a b-lactamase inhibitor sold together with the broad-spectrum antibiotic piperacillin, is one of the star examples of triazole-containing compounds. In fact, the triazole ring appears to be crucial to the efficacy of tazobactam and related triazole-containing compounds, which were initially shown to be powerful b-lactamase inhibitors with superior characteristics than clavulanic acid and sulbactam, in terms of potency [89, 90]. Triazoles have also been employed in the field of antibiotics to enhance the target drug's pharmacokinetic qualities. Using the triazole moiety as a link between the cephalosporin core and the triazole moiety, for instance, good oral availability cephalosporins were produced [91]. The formed 1,2,3-triazole is one of many bioisosteres of the amide moiety that has drawn growing interest in medication development. Figure 1.5 demonstrates the similarities between the two moieties which include the similarity in the size represented by the distance between substituents (3.8 - 3.9 A˚ in amides and 5.0 - 5.1 A˚ in 1,2,3-triazoles), an estimated dipolar moment of ≈ 4 Debye for amide and ≈ 5 Debye for 1,2,3-triazoles, along with the H-bond acceptor capacity characteristic [74]. Due to the lone pairs electrons in the 1,2,3-triazole rings' nitrogen atoms N(2) and N(3), which have undergone sp2 hybridization, they are able to function as weak hydrogen-bond acceptors. Furthermore, the high dipole moment of 1,2,3-triazole ring causes the C(5) proton to become polarized to the point where it could perform as a hydrogen-bond donor, the same action to the amide NH. Additionally, a substantial dipole on the 1,2,3-triazole ring may line up with the other amides in a certain peptide secondary structure. [92].

Figure 1.5

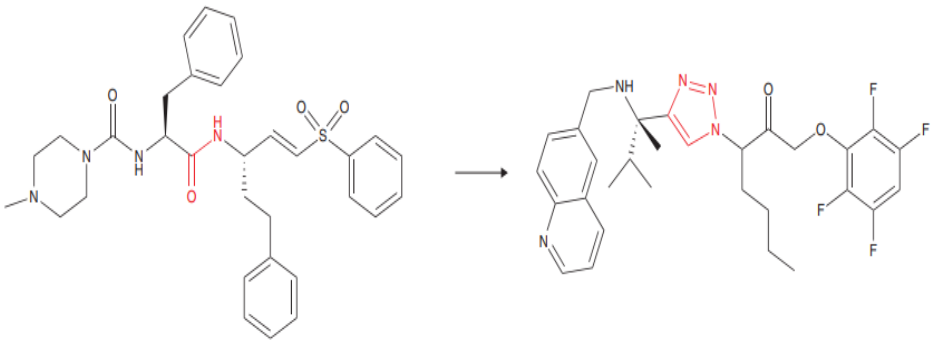
Concept of 1,2,3 triazole's ability to operate as a non-classic amide bioisoester



Yet 1,2,3-triazoles are incredibly resistant to hydrolysis, unlike amides. Many 1,2,3-triazoles are known to have biological activity because they may behave as stiff connecting components that mimic the electrical and atom replacement features of a peptide bond yet are resistant to hydrolysis. When the amide link is switched out for the 1,2,3-triazole moiety, certain prospective medications still exhibit biological activity. By substituting an amide bond with a 1,2,3-triazole unit, J. A. Ellman et al. described a group of non-peptidic Cruzain inhibitors as possible new drug leads for the chemotherapy treatment of Chagas disease. In fact, two hydrogen bonds were found in the cruzain-1 complex, one between the 1,2,3-triazole's N(3) atom and Gly66 and the other between its N(5) atom and Asp161 (scheme 1.7) [93]. The structure with the amide moiety acts as peptidic inhibitor whereas the other structure with 1,2,3 triazole ring functions as a nonpeptidic inhibitor.

Scheme 1.7

Example for 1,2,3 triazole acting as bioisoester of amide

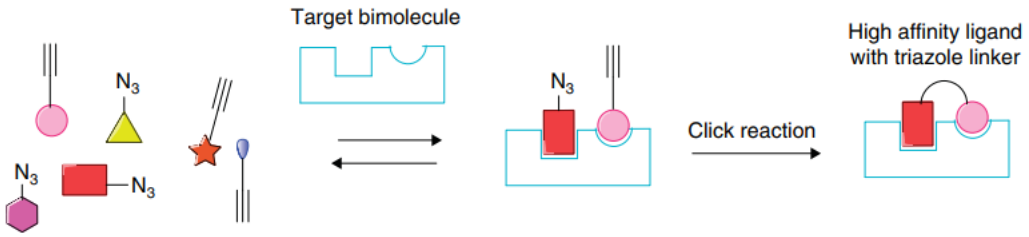


Also, 1,2,3-triazoles are considered basic aromatic heterocyclic compounds. Because of this, for aromatic rings, especially heteroaromatic rings, 1,2,3-triazoles are suitable isosteres. In addition, the 1,2,3-triazole ring is an isostere of the double bond. Even so, when compared to other organic molecules that include three neighboring nitrogen atoms, 1,2,3-triazoles are unexpectedly stable. Resveratrol is a phytoalexin that has a variety of biological actions. Using conventional click chemistry, Genazzani altered the double bond for a 1,2,3-triazole ring. Initial research revealed that this process may result in the synthesis of a chemical that has some of the resveratrol features but not all of them [94]. The advantages of click chemistry are its modularity, great efficiency (often a yield of 100%), tolerance for a wide range of functional groups, and biocompatibility. Under physiological conditions, the 1,2,3-triazole moiety that has been produced is extremely stable. These merits make the 1,2,3-triazole moiety an efficient linker. Twin medications, including "identical twin drugs" (homodimers) and "nonidentical twin drugs" (heterodimer) have been produced using the 1,2,3-triazole linker. The purpose of a homodimer is to produce a medication that is more powerful and/or more selective than the single entity. The heterodimer is made up of the union of a pair of distinct chromophores. The new molecule will have both of the initial pharmacological properties in this situation. An example: click chemistry was used to link the cores of the b-lactam and chloroquinoline molecules via 1,2,3-triazole, followed by the assessment of the in-vitro antimalarial activity of the resultant compounds [95]. By the usage of 1,2,3- triazole as a linker, it has also been possible to create bidentate inhibitors, which work by interacting with two distinct binding sites on the same target. This is especially useful in searching for small-molecule leads for enzymes (and other proteins) that have prolonged active site or multiple binding pockets. Using click chemistry, Yao et al. successfully designed and generated a sizable library of PTP1B (protein tyrosine phosphatase 1B) inhibitors [96]. Alkyl linkers with various lengths and aromatic rings with various polarity were selected as suitable peripheral groups. Cu(I) catalyst was used to create 66 distinct bidentate compounds, and the resultant 1,2,3-triazole products were tested without any additional purification. A promising potential PTP1B inhibitor was found with a recorded IC_{50} of 4.7 µM. Several researchers are using fragment-based techniques to uncover lead compounds more quickly in order to speed up the drug development process. An outstanding overview on in situ click chemistry has been published [97]. For instance, a library of 24 azide-containing

fragments (400 M) was incubated with an acetylene compound (60 M) in the existence of bovine carbonic anhydrase II. When the enzyme was present, a collection of 12 reagent combinations specifically produced a 1,2,3-triazole [98]. The process of in-situ click chemistry is shown in scheme 1.8.

Scheme 1.8

In-situ click chemistry process



The Huisgen reaction was revived, as was already indicated, by the employment of copper as a catalyst. Copper (II) salts, such as copper sulfate pentahydrate ($CuSO_4.5H_2O$) [79] or copper acetate ($Cu(CH_3COO)_2$) [99], are used in the traditional catalytic system when a reducing substance like sodium ascorbate ($C_6H_7NaO_6$) or metallic copper is present [79]. This maintains noticeably high quantities of the catalytic species while continuously converting copper (II) to copper (I). Tert-butanol ($(CH_3)_3COH$) and water are combined as the solvent since, in these circumstances, a base is not required to produce the copper acetylide (Cu_2C_2) species. It is crucial to note that this solvent is suitable for lipophilic compounds. When it is not possible to use aqueous conditions, organic solvents (such as tetrahydrofuran ($(CH_2)_4O$), toluene ($C_6H_5CH_3$), dichloromethane (DCM) (CH2Cl2), acetonitrile (ACN) (CH_3CN)) can be used in the presence of a equivalent ratio of copper (I) salts (such as copper iodide CuI [80], Tetrakis(acetonitrile)copper(I) hexafluorophosphate Cu(CH3CN)4PF6 [100], Bromotris(triphenylphosphine)copper(I) 18 CuBr(PPh3)4, or iodocopper triethyl phosphite CuIP(OEt)3 [101]) and an excess of a base, typically a tertiary amine (e.g., triethanolamine TEA, N, N'-diisopropylethylamine DIPEA) can be used. Because the 1,3-dipolar cycloaddition reaction involving azides and alkynes only produces the 1,4-disubstituted triazole ring, efforts have been undertaken to broaden the reaction's usefulness by creating an orthogonal cycloaddition that produces the 1,5-disubstituted product. As a result, this click chemistry reaction would be an authentic

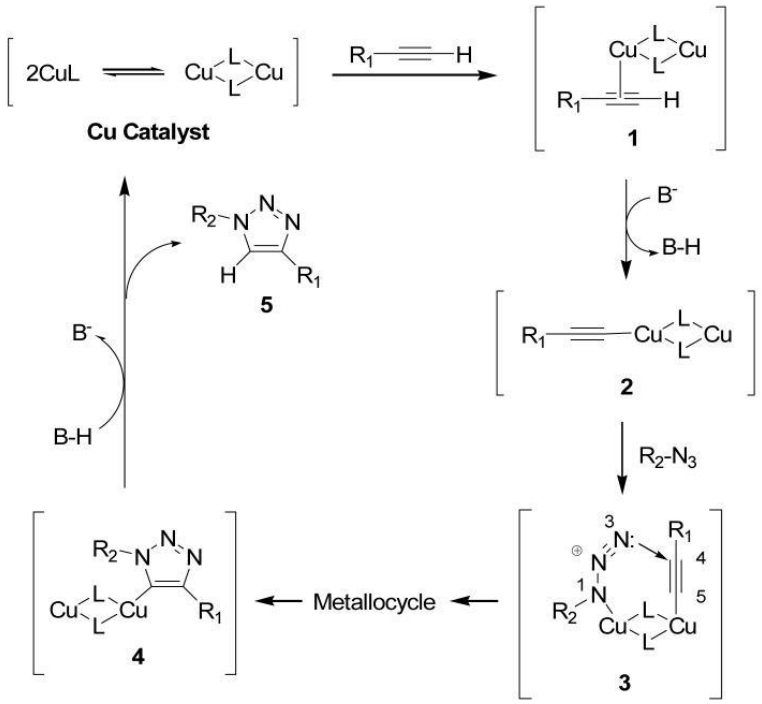
regiocomplementary reaction that, in the given circumstances, generates the required regioisomer. The formed ring has electronic merit which makes it resistant to hydrolysis, analysis, and many other cleavage odds [102]. Fluorescent tagging of alkyne and/or azide-containing biomolecules which in turn helps in providing a suitable tool to pursue many biological events [103, 104]. In addition, because of its mild conditions and the usage of aqueous solvents, it can be served as an effective tool for biorthogonal fluorescence tagging inside the living system [105].

