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Research Article

Synthetic Strategies for Warfarin and Its Deuterated Analogues: Implications for Anticoagulant Therapy

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ABSTRACT

Warfarin, a widely used oral anticoagulant, was synthesized along with its deuterated analogue (deuterated warfarin) to investigate the influence of deuterium substitution on physicochemical and spectral properties. The deuterated derivative was prepared using deuterated benzalacetone as the starting material. Comparative evaluation between warfarin and its deuterated form revealed distinct differences in physicochemical parameters, confirming the successful incorporation of deuterium. Spectroscopic analysis showed a shift in the C–H stretching frequency in the IR spectrum and the absence of C–H proton signals in the ¹H NMR spectrum of deuterated warfarin, both indicative of effective deuteration. These findings suggest structural modification at the molecular level due to isotope substitution. However, further drug metabolism studies employing liver microsomal enzyme CYP3A4 are required to validate the metabolic stability and elucidate the in vivo pharmacokinetic behavior of deuterated warfarin. This study provides a foundation for understanding the potential therapeutic advantages of deuterated analogues in improving drug stability and metabolic profiles.

INTRODUCTION

The heavier, non-radioactive stable isotope of hydrogen that occurs naturally is called deuterium. Deuterium was discovered in 1931 by Harold Urey, who was given the Nobel Prize in 1934 for

this discovery. An essential tool for understanding the mechanisms of chemical reactions is the deuterium isotope effect. One proton, one electron, and one neutron make up deuterium, thus doubling its mass without significantly changing its characteristics. The C-D bond, on the other hand,

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is a little shorter, has less electrical polarizability, and stabilizes surrounding bonds by hyper conjugation (including the developing anti-bonding orbital of a newly forming bond). It has the ability to inhibit van der Waals stabilization and to produce other difficult-to-predict properties like alterations. [1]

1.1 General introduction

The first deuterated molecule patent in the country was issued in the 1970s. Since then, patents for deuterated drugs have increased in frequency.[2] The application of the deuterium isotope effect has become more widespread throughout time and is currently applied in a variety of industries. [3]Research on pharmacokinetics and mechanistic investigations of drug metabolism (PK) are important. Efficacy, acceptability, bioavailability, and safety are also important considerations. Deuterated drug candidates first appeared in the year 2000. In the 1970s, deuterated metabolites were initially examined. The government finally

took action after more than 40 years, though. Deutetrabenazine was the first deuterated drug to receive FDA approval. Many publications have discussed this subject. [3]

The first deuterated molecule patent in the country was issued in the 1970s. [3]Since then, there have been numerous patents on deuterated medicines. The use of the deuterium isotope effect has gained prominence over time and is currently utilized frequently in many different fields. Research on pharmacokinetics and mechanistic investigations of drug metabolism (PK) are also important. Efficacy, acceptability, bioavailability, and safety are also important considerations. Deuterated drug candidates first appeared in the year 2000. In the 1970s, deuterated metabolites were initially examined. [3]. The government finally took action after more than 40 years, though Deutetrabenazine was the first deuterated drug to receive FDA approval. Many publications have discussed this subject. The advantages and disadvantages of deuterated medicine. [3].

	Proton		Deuterium
➤	In the nucleus of hydrogen atoms, there are no neutrons.	➤	The nucleus of deuterium atoms contains a neutron.
➤	Deuterium is a hydrogen isotope that is stable.	➤	The chemical element hydrogen has an atomic number of one.
➤	Atomic mass is 2.014u	➤	Atomic mass is 1.00794
➤	Abundance is 0.015	➤	Abundance is 99 %
➤	Not radioactive	➤	Not radioactive

1.2 Need of deuteration:

The conversion of a protonated drug into a deuterated form is an efficient way to change the physicochemical characteristics of many medicinal compounds. The interconversion of vitamin K and vitamin K epoxide, as well as vitamin K's function in the carboxylation of a number of clotting cascade proteins, are interfered with by this anticoagulant, which prevents the start of clotting. 1 Warfarin-containing medications

have been used to treat and prevent blood clots in the context of cardiac valve replacement, pulmonary embolism, venous thrombosis, and atrial fibrillation.[4]

1.3 Advantages of deuterated warfarin over warfarin

- Because a deuterated form of warfarin breaks down more slowly, it remains in the body for

longer and needs fewer doses (in terms of strength & regimen).

- Deuterated warfarin has a much lower rate of metabolism and a prolonged half-life due to the kinetic isotope effect.
- Deuterium warfarin may also lessen toxicity by lowering the production of harmful metabolites.
- In addition, there are fewer drug-drug interactions thanks to the deuterated warfarin's

increased stability in the presence of other medications.

1.4 Methods of deuteration

(A) Biodeuteration

Depending on the needed level of deuteration, the biodeuteration of molecules may involve the usage of heavy water (D_2O) in the host organism's growth medium. With the help of the ^{13}C and

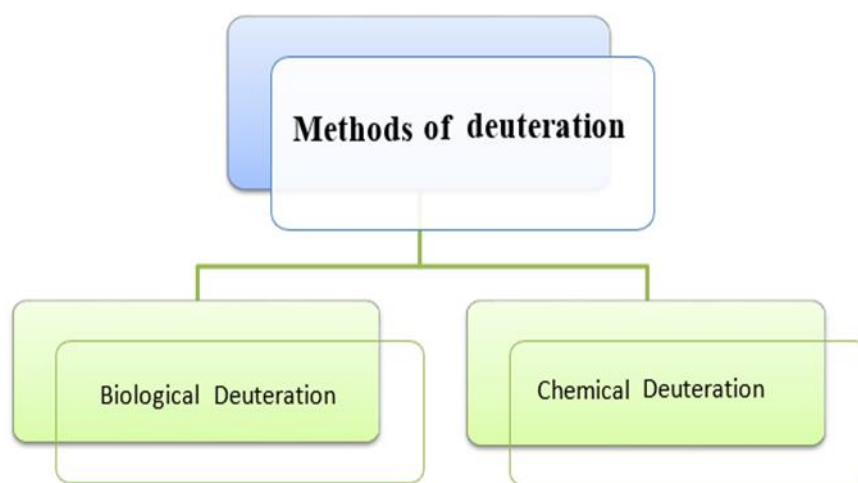


Fig.1.1 Methods of deuteration

^{15}N medium components, the target biomolecule can receive several labels. *Escherichia coli* are typically used as the host organism for recombinant protein expression, although a yeast host (*Pichia* sp.) can also be used, depending on the features of the target protein. For the insertion of gene sequences into the best expression vectors for deuteration, molecular biology knowledge is available. The biodeuteration laboratory has also had success producing various biopolymers in bacterial, yeast, and algal systems (such as chitosan, polyhydroxyalkanoates, and cellulose). Shaker culture systems create small volume flask cultures from transformation or seed Cultures.[5] The NDF does have a bioreactor with an energy photosynthetic system for growing algae and photoautotrophic microorganisms, a methanol

monitor, and feedback controls for yeast growth and initiating protein expression..[5]

(B) Chemical Deuteration

Chemical deuteration is the process of preparing a desired molecule by deuterating complete molecules or molecular building blocks by subjecting them to heavy water (deuterium oxide) at high temperatures and pressures while being in the presence of a catalyst. If necessary, molecules can be created using organic chemistry methods from the deuterated building blocks. All of the deuterated substances produced will be analyzed and characterized using the analytical tools at the chemical deuteration facility. Multinuclear NMR spectroscopy (1H , 2H , ^{13}C , and ^{31}P (for phospholipids)) is used to determine purity and

calculate the deuteration amount at various chemical sites. The isotopic abundances of the various isotopologues will also be calculated using MS to provide a measurement of the product's overall deuteration level.[5]

1.5 Brief introduction of warfarin Brand

Names-Coumadin, Jantoven

Chemical Formula-C₁₉H₁₆O

Molecular Weight- 308.3279

Absorption-Completely absorbed from the GI tract. The mean T_{max} for warfarin sodium tablets is 4 hours

Volume of distribution-0.14 L/kg of V_d was detected. Label, 17,9 Warfarin D's distribution phase lasts 6 to 12 hours. 17 It has been found to cross the placenta and obtain serum concentrations in the fetus that are similar to those in the mother. [5].

