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Synthesis, Characterization and in vitro kinetics of 6-Aminocaproic Acid Prodrugs

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Dedication

This thesis is dedicated to the teacher of teachers, Prophet of humanity Mohammad- peace be upon him.

I dedicate my thesis work to my family and many friends. A Special feeling of graduated to my loving parents whose words of encouragement and push for tenacity ring in my ears.

Last but not least, special thanks to my supervisor Professor Dr. Rafik Karaman for his unlimited support and cooperation, he has been my best mentor.

Fatima Thawabta

Declaration

I certify that the thesis submitted for the degree of master is the result of my own research, except where otherwise acknowledged, and that this thesis (or any part of the same) has not be submitted for a higher degree to any other university or institution.

Signed: ……..

Fatima Sadeq Abdallah Thawabta

Date: --, 2014

Acknowledgment

First and foremost, I am deeply thankful to Almighty **Allah** from whom I always receive help and protection.

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Abstract

Prodrug is pharmacologically inactive molecule of an active drug molecule that prior to exert its pharmacological effect requires an enzymatic and/or chemical transformation to release the active parent drug. Prodrugs can be used to bypass physicochemical, pharmaceutical, pharmacokinetic and pharmacodynamic barriers of drug formulation, such as poor aqueous solubility, chemical instability, insufficient oral absorption, rapid presystemic metabolism, inadequate tissue penetration, toxicity and local irritation.

In recent years, the design of drugs and prodrugs has increasingly relied on computer modeling techniques. This type of modeling is often referred to computer-aided drug design which uses computational chemistry to discover, enhance, or study drugs and related biologically active molecules. Several enzyme models were evaluated using computational chemistry for the purpose to design prodrugs by which their linkers mimic the chemistry of enzyme models. Using the enzyme model approach and based on DFT calculations three different 6-aminocaproic acid prodrugs were designed, synthesized and characterized by FT-IR, ^{1}H-NMR, LC-MS, and their in vitro intra-conversion to their parent drugs revealed that the $t_{1/2}$ value was largely affected by the pH of the medium. For 6-aminocaproic acid **ProD1** the experimental $t_{1/2}$ values in 1N HCl, buffer pH 2.5 and buffer pH 5 were 11 hours, 20.6 hours and 23.4 hours, respectively. 6-aminocaproic acid **ProD2** was found to be readily intraconverted at 1N HCl, while at pH 2.5 its half-life was 15 hours and at pH 5 it was 19.5 hours, whereas it was stable at pH 7.4. On the other hand, 6-aminocaproic acid **ProD3** was stable in all studied media.

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List of Abbreviations

Abbreviations	Definition
HPLC	High-performance liquid chromatography
LC-MS	Liquid chromatography-Mass spectrometry
m/z	Mass-to-Charge ratio
NMR	Nuclear magnetic resonance
ppm	Part per million
M.P	Melting point
$t_{1/2}$	Half life
N	Normal
FT-IR	Fourier transform infrared spectroscopy
D.W	Distilled water
THF	Tetrahydrofuran
nm	nano meter
HLB	Hydrophilic-lipophilic balance
UK	United Kingdom
USA	United States of America
A°	Angstrom
C°	Celsius

Introduction

Chapter One

Introduction

1.1 Background

The prodrug (predrug, proagent) term was introduced for the first time by Albert as a pharmacologically inactive moiety which is converted to an active form within the body [1]. This term has been successfully used to alter the physicochemical, pharmacokinetic properties, (absorption, distribution, excretion and metabolism) of drugs and to decrease their associated toxicity [2]. The prodrug approach is used to minimize the undesirable drug properties while retaining the desirable therapeutic activity. Currently, 5-7 % of all approved drugs worldwide can be classified as prodrugs and in the years 2001 and 2002 approximately 15 % of all new launched drugs were prodrugs [3].

The prodrug is pharmacologically inactive molecule of an active drug molecule that prior to exert its pharmacological effect requires an enzymatic and/or chemical transformation to release the active parent drug. Prodrug can be used to bypass physicochemical, pharmaceutical, pharmacokinetic and pharmacodynamic barriers of drug formulation, such as poor aqueous solubility, chemical instability, insufficient oral absorption, rapid presystemic metabolism, inadequate tissue penetration, toxicity and local irritation [3, 4].

Recently Drug design become increasingly relying on computer modeling techniques. This type of modeling is often referred to as computer-aided drug design by which computational chemistry methods are used to discover, enhance, or study drugs and related biologically active molecules [5]. Recently, numerous novel prodrugs have been designed by Karaman’s group for the treatment of Parkinson's disease (dopamine), malaria (atovaquone) , viral infections(amoxicillin) , hypertension (atenolol) and etc.. Using DFT (density functional theory) calculations methods, Karaman’s group studied a significant number of intermolecular processes (enzyme models). Based on the calculation results they designed linkers to be linked to amino- and hydroxyl- containing drugs such that the proposed prodrugs will have a moderate HLB value. The combination of both hydrophilic

and lipophilic groups is expected to give better bioavailability due to moderate hydrophilic- lipophilic balance value [6-8].

Applications of prodrug approach

A. Improving solubility and bioavailability

Poor solubility of drug will be a challenge when dissolution of the drug in a dosage form is the rate limiting step. Many techniques are used to overcome solubility problems as salt formation and solubility enhancer excipients. Prodrugs can also be used as an alternative technique to increase aqueous solubility of parent drug by attaching polar groups such as phosphate, amino acids and sugar moieties [9-11].

If the prodrug contains a phosphate group, the parent drug will be readily released by endogenous phosphatase as alkaline phosphatase, while if it contains amino acid esters or amides the prodrug will be cleaved by esterase or amidase available in plasma or tissues, and if it contains sugar moieties such as glucose, β-glucosidase will be utilized to convert the prodrug to its corresponding parent drug [12].

B. Increasing permeability and absorption

For a drug to be absorbed it should pass through many membranes. This requires a lipophilic group to be in the drug structure so if the drug contains polar groups it will have a limited permeability and then a low absorption profile. The prodrug strategy can be used to enhance permeability by masking the polar group as hydroxyl, thiol, carboxyl, phosphate or amino acid with more lipophilic alkyl or aryl esters and these prodrugs can be readily converted to the parent drugs via hydrolysis catalyzed by esterase enzyme [13].

C. Changing distribution profile

This approach can be accomplished using a prodrug to deliver a parent drug to a specific target utilizing site- selective endogenous enzymes to convert the prodrug to its active parent drug. In addition, this approach may also be used to decrease drug toxicity [12].

D. Improving taste

If a drug has unpleasant taste, it will decrease the patient compliance. Prodrugs can be used to improve the taste of the parent drugs either by decreasing their solubility in saliva or by masking the functional group that is responsible for the binding to the taste receptors located on the tongue and thus to reduce the unpleasant taste [14].

Successfully designed prodrug should be activated to its parent drug; activation process involves metabolism by enzymes distributed throughout the body, hydrolytic enzymes as esterases and amidases and non-hydrolytic enzymes such as cytochrome P450 are the most important enzymes involved in the bioactivation process of prodrugs.

Enzymes accelerate the rate of chemical reactions that the substrate (drug) might undergo in physiological environment. The reaction rate in the majority of enzymatic reactions is 10^{10} to 10^{18} fold than non-enzymatic reaction [15].

When the prodrug is designed to be activated by natural enzymes such as esterases and amidases, the prodrug might be tackled by a premature hydrolysis during the absorption phase in enterocytes of gastrointestinal tract. This might produce more polar and less permeable prodrug which results in a decreased bioavailability (50%). While if the prodrug is activated by cytochrom P450 enzymes which are responsible for 75% of the enzymatic metabolism of prodrugs, a genetic polymorphisms might persist and then lead to variability in prodrug activation and thus affect the efficacy and safety of designed prodrugs [15]. Thus, it might be difficult to predict the bioconversion rate of the enzymatic hydrolysis of the prodrug and hence a difficulty in predicting their pharmacological or toxicological effects.

In the past few decades, computational chemistry has been utilized in calculating physicochemical and molecular properties of compounds. Using molecular orbital (MO) and molecular mechanics (MM) methods prediction of chemical reactions can be done. This tool can be used to design prodrugs that can be chemically interconvert to their parent drugs without any involvement of enzyme catalysis. The release of the active drug will be solely dependent on the rate limiting step of the intramolecular process (prodrug chemical features) [16].

***Prodrugs classification*:**

The conventional method used to classify prodrugs is based on derivatization and the type of carriers attached to the drug. This method classified prodrugs into two sub-major classes:

(1) Carrier-linked prodrugs, in which the promoiety covalently linked to the active drug but it can be easily cleaved by enzymes (such as an ester or labile amide) or non-enzymatically to provide the parent drug. Ideally, the group removed is

pharmacologically inactive, nontoxic, and non-immunogenic, while the promoiety must be labile for in vivo efficient activation [17, 18].

Carrier-linked prodrugs can be further subdivided into: (a) bipartite which is composed of one carrier (promoiety) attached directly to the drug, (b) tripartite which utilizing a spacer or connect a group between the drug and a promoiety. In some cases bipartite prodrug may be unstable due to inherent nature of the drug-promoiety bond. This can be overcome by designing a tripartite prodrug and (c) mutual prodrugs, which are consisting of two drugs linked together.

(2) Bioprecursors which are chemical entities that are metabolized into new compounds that may be active or further are metabolized to active metabolites (such as amine to aldehyde to carboxylic acid). In this prodrug type there is no carrier but the compound should be readily metabolized to induce the necessary functional groups [2, 19, 20].

1.2. 6-Aminocaproic acid

6-Aminohexanoic acid (Figure 1) is a synthetic lysine analog that suppresses fibrinolytic activity by fitting into plasminogen´s lysine-binding site and preventing the binding of plasminogen to fibrin [21].

It is an anti fibrinolytic drug used to control bleeding that occurs when blood clots are broken down too quickly (such as during or after heart surgery; in people who have certain bleeding disorders; prostate, lung, stomach, or cervical cancer; and in pregnant women when the placenta separates from the uterus before the baby is ready to be born), it is also used to control bleeding in the urinary tract that may occur after prostate or kidney surgery [22].

Studies on this drug have showed a clinical benefit in decreasing mortality and morbidity in many traumatic cases and different types of surgery like coronary artery bypass grafting in which the administration of 6-aminocaproic acid resulted in a significant decrease in blood loss and blood transfusion requirements were significantly less [23].

Also the administration of this drug can reduce blood loss and consequently transfusion and transfusion-related risk in patient who undergo hip replacement [24].

As well as, it can reduce the incidence of secondary hemorrhage following traumatic hyphema [25]. It plays an important role in dental extraction in patients with hemophilia

[26], and in control hemorrhage in patients with amegakaryocytic thrombocytic thrombocytopenia [27].

There are other antifibrinolytic drugs, such as aprotinin, and tranexamic acid, used intraoperatively, postoperatively, and during extracorporeal membrane oxygenation (ECMO) to reduce bleeding and minimize the need for exogenous blood products. However, because of risk of renal failure, cardiac failure, stroke, or encephalopathy that are associated with the use of aprotinin, the latter was removed from the market., Hence, 6-aminocpaproic acid and tranexamic acid are potentially safer alternatives [22]. In the UK, tranexamic acid, a tissue plasminogen and plasmin inhibitor, is most commonly used, with evidence for benefit in cardiac, orthopaedic, urological, gynaecological, and obstetric surgery. In the USA, aminocaproic acid, which also inhibits plasmin, is commonly used [22].