A coordinated mechanism allows cycloadditions to happen. However, molecular modeling work [84] on the Huisgen dipolar cycloaddition HDC reaction and actual kinetic data [106] tend to indicate a sequential reaction pathway [81, 83]. According to studies using density functional theory, in one particular reaction, the activation barrier for a concerted HDC reaction that is catalyzed actually exceeds the barrier for a concerted reaction that is not catalyzed (27.8 kcal/mol vs. 26 kcal/mol). [84]. Additionally, the activation barrier for a concerted catalyzed reaction is 11 kcal/mol greater than for a stepwise-catalyzed HDC reaction. [81]. On the basis of experimental data [79, 83] and the observation that CuI can easily bind to terminal alkynes (the sonogashira reaction) [107], it is hypothesized that the reaction's initial phase is the complexation of a CuI dimer to the alkyne via π interactions (1 in scheme 1.9). The terminal hydrogen is then deprotonated, resulting in the formation of a Cu-acetylide [83]. Depending on the reaction conditions used, a variety of distinct types of Cu-acetylide complexes can arise; 2 represents just one possible option [84]. CuI complexation decreases the terminal alkyne's pKa by up to 9.8 pH units, enabling deprotonation to take place in a water-based solvent without the incorporation of a base [81]. It would be necessary to add a base, such as 2,6-lutidine or N,N′-diisopropylethylamine (DIPEA), if acetonitrile or another non-basic solvent were to be used [108]. Next, in the Cu-acetylide complex, N(1) separates one of the ligands from the second Cu, resulting in the formation of 3. As a result, the azide is "activated" for nucleophilic attack on C(5). The alkyne's C(4) is now easily attacked by N(3) due to proximity and electrical considerations, resulting in a metallocycle (not drawn for ease). The metallocycle then shrinks as N(1)'s lone pair of electrons attacks C(5) to create the corresponding triazole 4. As soon as 4 forms, the connected Cu dimer starts to combine with a second terminal alkyne. Yet since the structural shape of the complex is

undesirable, this second alkyne cannot proceed through a cycloaddition and instead dissociates upon protonation to regenerate 4. The CuI catalyst is finally liberated from the 1,2,3-triazole product 5 by a final protonation step, allowing it to engage in a second catalytic cycle with other substrates [81]. While interactions with a protonated external base and/or solvent are most likely the cause of both of these protonations, further research is required to definitively prove this [81].

Scheme 1.9

Huisgen dipolar cycloaddition reaction



The entire drug development process has been considerably aided by click chemistry, which makes it simple to synthesize the constituent parts of novel chemical entities (NMEs). Although it hasn't entirely replaced current drug discovery techniques, it has enhanced and supplemented them by assisting with lead discovery and optimization. Structure-based design is aided by click chemistry, which also enhances combinatorial chemistry approaches. By selecting the right building blocks, click chemistry may also create derivatives or imitate conventional pharmacophores, natural products, and pharmaceuticals [76, 86]. Triazole linkage is recognized as an active pharmacophore and binds aggressively to a variety of proteins in different ways because of the substantial dipole movement [109]. The hydroxyl groups of the serine and threonine amino acid residues on proteins are phosphorylated by a class of enzymes known as

protein kinases (PKs), which regulates the activity of other proteins. Anomalies in a kinase or its degree of expression are a common feature of many disorders, such as diabetes and cancer. Drug discovery efforts have focused mostly on PKs as therapeutic targets because of their major significance in several signal transduction pathways [110]. It was recently discovered that adding (1H-1,2,3,-triazol-1-yl)acetate or phosphonate to a basic 4-(40-fluorophenyl)imidazole via CuAAC enhanced the water solubility and lowered the lipophilicity of kinase inhibitors.

1.4.1 Literature click chemistry reaction applications

The endocrine glands produce sex hormones (progesterone, and estrogens) alongside mineralocorticoids and other adrenocortical hormones in order to sustain a living creature's proper physical function [111]. Extra intake of exogenous steroid hormones, on the other hand, have traditionally been thought of as endocrine disruptors (EDCs) [112]. In order to accelerate the growth and enhance the muscular mass of agricultural animals, several steroid hormones were specifically introduced illegally to animal meals [113]. Due to their potential for harmful consequences, there have been a number of concerns expressed in recent years over the possible existence of steroid hormones in foods of animal origin. Numerous research suggested that high levels of steroid hormones possibly impede healthy cell division, increasing the risk of certain illnesses [114]. The development of practical and efficient methods to identify trace hormone levels in food is crucial for ensuring food safety. A click reaction-triggered turn-on fluorescence labeling approach was created by Li et al., 2022 for the purpose of determining steroid hormones in meat samples. The labeling technique was based on the interaction of 3-Azido-7-hydroxycoumarin with the alkynyl group of steroid hormones leading to the successful conversion of steroid hormones into triazole compounds which exhibits a turn-on fluorescence response in the existence of copper (I) [115]. Then, the generated fluorescent products were identified by using HPLC-FLD. L-Ascorbic acid (L-AA), the catalytic substance (Copper sulfate pentahydrate) ($CuSO_4$), and the reducing reagent sodium carbonate (Na_2CO_3) were used in this click reaction. The steroidal hormones that were examined in the meat sample in this mentioned study are norgestrel (NGT), norethindrone (19-NTD), ethinylestradiol (EE2), gestodene (G), and norethisterone acetate (NA). A negligible fluorescence signal was produced by the 3-azido-7-hydroxycoumarin solution due to quenching effect. But following CuAAC

interaction with the steroidal alkyne moiety, there was a considerable fluorescence amplification of the emission at 470 nm (excitation at 395 nm). Under the optimum used circumstances, the suggested approach demonstrated outstanding performance with strong linearity (R2 0.9998) and a low detection limit (1.8-7.3 g /L).

Since ethinylestradiol (EE) has a relatively low C_{max} (75 pg/ml), it is difficult to detect its trace presence in biological matrices. Despite fluorometric techniques' excellent sensitivity, their ability to detect EE is limited by the latter's weak natural fluorescence. In order to accomplish the needed ultra-sensitive bioanalysis, it is therefore essential to derivatize it using a fluorogenic reagent. By using copper sulfate and L-ascorbic acid to interact with the alkyne group of EE, Aref et al., 2022 presented a potential click fluorescent probe, 4-azido-7-nitrobenzoxadiazole (NBD-AZ), which produces a highly fluorescent and steady 1,2,3-triazole derivative [116]. Calculations using density functional theory (DFT) showed how, in comparison to NBD-AZ, the triazole formation affects the fluorescence of the click reaction result. 470 and 540 nm ended up being selected as the excitation and emission wavelengths, respectively. The recommended strategy has demonstrated exceptional performance and great linearity at concentration ranges of (25-300 pg/ml) as well as Low detection limit of 7.5 pg/ml.

Ahmed and Abdallah, 2019 published a research paper which introduced Dansyl azide (DNS-AZ) as a new fluorescent labeling reagent for the catalyzed azide alkyne cycloaddition click chemical reaction for the determination of alkyne-containing molecules [117]. Rasagiline mesylate (RSM) was utilized in their study as a prototype for medications that might be detected in biological samples using the CuAAC reaction. Sodium ascorbate and copper (II) function as catalysts in the reaction between RSM and DNS-AZ to produce a stable 1,2,3-triazole derivative that can be detected by HPLC and fluorescence. The procedure enabled reliable and precise RSM measurement in the linearity range of 0.50–100 ng/ml. The procedure also was measured for it lower limit of detection which was 0.16 ng/ml for RSM in rat plasma.

1.5 Validation methods

It's critical to validate newly established methods to ensure they are appropriate for the goals for which they were designed. Usually, the methods' quality is assessed through validation, specifically to determine whether they are sufficient for the intended

application. Modern analytical chemistry laboratories place a high priority on technique validation. The US Food and Drug Administration (FDA) has amended draft guidelines and offered specific suggestions for the pharmaceutical industry's use of bioanalytical method validation. For the disciplines of pharmaceutical and biotechnological processes, as well as bioanalytical techniques, the International Conference on Harmonization (ICH) has offered definitions of validation concerns covered in "analytical procedures". Similarly, the US Pharmacopeia (USP) has released recommendations for method validation of analytical techniques for medicinal products. In contrast to the FDA's regulations, those from ICH and USP are less comprehensive [118-121].

1.5.1 Precision and accuracy

The extent of scattering between a set of measurements taken under specific conditions from serial sampling of identical homogeneous samples is how to determine how precise an analytical process is. The precision is measured using the relative standard deviation (RSD), or coefficient of variation (C.V.). There are three categories into which the measured RSD may be subdivided: repeatability (intra-day precision), intermediate precision (inter-day precision) and reproducibility (between laboratories precision) [118, 120, 122, 123]. Repeatability is established when the analysis is carried out in one laboratory by one analyst within the same day utilizing the same equipment. According to FDA, repeatability should be evaluated by analyzing at least five measurements at three distinct concentrations (low, medium, and high) within the range of expected values [118]. But in accordance with ICH [120], two ways are used to determine repeatability, one is done through the analysis of three measurements at three distinct concentrations, and the other is by performing six analyses at 100% of the test concentration. That second one is usually done for the analysis of pharmaceutical products, in which the concentration should be the same across all samples. The type of analysis to be verified has a considerable impact on the acceptance criteria for precision. Precision for chemical analysis in pharmaceutical quality control need to be higher than 2% [124]. On the other hand, with regard to the exception of the lower limit of quantification (LLOQ), a value that shouldn't be more than 20%, each concentration level should have precision values that are higher than 15%, as recommended by bioanalytical applications [118]. The intermediate precision displays the fluctuations

impacted by daily analysis, including those generated by different analysts, instruments, etc. Reproducibility, shows the precision attained across different laboratories. The accuracy of an analytical method, occasionally referred as trueness, is the level of agreement or closeness between the true value of the analytes in the sample and the value calculated by the method [123]. FDA claims that accuracy may be assessed through examining samples with known concentrations and correlating the measured resulted values with the actual values. A minimum of five measurements for at least three different concentrations (low, medium, and high) within the expected concentration range should be employed to properly evaluate the accuracy of the bioanalysis. Accordance to the true value, the mean value should be within 15%, with the exception of the LLOQ, where it should be within 20% [118]. The exact analytical experiments can be used to calculate precision and accuracy.

1.5.2 Selectivity/specificity

Selectivity or specificity are occasionally used interchangeably when describing a process, however there is uncertainty which term often to use. The distinctions between the two terminologies were highlighted by Vessman [125]. Selectivity describes a technique that can discriminate between the analyte(s) response and all other responses while providing responses for a variety of substances. Specificity refers to when a technique gives responds to just one particular analyte. The word "selectivity" is more relevant since it is uncommon for a procedure in chromatography using UV-detectors to give responds to only one analyte. In order to evaluate the method's selectivity, blank samples should be processed both with and without analytes, and then they should be injected to look for interferences. The method's selectivity is crucial for precise analyte quantification.

1.5.3 Limit of detection and quantification

The lower limit of quantification, also known as LLOQ, and the limit of detection, or LOD, should be clearly distinguished from one another. When analyte is introduced into a chromatographic system, the LOD is the concentration that produces a peak height that is three times the noise; the threshold at which an observed value exceeds its level of uncertainty. LOD stands for lowest detectable but not necessarily quantifiable level of an analyte in a sample. The least amount of analyte that can be precisely and

accurately measured in a sample is known as the lower limit of quantification (LLOQ). The accuracy of the LLOQ should be between the range of 80-120% and with a precision of maximum 20% [118].

1.5.4 Linearity and range

Linearity is known as the capacity of an analytical technique to produce test findings that are proportionate to the concentration of analyte in a sample either directly or through a clear mathematical transformation within a specified range [121]. The concentration range 80–120% of the predicted concentration range need to be used to determine linearity. The study's targeted samples and the calibration standards must be assembled in the same matrix [118]. Six to eight standard samples with non-zero values should make up the calibration curve [118]. The calibration curve shouldn't use either the zero sample or the blank sample, which are both matrix samples processed with and without internal standards (ISTD), respectively. When standards are prepared using independent samples rather than serial dilution, it is simpler to identify a mistake or error in any of the samples. Nevertheless, if a mistake was made when the highest standard sample was being prepared, a serial dilution may provide a misleading regression result. For a linear regression equation, an appropriate standard curve should have an intercept that is not statistically different from zero. According to the FDA, the linear correlation coefficient (r) for bioanalysis should be 0.95 or above [118]. Since the standards in the lowest range might depart from linearity even when the r is high, a strong linear correlation coefficient alone does not guarantee a linear standard curve. Instead, a graph showing the response/sample concentration vs the logarithmic sample concentrations should be included with the linear coefficient, and the y-axis variation shouldn't be more than 5% [126]. The interval that has been rigorously confirmed for accuracy, precision, and linearity is known as the concentration range of an analytical technique.