Route of elimination:

Warfarin is almost fully eliminated by metabolism, with just a little quantity excreted unaltered. 8 percent of the whole dose is eliminated in the urine, while the remaining 20% is excreted in the faeces.

Pharmacokinetics

- ☐ Highly bound to plasma proteins
- ☐ Metabolism occurs through liver
- ☐ Substrate of CYP450 enzyme
- ☐ Excreted in urine and stool

1 Background history of selected drug

Dicoumarol, the first coumarin, was found after which warfarin was synthesized. In 1948, warfarin was widely used for the first time as rat poison. In 1954, the USFDA approved the use of warfarin as a blood clot preventive in people. There are generic variants of the drug warfarin available. With nearly 14 million prescriptions given, it was the 50th most often prescribed medication in the US in 2019. Factor Xa inhibitors have recently become more well-liked as warfarin alternatives because to a decreased risk of side effects. Warfarin, an anticoagulant medication, prevents blood clots and migration. Warfarin was originally developed as a pesticide, but it has since become the most often administered oral anticoagulant in North America (D-Con, Rodex, and others). [5]

1.5.2 Adverse effects

- ☐ Osteoporosis, valve and
- ☐ Artery calcification,
- ☐ Toe syndrome,

Warfarin use has also been related to drug interactions. Warfarin does not really change blood viscosity; instead, it prevents the creation of multiple regulatory and physiologically active versions of various clotting factors, which are dependent on vitamin K.

1.6 Pharmacology Of Warfarin:

A coumarin derivative called warfarin prevents the cyclic interconversion of vitamin K and its 2,3 epoxide, which is how it works as an anticoagulant (vitamin K epoxide). For the carboxylation of glutamate residues to -carboxyglutamates (Gla) on the N-terminal sections of vitamin K-dependent proteins, vitamin K serves as a cofactor. 1–6 only after being - carboxylated by vitamin K are these proteins, which make up the coagulation factors II, VII, IX, and X, physiologically active. Warfarin



and the glutamic acid carboxylation of vitamin K-dependent coagulation proteins. Vitamin K1 from dietary sources is changed into vitamin KH2 by a vitamin K reductase that is resistant to warfarin.[5]

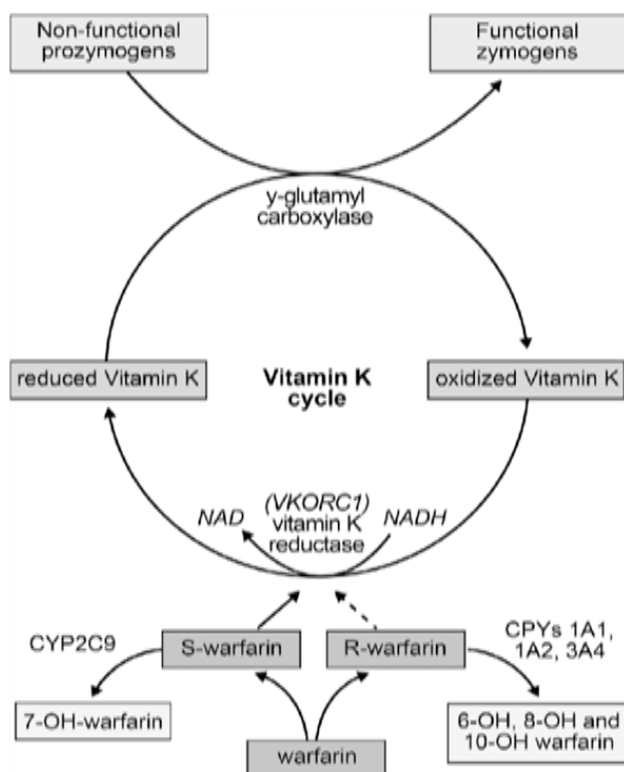


Fig.1.2 Mechanism of warfarin

Method of analysis of deuterated compound:

After synthesis, the deuteration must be verified, and then their medicinal qualities should be evaluated. For deuterated warfarin screening, a number of analytical techniques offer useful information. Deuterated warfarin is analyzed using a variety of physicochemical tests, chromatographic procedures, and spectroscopic techniques. Primary confirmation of salt

production is provided by physicochemical tests, while information is provided by spectroscopic techniques like IR and ^1H NMR[6].

Structural modifications that take place as deuterated warfarin is formed. Different methods for analysis of the deuterated warfarin are

Depicted in figure 1.3.

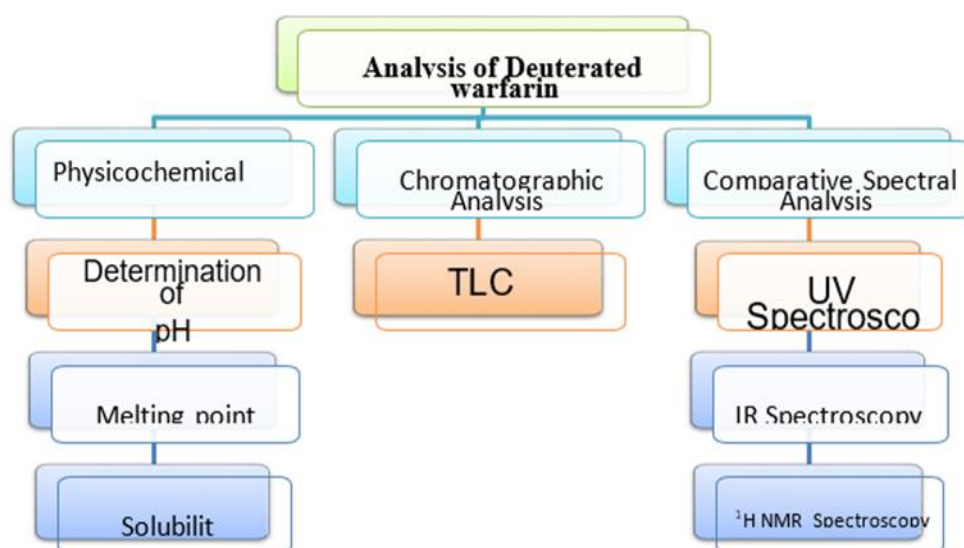


Fig 1.3. Method of analysis of deuterated warfarin

1.7. Physicochemical properties for characterization of a deuterated warfarin:

When compared to the original warfarin, deuterated warfarin exhibits various physicochemical features. Here, a few physicochemical characteristics for comparing warfarin with deuterated warfarin are discussed. The pH of the solution changes as deuterated warfarin that is water hyphen solubility forms. Therefore, measuring pH is very important. A drug's solubility is crucial to its capacity to dissolve, absorb, and be bioavailable. Since warfarin has a very low water solubility, one of the initial methods for improving the compound's water solubility is deuterated warfarin production. Finding a substance's melting point is another method for measuring the chemical changes. The changes in melting point indicate structural modifications brought on by reaction completion.