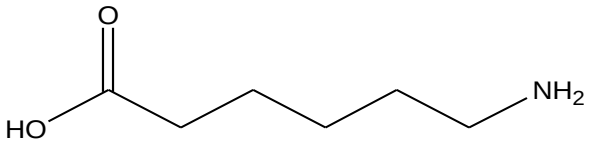


Figure 1. Chemical structure of 6-aminocaproic acid.

1.3. Research problem

The bioavailability of 6-aminocaproic acid is only 24%. This low value is attributed to the amino acid nature of the drug. At physiological pH, 6-aminocaproic acid will exist mainly in the ionized form; this ionization will decrease the ability of 6-aminocaproic acid to be transferred to the systemic blood circulation. This limited permeation results in poor bioavailability of the anti-bleeding drug [28].

Thus, using the prodrug approach to increase 6-aminocaproic acid bioavailability can be utilized. This can be achieved by making a covalent linkage between 6-aminocaproic acid and a non-toxic promoiety that increases the lipophilicity of 6-aminocaproic acid.

The proposed prodrug was designed such that the prodrug will be chemically intraconverted to 6-aminocaproic acid and a non-toxic moiety in a rate which is only dependent on the structural features of the inactive linker.

1.4. Thesis objectives

General Objective:

The main goal of this research was to synthesize and to study the kinetics for the following novel prodrugs: 6-aminocaproic acid maleate, 6-aminocaproic acid dimethyl maleate, 6-aminocaproic acid succinate.

Specific objectives:

1. To synthesize 6-aminocaproic acid prodrugs with the following characteristics:

- ✓ To be readily dissolved in physiological media.
- ✓ To have an increased bioavailability profile.
- ✓ To have moderate hydrophilic lipophilic balance (HLB) value.
- ✓ To be converted to 6-aminocaproic acid in a programmable manner.
- ✓ To give a safe non-toxic linker moiety after the chemical conversion process.

2. To perform in vitro kinetic studies of 6-aminocaproic acid prodrugs at different pHs mimicking the physiological media.

Literature Review

Chapter Two

2. Literature Review

2.1. Introduction

Nowadays, quantum mechanics (QM) such as *ab* initio, semi-empirical and density functional theory (DFT) and molecular mechanics (MM) are recommended to provide structure energy calculations for prediction of drugs and prodrugs alike [29-33].

The *a*b initio molecular orbital methods are based on rigorous use of Schrodinger equation with number of approximations. *ab* initio methods can be applied only on small system containing not more than thirty atoms. Semi-empirical method is applied on molecules with more than 50 atoms and gives information for practical application [34,35]. DFT method is a semi-empirical method used for medium sized systems and it is not restricted to the second row of the periodic table [36].

The mechanisms of some intramolecular processes that were advocated to understand enzyme catalysis (enzyme models) were investigated by Karaman's group to design novel prodrug linkers [37, 38-56]. Among the studied intramolecular processes:

- Acid-catalyzed lactonization of hydroxy-acids as researched by Cohen [31-57, 58] and Menger [59-65].
- S_N2-based ring closing reactions as studied by Brown, Bruice, and Mandolini [32, 33, 66].
- Proton transfer between two oxygens in Kirby's acetals [67-71], and proton transfer between nitrogen and oxygen in Kirby's enzyme models [67-71] and proton transfer from oxygen to carbon in some of Kirby's enol ethers [67-71].

The computational calculations using DFT and *ab* initio methods carried by Karaman's group revealed the followings: (i) rates acceleration in intramolecular processes are a result of both entropy and enthalpy effects. In intramolecular ring-closing reactions where enthalpic effects were predominant, steric effects were the determining factor for the acceleration, whereas proximity orientation was the determining factor in proton-transfer reactions. (ii) The distance between the two reacting centers is the main factor in determining whether the reaction type is intermolecular or intramolecular. When the distance exceeded 3 Å, an intermolecular engagement was preferred because of the engagement with a water molecule (solvent). When the distance between the electrophile and nucleophile was < 3 Å, an intramolecular reaction was dominant [38-55, 72].

Based on previous studies on enzyme models a design of a prodrug with modified pharmacokinetics properties that can releases the parent drug in a slow or fast manner could be accomplished [73].

2.2. Design of 6-aminocaproic acid prodrug using Kirby`s enzyme model (Proton transfer in N-alkylmaleamic acids)

Acid-catalyzed hydrolysis of N-alkylmaleamic acids **1-9** (Figure 2) was kinetically studied by Kirby's group; they concluded that the amide bond cleavage occurs due to intramolecular nucleophilic catalysis by the adjacent carboxylic acid group and the rate-limiting step is the tetrahedral intermediate breakdown [74].

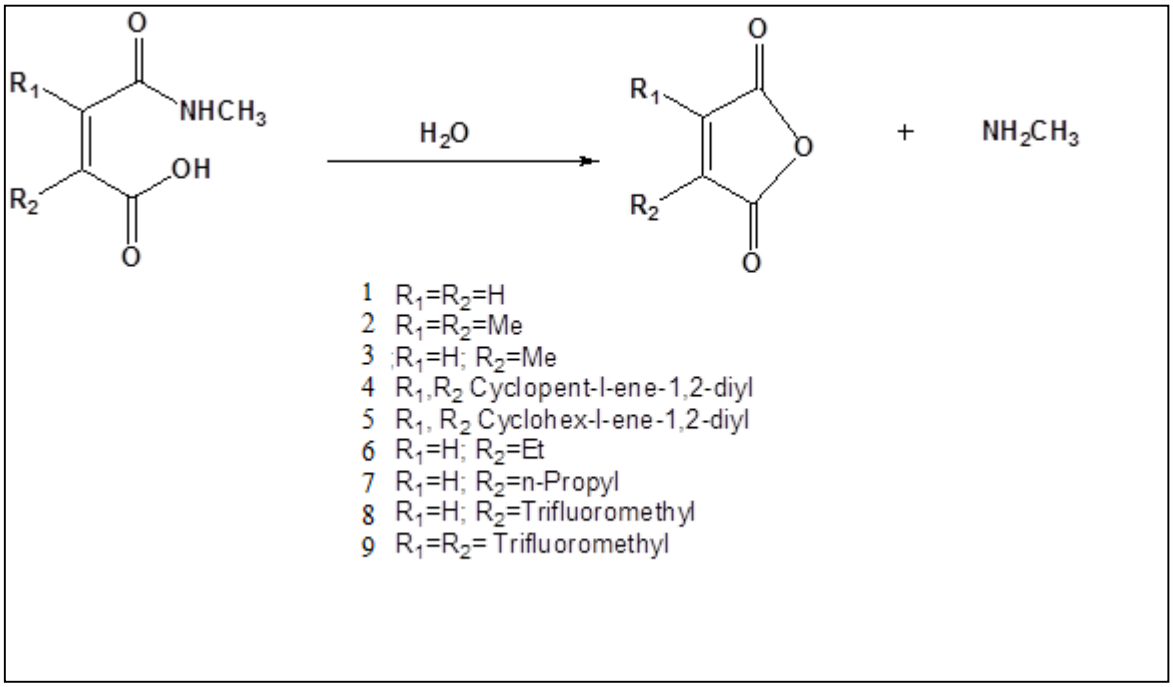


Figure 2. Acid-catalyzed hydrolysis of N-alkylmaleamic acids.

DFT calculations on the acid-catalyzed hydrolysis of Kirby`s N-alkylmaleamic acids that were done by Karaman's group showed that the rate limiting step in aqueous medium is the collapse of the tetrahedral intermediate whereas in the gas phase the rate limiting step is the formation of the tetrahedral intermediate. Furthermore, Karaman's calculations revealed a correlation between the acid-catalyzed hydrolysis efficiency and the following parameters:

1. The difference in the strain energies of the ground states along the reaction pathway, reactants, intermediates and products..
2. The distance between the hydroxyl oxygen of the carboxylic group and the amide carbonyl carbon.
3. The attack angle of the carboxylate oxygen and the amide carbonyl carbon.

The calculations also demonstrated that the acid catalyzed reaction involves three steps: (1) proton transfer from the carboxylic group to the adjacent amide carbonyl oxygen, (2) nucleophilic attack of the carboxylate anion onto the protonated carbonyl carbon; and (3) dissociation of the tetrahedral intermediate to provide products (Figure 3)

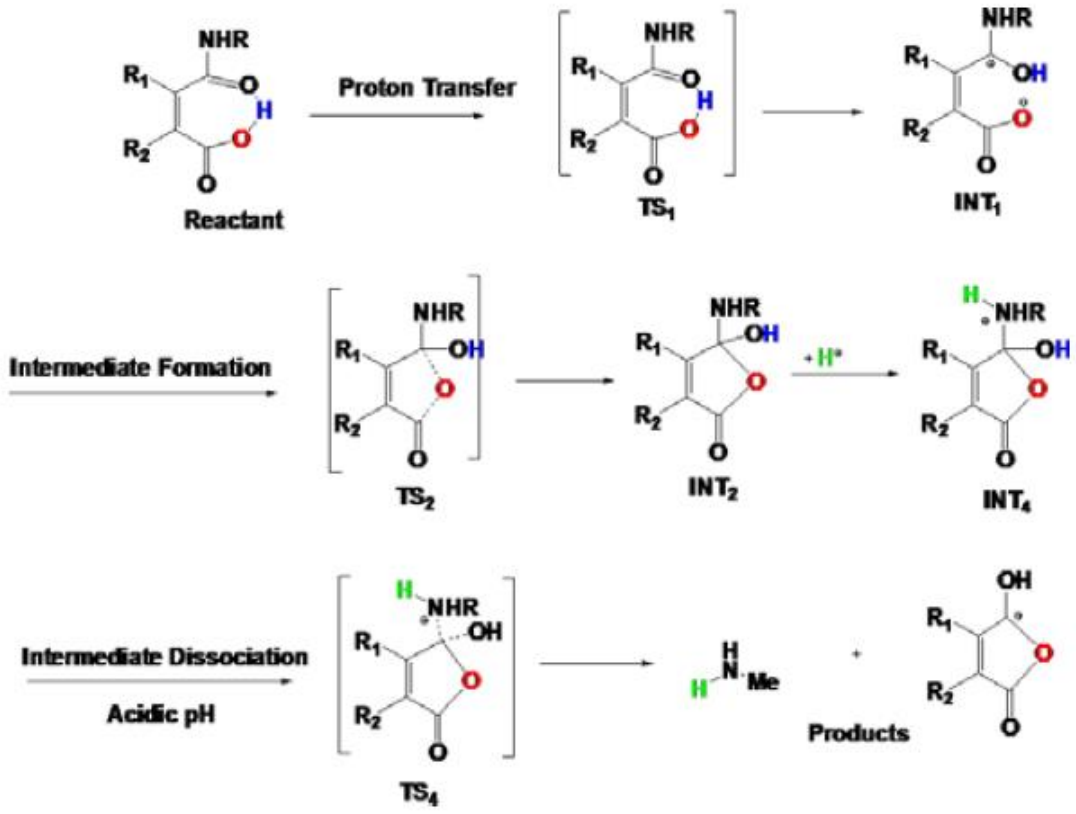


Figure 3. Proposed mechanism for the acid-catalyzed hydrolysis of N-alkylmaleamic acids.