1.5.5 Robustness

Both the ICH and the USP standards describe the robustness of an analytical technique as a measure of its potential to be unaffected by minor but deliberate modifications in procedural parameters mentioned in the documentation, demonstrating the suitability and dependability of the method or approach under typical application and normal usage

[127, 128]. Robustness studies are also utilized to set system suitability parameters in order to ensure that the reliability of the entire system—including the instrument and the method—is upheld throughout installation and usage. It makes sense to evaluate robustness throughout development since it is simple to identify factors that impact the technique when they are changed for selectivity or optimization. Assessing robustness at the start of the formal method validation procedure or before it begins means a little time spent now will pay off later on in terms of time, effort, and cost savings [129]. In a robustness study, the parameters of the approach are deliberately changed to examine if the results are impacted. Examples of common variances in liquid chromatography (LC) include: mobile phase composition, proportion of organic solvents, flow rate, wavelength, temperature, pH of the mobile phase and different column lots [130, 131]. A factorial experiment may include evaluating these parameters concurrently or one component at a time [132].

1.5.6 System suitability

Testing for system suitability is a crucial step in many analytical processes. The tests are founded on the idea that the tools, electronics, analytical processes, and test samples make up a whole system that may be assessed as such [133]. With the use of these tests, it can be determined whether the chromatographic system's detection sensitivity, resolution, and reproducibility are sufficient for the intended analysis. The parameters that were used in the system suitability testing (SST) report are: capacity factor (k'), relative retention (α), resolution (R_s), tailing factor (T), theoretical plate number (N) [134].

1.6 Aims of the study

In this thesis, we want to develop a click reaction-based norethindrone derivative employing the appropriate chromophore. Hence, it is crucial to comprehend the pharmacokinetics of medications that may result in positive or negative side effects, even in little doses. Thus, it is crucial to employ a sensitive and accurate analytical approach for identifying tiny quantities of medicines in biological samples. High-performance liquid chromatography (HPLC) detection is a potent analytical method for identifying pharmaceuticals due to its great sensitivity and selectivity. Unfortunately, many important compounds cannot be identified with HPLC due to the absence of the

essential chromophoric, fluorophoric, or redox groups. To do this, we develop a straightforward chemical process for derivatizing norethindrone that relies on the click reaction with an azide-conjugated alkyne to produce a five-membered heterocycle (1,2,3-triazole) and an extended conjugation. This will change the drug's absorbance to a more hyperchromic and bathochromic shift and, as we shall demonstrate, widen the UV detection window, making it easier to develop a new analytical detection technique.

1.7 Objectives

1. Create a derivative of norethindrone based on the click reaction. Moreover, a suitable chromophore must be developed, and an accurate and sensitive HPLC technique must be created for its confirmation.
2. The formation of a pure chemical derivative of norethindrone in order to be used as a standard during the method development.
3. Validation of the developed HPLC technique utilizing accepted metrics, such as linearity, range, accuracy, precision, limit of detection (LOD), limit of quantification (LOQ), and robustness/ruggedness.

Chapter Two

Methodology

2.1 Reagents and materials

Norethindrone (Cas. #: 68-22-4), 4-(azidomethyl) benzonitrile (Cas. #: 84466-87-5), N, N-Diisopropylethyl-amine (Cas. #: 7087-68-5), were attained from Sigma-Aldrich Co., USA. Silica gel (Pore size 60 Å, 40-63 µm particle size, 230-400 mesh particle size, Sigma Aldrich Co.) was used to purify the product using column chromatography. The reaction was examined using thin layer chromatography (TLC) using the DC-Fertigfolien Alugeram ® Sil 6 G/UV 245 kit from the Macherey Nagel Company in Germany. The greatest purity level is employed in using all materials and reagents.

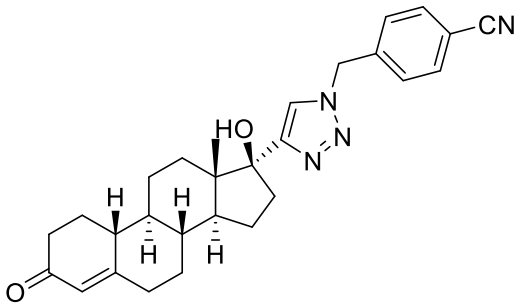
Ethyl acetate 99.5% (EtOAc), hexane (Hex), and dichloromethane (DCM) were purchased from C.S. Company, Haifa, acetonitrile supragradient grade for chromatography (ACN) was purchased from SDFCL, India, Copper(I) iodide (CuI) was purchased from Merck Millipore, USA, and Acetic acid and methanol were purchased from El Shams Co, Palestine.

2.2 Instrumentation

A binary HPLC pump (Waters 1525, Singapore) and a photodiode array detector were used. A Bruker 500 MHz-Avance III NMR spectrometer in Switzerland was used to obtain the spectral data. 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 carried out in the Pharmaceutical Laboratory (An-Najah National University, Nablus), except for NMR, which was carried out at the University of Jordan.

2.3 Synthesis of norethindrone derivative



2 mL of dichloromethane was put on a mixture of norethindrone (20 mg, 0.067 mmol), CuI (2.55 mg, 0.0134 mmol), DIPEA (3.46 mg 4.5 µl, 0.0034 mmol) acetic acid (0.5µL, 0.002 mmol) and 4-(azidomethyl) benzonitrile (147.4 µl, 0.0737 mmole) which were all stirred at room temperature for an overnight. Several derivatizing reagents such as benzyl azide and 4-azidoaniline were used and tried before we finally chosen the 4-(azidomethyl) benzonitrile. The superiority in choice was the usage of the latter because it gave a complete reaction with norethindrone within short time and a resultant product with very high yield. The TLC was used to monitor the reaction using mobile phase (DCM15: MeOH 1), and when it indicated that the reaction had finished, it was terminated. Then, DCM and 1M HCl (x3) were used to treat the reaction, and the organic layer that was collected and evaporated by a rotary evaporator. 1M HCl was used in order to convert the base in the product to a corresponding salt making it more water soluble which will partition to the aqueous layer and so getting rid of the unwanted impurities from being existed in the collected organic layer. The produced crude product was then separated using a flash chromatography on silica gel using mobile phase of (ethyl acetate 3: petrolatum ether 1) obtaining a semi-solid white product with a yield of 90%. Rf is 0.25 (EtOAc: petrolatum ether 3:1). ^{1}H NMR (500 MHz, DMSO): δ 7.91 (s, 1H, H triazole), 7.86 (d, 2H, J = 5Hz, Ar), 7.44 (d, 2H, J = 10 Hz, Ar), 5.68 (s, 3H, CH_2Ar, OH), 5.12 (s, 1H, CHCO), 2.43-1.15 (m, 20H, steroid), 0.95 (s, 3H, CH_3).

2.4 HPLC analytical method development

2.4.1 Analytical method development

A working standard solution containing 1mg/ml was prepared for the purpose of determining the ideal value for the norethindrone derivative's λ_{max}. Following that, acetonitrile was used as a blank to scan this solution in the ultraviolet spectrum between 200 and 400 nm. Since the medication's greatest absorbance was found to occur at 245 nm, this wavelength was chosen as the detecting wavelength for the purpose of calculating the concentration of norethindrone derivative. Several mobile and stationary phases were tested in order to get the best and most acceptable HPLC conditions. Three distinct compositions were used in the mobile phase's preparation: (1) Acetonitrile and water in a ratio of 50:50; (2) Acetonitrile and water in a ratio of 75:25; (3) Methanol and water in a ratio of 60:40. A short and tall C18 columns were tried and used in the separation method. A flow rate of between 0.9 and 1 ml/min were used to run the mobile phase.

2.4.2 Analytical method validation

We complied with the FDA and ICH guidelines in the validation process that has being developed [118, 122]. Testing was done on the following validation parameters: linearity, range, accuracy, precision, robustness, and ruggedness [128, 135]. The HPLC-developed technique underwent the system suitability test, where the eluted peaks were examined for characteristics including symmetry, theoretical plates, retention factor and resolution.

2.4.2.1 Stock solutions and standard solutions preparations used in validation method parameters assessment

four concentrations (10mg, 20mg, 30mg, 40mg) of norethindrone click reaction were prepared as stock solutions. Then, several standard solutions were prepared from these stock solutions through dilution of 1ml of each stock solutions in 4ml acetonitrile. Standard solutions concentrations of 2, 4, 6 and 8mg/ml were attained and used throughout HPLC method validation.

2.4.3 Precision

Two stages were taken to accomplish and assess the method's precision. Firstly, the instrument's precision evaluation, a standard solution in a concentration of 1 mg/ml was prepared by taking 1 ml of the standard solution of a concentration of (2 mg/ml) adding to a 1 ml acetonitrile solvent. Five injections of this prepared 1 mg/ml standard solution were conducted on the HPLC instrument. The chromatogram's produced peaks' percent relative standard deviation (RSD) was then computed. Intraday accuracy was investigated using concentration of 2 mg/ml. Later, the %RSD was calculated. The established method is considered precise if the RSD falls below 2% [57].

$$Standard\ Deviation\ (s) = \frac{\sqrt{\Sigma(x - \bar{x})}}{n - 1}$$

$$Relative\ Standard\ Deviation\ (\%RSD) = \frac{s}{\bar{x}} \times 100\%$$

2.4.4 Accuracy

The similarity of the readings from the regression line obtained from the HPLC's AUC values to the utilized concentrations indicates how accurate a procedure is [136]. A reaction containing norethindrone at a concentration of 4 mg/ml was employed to determine the accuracy. In order to assess this, the average AUC_{sample} (peak area of 4 mg of norethindrone) is used to calculate the accuracy of the procedure, which was then applied on the regression line equation to determine its concentration, followed by division on the real reaction concentration and multiplied by 100%.

2.4.5 Linearity and range

The linearity and range were assessed through the preparation of four reaction mixtures of norethindrone in acetonitrile solvent with concentrations of 1, 2, 6, and 8 mg/ml and each of them were injected three times into the HPLC. A calibration curve was generated showing the concentration vs the mean area under the curve (AUC) which allows the computation of the regression equations and the square correlation coefficient (r^2) via excel sheet by plotting concentration strengths on the x-axis and the average area under the curve for each concentration on the y-axis.

2.4.6 Limit of detection and quantification

These two detection thresholds serve as the benchmarks against which sensitivity is evaluated. The signal-to-noise ratio (S/N) of the norethindrone's derivative in the HPLC baseline is used to calculate these two values. The determined LOD and LOQ values had (S/N) values of 3 to 1 and 10 to 1, respectively [137]. To evaluate this, ten serial dilutions of a dilution factor of 10 for a norethindrone reaction mixture concentration of 2 mg/ml were injected and determined for their signal-to-noise ratio (S/N).

2.4.7 Robustness

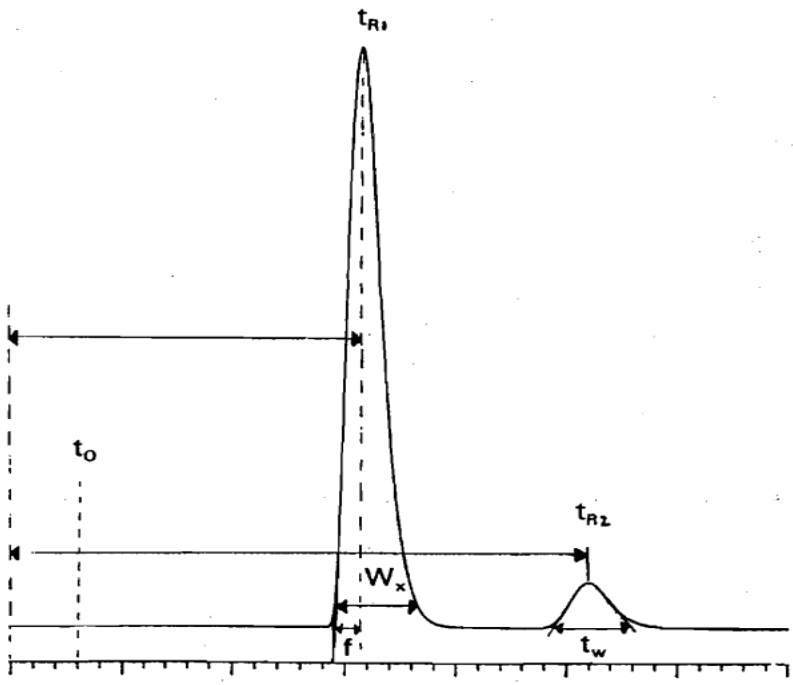
The robustness of the proposed technique was evaluated by doing small changes to the mobile phase composition, detecting wavelength, and mobile phase flow rate. We evaluated the impacts of the detection wavelength ±1 (244 nm and 246 nm), and the mobile phase flow rate −1 (0.8 mL/min) using a norethindrone reaction mixture concentration of 2 mg/ml. Also, the impact of the mobile phase composition ±1 was evaluated using a norethindrone reaction mixture concentration of 1 mg/ml and 0.5mg/ml for 59:41 and 61:39, respectively.