1.8. Chromatographic Techniques for Analysis of deuterated warfarin

1.8.1. Thin Layer Chromatography (TLC):

Because of its convenience of use, availability, high sensitivity, and quick separation time, thin

layer chromatography (TLC) is a regularly used analytical technique. The basis of TLC is the idea that a compound will have varying affinities for the mobile and stationary phases, which will influence how quickly it migrates. The components will move along the plate at varying speeds because some components have a larger attraction for the extremely polar silica gel (stationary phase), while others will move to the solvent more quickly. TLC analysis can be used to monitor a reaction's progress, separate a mixture, analyze purity, and purify small quantities of a compound on a preparative scale. Individual components emerge because of the separation.

The formula for RF is,

$$RF = \frac{\text{Distance traveled by sample}}{\text{Distance traveled by solvent}}$$

Because they are specific to each compound, the RF value can be used to identify substances. When comparing two substances under identical circumstances, the substance with a higher RF value is less polar because it does not stick to the stationary phase for as long as the polar substance, which would have a lower RF value.[7]

1.9. Spectroscopic Techniques for analysis of deuterated warfarin

1.9.1. Ultra-Violet (UV) Spectroscopy:

A portion of the light energy would be absorbed by the sample when it is exposed to a wavelength that corresponds to the energy of the electronic transitions within the molecule, and the electrons would then be moved to the higher energy state orbital. A spectrometer logs a sample's absorbance at various wavelengths. The term "max" refers to the wavelength at which the sample absorbs the most light. The Beer-Lambert law controls how much light molecules absorb. The Synthesis and Analysis of Deuterated Warfarin 10 is Related by Beer's Law Lambert's law connects the total absorbance to the optical path length, and absorbance to the concentration of the absorbing solute.

1.9.2. Infrared (IR) spectroscopy: Some infrared light frequencies are absorbed as it passes through a sample of an organic chemical, while other frequencies pass through the sample undisturbed. An infrared spectrum is produced by plotting the absorbance or transmittance against wave number. Since infrared spectroscopy is essentially vibrational spectroscopy, the main benefit of the method to organic chemists is related to the next finding. Different bonds (C-C, C=C, C≡C, C-O, C=O, O-H, and N-H) have different Vibrational frequencies, and we may recognize these unique frequencies as an absorption band in the infrared spectrum to determine the presence of these bonds in an organic molecule. Synthesis and Analysis of deuterated warfarin the well-defined IR regions are given in table 1.2.

Table 1. 2: Different regions of Infra-red spectroscopy

Sr. No	IR region	Significance
1	Near-Infrared (14000-4000cm ⁻¹)	<ul style="list-style-type: none"> It is poor in specific absorption, consists of overtones and combination bands resulting from vibrations in the mid-IR region of the spectrum
2	Mid-Infrared (4000-400cm ⁻¹)	<ul style="list-style-type: none"> It provides structural information for most organic molecules therefore widely used for sample □ Characterization.
3	Fingerprint (1500-400cm ⁻¹)	□ Absorption pattern in this region is highly complex but unique to each organic structure.
4	Far-Infrared (400-10cm ⁻¹)	□ It is less investigated than other two regions; however, it has been used □ For inorganic molecules.

Uses of IR spectrum:

No two molecules of a different structure have exactly the same infrared spectrum because every type of bond has a unique inherent frequency of vibration and because two of the same type of bonds in two distinct compounds are in two slightly different surroundings. We can determine whether two substances that are believed to be

identical are indeed identical by comparing the infrared spectra of the two substances.

Finding out a molecule's structural details is a second, more crucial usage of the infrared spectrum. Each type of bond's absorption—N-H, C-H, O-H, C-X, C=O, C-O, C-C, and C=C—is typically only observed in a tiny section of the



Vibrational IR area. For each kind of link, a constrained range of absorption can be established.

1.9.3.¹H Nuclear Magnetic Resonance spectroscopy:

NMR, or nuclear magnetic resonance, is the most efficient method for acquiring structural information. The number of atoms of the type being examined that are mechanically distinct is indicated by NMR. When we use NMR spectroscopy to study a molecule, we may note changes in the magnetic properties of the different nuclei that are present and estimate, to a significant extent, where these nuclei are located inside the

molecule. It would be simple to calculate the amount of each different type of hydrogen nuclei and the characteristics of each type's immediate environment when hydrogen (proton) nuclei are studied..[9]

Because it has both an electric charge and a mechanical spin, the proton, the nucleus of the Predicting the ¹H NMR spectrum of an organic compound begins with predicting the chemical shift, Positions for the different hydrogen are in the molecule.

Figure 1.4 is a useful chart containing approximate proton classification

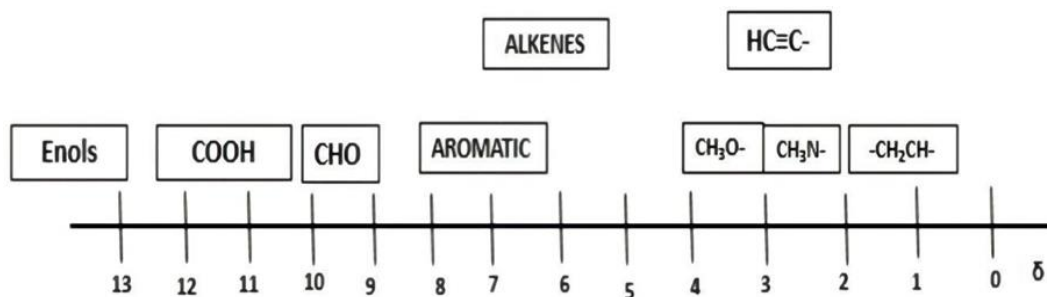


Figure.1.4: Approximate chemical shift positions for protons in organic molecules. Introduction to the present work:

Commonly utilized analytical methods such ¹H NMR, IR, UV, and TLC were used in the current investigation. The project's objective was to create deuterated warfarin and then use several analytical methods to examine the created substance. The deuterated version of warfarin, known as deuterated warfarin, is frequently used as metabolically effective oral anticoagulant drug.. (Figure1.5).

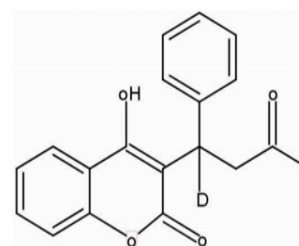
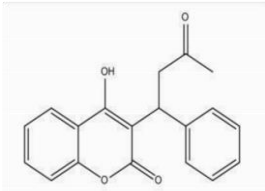
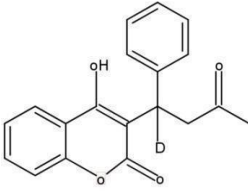


Figure.1.5 Structure of deuterated warfarin

Drug profiles of warfarin and deuterated warfarin are given in table no 1.3

Table 1.3 Drug profile of selected drug

Compound	Warfarin	Deuterated warfarin
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Structure		
Chemical formula	C ₁₉ H ₁₆ O ₄	C ₁₉ H ₁₅ D ₁₀ O ₄
Molecular weight	308.3	309.3
IUPAC name	2-oxo-3-(3-oxo-1-phenylbutyl)chromen-4-olate	4-hydroxy-3-[3-oxo-1-(phenyl-d ₅)butyl]-2H-1-benzopyran-2-one
Solubility	Insoluble in water, readily soluble in acetone and dioxane.	Soluble in ethanol, moderately soluble in water.
Physical description	Warfarin is a colorless, odorless, and tasteless substance. For Norway rats and house mice, it's Used as a rodenticide.[10]	Deuterated warfarin is a colorless, odorless, and tasteless substance
Melting point	161°C-162°C	150°C-160°C
Drug category	Anticoagulant, cardiovascular	Anticoagulant, cardiovascular

Aim And Objectives

Aim of the work: To synthesize the deuterated warfarin and to perform its analysis.