Based on the calculation results of Kirby`s model (proton transfer in N-alkylmaleamic acids) we propose three 6-aminocaproic acid prodrugs, 6-aminocaproic acid **ProD1- 3** (Figure 4).

As shown in Figure 4, 6-aminocaproic acid ProD1-3 have a carboxylic group (hydrophilic moiety) and a lipophilic moiety (the rest of the prodrug), where the combination of both moieties secures a relatively moderate HLB.

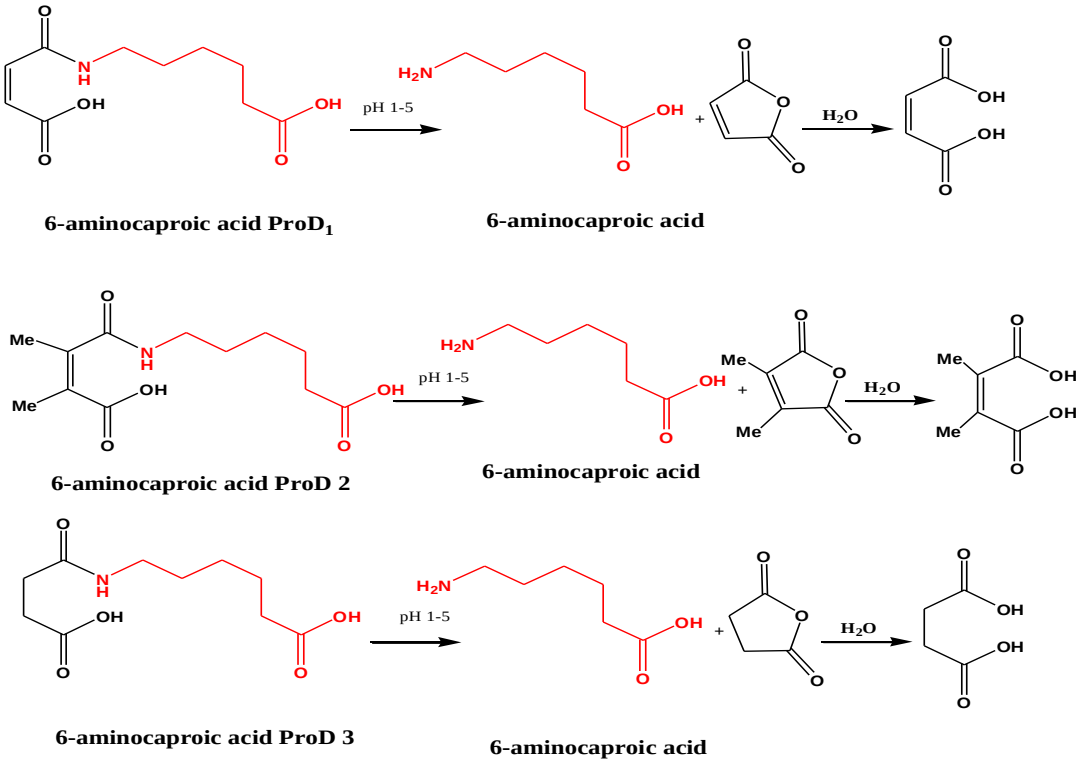
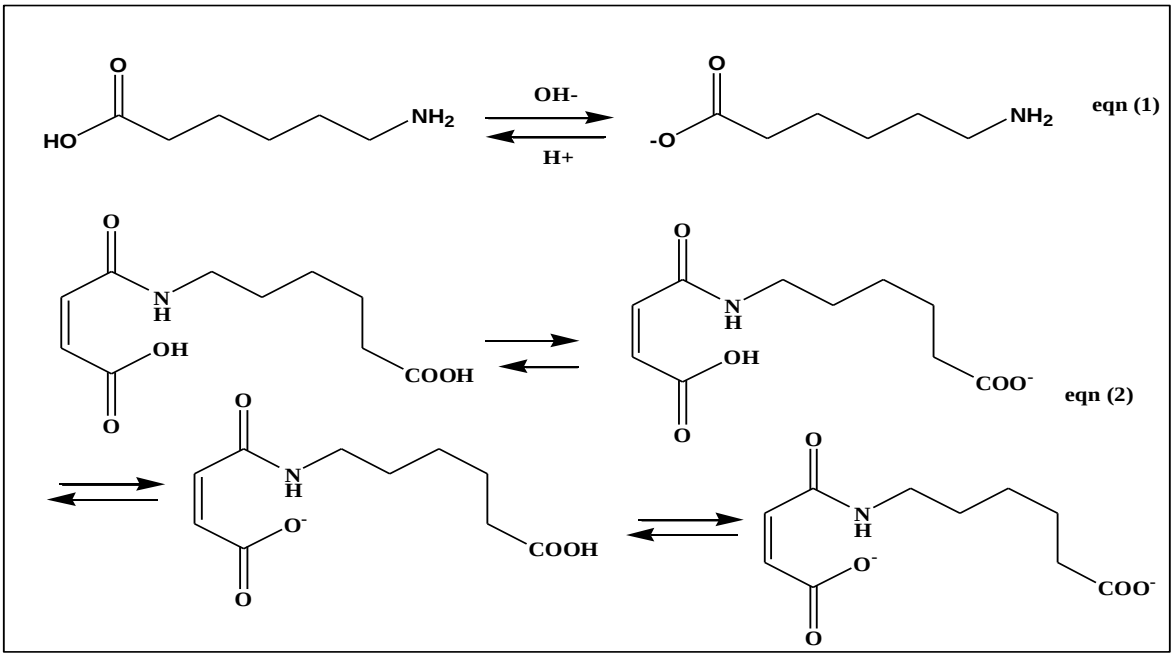


Figure 4. Acid-catalyzed hydrolysis of 6-aminocaproic acid **ProD1- 3**.

In most of the physiologic environments (pH 1- 8.0) 6-aminocaproic acid will exist primary in the ionized forms (eq.1) while its prodrugs, 6-aminocaproic acid **ProD1- 3**, will equilibrate between the ionic and the free acid forms (eq. 2) especially in a physiological environment of pH 5.5-6.8 (intestine). Thus, it is expected that 6-aminocaproic acid **ProD1- 3** may have a better bioavailability than the parent drug due to neutralizing the ionized amine group which results in absorption improvement. In addition, these prodrugs may be used in different dosage forms (i.e. enteric coated tablets, topical use and etc.) because of their potential solubility in organic and aqueous media due to the ability of the carboxylic group to be converted to the corresponding carboxylate anion in a physiological pH of around 6.0.



It should be emphasized that at pH 5.5-6.5 (SC, skin, mouth cavity and intestine physiologic environments), the carboxylic group of the prodrugs will equilibrate with the corresponding carboxylate form (eq. 2). Subsequently, the free acid form will undergo proton transfer reaction (rate limiting step) to yield the antifibrinolytic drug, 6-aminocaproic acid, and the inactive linker as a by-product (Figure 4).

It is worth noting that our proposal is to exploit 6-aminocaproic acid prodrugs **ProD1- 3** for oral use via enteric coated tablets. At this physiologic environment, these prodrugs will exist in the acidic and ionic forms where the equilibrium constant for the exchange between the two forms is dependent on the pK_a of the given prodrug (eq. 2). The experimental determined pK_{a1} for 6-aminocaproic acid **ProD1- 3** linkers is in the range of 4-6. Therefore, it is expected that the pK_as of the corresponding prodrugs will have similar pK_a range as for the carboxylic linkers. Since the pH for the small intestine lies in the range of 5.5-6.8, the calculated unionized (acidic) /ionized ratio will be in the range of 10-50%. Although the percentage of the acidic form is not significantly high, we expect that these prodrugs to undergo an efficient proton transfer (rate limiting step) to yield the antifibrinolytic drug, 6-aminocaproic acid. In the blood circulation at pH 7.4, the calculated acidic form is around 1% and it is expected that the efficiency for delivering the parent drug will be relatively low. Improving the efficiency could be achieved by using carboxylic linkers having pK_a close to that of the blood circulation (pH 7.4

Experimental

Chapter Three

3.1 Materials and methods

This chapter contains three parts: the first part is devoted to the description of the chemicals and reagents used throughout the study, the second part describes the instruments used, and the last part deals with specific preparation and analysis of prodrugs 6-aminocaproic acid with maleic anhydride, 6-aminocaproic acid with dimethyl maleic anhydride and 6-aminocaproic acid with succinic anhydride.

Part one

3.1.1 Chemicals and Reagents

Pure standards of 6-aminocaproic acid, maleic anhydride, 2,3- dimethyl maleic anhydride, succinic anhydride, sodium hydroxide (NaOH), tetrahydrofuran (THF), ethyl acetate, distilled water (D.W), methanol, chloroform, acetone, and water for analysis were for HPLC grade and were purchased from Sigma Aldrich Company.

Part two

3.2 Instrumentation

3.2.1 High Pressure Liquid Chromatography

High Pressure Liquid Chromatography (HPLC-PDA) system consists of an alliance 2695 HPLC from (Waters: Israel), and a waters Micromass® Masslynx ™ detector with Photo diode array (PDA) (Waters 2996: Israel). Data acquisition and control were carried out using Empower ™ software (Waters: Israel). Analytes were separated on a 4.6 mm x150mm C18 XBridge ® column (5 µm particle size) used in conjunction with a 4.6mmx20 µm XBridge ™ C18 guard column. Microfilter was used with 0.45 µm (Acrodisc® GHP, Waters).

3.2.2 UV-Spectrophotometer

The concentrations of the samples were determined spectrophotometrically (UV-spectrophotometer, Model: UV-1601, Shimadzu, Japan) by monitoring the absorbance at the λ_{max} for the drug and prodrug.

3.2.3 pH meter

pH values were recorded on pH meter model HM-30G: TOA electronics ™ was used in this study to measure the pH value for each sample.

3.2.4 Fourier Transforms Infrared Spectroscopy (FTIR):

All infrared spectra were obtained from a KBr matrix (4000–400 cm^{-1}) using a PerkinElmer Precisely, Spectrum 100, FT-IR spectrometer.

3.2.5 ^{1}HNMR

Data were collected using Varian Unity Inova 500 MHz spectrometer equipped with a 5-mm switchable and data were processed using the VNMR software. For ^{1}H-NMR, chemical shifts are reported in parts per million (ppm, δ) downfield from tetramethylsilane (TMS). Spin multiplicities are described as s (singlet), brs (broad singlet), t (triplet), q (quartet), and m (multiplet).

3.2.6 Rotary Evaporator

Speed Control: 20-270 rpm, Digital bath for water or oil 20-180^{o}C, ±1^{o}C, Bath can also be used separately, Distillation glass set type G1 (Standard diagonal condenser).

3.2.7 LC-MS

HPLC–MS/MS measurements were performed employing a Shimadzu prominence high performance liquid chromatography system (Shimadzu corp. Japan).

Part three *(Scheme1)*

3.3 Synthesis methods

3.3.1 6-aminocaproic acid ProD1

In a 250 ml RBF, 20 mmol of 6-aminocaproic acid white powder was dissolved in THF (100 ml), 0.5 gm of sodium hydroxide (NaOH) were dissolved in 2ml D.W and the resulting solution was added and the reaction mixture was stirred, after 30 minutes 20 mmol of maleic anhydride was slowly added to the reaction mixture and stirred at room temperature for 24 hours, then the solvent was evaporated till dryness. The resulted white powder was washed using ethyl acetate and dried. the reaction was monitored using TLC with eluent (chloroform: methanol 3:1). Then final product was characterized using ^{1}H-NMR, LC-MS and IR.