2.4.8 System suitability

System suitability testing was done to ensure that the system was suitable for the analysis to be carried out. This was investigated by calculating resolution, peak symmetry, retention factor and theoretical plates for the norethindrone derivative spectral HPLC peak. Figure 2.1 represents the main peak values used to assess system suitability followed by the equations of each system suitability parameter used in the calculations [138].

Figure 2.1

The main peak values used to assess system suitability



W_x = width of the peak determined at either 5% (0.05) or 10% (0.10) from the baseline of the peak height.

f = distance between peak maximum and peak front at W_x

t_o = elution time of the void volume or non-retained components.

t_R = retention time of the analyte.

t_w = peak width measured at baseline of the extrapolated straight sides to baseline.

$$resolution\ (Rs) = \frac{(\mathrm{tr}_2 - \mathrm{tr}_1)}{0.5\ (tw_1 + tw_2)}$$

$$symmetry\ of\ peak\ (tailing\ factor) = \frac{\mathrm{W_x}}{2f}$$

$$theoritical\ plates\ (N) = 5.54\ \left(\frac{t_r}{\mathrm{W1/2}}\right)2$$

$$retention\ \text{factor}\ (\mathrm{k}') = \frac{(t_r - t_0)}{t_0}$$

Chapter Three

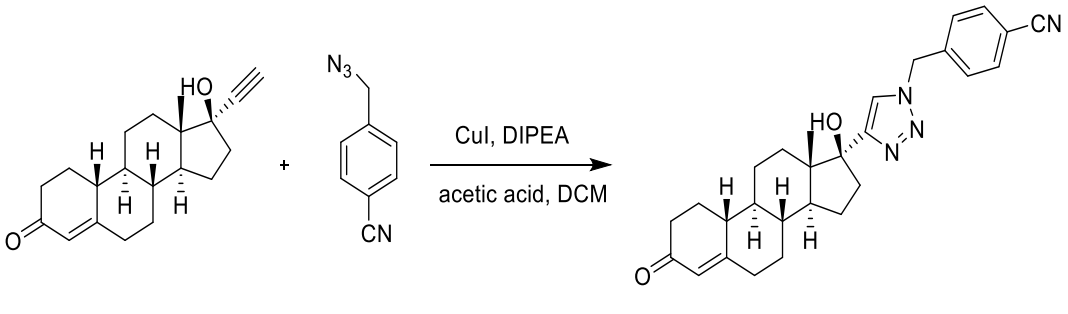
Results

3.1 Synthesis of norethindrone derivative

A novel pre-column derivatization method for the chromatographic investigation of alkyne compounds was inspired by the click reaction, a cycloaddition reaction between an alkyne and an azide in the presence of Cu(I). Click reaction is one of the most efficient ways to produce a highly pure product with a high yield. When azide and alkyne are conjugated, a five-membered heterocycle known as the 1,2,3-triazole is created results in an extended conjugation that shifts the drug's absorbance toward a more hyperchromic and bathochromic shift. As norethindrone is an alkyne, and 4-(azidomethyl) benzonitrile is an azide, thus we may use these groups to catalyze the interaction between complementary chromophores in a unique way to increase their UV detection. Scheme 3.1 shows the synthesis of the reaction while it was being monitored by the TLC to help decide when it would be finished most efficiently. After multiple tests, it was found that an overnight reaction time was the optimum time for reaction completion. An analysis using 1H NMR affirmed the product's structure, as seen in figure 3.1. It noticed the peak of the triazole proton which confirms the successful synthesis of the triazole ring through click reaction as we can found it at 7.91 ppm. Moreover, the four protons of the aromatic ring it can be found at 7.86 and 7.44 ppm. Finally, the steroidal proton can be seen in the range of 2.42 to 1.15 ppm.

Scheme 3.1

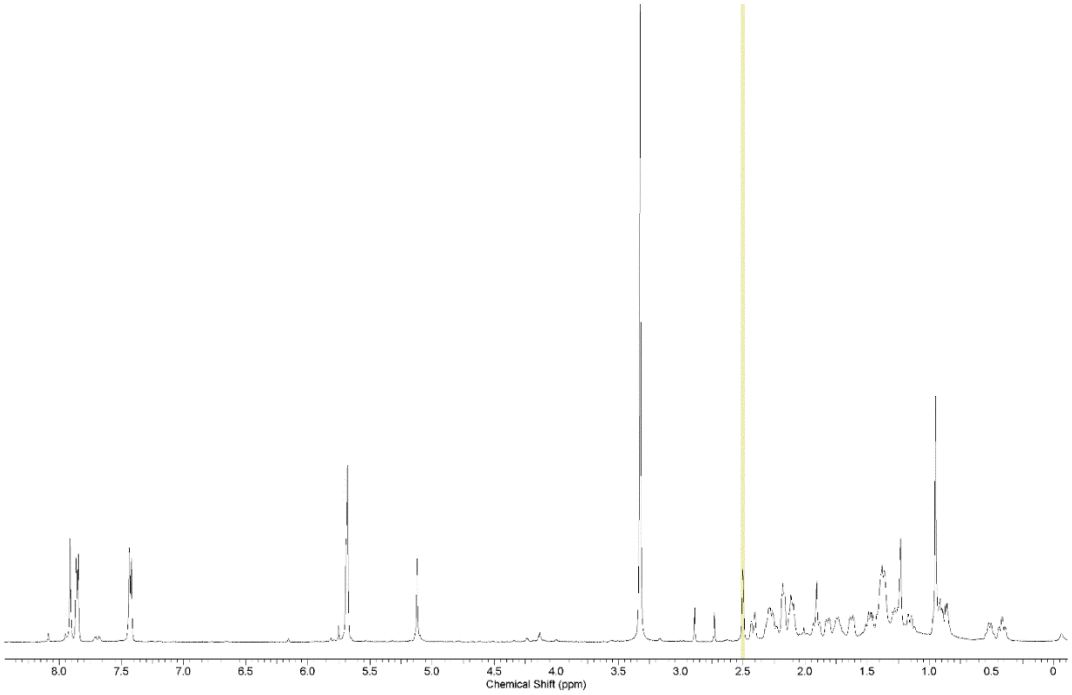
Synthetic scheme Norethindrone derivative



norethindrone 4-(azidomethyl)benzonitrile

Figure 3.1

1H NMR spectrum of norethindrone derivative



3.2 HPLC analytical method development

3.2.1 Method development

According to ICH criteria, a reverse-phase HPLC analytical technique was created and verified. Detailed specifications for the developed HPLC system for reverse-phase chromatography. It was discovered that the medication exhibited maximum absorbance at 245 nm; hence, this wavelength was chosen as the detecting wavelength for the purpose of determining the norethindrone derivative concentration. A short C18 column and a 245 nm detection method were both employed with the XTERRA® MS system. A mobile phase composition of MeOH: water 60:40 was chosen as it gave best separation with no interlapping peaks. A mobile phase flow rate of 0.9ml/min was selected for HPLC system as it gave peaks with acceptable system suitability parameters values. Table 3.1 displays the HPLC parameters. Figure 3.2 represents that the peaks were symmetrical with no broadening and their elution was at distinct retention times, 8.580 min for norethindrone, 6.541 min for the derivative, and 4.834 min for 4-(azidomethyl) benzonitrile.

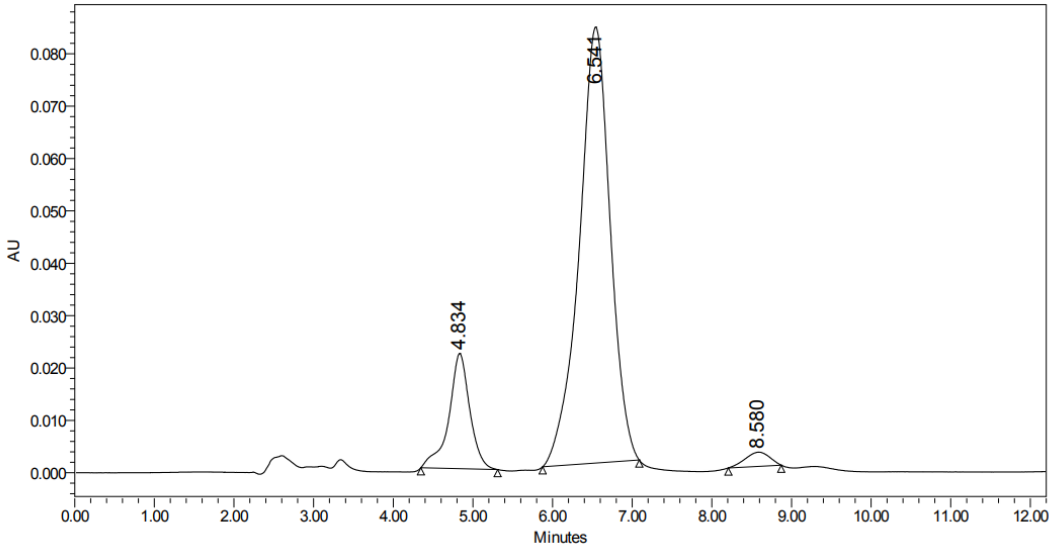
Table 3.1

The HPLC chromatographic conditions

HPLC Chromatographic	conditions
Mobile phase composition	MeOH: water 60:40 v/v
Flow rate	0.9 mL/min
Wavelength (λ)	245 nm
Stationary phase	XTERRA® MS C18, 5µm, 4.6×250 mm cartridge
Column T	25°C
Injection V	10 µL
Run time	13min

Figure 3.2

A component mixture of eluted peaks form the Chromatogram



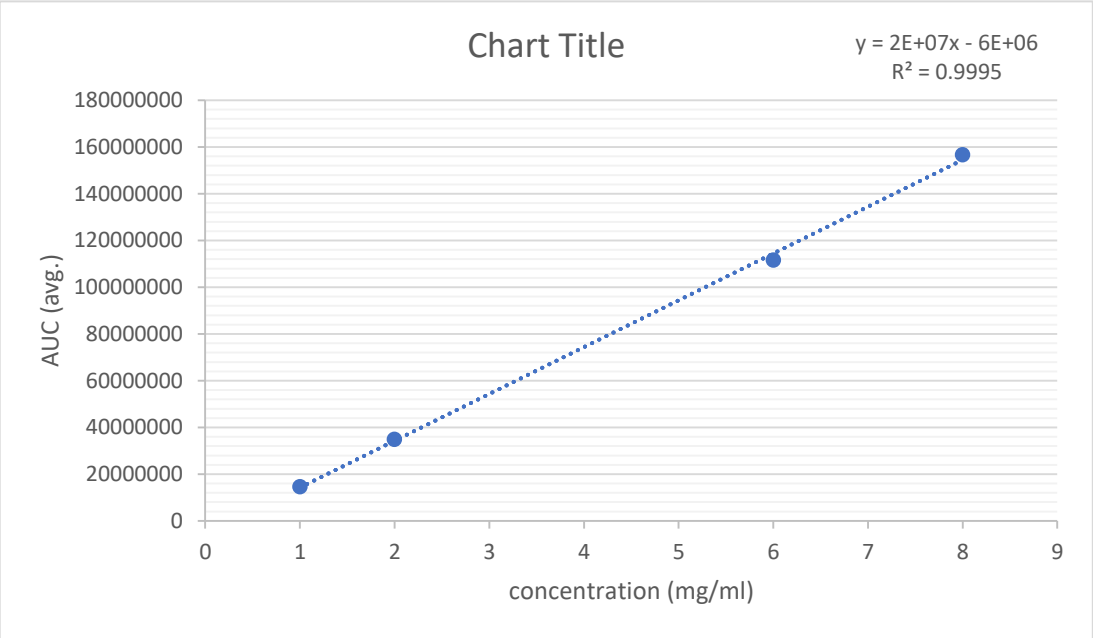
3.3 Method validation

3.3.1 Linearity and range

The linearity of the procedure was assessed by a graph showing the calculated response/sample concentration vs the sample concentrations. Derivative's regression line graph is shown in Figure 3.3. An R2 value of 0.9995 demonstrated a linear relationship between the observed peak area and sample concentration. According to ICH and FDA guidelines, which state that the value must be greater than 0.970 and 0.95, respectively [118, 135]; this is within the permitted range.