Objective of the work: The objectives of the work are listed below: Optimize the process parameters for synthesis of deuterated warfarin. When

compared to protonated warfarin, deuterated warfarin has a reduced toxicity

EXPERIMENTAL WORK

4.2 Chemicals and reagents: Chemicals used during the project work are mentioned in

Table: 4.2 Table 4.2 Chemicals used for the project

No	Chemicals	Grade	Make
1	Ethyl acetate	AR	S.D.Fine.Chemicals
2	Hexane	AR	S.D.Fine.Chemicals
3	Sodium hydroxide	AR	S.D.Fine.Chemicals
4	Hydrochloric acid	AR	S.D.Fine.Chemicals
5	Sodium sulphate	AR	Loba chemie.Pvt.Ltd
6	Acetone	AR	S.D.Fine.Chemicals
7	Sodium chloride	LR	Loba chemie.Pvt.Ltd
8	Zinc dust	LR	Loba chemie.Pvt.Ltd
9	Glacial acetic acid	AR	Loba chemie.Pvt.Ltd
10	Distilled water	LR	-
11	Ammonia	LR	Loba chemie.Pvt.Ltd
12	Chloroform-D	LR	S.D.Fine.Chemicals
13	Methanol	AR	S.D.Fine.Chemicals
14	Ethanol	AR	S.D.Fine.Chemicals
15	Phenol crystals	LR	S.D.Fine.Chemicals
16	Acetonitrile	HPLC	Qualigens



4.3 General procedure:

4.3.1. Method of preparation of warfarin

Step 1. Synthesis of benzalacetone

8.0 g (11.0 moles, 800 cc) of acetone and 4.02 g (400 cc, 4.0 moles) of benzaldehyde were combined with 4 ml (0.22 moles) of water in a round bottom flask equipped with a mechanical stirrer. To this, 1 ml (0.025 moles) sodium

hydroxide (10%) was added. The solution was stirred for 3.5 hours. Then the solution was cooled. Completion of the reaction was confirmed by thin layer chromatography. The reaction was quenched using diluted HCl. Ethylacetate was added to this solution and the organic layer was separated and dried over sodium sulphate. Ethylacetate was evaporated to obtain the titled compound.[9] Benzalacetone was synthesized from benzaldehyde and acetone. The scheme of synthesis depicted in **fig 4.1**

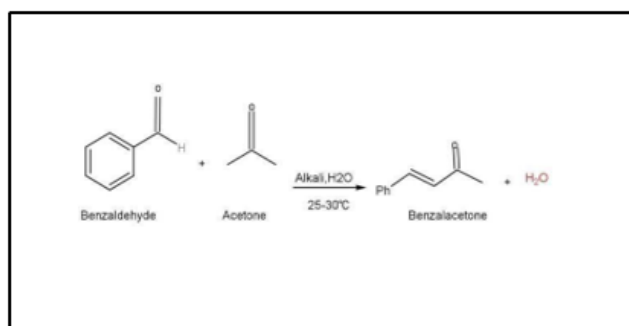


Fig.4.1. Synthesis of benzalacetone

Step 2 Synthesis of Warfarin:

Using a reflux condenser and stirrer connected to a flask, added the 4 hydroxycoumarin (0.308 moles, 5.0 gm) and benzalacetone (0.0342 moles, 5.0 gm), 35 ml H₂O (1.942 moles), and ammonia (0.006 moles, 0.11 ml) all added the same time. The solution was boiled and kept at a constant temperature for 2.5 hours, during which time a thick precipitate formed. Refluxing was continued for an hour with mild agitation. Reduced

the temperature of the reaction mixture to room temperature. The product was filtered to separate the solid. [11]. The solution was floated in fresh water until it was completely dry. Hydrochloric acid was used to neutralise the base NaOH (dilute). Using distilled water, the product was washed several times. The final product was isolated and characterized using various spectral, chromatographic studies. Scheme of synthesis is depicted in **fig 4.2**

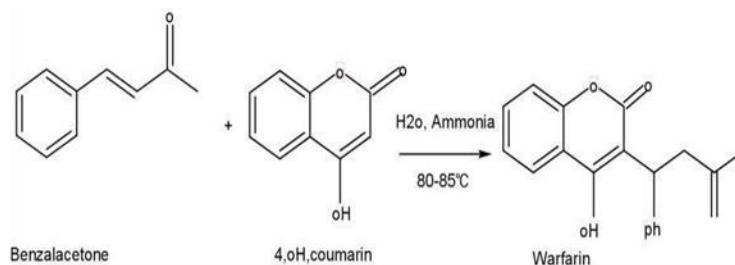


Fig 4.2. Synthesis of warfarin

The % yield of synthesized warfarin was calculated to be 75%

4.3.2 Method of preparation of deuterated warfarin

Step1: Synthesis of deuterated salicylaldehyde:

A solution of potassium butoxide (0.20g, 0.2303 moles) in D₂O (4.15g, 0.2303 moles) was prepared and rapidly added to phenol (1.0g, 0.0078 moles). The resultant solution was heated on a water bath between 70°C and 80°C. CDCl₃ is added gradually drop by drop over 1.5 hours. Then stirring was further continued for a further an hour. The reaction mixture was allowed to cool down. After cooling 1.74g (0.04 moles) of HCl was added

while vigorously swirling the mixture to make it acidic. The separated oil was combined with a sizable amount of NaCl (1.5g). Enough water (D₂O) was added to completely dissolve the ingredients.

The product was taken from the oil and hot water was used to wash it multiple times. The product was recrystallized using ethanol (0.75g, 0.0162moles).[11].

Chemical name	Quantity	moles
Phenol	1.0g	0.0078
Potassium tert.butoxide	0.20g	0.005
D2O	4.05g	0.2303
CDCl3	1.31g	0.0108
HCl	1.74g	0.046
Ethanol	0.75g	0.0162

Step 2: Synthesis of deuterated benzaldehyde

The ratio of salisaldehyde to zinc dust for the preparation of benzaldehyde should be 1:1.2. For each milliliter of salisaldehyde, was added 0.823g (0.0067moles) of zinc. Solution was heated in a water bath to 130°C. Utilizing Whatmann filter paper, the solution was filtered and was allowed to cool. Ethyl acetate was added and the solution was kept overnight. Ethyl acetate was evaporated. And the product was collected.[12]

Step 3: Synthesis of deuterated benzalacetone:

8.0g (11.0 moles, 800 cc) of acetone and 4.02 g (400cc, 4.0 moles) of deuterated benzaldehyde were combined with 4(0.22moles) ml of water in a round bottom flask equipped with a mechanical stirrer. To this, 1 ml sodium hydroxide (10%) was added. The solution was stirred for 3.5hours. Then

the solution was cooled. Completion of the reaction was confirmed by thin layer chromatography. The reaction was quenched using diluted HCl. Ethylacetate was added to this solution and the organic layer was separated and dried over sodium sulphate. Ethylacetate was evaporated to obtain the deuterated benzalacetone.

Step 4: Synthesis of deuterated warfarin:

Using a reflux condenser and stirrer connected to a flask, added to the 4 hydroxycoumarin (0.0061moles,1.0gm) and deuterated benzalacetone (0.0068 moles,1.0gm), 7ml H₂O(0.388moles), and ammonia (0.012moles,0.022ml) were added. The solution was boiled and kept at a constant temperature for 2.5 hours, during which time a thick precipitate was formed. Refluxing was continued for an hour with mild agitation. The reaction mixture was



allowed to cool to room temperature. The product was filtered to separate the solid. The solution was floated in fresh water until it was completely dry. Hydrochloric acid was used to neutralise the base NaOH (dilute). Using distilled water, the product was washed several times. Final product was

isolated and characterized using various spectral, chromatographic studies.[11] Warfarin was synthesized from benzalacetone and 4-hydroxycoumarin. Scheme of synthesis depicted in fig.4.3.