6-aminocaproic acid **ProD1:** White solid with M.P**.** 85 C^{o}**;** ^{1}H-NMR δ (ppm) CD_3OD- 1.42 (m, 2H, CH_2-$\underline{CH_2}$- CH_2), 1.56 (m, 2H, CH_2-CH_2-$\underline{CH_2}$-CH_2), 1.60 (m, 2H, CH_2-$\underline{CH_2}$-CH_2-CH_2), 2.20(t, 2H, COO-$\underline{CH_2}$-CH_2), 2.96 (t, 2H, CH_2-$\underline{CH_2}$-NH), 6.10 (d, 1H, J = 10 Hz, $\underline{HC}$=CH), 6.27 (d, 1H, J = 12 Hz, HC=$\underline{CH}$). IR (KBr/ν_{max} cm^{-1}) 1782 (C=O), 1707 (C=O), 1570 (C=C), 1404, 1283, 1189, 1104, 1062. *m*/*z* 252 $(M+Na)^{+}$. Yield: 96% (4.8 g).

3.3.2 6-aminocaproic acid ProD2

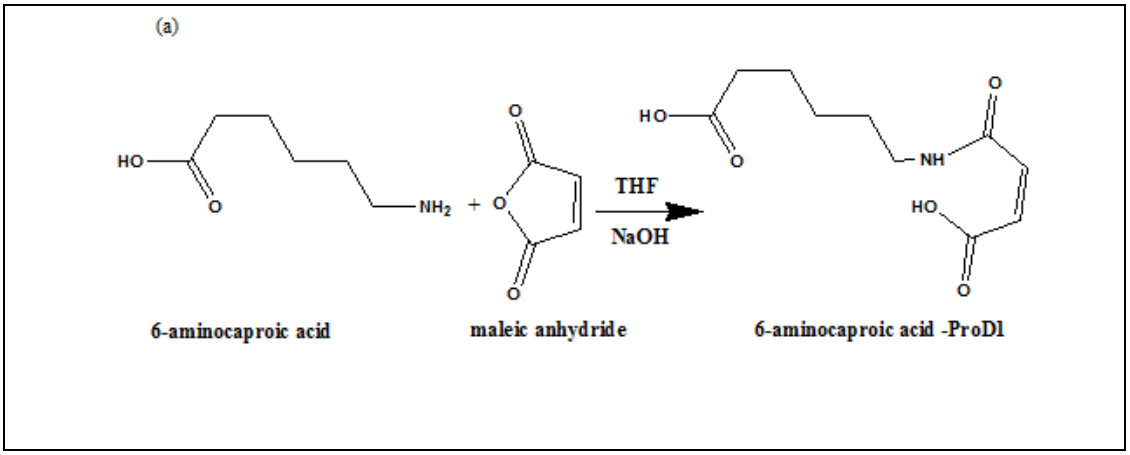
In a 250 ml RBF, 20 mmol of 6-aminocaproic acid was dissolved in THF (100 ml), 0.5 gm of sodium hydroxide (NaOH) were dissolved in 2ml D.W and the resulting solution was added and the reaction mixture was stirred, after 30 minutes 20 mmol of dimethyl maleic anhydride was slowly added to the reaction mixture and stirred at room temperature for 24 hours, then the solvent was evaporated till dryness. The resulted white powder was washed using ethyl acetate and dried. the reaction was monitored using TLC with eluent (chloroform: methanol 3:1) . Then final product was characterized using ^{1}H-NMR, LC-MS and IR.

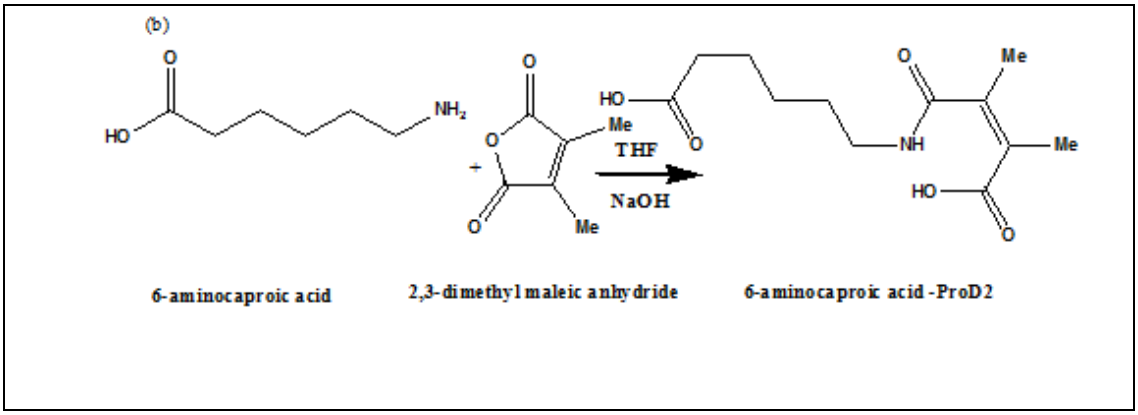
6-aminocaproic acid **ProD2:** White solid with M.P**.** 158 C^{o}**;** ^{1}H-NMR δ (ppm) CD_3OD- 1.42 (m, 2H,CH_2- $\underline{CH_2}$- CH_2-CH_2), 1.56 (m, 2H, CH_2-CH_2-$\underline{CH_2}$-CH_2), 1.62 (m, 2H, CH_2-$\underline{CH_2}$-CH_2-CH_2),1.93 (s,6H, $\underline{CH_3}$-C=C-$\underline{CH_3}$), 2.20 (t, 2H, COO-$\underline{CH_2}$ -CH_2), 2.96(t, 2H, CH_2-$\underline{CH_2}$-NH), IR (KBr/ν_{max} cm^{-1}) 1850 (C=O), 1782(C=O), 1581(C=C), 1389, 1282, 1204, 1105,1057. *m*/*z* 279 $(M+Na)^{+}$. Yield: 91.4% (2.35 g).

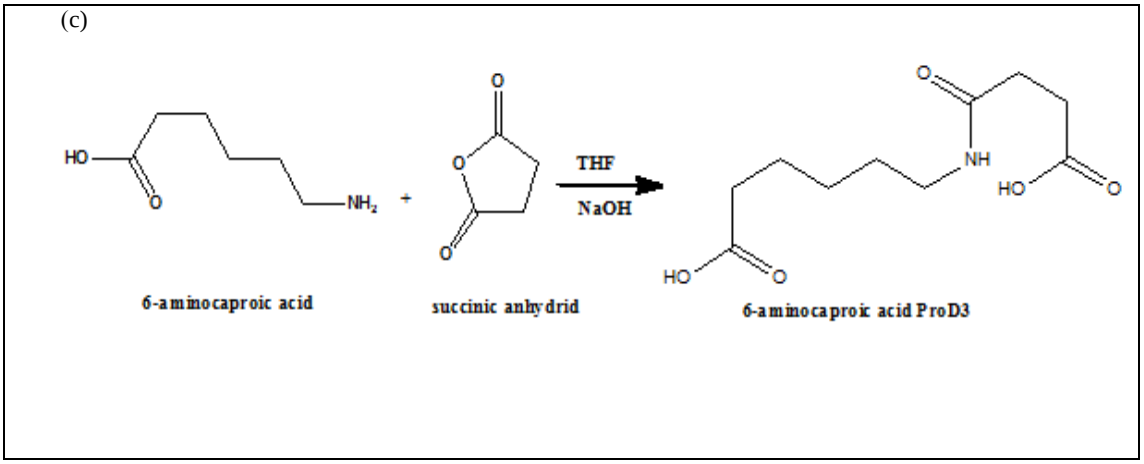
3.3.3 "6-aminocaproic acid ProD3"

In a 250 ml RBF, 20 mmol of 6-aminocaproic acid was dissolved in THF (100 ml), 0.5 gm of sodium hydroxide (NaOH) were dissolved in 2ml D.W and the resulting solution was added and the reaction mixture was stirred, after 30 minutes 20 mmol of succinic anhydride was slowly added to the reaction mixture and stirred at room temperature for 24 hours, then the solvent was evaporated till dryness. The resulted white powder was washed using ethyl acetate and dried. the reaction was monitored using TLC with eluent (chloroform: methanol 3:1) . Then final product was characterized using ^{1}H-NMR, LC-MS and IR.

6-aminocaproic acid **ProD3:** White solid with M.P. 100 C°**;** ^{1}H-NMR δ (ppm) CD_3OD-1.35 (m, 2H CH_2-$\underline{CH_2}$-CH_2), 1.50 (m, 2H, CH_2-CH_2-$\underline{CH_2}$-CH_2), 1.6 5(m, 2H, CH_2-$\underline{CH_2}$-CH_2-CH_2), 2.24(t, 2H, COO-$\underline{CH_2}$ -CH_2), 2.45 (t, J = 6 Hz, 2H,$\underline{CH_2}$-CH_2), 2.51 (t, 2H, J = 6.4 Hz, CH_2-$\underline{CH_2}$), 2.96 (t, 2H, $\underline{CH_2}$-NH). IR(KBr/ν_{max} cm^{-1}) 1632(C=O), 1554(C=O), 1474, 1414, 1366, 1239, 1200, 1166, 1028. *m*/*z* 254 $(M+Na)^+$. Yield: 94% (4.36 g).







Scheme 1: Synthesis of (a) 6-aminocaproic acid **ProD1**, (b) 6-aminocaproic acid **ProD2,** (c) 6-aminocaproic acid **ProD3.**

3.4 Kinetic Methods

3.4.1 Buffer Preparation

6.8 g potassium dihydrogen phosphate were dissolve in 900 ml water for HPLC, the pH of buffer pH 2.5 was adjusted by diluted o- phosphoric acid and water was added to a final volume of 1000 ml (0.05M). The same procedure was done for the preparation of buffers pH 5 and 7.4. However, the required pH was adjusted using 1 N NaOH.

Inter conversion of 500 ppm 6-aminocaproic acid **ProD1-3** solutions, in 1N HCl, buffer pH 2.5, buffer pH 5 or buffer pH 7.4, to its parent drug, 6-aminocaproic acid, was followed by HPLC at a wavelength of 210 nm. Conversion reactions were run mostly at 37.0 ˚C.

3.4.2 Calibration curve

A 100 ml stock solution of 6-aminocaproic acid **ProD1-3** with a final concentration of 50 ppm were prepared by dissolving 500 mg from each prodrug in 100 ml methanol. The following diluted solutions were prepared from the stock solution: 100, 200, 300 and 400 ppm. Each solution was then injected to the HPLC apparatus using 6 mm x 250 mm, 5 µm C18 XBridge ® column, mobile phase contains (pH 2.5 using diluted phosphoric acid), a flow rate of 1 ml min^{-1} and UV detection at a wavelength of 210 nm.

Peak area vs. concentration of the pharmaceutical (ppm) was then plotted, and R^2 of the plot was recorded.