Figure 3.3

Linearity curves for the product



3.3.2 Accuracy and selectivity

The accuracy of the method is computed using the average AUC_{sample} (average peak area of 4 mg of norethindrone reaction mixture) and then applying this avg. AUC_{sample} on the regression line equation to find its concentration followed by division on the actual reaction concentration multiplied by 100 which then resulted in 101.52%. When the value of 4 mg/ml was used to compute the value, a discrepancy of 1.52% was discovered between the measured and calculated values. These findings show the accuracy of the regression line parameters that were estimated as well as the established linear relationship between sample concentration and observed AUC. As per the ICH recommendations [139], the requirements for acceptance with a range recovery of 80-120% and 90-110% were satisfied with a result of 101.52%.

3.3.3 Precision

We looked at several ranges in the assessment of method's precision; the %RSD was found to be 1.07% by injecting 1 mg/mL 5 times on HPLC, demonstrating the precision of the instrument. Calculations for the intraday precision were done by injecting a norethindrone reaction mixture of 2 mg/ml twice on another day. The results showed that the %RSD was less than 2.0. Table 3.2 lists the precision findings. In accordance

with the ICH guidelines [139], we discovered that our outcomes fell inside the permitted range. Individual findings were also determined to be within the validation range and to accord with the preset RSD, which was found to be 2%.

Table 3.2

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

Precision parameter	Conc.mg/ml	AUC	Average	%RSD
Intraday precision	2	27608076 28145597	27876836.5	1.36%
Instrument precision	1	12233165 12333166 12505735 12430542 12563957	12413313	1.07%

3.3.4 Detection and quantification limit

The lowest amount of analyte in a sample that can be recognized is known as the detection limit, or LOD. It may be stated as a concentration that provides an approximately 3:1 signal-to-noise ratio. Whereas, the lowest amount of analyte in a sample that can be measured with acceptable precision and accuracy employing a signal-to-noise ratio of around 10:1 is known as the limit of quantification, or LOQ. For the product, the LOD and LOQ were $2*10^{-6}$ mg/ml and, $2*10^{-4}$ mg/ml, respectively. According to ICH guidelines [139], it is justified to estimate the limit of detection using such a signal-to-noise ratio of 3:1. Also, a 10:1 ratio for the LOQ is the preferred value. A predetermined peak area should be used to calculate the signal-to-noise ratio for a chromatographic operation. The ratio should be evenly distributed around the peak of interest for the best results. Our findings indicate that these were satisfied.

3.3.5 Robustness

Analytical methods can be assessed based on how well they hold up to routine usage and how little they alter when subjected to tiny and deliberate adjustments to the technique parameters. Various wavelengths, flow rates, and mobile phase compositions were investigated in this experiment. Robustness of the flow rate and wavelengths were done by injecting reaction of 2 mg/ml into HPLC, whereas, robustness of the different mobile phase compositions was investigated by injecting 1 mg/ml (resulted from the dilution of the remaining 2mg/ml reaction which was used in the same section) for the 59:41 mobile phase composition and 0.5mg/ml (resulted from dilution of the remaining

1 mg/ml) for 61:39 mobile phase composition. The data are shown in Table 3.3. Take notice of the variance in an area brought on by the various injection volumes of the sample. As per ICH guidelines [139], the acquired results comply with requirements, and each of the individual results fall within the validation range and agrees to a set RSD, 2%.

Table 3.3

Results of the robustness validation of the wavelength of the maximal absorption parameter, the flow rate parameter and the mobile phase composition parameter

Chromatographic parameter	Conditions	AUC	Average	%RSD
Wavelength	246 nm	86733468	86327161	1.92%
		85577842		
	244 nm	84563973		
		88433359		
Flow rate	0.8 mL/min	88947692	87375908	1.75%
		87289292		
		85890740		
Mobile phase composition	59:41	40294766	40519003	0.78%
		40743239		
	61:39	21182003	21386276	1.35%
		21590548		

3.3.6 System suitability

System suitability tests are performed in order to ensure reliability in the analytical method and the findings acquired by confirming that a system is operating as intended. As seen in Figure 3.4 and the findings reported in Table 3.4, the established technique demonstrated that all standard system suitability criteria for the norethindrone's derivative peak in relative to the derivatizing reagent (4-(azidomethyl) benzonitrile), including the resolution, symmetry of the peaks, theoretical plates, and retention factor (K), are within acceptable ranges according to ICH guidelines [11].

Figure 3.4

Chromatogram for system suitability

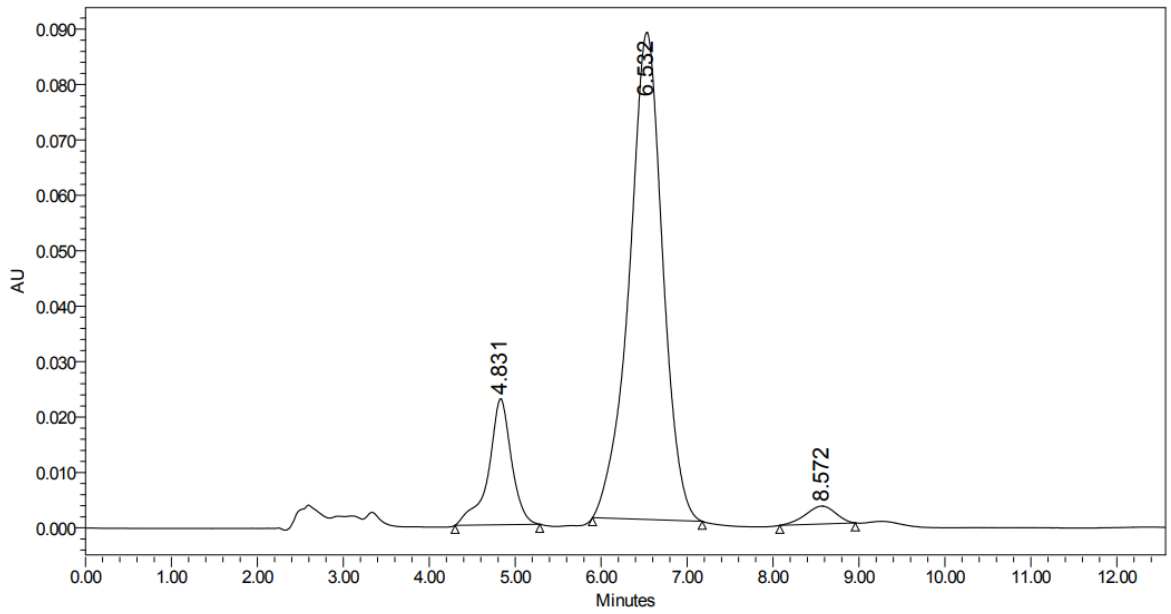


Table 3.4

System suitability

Norethindrone derivative		Acceptance criteria values
Resolution (R) BP	1.54	≥ 1.5
Symmetry of peaks	1.03	0.9-1.1
Theoretical plates (N)	2626	> 2000
Retention factor (K)	0.98	< 1

Chapter Four

Discussions and Conclusions

Based on the results we have, the derivatization technique of norethindrone using one of the most commonly followed click chemistry techniques known as the huisgen 1,3-dipolar cycloaddition (CuAAC), in which an azide (4-(azidomethyl) benzonitrile) is added to an alkyne-containing molecule (norethindrone) allowing the formation of 1,2,3-triazole moiety [78]. Our method proves to be applicable, easy, and straightforward with few steps to be followed. Our developed technique was then validated according to FDA and ICH's guidelines, which gave recorded values that indicate our method's precision, accuracy, linearity, and sensitivity. In literature, there are several studies focused on the development and validation of UV-spectrophotometric, LC-MS-MS with/without followed derivatization techniques, LC-FLD with/without derivatization techniques for the analysis of norethindrone and/or other steroidal hormones. Table 4.1 illustrates the most highlighted comparisons between the results and conditions of our new study with other related literature published UV-spectrophotometric report studies for norethindrone analysis.

Table 4.1

Literature published UV-spectrophotometric report studies for norethindrone analysis

Sample	Λ_{max} (nm)	Derivatizing reagent	LODs (mg/ml)	Linearity (r^2)	Ref.
commercially available tablets	270	-	19.87	0.999	Reddy, A., et al. [26]
commercially marketed formulation	475	2,4 dinitrophenyl hydrazine	$2.9*10^{-4}$	0.998	Lakshmi, R., and R. Swamy, 2020
commercially available tablets	256	-	$9*10^{-4}$	0.9979	Manasa, M., et al. [27]

Although, UV-spectrophotometric analysis is easy to operate at laboratory scale and can be finished faster and cheaper than analysis using HPLC/UV detector, however, it is more preferable to analyze substances via HPLC/UV detectors rather than using UV-spectrophotometric methods alone. In HPLC, measurement of absorbance and separation of samples with many absorbing components happens at once, however, in UV-spectrophotometry the assessment of samples' absorbance undergoes when just one absorbing component is present. Also, the usage of a mobile phase that combines either a buffer and a polar solvent or two polar solvents is applicable in HPLC apparatus. Whereas, in UV-spectrophotometry apparatus, it is appropriate to use polar solvent only. Regarding spectral analysis of compounds, UV-spectrophotometry offers the analysis of polar compounds with λ_{max} values of 200 to 400, on the other hand, HPLC has a broader range of detectors, as a result, compounds can be studied beyond the limit offered by the UV technique, and so, a greater sensitivity can be accomplished. Greater resolution of the peaks is attained with HPLC as a wide variety of stationary phases can be used, in contrary to the need for first and second derivative spectrophotometric methods in UV analysis to attain a comparable resolution. HPLC serves as an accurate identification test for a certain substance and is helpful to determine the quantitative characteristics. UV-spectrophotometer determines the qualitative characteristics and may be used as a final examination for a particular substance.

Concerning limit of detection (LOD) values of the mentioned UV-spectrophotometric methods, our validated HPLC method shows much lower LOD value of $2*10^{-6}$ mg/ml, demonstrating a higher sensitivity method property.

Other comparisons between our study and literature published studies involving analysis of norethindrone/ norethindrone acetate or ethinylestradiol via different HPLC and LC-MS/MS technique are displayed in table 4.2.

Table 4.2

Literature published studies regarding analysis of norethindrone/ norethindrone acetate or ethinylestradiol via HPLC technique

Sample	Detection	Λ_{max} (nm)	Derivatizing reagent	LOD (mg/ml)	Linearity (r^2)	System suitability (N, symmetry of peaks)	Ref.
NEA in pharmaceutical formulation	RP-HPLC C18 column	220	-	$2.7*10^{-4}$	0.982	2000, 1.5	koneru, A., et al., 2015
NEA in plasma, tablets and in mixture with other steroids	RP-HPLC C18 column	240	-	$6.25*10^{-5}$	0.999	17078 (pro meter), 1.964	Hashem, H., S. Abd EL-hay, and T. Jira [40]
EE in rhesus monkey plasma	LC-MS/MS	-	Dansyl chloride	0.2/sample	0.999	-	Anari, M.R., et al. [69]
Estrogens from human plasma	LC-MS/MS	-	2-fluoro-1-methyl pyridinium sulfonate	0.2/sample	0.999	-	Faqehi, A.M., et al. [70]
EE in 1 ml human plasma sample	LC-MS/MS	-	Dansyl chloride	$2.5*10^{-10}$	0.998	-	Shou, W.Z., X. Jiang, and W. Naidong [68]
NE in 0.5ml plasma sample	LC-MS/MS	-	Dansyl chloride	$5*10^{-8}$	0.998	-	Li, W., et al. [71]

In our method, the norethindrone derivative is detected on λ_{max} = 245 nm, which when compared to the λ_{max} in the first two literature HPLC methods of underivatized norethindrone analytical techniques (220 nm, 240 nm), an affirm can be taken on the advantage of the followed 1,3 dipolar cycloaddition and the success of our derivatizing methodology in producing a derivatized norethindrone with a more hyperchromic and bathochromic shift in the UV absorbance range. Also, a much lower limit of detection (LOD) is achieved in our method, hence, a higher sensitivity can be accomplished in the analysis of norethindrone from pharmaceutical samples. Concerning system suitability values, our methodology has superior values of theoretical plates (N) and more symmetrical peaks.