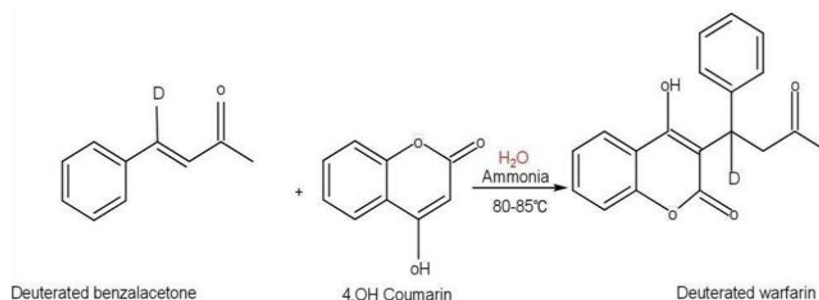


Fig.4.3. Synthesis of deuterated warfarin

The % yield of deuterated warfarin was calculated to be 76%.

Another method used for benzalacetone synthesis:

Method 1:

In a 100 ml conical flask, benzaldehyde (2.2345 mol, .2 ml) and acetone (2.2324 mol, 2.6 ml) were added. NaOH (2 g) pellets were then added to ethanol (20 ml) and distilled water (20 ml) and thoroughly mixed. Then, this solution was added to the acetone and benzaldehyde combination, and it was continually swirled for 38 minutes. Using distilled water as a subpar solvent, the result was then suction filtered from the filter paper. Before the solvent was removed using suction filtration, the crude product was recrystallized using hot ethyl acetate as the solvent heated to 150°C. On a piece of filter paper placed on a watch glass, the crystals were spread out and cured under a lamp for eight minutes. Yield and melting point information for the.[11]

Method 2:

At room temperature, benzaldehyde (0.02 mol, 2.12 g) was added drop wise to a solution of NaOH (0.05 mol, 2 g) in aqueous ethanol (1:1). Acetone (0.02 mol, 1.17g) was added drop wise and swirled for 30 minutes after continuing to mix for another 10 minutes. The reaction mixture received water (20 ml), which was then filtered. The item was purified by re-crystallizing from ethanol, rinsed with water (20 ml x 3), and then allowed to dry.

Another method used for warfarin synthesis:

Method 1: About 8 hours at 50 °C were used mixing 4-Hydroxycoumarin (1 mmol), benzalacetone (2 mmol), and [brim] BF₄ (1 mmol). Ethyl acetate was used to extract the final product 3 after water (5 ml) was added (2.5mL). The organic phase was dried using Na₂SO₄ anhydride. In order to produce warfarin 3, the solvent was evaporated. Melting point and TLC were used to identify compound 3 after synthesis..[11]

Method 2: In a flask connected with a reflux condenser and a stirrer, the following reagents were combined: 1.5 g of 4-hydroxycoumarin, 1.5 g of benzalacetone, 35 cc of water, and 0.11 cc of ammonia. A sizable precipitate formed after the

mixture was heated to a boil and kept at reflux for 2:30 hours. During refluxing, the reaction mixture was rapidly stirred for a further hour before being cooled to room temperature. The solid crude product was removed through filtration, after which it was floated in fresh water and as dryly as possible sucked. The solid crude was dissolved in benzene, refluxed for 45 minutes with stirring, cooled, filtered, rinsed with new benzene while on the filter, and sucked as dry as possible..[11]

Method 3: 4-hydroxycoumarin (1 mmol), benzalacetone (2 mmol), and [brim] Br (1 mmol) were combined for 5 hours at room temperature. The resultant product 3, which contained water, was then extracted using ethyl acetate (2x5mL). The organic phase was dried using Na₂SO₄ anhydride. Additionally, the solvent was evaporated to get pure warfarin 3.[11]

4.3.3. Determination of physicochemical properties: The general procedures to evaluate the physicochemical properties of warfarin and deuterated warfarin are discussed in below:

4.3.3.1. Determination of pH: The pH of warfarin and deuterated warfarin was determined by using digital pH meter. The pH meter was calibrated with a standard buffer solution (pH 4 and pH 7) before use.

4.3.3.2. Melting point: The capillary containing the sample was placed in the digital melting point apparatus. The temperature was gradually increased at the rate of 20°C per min, till the temperature reached 90°C and thereafter at the rate of 10°C per min. The temperature was noted when the sample starts melting. This was done in triplicate for both samples.

4.3.3.3. Solubility: Solubility was determined as per the Indian Pharmacopoeia (IP). 1 gm of substance was dissolved in Different solvents,

such as water, methanol, chloroform, tetrahydrofuran (THF), dimethylsulfoxide (DMSO), etc. The amount of solvent required dissolving warfarin and deuterated warfarin was noted as per solubility criteria of I.P. and U.S.P.

4.3.3.4. Chromatographic Analysis: Chromatographic analysis was done using TLC to get resolution between warfarin and deuterated warfarin.

4.3.3.5. Thin Layer Chromatography (TLC): To resolve the situation between warfarin and deuterated warfarin, different movable phases were tried. Because deuterated warfarin is more polar than warfarin, which is a less polar molecule, it was suggested that it should move up the TLC plate slowly or not at all (lower R_F value). A, few representative trials are shown in the table 5.3 of section 5.2.1.

4.3.3.6. Spectroscopic techniques for analysis: Spectral analysis of deuterated warfarin and warfarin was done by using UV spectroscopy, IR spectroscopy, ¹H NMR spectroscopy. The product was characterized partly in-house and partly outsourced to Diya Labs and Dr. M. K. Rangnekar Memorial Drug Testing Laboratory.

4.3.3.6.1. Ultra-Violet spectroscopy: UV spectra of warfarin and deuterated were compared. The procedure for preparation of 10 ppm solutions of deuterated warfarin; warfarin is discussed in section 5.3.1 (A) of this chapter. The UV Spectra of warfarin (10 ppm), deuterated warfarin (10ppm) in Acetonitrile: water (1:1) was compared in range of 200-400 nm.[8]

4.3.3.6.2. Infra-Red Spectroscopy: Using an electrically powered KBr press model, KBr was combined with warfarin (100%), deuterated warfarin (100%), and a mixture of deuterated warfarin and warfarin (1:1). The manufactured



disc containing deuterated warfarin, warfarin, and a combination was utilized to get the IR spectra using a Jasco FT/IR-4100 type A Fourier transform spectrophotometer. There was a 4000-400 cm⁻¹ scanning range. In order to establish the structural changes in deuterated form, IR analyses of warfarin, warfarin deuterated, and combination (50 percent warfarin deuterated by weight) were conducted. Results were seen in the mid-IR (4000-400 cm⁻¹) and fingerprint region of IR (1500-400 cm⁻¹) of the IR. Results of IR are given in section 5.3.2.

4.3.3.6.3. Proton Nuclear Magnetic Resonance (¹H NMR) Spectroscopy: One of the most important methods for interpreting structural data is NMR spectroscopy. In order to conduct analysis at 400 MHz and identify structural changes,

deuterated warfarin and warfarin were dissolved in a suitable solvent, such as deuterated dimethyl sulfoxide (DMSO-d₆). Warfarin's and deuterated warfarin's spectra were examined for the presence of the methyl (-CH) proton. Warfarin would contain the -CH proton but not deuterated warfarin. Results of ¹H NMR are given in section 5.3.3.