3.4.3 Preparation of standard and sample solution

6-aminocaproic acid prodrugs

A 500 ppm of standard 6-aminocaproic acid was prepared by dissolving 50 mg of 6-aminocaproic acid in 100 ml of 1N HCl, buffer pH 2.5, buffer pH 5 or buffer pH 7.4, then each sample was injected into HPLC to detect the retention time of 6-aminocaproic acid.

A 500 ppm of standard linker (maleic anhydride, 2,3-dimethyl maleic anhydride, succinic anhydride) was prepared by dissolving 500 mg of each linker in 100 ml of 1N HCl, buffer pH 2.5, buffer pH 5 or buffer pH 7.4, then each sample was injected into HPLC to detect the retention time of linker.

A 500 ppm of each 6-aminocaproic acid **ProD1-3** was prepared by dissolving 50 mg of the 6-aminocaproic acid **ProD1-3** in 100 ml of 1N HCl, buffer pH 2.5, buffer pH 5 or buffer pH 7.4 then each sample was injected into HPLC to detect the retention time.

The progression of reaction was followed by monitoring the disappearance of the prodrug and the appearance of the linkers, maleic anhydride, 2,3-dimethyl maleic anhydride and succinic anhydride with time.

Results and Discussion

Chapter Four

4. Results and Discussion

6-aminocaproic acid prodrugs were synthesized, purified and characterized using different spectroscopic and chromatographic techniques.

4.1. Characterization

6-aminocaproic acid and 6-aminocaproic acid **ProD1- 3** were characterized by FT-IR, LC-MS and ^{1}H-NMR spectroscopy.

6-aminocaproicacid ($C_6H_{13}NO_2$). The IR spectrum (Figure 5a) shows an absorbance at 1655 cm^{-1} corresponds to C=O, 1453 cm^{-1}, 1291 cm^{-1}, 1212 cm^{-1}, 1104 cm^{-1}, 1044 cm^{-1}. The ^{1}H-NMR (Figure 6 b) peaks correspond to 1.42 ppm (m, 2H, CH_2-$\underline{CH_2}$-CH_2), 1.55 ppm (m, 2H, CH_2-$\underline{CH_2}$-CH_2-CH_2), 1.56 ppm (m, 2H, CH_2-CH_2-$\underline{CH_2}$-CH_2), 2.23 ppm (t, 2H, COO-$\underline{CH_2}$-CH_2-), 2.96 ppm (t, 2H,$CH_2\underline{CH_2}$-NH_2).

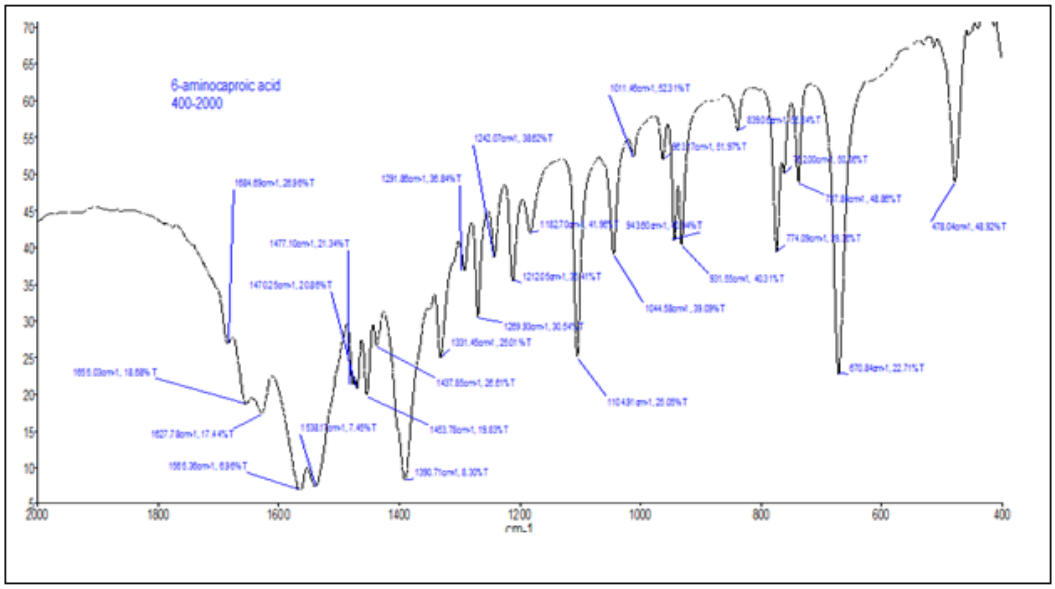


Figure 5a. FT-IR spectrum of 6-aminocaproic acid.

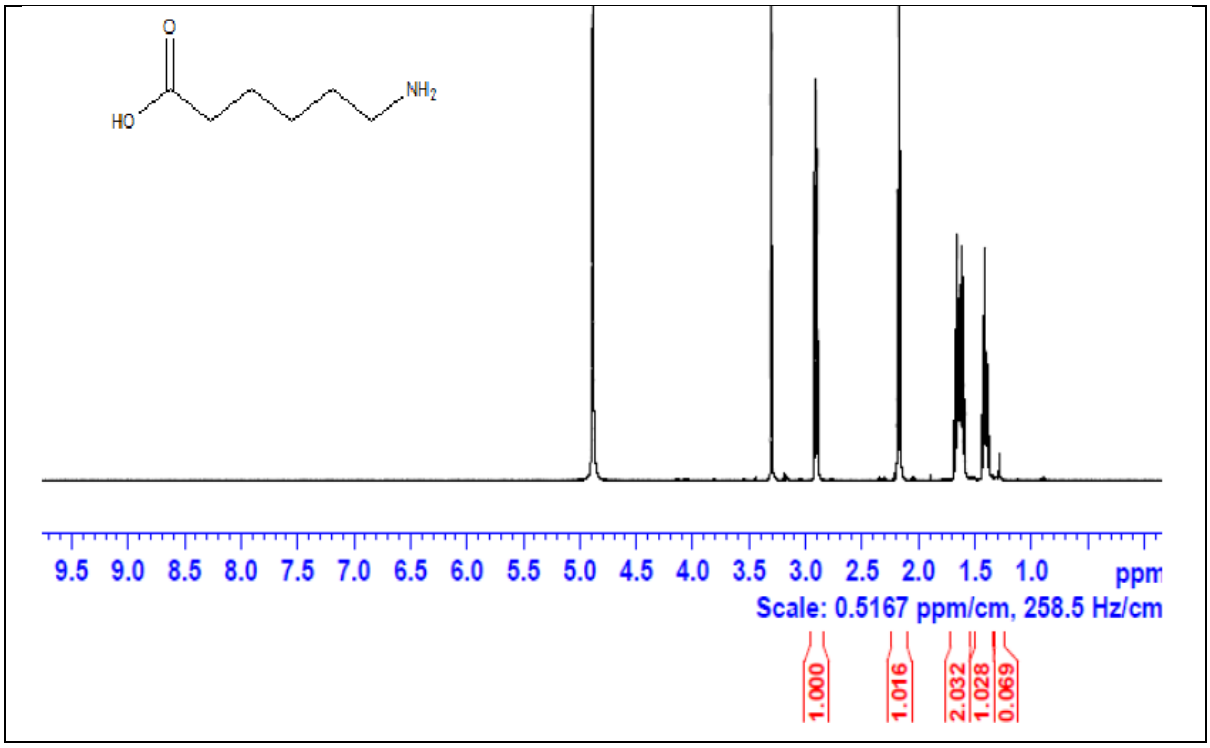


Figure 5b. ^{1}H-NMR spectrum of 6-aminocaproic acid in CD_3OD.

6-aminocaproicacid ProD1 (**$C_{10}H_{15}NO_5$**).The zoomed IR spectrum (Figure 6a) shows an additional signals with absorbance 1782 cm^{-1}, 1707 cm^{-1} corresponds to C=O of the maleate moiety. A high resolution LC-MS (Figure 6 b) shows a protonated peak adduct of $[M+Na]^+$ was appeared at *m/z* of 252.0839. The ^{1}H-NMR (Figure 6 c) the alkene cis protons show doublet peaks at 6.10 ppm and 6.27 ppm with coupling constant of 10 Hz, indicates the cis arrangement of these protons.

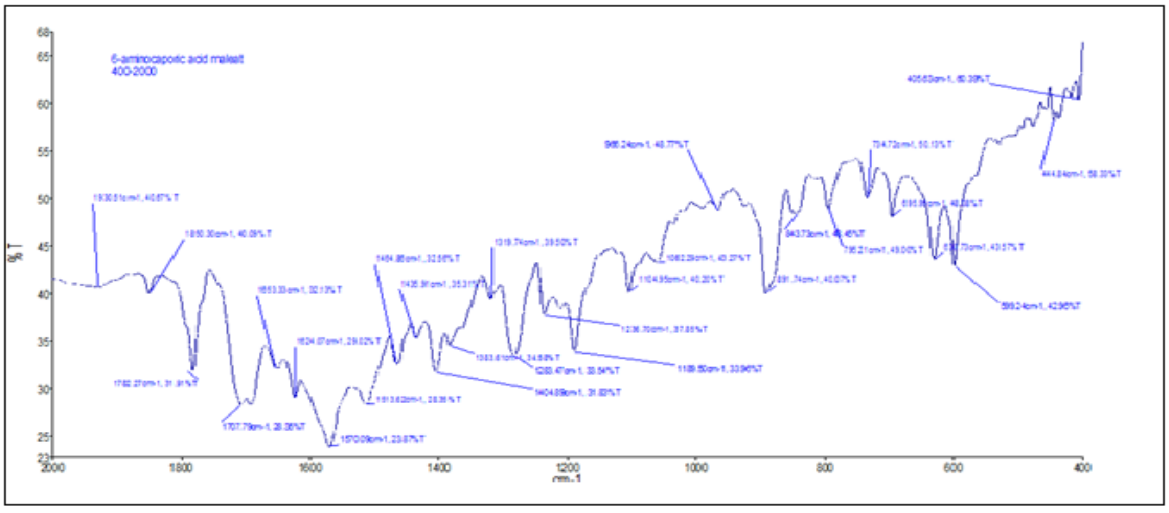


Figure 6a. FT-IR spectrum of 6-aminocaproic acid **ProD1**.

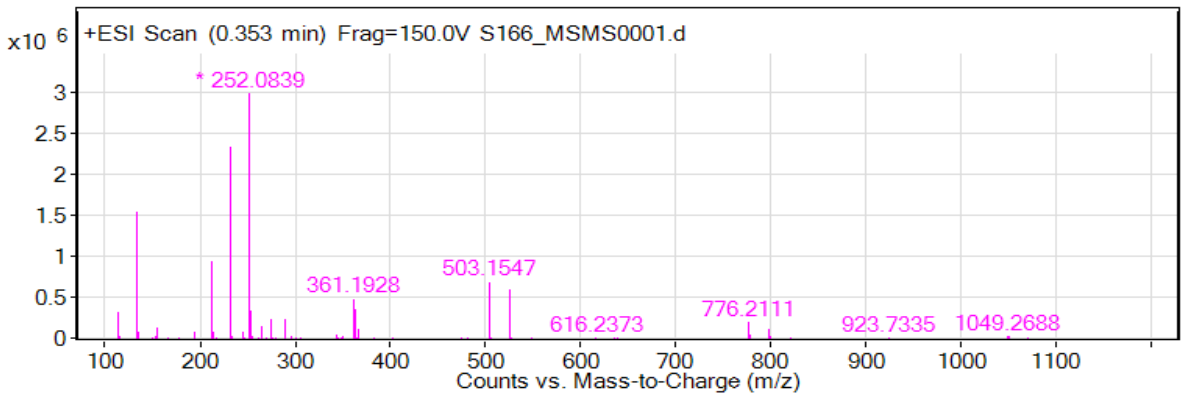


Figure 6b. LC-MS spectrum of 6-aminocaproic acid **ProD1**.