Negligible contrast can be made between our study and the last four studies in table 4.2 because the samples that had been used for analysis in these four mentioned studies are plasma samples, whereas, our study concerned about the analysis of norethindrone derivative as a pure substance or in a mixture without the need for an extraction from plasma or biological samples. However, a general conclusion could be taken about the advantage and importance of chemical derivatization with dansyl chloride and 2-fluoro-1-methyl pyridinium sulfonate with the hydroxyl and keto group of EE and NEA, respectively, to produce derivatives that allow easy detection of low concentration in biological samples, if needed. LC-MS/MS analytical technique is more sensitive to follow in screening substances from biological samples with more steps to be followed in the sequence of analysis, when compared to HPLC with UV detectors.

In the last few years, many researches have been published in the development and synthesis of a newly derivatized compounds via click reaction (huisgen 1,3 dipolar cycloaddition) in order to add fluorometric moiety to the original compound, allowing it to be more detectable on the fluorescence detector following HPLC separation. Table 4.3 summarizes three published papers of 1,3 dipolar cycloaddition click reaction on different drug substances.

Table 4.3

Published papers of 1,3 dipolar cycloaddition click reaction on different drug substances

Sample	Derivatizing reagent	LOD	Ref.
NE, NEA, gestodene, ethinylestradiol and norgestre in meat sample	3-azido-7-hydroxycoumarin	1.8 mg/ml	Li, Q., et al. [115]
EE in Spiked plasma samples	4-azido-7-nitrobenzene oxadiazole	7.5 pg/ml	Salama, I., et al. [116]
Rasageline (RSM) in biological rat plasma samples	Dansyl Azide (DNS-Az)	$1.6*10^{-7}$ mg/ml	Ahmed, S. and N.A. Abdullah [117]

There is no literature published data about click derivatization of NE for enhancing UV detection after HPLC separation from a mixture or from pharmaceutical formulations which in turn gives the novelty for our development of an easy and straightforward synthesis of norethindrone derivative through click reaction with 4-azidomethyl benzonitrile and our method's good achievable accuracy, linearity, precision, sensitivity, robustness and system suitability according to the followed validation parameters of the FDA's and ICH guidelines.

Conclusion

In this dissertation, an HPLC analytical technique for norethindrone estimation in a formulation has been developed and validated. The proposed method is accurate, precise, straightforward, quick, and specific. Due to the suggested method's ease of use, routine norethindrone analysis may be carried out in low-tech, low-budget laboratories without the need for specialized analytical equipment. The suggested HPLC technique with a UV/Vis detector was found to fulfill the requirements for drug system suitability, resolution, and peak integrity. Also, we demonstrated that the approach is determined to be sensitive by the limits attained in quantification and detection. According to the analysis, the derivatization of norethindrone was adjusted to be assessed at λ_{max} = 245 nm, for which the norethindrone derivatize eluted peak was isolated from other reagents. The method was subsequently validated and reported to be linear (R2 = 0.9995), precise (RSD = 1.07%), accurate (%recovery = 101.52%), sensitive to LOD ($2*10^{-6}$ mg/ml) and LOQ ($2*10^{-4}$ mg/ml), and within the acceptable limits. The high sensitivity of this developed method makes it a perfect choice for bioanalysis especially when analysis involve bioequivalence and pharmacokinetics studies which require accurate quantification of drugs at very low concentration. This study can be applied for such bioanalytical test for norethindrone and other similar cyano compounds. Our developed method can be a promising method for the analysis of norethindrone from biological and macro matrices as it gave good sensitivity values.

List of Abbreviations

Abbreviation	Meaning
UV-visible	Ultraviolet-visible spectroscopy
HPLC	High-performance liquid chromatography
TLC	Thin Layer Chromatography
FLD	Fluorescence detector
MP	Mobile phase
SP	Stationary phase
NE	Norethindrone
LH	Luteinizing Hormone
OC	Oral Contraceptive
HRT	Hormonal Replacement Therapy
EE	Ethinylestradiol
LN	Levonorgestrel
USP	United States Pharmacopeia
λ_{max}	Lambda max
MeOH	Methanol
ISTD	Internal Standard
LMWA	Low Molecular Weight Aldehydes
AA	Acetic Acid
Log K_{ow}	Decimal logarithm of octanol-water partition
LLE	Liquid-liquid extraction
DCM	Dichloromethane
MTF	Metformin
ESI	Electrospray tandem mass spectrometry
LC-MS/MS	Liquid chromatography tandem mass spectrometry
LOD	Limit of Detection
LOQ	Limit of Quantification
DIPEA	N, N'-diisopropylethylamine
HDC	Huisgen Dipolar Cycloaddition
CuI	Copper iodide
NMEs	Novel Chemical Entities

FDA	Food and Drug Administration
LLOQ	Lower Limit of Detection
NEA	Norethindrone acetate
HCl	Hydrochloric acid
EtOAc	Ethyl acetate

References

[1] Rina, R., M. Baile, and A. Jain, *A Review: Analytical Method Development and Validation.* Systematic Reviews in Pharmacy, 2021. 12(11): p. 3601-3605.

[2] Kenkel, J., *Analytical chemistry for technicians*. 2002: CRC Press.

[3] Kissinger, P.T., *Instant Notes: Analytical Chemistry. D. Kealey and PJ Haines. Oxford: Bios Scientific Publishers Limited, 2002, 352 pp.,£ 14.99, softcover. ISBN 1-859961-89-4.* Clinical Chemistry, 2002. 48(12): p. 2303-2303.

[4] Harvey, D., *Modern analytical chemistry*. Vol. 1. 2000: McGraw-Hill New York.

[5] Chatwal, G. and S. Anand, *Nuclear Magnetic Resonance.* Instrumental methods of Chemical Analysis, Himalayan Publishing House, Mumbai, 2002: p. 188-212.

[6] Kumar, S., *Spectroscopy of organic compounds*. 2008.

[7] Luxminarayan, L., et al., *A review on chromatography techniques.* Asian Journal of Pharmaceutical Research and Development, 2017: p. 1-08.

[8] Rao, B.V., et al., *A review on stability indicating HPLC method development.* World journal of pharmacy and pharmaceutical sciences, 2015. 4(8): p. 405-423.

[9] Chawla, G. and K.K. Chaudhary, *A review of HPLC technique covering its pharmaceutical, environmental, forensic, clinical and other applications.* Int J Pharm Chem Anal, 2019. 6(2): p. 27-39.

[10] Vidushi, Y., B. Meenakshi, and M. Bharkatiya, *A review on HPLC method development and validation.* Res J Life Sci, Bioinform, Pharm Chem Sci, 2017. 2(6): p. 178.

[11] Ravisankar, P., et al., *A review on step-by-step analytical method validation.* IOSR J Pharm, 2015. 5(10): p. 7-19.

[12] Benc, D., et al., *Glucocorticoid therapy and adrenal suppression.* Medicinski pregled, 2017. 70(11-12): p. 465-471.

[13] Rudolph, L.M., et al., *Actions of steroids: new neurotransmitters.* Journal of Neuroscience, 2016. 36(45): p. 11449-11458.

[14] GÖrÖg, S., *Recent advances in the analysis of steroid hormones and related drugs.* Analytical sciences, 2004. 20(5): p. 767-782.

[15] Hammond, G.L., T. Rabe, and J.D. Wagner, *Preclinical profiles of progestins used in formulations of oral contraceptives and hormone replacement therapy.* American Journal of Obstetrics & Gynecology, 2001. 185(2): p. S24-S31.

[16] Schoonen, W., et al., *Hormonal properties of norethisterone, 7α-methyl-norethisterone and their derivatives.* The Journal of steroid biochemistry and molecular biology, 2000. 74(4): p. 213-222.

[17] Rivera, R., I. Yacobson, and D. Grimes, *The mechanism of action of hormonal contraceptives and intrauterine contraceptive devices.* American journal of obstetrics and gynecology, 1999. 181(5): p. 1263-1269.

[18] SATISH, J., P. RADHAKRISHNANAND, and K.S. BABU, *METHOD DEVELOPMENT AND VALIDATION OF NORETHINDRONE ACETATE ASSAY AND ITS RELATED IMPURITIES IN API AND PHARMACEUTICAL FORMULATION WITH ORTOGONAL DE-TECTOR TECHNQUES.*

[19] Compendium, E.M. *Norethisterone 5mg Tablets*. 24 May 2023]; Available from: https://www.medicines.org.uk/emc/product/1494/smpc#gref.

[20] Sitruk-Ware, R., *Reprint of pharmacological profile of progestins.* Maturitas, 2008. 61(1-2): p. 151-157.

[21] PubChem. *norethindrone*. Available from: https://pubchem.ncbi.nlm.nih.gov/compound/Norethindrone.

[22] drugbank. *Norethisterone*. Available from: https://go.drugbank.com/drugs/DB00717.

[23] Electronic Medicines Compendium. *Norethisterone 5mg Tablets*. 30 june 2023]; Available from: https://www.medicines.org.uk/emc/product/1494/smpc#gref.

[24] Hazardous Substances Data Bank (HSDB). *norethindrone* Available from: https://pubchem.ncbi.nlm.nih.gov/source/hsdb/3370.

[25] Shroff, A.P. and E.S. Moyer, *Norethindrone*, in *Analytical Profiles of Drug Substances*. 1975, Elsevier. p. 268-293.

[26] Reddy, A., et al., *UV Spectrophotometric Determination of Norethisterone in Tablets.* Asian Journal of Research in Chemistry, 2010. 3(1): p. 172-174.

[27] Manasa, M., et al., *METHOD DEVELOPMENT AND VALIDATION OF NORETHINDRONE BY UV-VISIBLE SPECTROPHOTOMETER IN BULK AND PHARMACEUTICAL DOSAGE FORM.* 2018.

[28] Loo, J. and R. Brien, *Analysis of norethindrone in plasma by high-performance liquid chromatography.* Journal of Liquid Chromatography, 1981. 4(5): p. 871-877.

[29] Licea-Perez, H., et al., *A semi-automated 96-well plate method for the simultaneous determination of oral contraceptives concentrations in human plasma using ultra performance liquid chromatography coupled with tandem mass spectrometry.* Journal of chromatography B, 2007. 852(1-2): p. 69-76.

[30] Keam, S.J. and A.J. Wagstaff, *Ethinylestradiol/drospirenone: a review of its use as an oral contraceptive.* Treatments in endocrinology, 2003. 2(1): p. 49-70.

[31] Kubba, A., *Contraception: developments reviewed.* Inpharma, 2000. 1267: p. 9.

[32] Oddsson, K., et al., *Superior cycle control with a contraceptive vaginal ring compared with an oral contraceptive containing 30 μg ethinylestradiol and 150 μg levonorgestrel: a randomized trial.* Human Reproduction, 2005. 20(2): p. 557-562.

[33] Bagon, K.R. and E. Hammond, *Determination of ethinyloestradiol in single tablets and its separation from other steroids by high-performance liquid chromatography.* Analyst, 1978. 103(1223): p. 156-161.

[34] Gluck, J. and E. Shek, *Determination of ethinylestradiol and norethisterone in an oral contraceptive capsule by reversed-phase high performance liquid chromatography.* Journal of Chromatographic Science, 1980. 18(11): p. 631-636.

[35] Swynnerton, N.F. and J.B. Fischer, *Determination of Ethynylestradiol and Norethindrone in Synthetic Intestinal Fluid and in Timed-Release Oral Formulations.* Journal of Liquid Chromatography, 1980. 3(8): p. 1195-1204.

[36] Fayad, P.B., M. Prévost, and S. Sauvé, *On-line solid-phase extraction coupled to liquid chromatography tandem mass spectrometry optimized for the analysis of steroid hormones in urban wastewaters.* Talanta, 2013. 115: p. 349-360.

[37] Gonzalo-Lumbreras, R. and R. Izquierdo-Hornillos, *High-performance liquid chromatographic optimization study for the separation of natural and synthetic anabolic steroids. Application to urine and pharmaceutical samples.* Journal of Chromatography B: Biomedical Sciences and Applications, 2000. 742(1): p. 1-11.