RESULTS AND DISCUSSION

5.1: Evaluation of physicochemical properties: The synthesized batches of deuterated warfarin as well as warfarin were evaluated for Physicochemical properties such as pH, melting point, solubility. The results of tests are shown in **table 5.2**

Table 5.2: Evaluation of physicochemical properties of deuterated warfarin and warfarin

1	pH	6.33	6.22
		6.32	6.35
		6.25	7.32
		%RSD=0.69%	%RSD=1.08%
2	Solubility	Practically insoluble in water(0.017mg/ml)	Insoluble in water(0.017mg/ml)
		Readily soluble in acetone and dioxane	Soluble in organic solvents like acetone,toluene,ethyl acetate
		Moderately soluble in alcohols	Moderately soluble In methanol
		Soluble in DMSO	Soluble in DMSO
3	Melting point	155	150
		160	153
		161	155
		%RSD=2.03%	%RSD=1.65%

Discussion: The following physicochemical properties of warfarin and deuterated warfarin were visually evaluated and compared.

pH- Therefore, the pH of deuterated warfarin was found to be 6.5, whereas the pH of warfarin was 6.

Melting point- The melting point of warfarin and deuterated warfarin was found to be in the range of 155-160 °C and 150-155°C respectively. The shifts

in melting point indicate the changes in structure due to the completion of reaction.

Solubility- deuterated warfarin was insoluble in water and soluble organic solvents. Both warfarin and deuterated warfarin was moderately soluble in methanol but was insoluble in water. It was found that both the compounds were soluble in polar




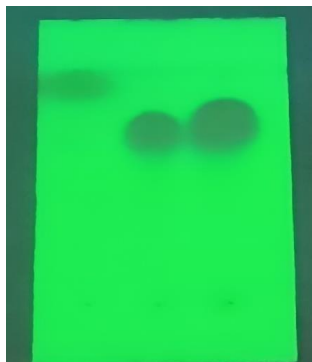
aprotic solvents, such as tetrahydrofuran and dimethyl sulfoxide.


5.2. Chromatographic analysis of warfarin and deuterated warfarin using TLC:

Chromatographic techniques were conducted to obtain resolution between warfarin and deuterated warfarin. The results of TLC are discussed in the following sections.

5.2.1. Thin Layer Chromatography (TLC): The affinities of substances for the stationary and mobile phases are the base of TLC, as they are for other chromatographic techniques. Due to the mobility, substances that have a higher affinity for the stationary phase travel slowly while the other substances move more slowly. Between warfarin and deuterated warfarin. To achieve resolution between Deuterated warfarin and warfarin, numerous mobile phases were considered. Few representative trials are shown below in table 5.5.

Table 5. 3: Representative Thin layer chromatographic trails

No	Mobile phase	TLC plate	Observations	Inference
1	Ethylacetate: Hexane (7:3)		When compared to deuterated warfarin, warfarin becomes much. Every area exhibits minimal tailing	Warfarin's Rf value was insufficient to distinguish it from deuterated warfarin.
2	Ethyl acetate: Hexane: Acetic acid (8:2:1)		Both warfarin and deuterated warfarin shows same Rf value. One extra spot of warfarin observed near the solvent Front.	Little acetic acid is used to increase the Rf value and minimize tailing.

3	Ethyl acetate: Hexane(9:1)		Both spots have same Rf value	Due to similar chemical properties, deuterated warfarin exhibits the same Rf as warfarin. To prevent tailing, a single drop of acetic acid was utilized.
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5.3: Comparative spectral analysis between warfarin and deuterated warfarin

Comparative spectral analysis was done to check for the absence of any interference. Different spectroscopic techniques, such as Ultra Violet (UV) spectroscopy, Infra-Red (IR) spectroscopy, and Proton Nuclear Magnetic Resonance (^1H NMR) spectroscopy were tried. Results of each technique are given in the sections below.

5.3.1. Ultra Violet (UV) spectroscopy: The UV spectra of deuterated warfarin ($10\text{ }\mu\text{g/mL}$), warfarin ($10\text{ }\mu\text{g/mL}$), were compared. The sample preparation of $10\text{ }\mu\text{g/mL}$ solutions of, warfarin and deuterated warfarin are given in section 4.3.3.1 (A) of chapter. UV spectra for deuterated warfarin (mg/mL), warfarin (mg/mL) sample mentioned above are depicted in figures 5.7 to 5.8 and results of absorbance are depicted in table 5.6. ACN: Water (1:1) was chosen as diluents since both deuterated warfarin well as warfarin had to be solubilized. UV.[8]

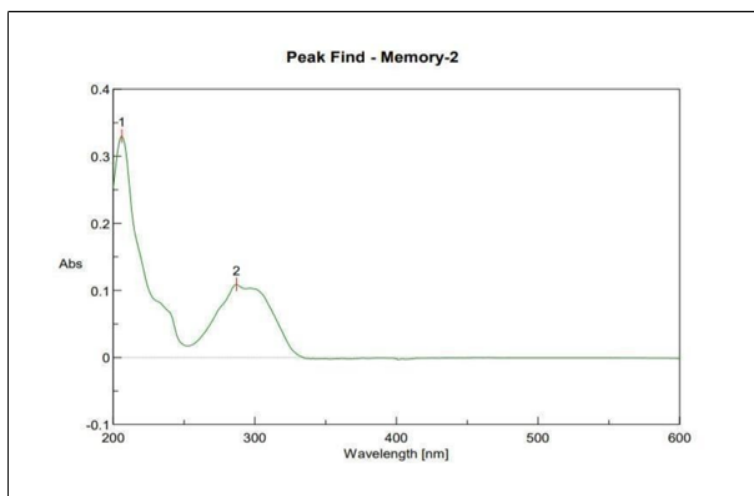


Fig.5.2 UV spectra of warfarin (10ppm)

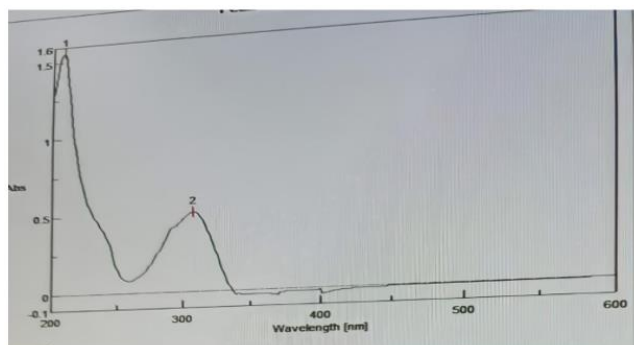


Fig 5.3. UV spectrum of (10µg/mL) Table.5.4 Results of UV analysis

Sr. No	Wavelength	Absorbance	
		Warfarin	Deuterated Warfarin
1	280	0.326	0.332
2	305	0.0890	0.0800
3	310	0.0727	0.0714

DISCUSSION:

The UV spectra of warfarin and deuterated warfarin were comparable. Deuterated warfarin displayed a lower absorbance than warfarin. **5.3.2**

Infra-Red Spectroscopy: IR spectra of warfarin and deuterated warfarin was recorded and results

were observed in (400cm⁻¹- 4000 cm⁻¹) and fingerprint region between 400cm⁻¹-1500cm⁻¹ to confirm the structural changes in deuteration. Results of IR analysis are shown in **fig 3** and comparison of bands present in warfarin and deuterated warfarin is shown in **fig 5.4** and **fig 5.5**.