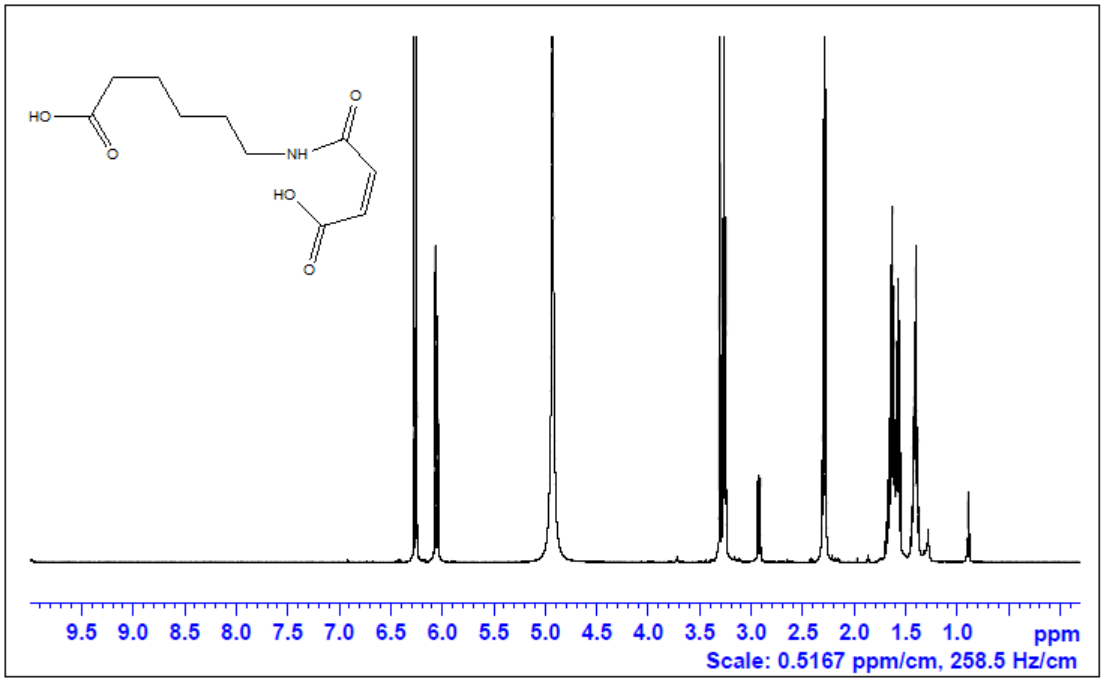


Figure 6c.[1]H-NMR spectrum of 6-Aminocaproic acid **ProD1**in CD_3OD.

6-aminocaproic acid ProD2 (**$C_{12}H_{19}NO_5$**). The zoomed IR spectrum (Figure 7a) shows an absorbance of the additional carbonyl group (C=O) for the dimethyl maleate moiety at 1850 cm^{-1} and 1782 cm^{-1}. A high resolution LC-MS (Figure 7 b) shows a protonated peak for adduct $[M+Na]^+$ at *m/z 279*.1605. The [1]H-NMR (Figure 7 c) an additional singlet peak occurs at 1.93 ppm represenst the two methyl groups on C=C of the dimethyl maleate moiety.

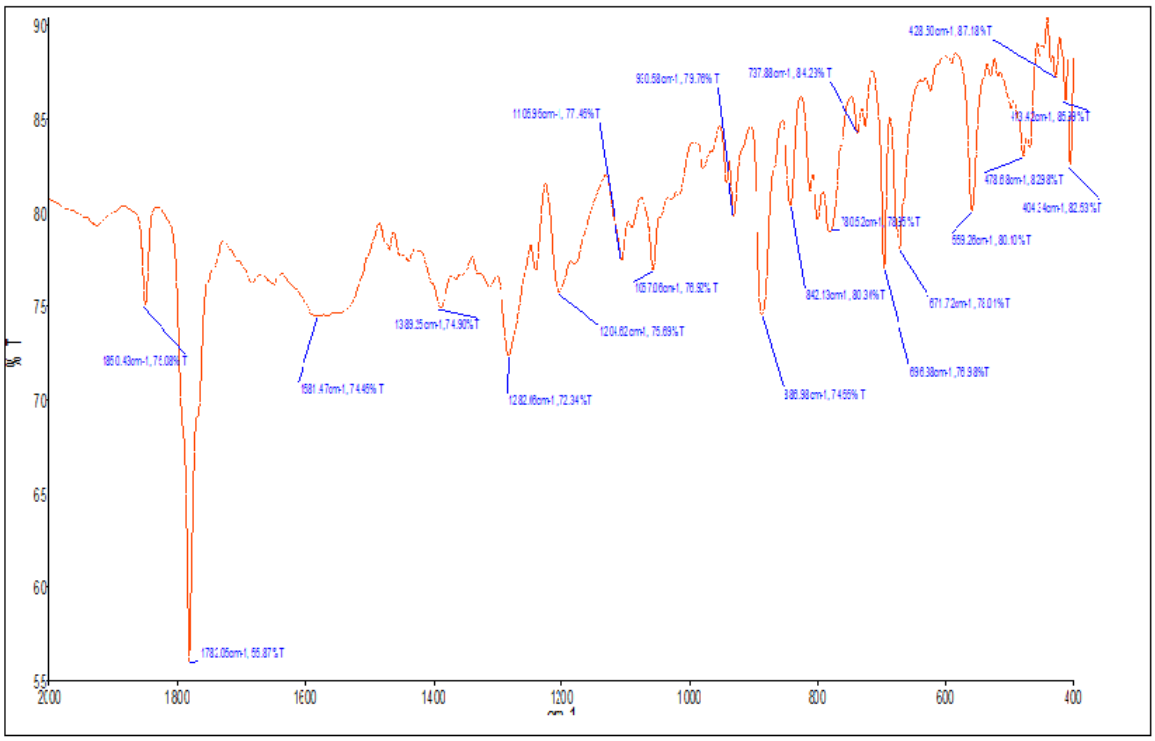


Figure 7a. FT-IR spectrum of 6-aminocaproic acid **ProD2**.

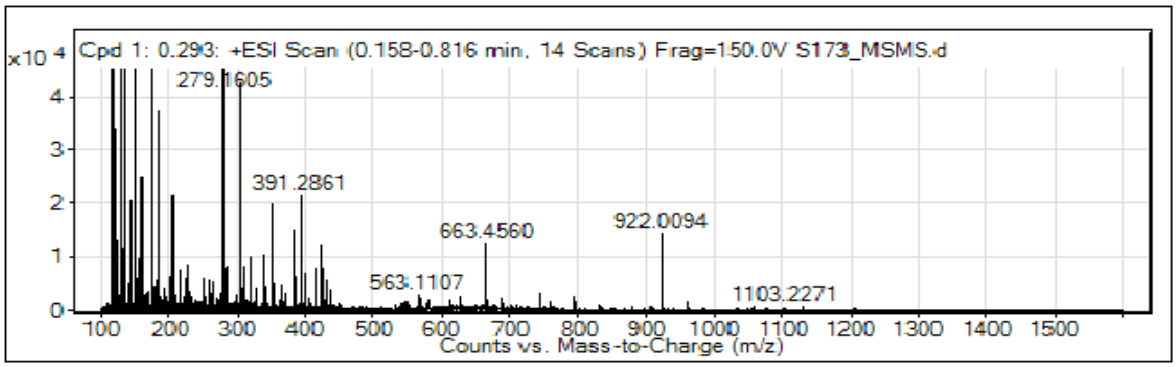


Figure 7b. LC-MS spectrum of 6-aminocaproic acid **ProD2**.

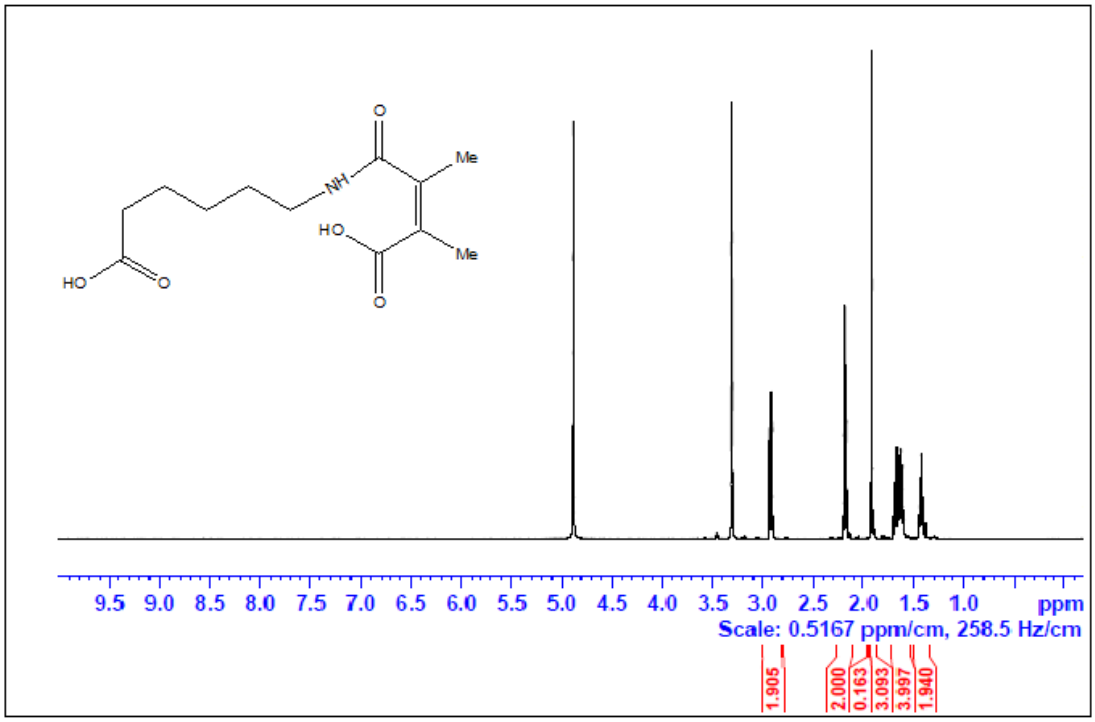


Figure 7c. ^{1}H-NMR spectrum of 6-aminocaprroic acid **ProD2** in CD_3OD.

6-amincaproic acid ProD3 (**$C_{10}H_{17}NO_5$**). The IR spectrum (Figure 8a) shows an absorbance at 1632 cm^{-1} corresponds to the additional carbonyl group (C=O) for the succinate moiety. A high resolution LC-MS shows a protonated peak at adduct of $[M+Na]^+$ *m/z* 254.0999 (Figure 8b). The ^{1}H-NMR (Figure 8c) shows an additional triplet signal for the succinate moiety protons which show a singlet peak at 2.45 ppm with coupling constant of 6 Hz.