[38] Bagócsi, B., et al., *OPLC, a method between TLC and HPLC, for purity testing of norethisterone bulk drug substance and tablet.* JPC-Journal of Planar Chromatography-Modern TLC, 2003. 16(5): p. 359-362.

[39] Krishna, P.M., et al., *Development and validation of method for the determination of related substances of norethindrone in norethindrone tablets and degradation studies.* Int J ChemTech Res, 2011. 3(1): p. 143-148.

[40] Hashem, H., S. Abd El-hay, and T. Jira, *A rapid stability indicating HPLC-method for determination of norethisterone acetate in plasma, tablets and in a mixture with other steroids.* Int J Pharm Pharmaceut Sci, 2015. 7: p. 279-82.

[41] Hill, H., *Bioanalysis in drug discovery.* Bioanalysis, 2011. 3(19): p. 2155-2158.

[42] Hansen, F., E.L. Øiestad, and S. Pedersen-Bjergaard, *Bioanalysis of pharmaceuticals using liquid-phase microextraction combined with liquid chromatography–mass spectrometry.* Journal of pharmaceutical and biomedical analysis, 2020. 189: p. 113446.

[43] Moldoveanu, S. and V. David, *Modern sample preparation for chromatography*. 2021: Elsevier.

[44] Ahmad Dar, A., P. Sangwan, and A. Kumar, *Chromatography: An important tool for drug discovery.* Journal of separation science, 2020. 43(1): p. 105-119.

[45] Adegoke, O.A., *Chemical derivatization methodologies for UV-visible spectrophotometric determination of pharmaceuticals.* Int. J. Pharm. Sci. Rev. Res, 2012. 14(2): p. 6-24.

[46] David, V., S.C. Moldoveanu, and T. Galaon, *Derivatization procedures and their analytical performances for HPLC determination in bioanalysis.* Biomedical Chromatography, 2021. 35(1): p. e5008.

[47] Lawrence, J.F., *Derivatization in chromatography introduction practical aspects of chemical derivatization in chromatography.* Journal of Chromatographic Science, 1979. 17(3): p. 113-114.

[48] Danielson, N.D., P.A. Gallagher, and J.J. Bao, *Chemical reagents and derivatization procedures in drug analysis.* Encyclopedia of Analytical Chemistry, 2000: p. 7042-7076.

[49] Perez, H.L. and C.A. Evans, *Chemical derivatization in bioanalysis*. 2015, Future Science. p. 2435-2437.

[50] Deng, P., et al., *Derivatization methods for quantitative bioanalysis by LC–MS/MS.* Bioanalysis, 2012. 4(1): p. 49-69.

[51] Medvedovici, A., A. Farca, and V. David, *Derivatization reactions in liquid chromatography for drug assaying in biological fluids.* Adv. Chromatogr, 2009. 47: p. 283-314.

[52] Medvedovici, A., E. Bacalum, and V. David, *Sample preparation for large-scale bioanalytical studies based on liquid chromatographic techniques.* Biomedical Chromatography, 2018. 32(1): p. e4137.

[53] Moldoveanu, S. and V. David, *Chapter 9—The Role of Derivatization in Chromatography.* Modern Sample Preparation for Chromatography; Moldoveanu, S., David, V., Eds, 2015: p. 307-331.

[54] McMahon, D.H., *Methods development guidelines for chemical derivatization in gas chromatography*. Journal of chromatographic science, 1985. 23(9): p. 426-428.

[55] Bartzatt, R., *Dansylation of hydroxyl and carboxylic acid functional groups.* Journal of Biochemical and Biophysical Methods, 2001. 47(3): p. 189-195.

[56] Sun, Y., H. Tang, and Y. Wang, *Progress and challenges in quantifying carbonyl-metabolomic phenomes with LC-MS/MS.* Molecules, 2021. 26(20): p. 6147.

[57] Abualhasan, M., et al., *HPLC Analytical Method Development and Validation of Gabapentin through Chemical Derivatization with Catechol as a Chromophore.* International Journal of Analytical Chemistry, 2022. 2022.

[58] Tashev, K., V. Ivanova-Petropulos, and M. Stefova, *Optimization and validation of a derivatization method for analysis of biogenic amines in wines using RP-HPLC-DAD.* Macedonian Journal of Chemistry and Chemical Engineering, 2016. 35(1): p. 19-28.

[59] Tan, A. and K. Awaiye, *Use of internal standards in LC-MS bioanalysis.* Handbook of LC-MS Bioanalysis: Best Practices, Experimental Protocols, and Regulations, 2013: p. 217-227.

[60] Aubry, A.-F. and N. Weng, *So you think your assay is robust?* 2015, Future Science. p. 2969-2971.

[61] Mandal, P., et al., *Internal Standard an Important Analyte Use in Drug Analysis by Liquid Chromatography Mass Spectrometry-An Article.* International Journal of Pharmaceutical and Bio Medical Science, 2022. 2(01): p. 10-17.

[62] Mezzar, S., E. De Schryver, and P.P. Van Veldhoven, *RP-HPLC-fluorescence analysis of aliphatic aldehydes: application to aldehyde-generating enzymes HACL1 and SGPL1.* Journal of lipid research, 2014. 55(3): p. 573-582.

[63] Moldoveanu, S.C. and V. David, *Selection of the HPLC method in chemical analysis*. 2016: Elsevier.

[64] Poole, C.F. and S.N. Atapattu, *Determination of physicochemical properties of small molecules by reversed-phase liquid chromatography.* Journal of Chromatography a, 2020. 1626: p. 461427.

[65] McGinnis, A.C., E.C. Grubb, and M.G. Bartlett, *Systematic optimization of ion-pairing agents and hexafluoroisopropanol for enhanced electrospray ionization mass spectrometry of oligonucleotides.* Rapid Communications in Mass Spectrometry, 2013. 27(23): p. 2655-2664.

[66] Winther, J.R. and C. Thorpe, *Quantification of thiols and disulfides.* Biochimica et Biophysica Acta (BBA)-General Subjects, 2014. 1840(2): p. 838-846.

[67] Tache, F., et al., *HPLC-DAD determination of Metformin in human plasma using derivatization with p-nitrobenzoyl chloride in a biphasic system.* Microchemical journal, 2001. 68(1): p. 13-19.

[68] Shou, W.Z., X. Jiang, and W. Naidong, *Development and validation of a high-sensitivity liquid chromatography/tandem mass spectrometry (LC/MS/MS) method with chemical derivatization for the determination of ethinyl estradiol in human plasma.* Biomedical Chromatography, 2004. 18(7): p. 414-421.

[69] Anari, M.R., et al., *Derivatization of ethinylestradiol with dansyl chloride to enhance electrospray ionization: application in trace analysis of ethinylestradiol in rhesus monkey plasma.* Analytical chemistry, 2002. 74(16): p. 4136-4144.

[70] Faqehi, A.M., et al., *Derivatization of estrogens enhances specificity and sensitivity of analysis of human plasma and serum by liquid chromatography tandem mass spectrometry.* Talanta, 2016. 151: p. 148-156.

[71] Li, W., et al., *Simultaneous determination of norethindrone and ethinyl estradiol in human plasma by high performance liquid chromatography with tandem mass spectrometry—experiences on developing a highly selective method using derivatization reagent for enhancing sensitivity.* Journal of chromatography B, 2005. 825(2): p. 223-232.

[72] Holländer, A., *Why do we need chemical derivatization?* Plasma Processes and Polymers, 2017. 14(7): p. 1700044.

[73] Becer, C.R., R. Hoogenboom, and U.S. Schubert, *Click chemistry beyond metal-catalyzed cycloaddition.* Angewandte Chemie International Edition, 2009. 48(27): p. 4900-4908.

[74] Tron, G.C., et al., *Click chemistry reactions in medicinal chemistry: Applications of the 1, 3-dipolar cycloaddition between azides and alkynes.* Medicinal research reviews, 2008. 28(2): p. 278-308.

[75] Devaraj, N.K. and M. Finn, *Introduction: click chemistry*. 2021, ACS Publications. p. 6697-6698.

[76] Kolb, H.C., M. Finn, and K.B. Sharpless, *Click chemistry: diverse chemical function from a few good reactions.* Angewandte Chemie International Edition, 2001. 40(11): p. 2004-2021.

[77] Huisgen, R., G. Szeimies, and L. Möbius, *1.3-Dipolare cycloadditionen, XXXII. Kinetik der Additionen organischer azide an CC-mehrfachbindungen.* Chemische Berichte, 1967. 100(8): p. 2494-2507.

[78] Huisgen, R. and A. Padwa, *1, 3-Dipolar cycloaddition chemistry.* Wiley, New York, 1984. 1(2): p. 55-92.

[79] Rostovtsev, V.V., et al., *A stepwise huisgen cycloaddition process: copper (I)-catalyzed regioselective "ligation" of azides and terminal alkynes.* Angewandte Chemie, 2002. 114(14): p. 2708-2711.

[80] Tornøe, C.W., C. Christensen, and M. Meldal, *Peptidotriazoles on solid phase:[1, 2, 3]-triazoles by regiospecific copper (I)-catalyzed 1, 3-dipolar cycloadditions of terminal alkynes to azides.* The Journal of organic chemistry, 2002. 67(9): p. 3057-3064.

[81] Bock, V.D., H. Hiemstra, and J.H. Van Maarseveen, *CuI-catalyzed alkyne–azide "click" cycloadditions from a mechanistic and synthetic perspective.* European Journal of Organic Chemistry, 2006. 2006(1): p. 51-68.

[82] Huisgen, R., *1.3-Dipolare Cycloadditionen Rückschau und Ausblick.* Angewandte Chemie, 1963. 75(13): p. 604-637.

[83] Rodionov, V.O., V.V. Fokin, and M. Finn, *Mechanism of the Ligand-Free CuI-Catalyzed Azide–Alkyne Cycloaddition Reaction.* Angewandte Chemie, 2005. 117(15): p. 2250-2255.

[84] Himo, F., et al., *Copper (I)-catalyzed synthesis of azoles. DFT study predicts unprecedented reactivity and intermediates.* Journal of the American Chemical Society, 2005. 127(1): p. 210-216.

[85] Bräse, S., et al., *Organic azides: an exploding diversity of a unique class of compounds.* Angewandte Chemie International Edition, 2005. 44(33): p. 5188-5240.

[86] Kolb, H.C. and K.B. Sharpless, *The growing impact of click chemistry on drug discovery.* Drug discovery today, 2003. 8(24): p. 1128-1137.

[87] Zhan, W.-h., et al., *Synthesis of hemicyanine dyes for 'click'bioconjugation.* Tetrahedron letters, 2005. 46(10): p. 1691-1695.

[88] Bourne, Y., et al., *Freeze-frame inhibitor captures acetylcholinesterase in a unique conformation.* Proceedings of the National Academy of Sciences, 2004. 101(6): p. 1449-1454.

[89] BROOKS, G., et al., *6-(Substituted methylene) penems, potent broad spectrum inhibitors of bacterial. BETA.-lactamase. V. Chiral 1, 2, 3-triazolyl derivatives.: V. CHIRAL 1, 2, 3-TRIAZOLYL DERIVATIVES.* The Journal of Antibiotics, 1991. 44(9): p. 969-978.

[90] Kharb, R., P.C. Sharma, and M.S. Yar, *Pharmacological significance of triazole scaffold.* Journal of enzyme inhibition and medicinal chemistry, 2011. 26(1): p. 1-21.

[91] Pertino, M.W., et al., *1, 2, 3-Triazole-substituted oleanolic acid derivatives: Synthesis and antiproliferative activity.* Molecules, 2013. 18(7): p. 7661-7674.

[92] Palmer, M.H., R.H. Findlay, and A.J. Gaskell, *Electronic charge distribution and moments of five-and six-membered heterocycles.* Journal of the Chemical Society, Perkin Transactions 2, 1974(4): p. 420-428.

[93] Brak, K., et al., *Nonpeptidic tetrafluorophenoxymethyl ketone cruzain inhibitors as promising new leads for Chagas disease chemotherapy.* Journal of medicinal chemistry, 2010. 53(4): p. 1763-1773.

[94] Pagliai, F., et al., *Rapid synthesis of triazole-modified resveratrol analogues via click chemistry.* Journal of medicinal chemistry, 2006. 49(2): p. 467-470.