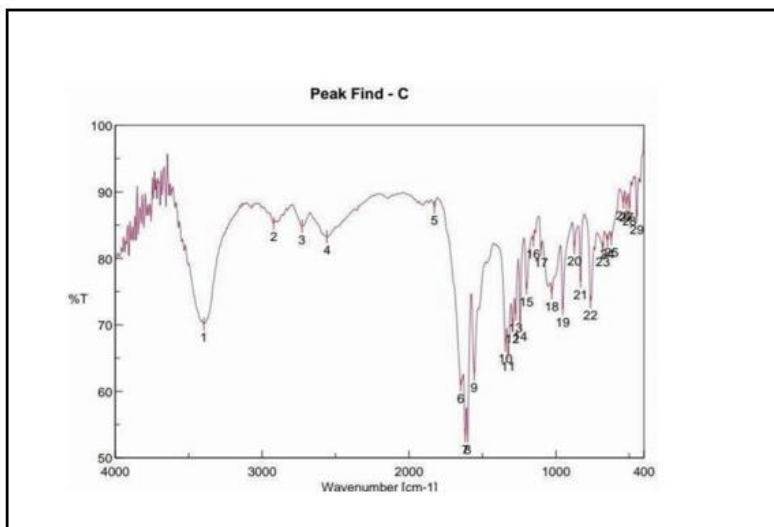


Figure 5.4. IR spectra of warfarin (10ppm)

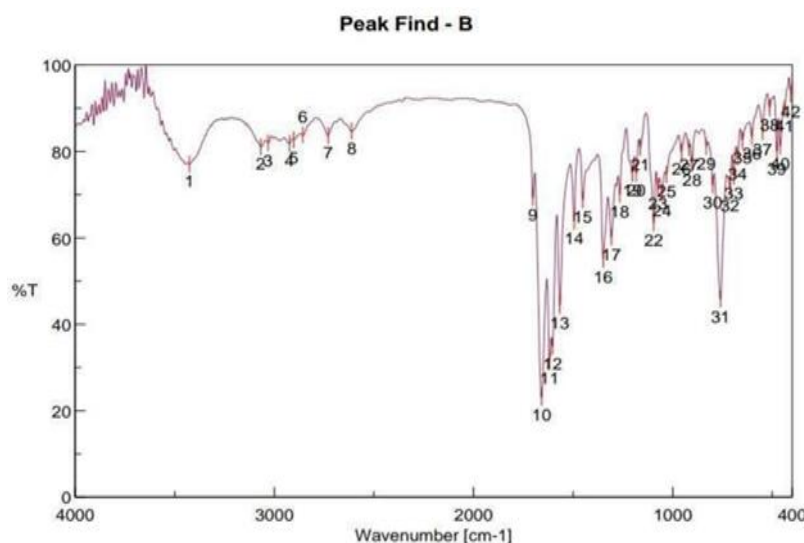


Fig.5.5.IR spectra of deuterated warfarin (10ppm)

Table 5.5: Major characteristic bands are present in warfarin and deuterated warfarin

Sr. No	Vibrational frequency(cm^{-1}) in warfarin	Vibrational Frequency (cm^{-1}) in deuterated warfarin	Molecular stretching
1	3279	Absent	O-H Stretching
2	3062	3068	C-H stretch in alkenes
3	1611	1617	C-H bending in aromatic compound
4	1618	Absent	C=C Stretch
5	1747	1701	C=O Stretch

5.3.3. Proton Nuclear Magnetic Resonance

It was attempted to detect structural alterations using ^1H NMR analysis. Additionally, warfarin rather than deuterated warfarin would show the C-

H proton on each carbon. Warfarin and deuterated warfarin's ^1H NMR analyses are depicted in fig, respectively. Interpretation of NMR spectrum of deuterated warfarin is given in table no 5.6 and 5.7.

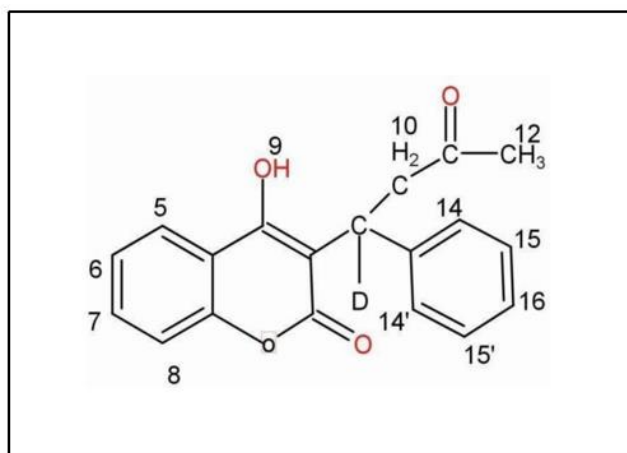


Fig.5.6 Structure of deuterated warfarin

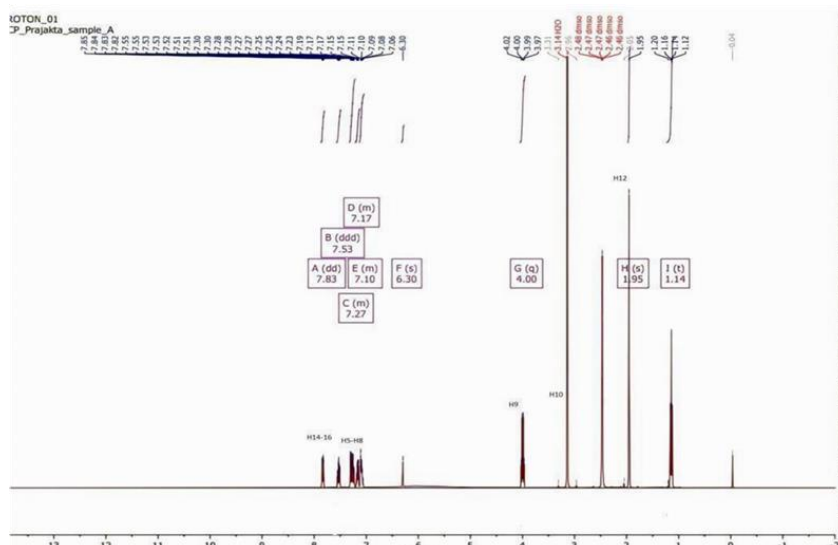


Fig. 5.7.1H NMR spectrum of deuterated Warfarin in DMSO-d6 measured at 400 MH

Table 5.6. Interpretation of NMR spectrum of deuterated warfarin

No	Chemical shift(ppm)	Interpretation
1	3.9	Hydroxyl protons at C9
2	6.97-7.94(multiple)	H attached to benzene ring
3	2.2(doublet)	H attached to C12 in structure
4	3.2(singlet)	H attached to C10 in structure

Interpretation of deuterated warfarin NMR spectra:

Methyl protons of carbonyl group (C12) appear as uncoupled singlet at δ 2.22 ppm whereas the C7 proton appears at δ 7.36 ppm (triplet). Donating electron density causing the C6 proton to shift up field to δ 6.98 ppm (doublet) while the C8 proton appears at δ 7.19 ppm (triplet) as stated in figure. While C12 protons appear as δ 2.2 (doublet) and C10 protons appear as δ 3.2 (singlet).