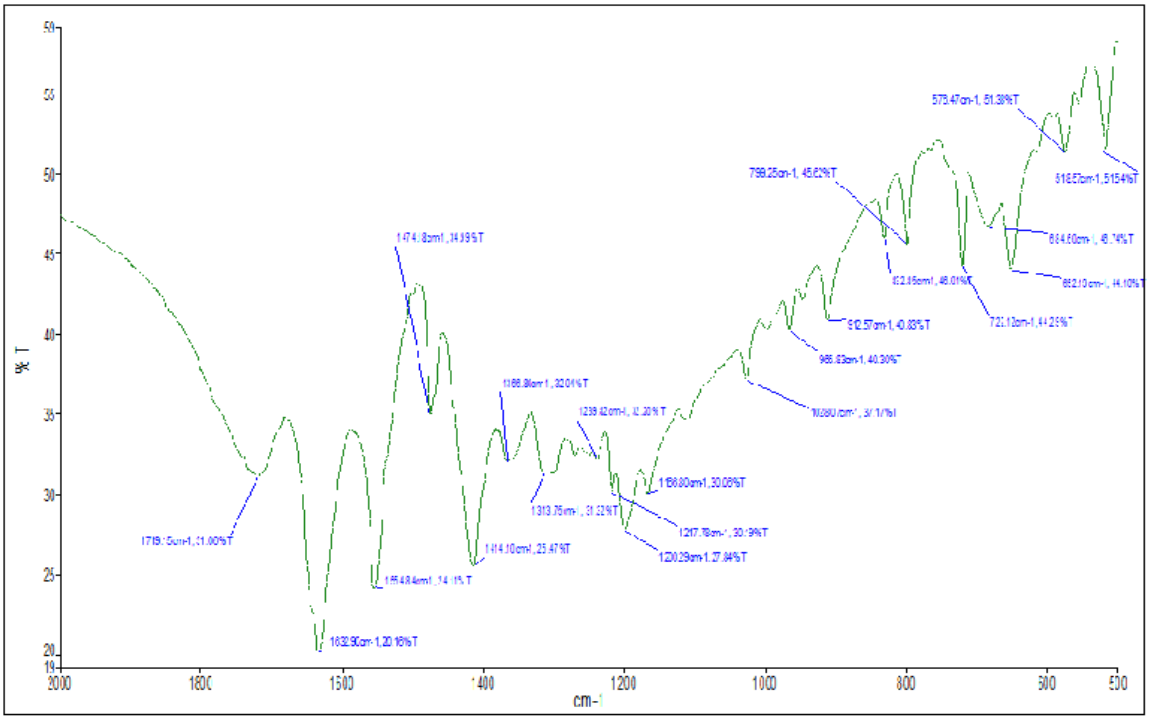


Figure 8a. FT-IR spectrum of 6-aminocaproic acid **ProD3**.

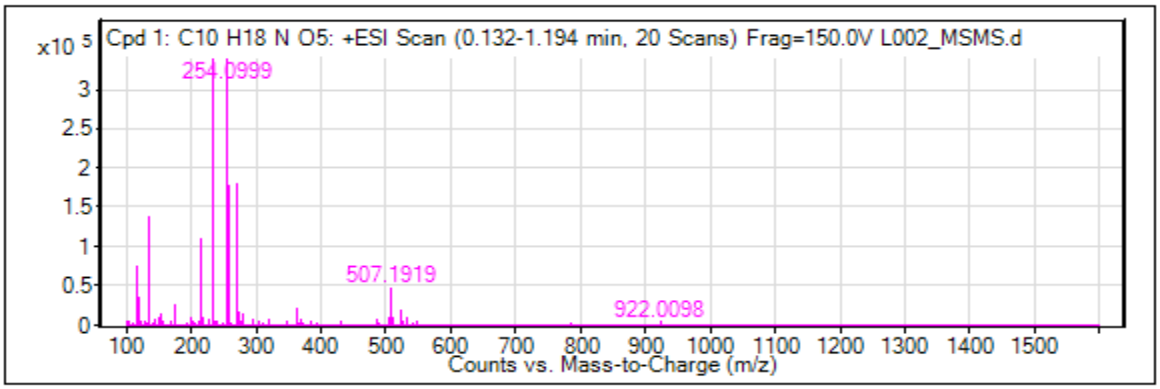


Figure 8b. LC-MS spectrum of 6-aminocaproic acid **ProD3**.

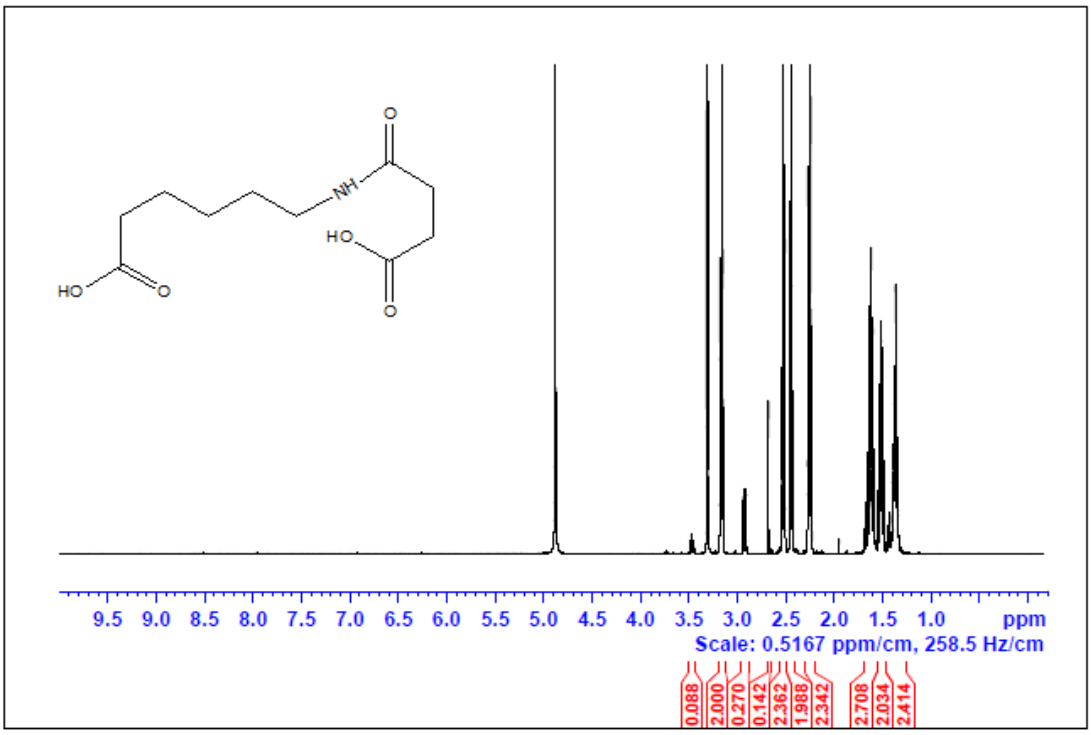


Figure 8c. ^{1}H-NMR spectrum of 6-aminocaproic acid **ProD3** in CD_3OD.

The IR, ^{1}H-NMR and LC-MS spectra results mentioned before, confirmed that the desired prodrugs are obtained from each reaction.

4.2 Calibration curve for 6-aminocaproic acid ProD1- 2

In order to follow the prodrugs kinetics, calibration curves were obtained by plotting the HPLC peak area versus concentration for each prodrug as displayed in Figures 9a and 9b. As shown in the figures, excellent linearity with correlation coefficient (R^2) of 0.992 6-aminocaproic acid **ProD1** (Figure 9a) and 0.983 for 6-aminocaproic acid **ProD2** (Figure 9b) were obtained.

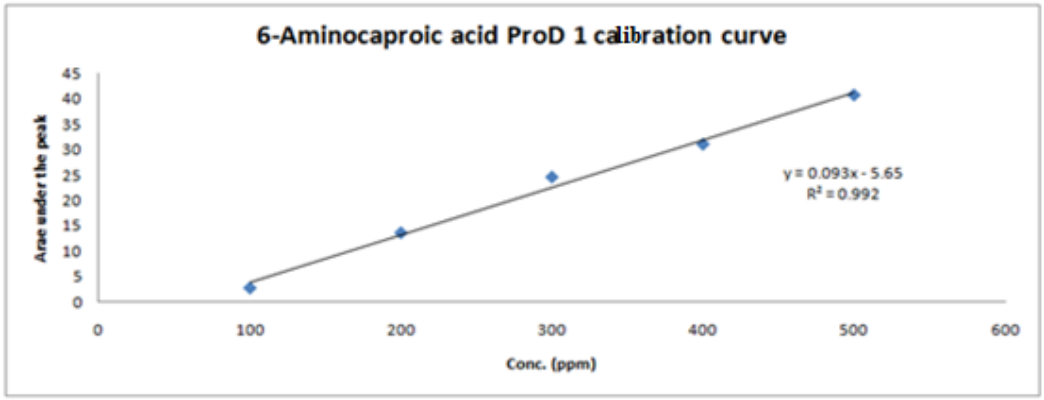


Figure 9a. Calibration curves for 6-aminocaproic acid **ProD1**.

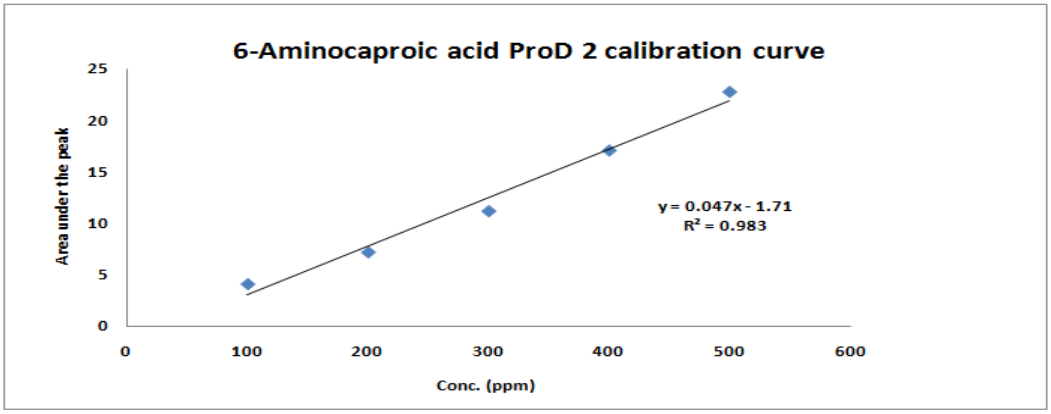


Figure 9b. Calibration curves for 6-aminocaproic acid **ProD2**.

4.3 Hydrolysis studies

4.3.1 6-aminocaproic acid ProD1-3

The kinetics of the acid-catalyzed hydrolysis studies were carried out in aqueous buffer in the same manner as that done by Kirby on Kirby's enzyme model 1-9 [73]. This is in order to explore whether the prodrug hydrolyzes in aqueous medium and to what extent, suggesting the fate of the prodrug in the system. Acid-catalyzed hydrolysis kinetics of the synthesized 6-aminocaproic acid **ProD1-3** were studied in four different aqueous media, 1N HCl, buffer pH 2.5, buffer pH 5 and buffer pH 7.4.