[95] Singh, P., et al., *Synthesis, docking and in vitro antimalarial evaluation of bifunctional hybrids derived from β-lactams and 7-chloroquinoline using click chemistry.* Bioorganic & medicinal chemistry letters, 2012. 22(1): p. 57-61.

[96] Srinivasan, R., M. Uttamchandani, and S.Q. Yao, *Rapid assembly and in situ screening of bidentate inhibitors of protein tyrosine phosphatases.* Organic letters, 2006. 8(4): p. 713-716.

[97] Mamidyala, S.K. and M. Finn, *In situ click chemistry: probing the binding landscapes of biological molecules.* Chemical Society Reviews, 2010. 39(4): p. 1252-1261.

[98] Mocharla, V.P., et al., *In situ click chemistry: enzyme-generated inhibitors of carbonic anhydrase II.* Angewandte Chemie, 2005. 117(1): p. 118-122.

[99] Dörner, S. and B. Westermann, *A short route for the synthesis of "sweet" macrocycles via a click-dimerization–ring-closing metathesis approach.* Chemical communications, 2005(22): p. 2852-2854.

[100] Aucagne, V. and D.A. Leigh, *Chemoselective formation of successive triazole linkages in One Pot:"Click− Click" chemistry.* Organic letters, 2006. 8(20): p. 4505-4507.

[101] Wu, P., et al., *Efficiency and fidelity in a click-chemistry route to triazole dendrimers by the copper (I)-catalyzed ligation of azides and alkynes.* Angewandte Chemie, 2004. 116(30): p. 4018-4022.

[102] Hansen, T.V., et al., *Just click it: Undergraduate procedures for the copper (I)-catalyzed formation of 1, 2, 3-triazoles from azides and terminal acetylenes.* Journal of chemical education, 2005. 82(12): p. 1833.

[103] Nwe, K. and M.W. Brechbiel, *Growing applications of "click chemistry" for bioconjugation in contemporary biomedical research.* Cancer Biotherapy and Radiopharmaceuticals, 2009. 24(3): p. 289-302.

[104] Gonçalves, M.S.T., *Fluorescent labeling of biomolecules with organic probes.* Chemical reviews, 2009. 109(1): p. 190-212.

[105] McKay, C.S. and M. Finn, *Click chemistry in complex mixtures: bioorthogonal bioconjugation.* Chemistry & biology, 2014. 21(9): p. 1075-1101.

[106] Mykhalichko, B.M., O.N. Temkin, and M.G. Mys' kiv, *Polynuclear complexes of copper (I) halides: coordination chemistry and catalytic transformations of alkynes.* Russian Chemical Reviews, 2000. 69(11): p. 957-984.

[107] Chinchilla, R. and C. Nájera, *The Sonogashira reaction: a booming methodology in synthetic organic chemistry.* Chemical reviews, 2007. 107(3): p. 874-922.

[108] Horne, W.S., C.D. Stout, and M.R. Ghadiri, *A heterocyclic peptide nanotube.* Journal of the American Chemical Society, 2003. 125(31): p. 9372-9376.

[109] Sharpless, K. *Secret life of enzymes: An aggressive strategy for drug discovery*. in *ABSTRACTS OF PAPERS OF THE AMERICAN CHEMICAL SOCIETY*. 2005. AMER CHEMICAL SOC 1155 16TH ST, NW, WASHINGTON, DC 20036 USA.

[110] Cheng, H.-C., et al., *Regulation and function of protein kinases and phosphatases.* Enzyme research, 2011. 2011.

[111] Hirpessa, B.B., B.H. Ulusoy, and C. Hecer, *Hormones and hormonal anabolics: residues in animal source food, potential public health impacts, and methods of analysis.* Journal of Food Quality, 2020. 2020: p. 1-12.

[112] Avar, P., et al., *HPLC-MS/MS analysis of steroid hormones in environmental water samples.* Drug Testing and Analysis, 2016. 8(1): p. 123-127.

[113] Fuentes, N. and P. Silveyra, *Estrogen receptor signaling mechanisms.* Advances in protein chemistry and structural biology, 2019. 116: p. 135-170.

[114] Janesick, A. and B. Blumberg, *Obesogens, stem cells and the developmental programming of obesity.* International journal of andrology, 2012. 35(3): p. 437-448.

[115] Li, Q., et al., *Click reaction triggered turn-on fluorescence strategy for highly sensitive and selective determination of steroid hormones in food samples.* Food Chemistry, 2022. 374: p. 131565.

[116] Salama, I., et al., *Novel Clickable Fluorescence Probe: Benzonitrofurazan-Azide for Trace and Selective Quantification of Ethinylestradiol in Spiked Human Plasma.*

[117] Ahmed, S. and N.A. Abdallah, *Dansyl azide as a selective fluorescence tagging probe for click chemistry reactions and its application to monitor rasagiline in pharmacokinetic studies.* Journal of Pharmaceutical and Biomedical Analysis, 2019. 165: p. 357-365.

[118] FDA, R.G., *Integration of Study Results to Assess Concerns about Human Reproductive and Developmental Toxicities (http://www. fda. gov/cder/guidance/4625dft. pdf)*. 2001, October.

[119] Gad, S.C., *Regulatory toxicology*. 2001: CRC Press.

[120] Guideline, I.H.T., *ICH Topic Q2B, Validation of analytical Procedures: Methodology, ICH, London 1996.* Klju~ ne rije~ i.

[121] Huber, L., *Validation and qualification in analytical laboratories*. 2007: CRC Press.

[122] Branch, S.K., *Guidelines from the international conference on harmonisation (ICH).* Journal of pharmaceutical and biomedical analysis, 2005. 38(5): p. 798-805.

[123] Lindholm, J., *Development and validation of HPLC methods for analytical and preparative purposes*. 2004, Acta Universitatis Upsaliensis.

[124] Johansson, E., A. Karlsson, and J.W. Ludvigsson, *Ultra high performance liquid chromatography method development for separation of omeprazole and related substances on core-shell columns using a Quality by Design approach.* Journal of separation science, 2020. 43(4): p. 696-707.

[125] Vessman, J., *Selectivity or specificity? Validation of analytical methods from the perspective of an analytical chemist in the pharmaceutical industry.* Journal of pharmaceutical and biomedical analysis, 1996. 14(8-10): p. 867-869.

[126] Bidlingmeyer, B., *Detector linearity.* Journal of Chromatography Sciences, 1993. 31: p. 294.

[127] Swartz, M. and I. Krull, *Method validation and robustness.* LCGC North America, 2006. 24(5): p. 480–490-480–490.

[128] Guideline, I.H.T., *Validation of analytical procedures: text and methodology.* Q2 (R1), 2005. 1(20): p. 05.

[129] Swartz, M. and I. Krull, *Validation, qualification, or verification?* LCGC North America, 2005. 23(10): p. 1100–1109-1100–1109.

[130] Vander Heyden, Y., et al., *Ruggedness tests on the high-performance liquid chromatography assay of the United States Pharmacopeia XXII for tetracycline hydrochloride. A comparison of experimental designs and statistical interpretations.* Analytica chimica acta, 1995. 312(3): p. 245-262.

[131] Vander Heyden, Y., et al., *Ruggedness tests for a high-performance liquid chromatographic assay: comparison of an evaluation at two and three levels by using two-level Plackett-Burman designs.* Analytica Chimica Acta, 1995. 316(1): p. 15-26.

[132] Shabir, G.A., *Step-by-step analytical methods validation and protocol in the quality system compliance industry.* Journal of validation technology, 2005. 10: p. 314-325.

[133] Agency, E.M., *ICH Topic Q 2 (R1) Validation of Analytical Procedures: Text and Methodology.* Prescrire Int, 1995. 20: p. 278.

[134] Guidance, R., *Validation of chromatographic methods.* Center for Drug Evaluation and Research (CDER), Washington, 1994. 2.

[135] Shabir, G.A., *Validation of high-performance liquid chromatography methods for pharmaceutical analysis: Understanding the differences and similarities between validation requirements of the US Food and Drug Administration, the US Pharmacopeia and the International Conference on Harmonization.* Journal of chromatography A, 2003. 987(1-2): p. 57-66.

[136] Abualhasan, M., et al., *Synthesis of Rutin Derivatives to Enhance Lipid Solubility and Development of Topical Formulation with a Validated Analytical Method.* Current Drug Delivery, 2022. 19(1): p. 117-128.

[137] Krull, I.S. and M. Swartz, *Analytical method development and validation for the academic researcher.* 1999.

[138] Food and D. Administration, *Reviewer Guidance: Validation of chromatographic methods.* cited November, 1994.

[139] Guideline, I.H.T., *Analytical Procedure Development Q14.* International Council for Harmonisation of Technical Requirements for Pharmaceuticals for Human Use: Geneva, Switzerland, 2022.

جـامعـــة النجاح الوطنية

كليـــة الدراســـات العليـــا

الاشتقاق الكيميائي للنورثيندرون بناءً على تفاعل كليك لتطوير طريقة تحليلية جديدة

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

2023

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الملخص

نوريثيندرون هو بروجستين اصطناعي يحاكي تأثيرات البروجسترون الطبيعية للإناث ولكن بقوة أكبر. تستخدم حبوب البروجستين التي تحتوي على نوريثيندرون بشكل أساسي كأقراص منع الحمل عن طريق زيادة ترقق بطانة الرحم وزيادة سماكة مخاط عنق الرحم. كما أنه يستخدم كعلاج هرموني بديل إلى جانب هرمونات الستيرويد الأخرى في علاج الحالات التي تسببها المستويات الهرمونية غير الطبيعية. نوريثيندرون له خصائص كبيرة في تحديد الجرعة، مما يؤدي إلى نافذة علاجية صغيرة. وبالتالي، هناك حاجة لطرق تحليلية تسمح بقياسات فعالة وموثوقة للنوريثيندرون. يجب أن يكون للطرق التحليلية المتوافقة ما يكفي من الحساسية أو الانتقائية أو كليهما من أجل تقييم العمليات الكيميائية المختلفة بسهولة ودقة أكبر. تتضمن إحدى الاستراتيجيات التكيفية المستخدمة في الاشتقاق الكيميائي تحويل المجموعات الوظيفية داخل الجزيء. في هذه الأطروحة، قدمنا اقتراحًا لإنشاء تقنية تحليلية جديدة للاشتقاق الكيميائي للنوريثيندرون. تم تحقيق ذلك باستخدام تفاعل نقرة مع ألكين مترافق مع أزايد، والذي أنتج حلقة غير متجانسة من خمسة أعضاء (1،2،3–تريازول) واقتران ممتد. كان هدفنا إنشاء طريقة تحليلية حساسة وانتقائية. تم إنشاء الطريقة التحليلية باستخدام HPLC مع كاشف UV/Vis والتحقق من صحتها باستخدام معلمات مثل الدقة والخطية والمدى والدقة وحد الكشف وحد الكميات وفقًا لإرشادات ICH وFDA. تم تحليل الطريقة المطورة باعتماد طول موجه 245 نانوميتر لقياس نوريثيندرون المشتق. استخدمت الطريقة HPLC باستخدام موبايل فاير ميثانول: ماء 60:40. بحيث تم فصل قمة

النوريثيندرون المشتق عن كواشف الاشتقاق المستخدمة الأخرى. ثم تم التحقق من صحة الطريقة التحليلية، ووجدت معاملات التحقق ضمن الحدود المسموح بها. أثبتت الطريقة المطورة أنها دقيقة (RSD = 1.07) وخطية (R2=0.999) و(نسبة الاسترداد = 106.5٪). علاوة على ذلك، كانت الطريقة المطورة حساسة لـLOD (2 * 6-10 مجم / مل) و LOQ (2 * 4-10 مجم / مل). لذلك، فإن الطريقة المطورة بسيطة وعملية ولديها حساسية وانتقائية ممتازة. يمكن تحليل نوريثيندرون في مجموعة متنوعة من أشكال الجرعات والمواد الخام، بما في ذلك المكونات الصيدلانية النشطة. تشير قيم الحساسية الجيدة لطريقتنا المطورة إلى أنه يمكن تطبيقها لتحليل نوريثيندرون في النظم البيولوجية.

الكلمات المفتاحية: الكيمياء النقرية، الاشتقاق الكيميائي، نورثيندرون، تطوير الطريقة التحليلية، كروماتوغرافيا السائل عالية الدقة.