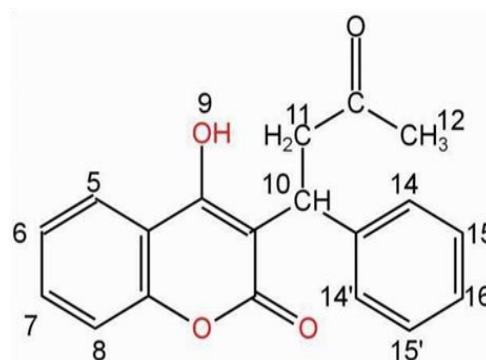


Fig.5.8. Structure of Warfarin

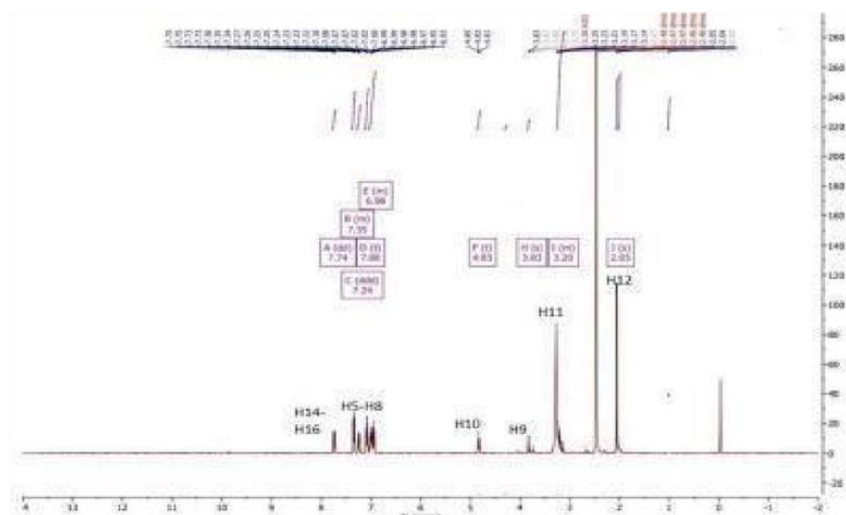


Fig 5 9.1H NMR spectrum of Warfarin in DMSO-d6 measured at 400 MHZ

Table 5. 7: Interpretation of NMR spectrum of warfarin

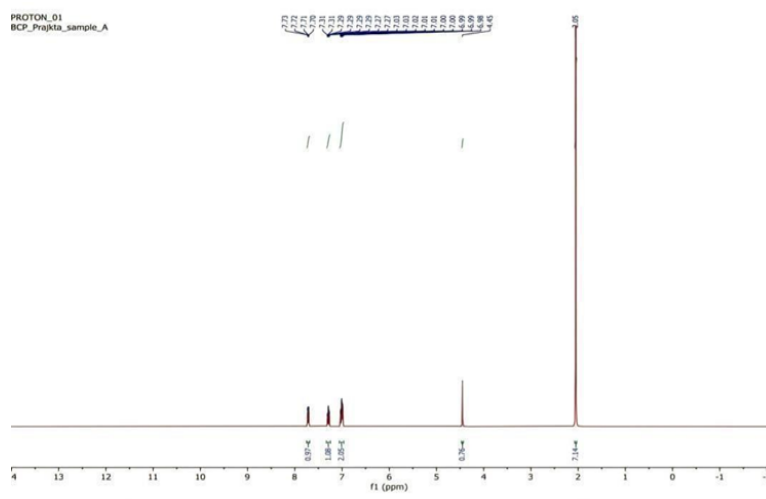
Sr. No	Chemical shift(ppm)	Interpretation
1	3.8	Hydroxyl protons at C9
2	6.97-7.94(multiplet)	H attached to benzene ring
3	2.2(doublet)	H attached to C12 in structure
4	3.2(singlet)	H attached to C11 in structure
5	4.8(singlet)	H attached to C10 in structure

Interpretation of warfarin NMR spectra:

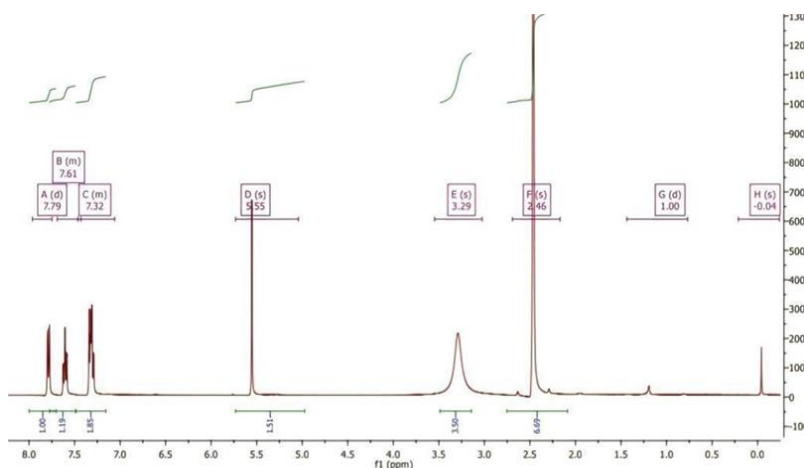
Warfarin's deuteration can be distinguished by a distinctive downfield chemical change at 4.8 ppm. When compared to the C7 proton, methyl protons of the carbonyl group (C12) emerge as uncoupled singlets at 2.22 ppm (triplet). The C6 proton shifts up field to 6.98 ppm (doublet) due to the electron

density donation, but the C8 proton appears at 7.19 ppm (triplet), as shown in the figure. While C10 protons appear as 3.2 and C12 protons as 2.2 (doublet), respectively (singlet).

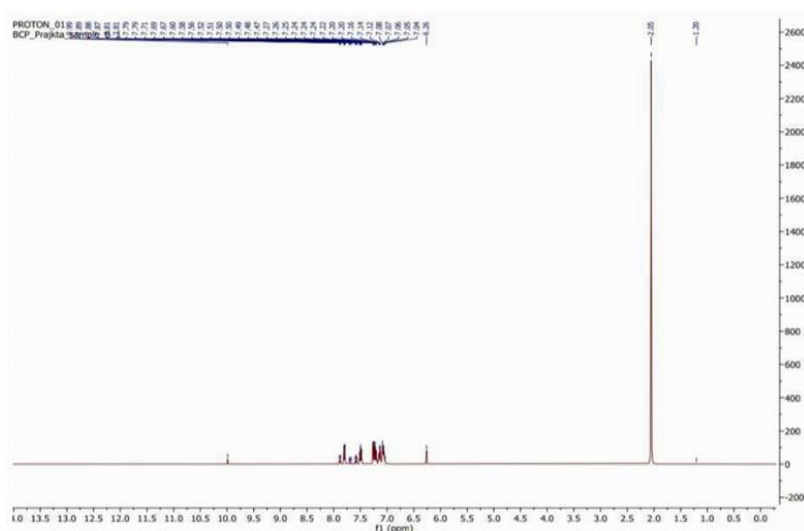
Some representative trials of 1H NMR of Warfarin:



Trial 1) 1 H NMR Of deuterated warfarin



Trial 2). ^1H NMR Of deuterated warfarin



Trial 3) Fig. ^1H NMR of deuterated warfarin

CONCLUSION:

The comparative ^1H NMR analysis of warfarin and deuterated warfarin confirms successful deuteration at the chiral carbon center. In warfarin, the proton attached to the chiral carbon exhibits a broad signal around δ 4.8 ppm, whereas this signal is absent in deuterated warfarin, indicating effective substitution of hydrogen with deuterium. Both compounds display characteristic resonances corresponding to aliphatic, aromatic, and carbonyl group protons. The methyl protons show a doublet at δ 2.04 ppm due to germinal coupling with acetone, while signals at δ 2.5 and δ 2.48 ppm arise from the solvent DMSO- d_6 . The aromatic protons

appear as a series of multiplets, the carbonyl-linked methylene protons exhibit a multiplet at δ 3.4 ppm, and the hydroxyl proton gives a singlet at δ 3.8 ppm. Overall, the spectral data clearly demonstrate successful deuterium incorporation in warfarin, validating the synthetic approach used for preparing its deuterated analogue.

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