Under the experimental conditions, the target compounds hydrolyzed to release the parent drug as evident by HPLC analysis. At constant pH and temperature, the reaction displayed strict first order kinetics as the *k*obs was fairly constant and a straight plot was obtained on plotting log concentration of residual prodrug vs. time. The rate constant (*k*obs) and the corresponding half-lives ($t_{1/2}$) for 6-aminocaproic acid prodrugs **ProD1-3** in the different media were calculated from the linear regression equation correlating the log concentrationof the residual prodrug vs. time. The 1N HCl and pH 2.5 were selected to

examine the intra-conversion of the 6-aminocaproic acid prodrugs in pH as of stomach, because the mean fasting stomach pH of adult is approximately 1-2. In addition, buffer pH 5 mimics the beginning small intestine pathway. pH 7.4 was selected to examine the intra-conversion of the tested prodrug in blood circulation system. Acid-catalyzed hydrolysis of 6-aminocaproic acid **ProD1** was found to be higher at 1N HCl than at pH 2.5 and 5 (Figures 10, 11 and 12). At 1N HCl, the half-life of the prodrug was 11 hours. On the other hand, at pH 7.4, the prodrug was entirely stable and no release of the parent drug was observed. Since the pK_a of 6-aminocaproic acid **ProD1** is in the range 3-4, it is expected that at pH 5 the anionic form of the prodrug will be dominant and the percentage of the free acid form that undergoes the acid-catalyzed hydrolysis will be relatively low. At 1N HCl and pH 2.5, most of the prodrug will exist as the free acid form and at pH 7.4 most of the prodrug will be in the anionic form. Thus the discrepancy in rates at the different pH buffers, the kinetic data are listed in Table 1.

Acid catalyzed hydrolysis of 6-aminocaproic acid **ProD2** was found to be readily intraconverted at 1N HCl, while at pH 2.5 the half-life was 15 hours (Figures13) and at pH 5 it was 19.5 hours (Figures14), while it was stable at pH 7.4; according to structural feature of 2,3-dimethyl maleic moiety, it contains two methyl groups on the C-C double bond (strained system) which results in a decrease of the distance between the two reactive centers (hydroxyl oxygen of the carboxylic group and the amide carbonyl carbon). Hence, the hydrolysis in 1N HCl and pH 2.5 of 6-aminocaproic acid **ProD2** is faster than that of 6-aminocaproic acid **ProD1** (Figures 15 and 16)

Table 2 summarized the kinetic data for 6-aminocaproic acid **ProD2**.

In the case of 6-aminocaproic acid **ProD3** the interatomic distance between the nucleophile (OH) and electrophile (C=O) is too high to make the nucleophile attack accessible. Hence, no reaction was observed with this prodrug in all different tested buffers (Table 3).

Table 1: The observed *k* value and$_{1/2}$ for the intraconversion of 6-aminocaproic acid **ProD1** in 1N HCl and at pH 2.5, pH 5 and pH 7.4.

Medium	k_{obs} **(h^{-1})**	$t_{½}$ **(h)**
1N HCl	1.39 x 10-^{4}	11
Buffer pH 2.5	1.70 x 10-^{4}	20.6
Buffer pH 5	9.6x 10-^{5}	23.4
Buffer pH 7.4	No reaction	No reaction

Table 2: The observed *k* value and $t_{1/2}$ for the intraconversion of 6-aminocaproic acid **ProD2** in 1N HCl and at pH 2.5, pH 5 and pH 7.4.

Medium	k_{obs} (h^{-1})	$t_{½}$ (h)
1N HCl	Readily released	Readily released
Buffer pH 2.5	1.01×10^{-4}	15
Buffer pH 5	5.8×10^{-5}	19.5
Buffer pH 7.4	No reaction	No reaction

Table 3: The observed *k* value and $t_{1/2}$ for the intraconversion of 6-aminocaproic acid **ProD3** in 1N HCl and at pH 2.5, pH 5 and pH 7.4.

Medium	k_{obs} (h^{-1})	$t_{½}$ (h)
1N HCl	No reaction	No reaction
Buffer pH 2.5	No reaction	No reaction
Buffer pH 5	No reaction	No reaction
Buffer pH 7.4	No reaction	No reaction

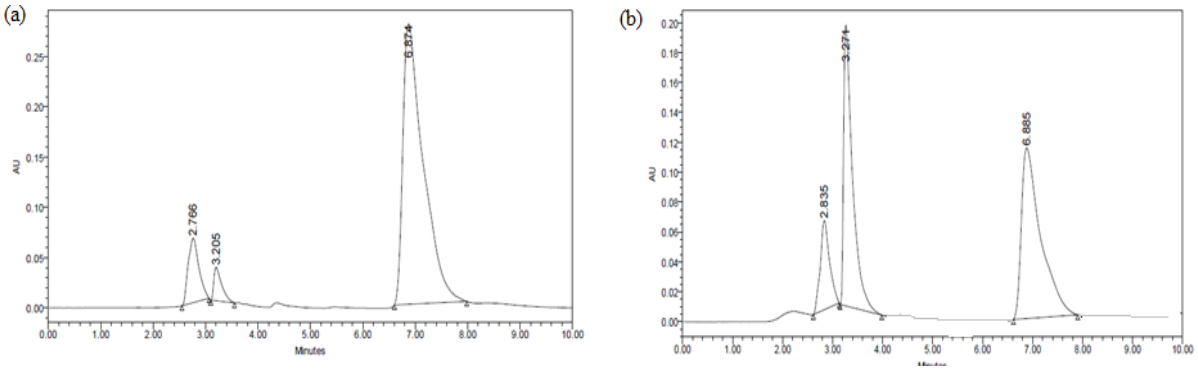


Figure 10. Chromatograms showing the intra-conversion of 6-aminocaproic acid **ProD1** in 1N HCl (a) at zero time, (b) at the end of reaction.

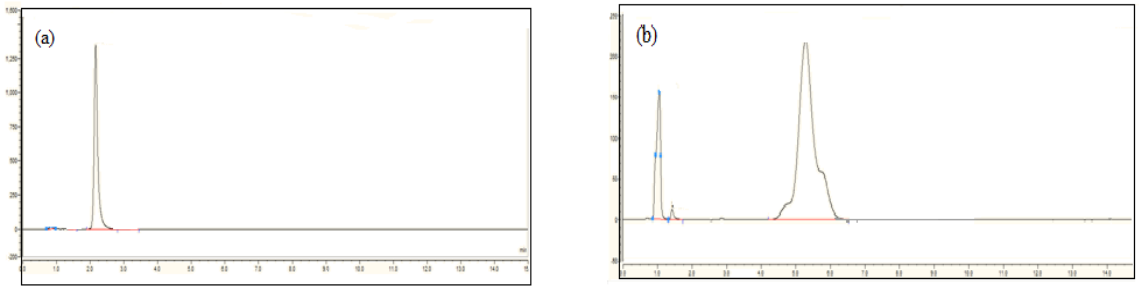


Figure 11. Chromatograms showing the intra-conversion of 6-aminocaproic acid **ProD1** at pH 2.5 (a) at zero time, (b) at the end of reaction**.**

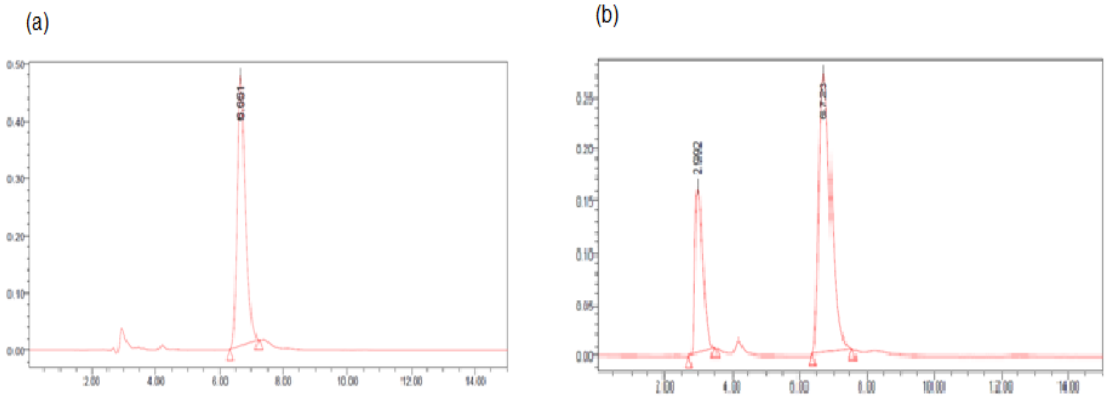


Figure 12. Chromatograms showing the intra-conversion of 6-aminocaproic acid **ProD1** at pH 5 (a) at zero time, (b) at the end of reaction**.**

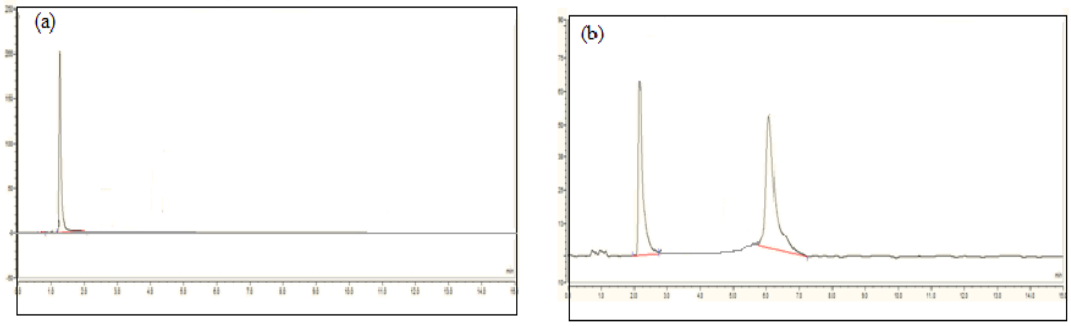


Figure 13. Chromatograms showing the intra-conversion of 6-aminocaproic acid **ProD2** at pH 2.5 (a) at zero time, (b) at the end of reaction**.**

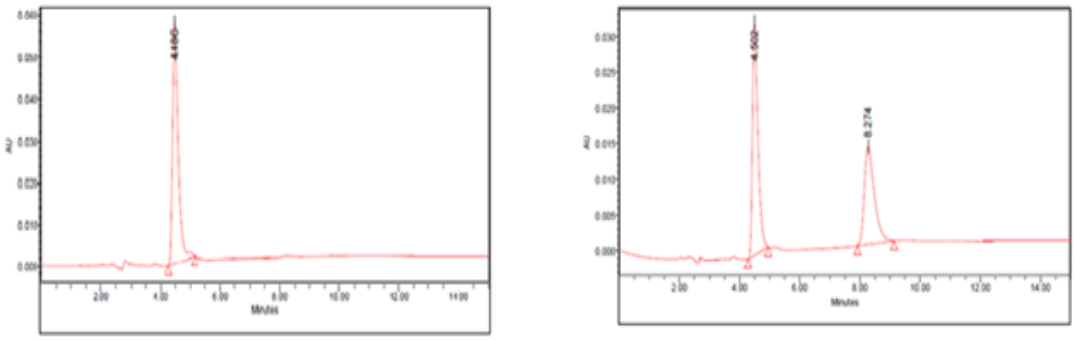


Figure14. Chromatograms showing the intra-conversion of 6-aminocaproic acid **ProD2** at pH 5 (a) at zero time, (b) at the end of reaction

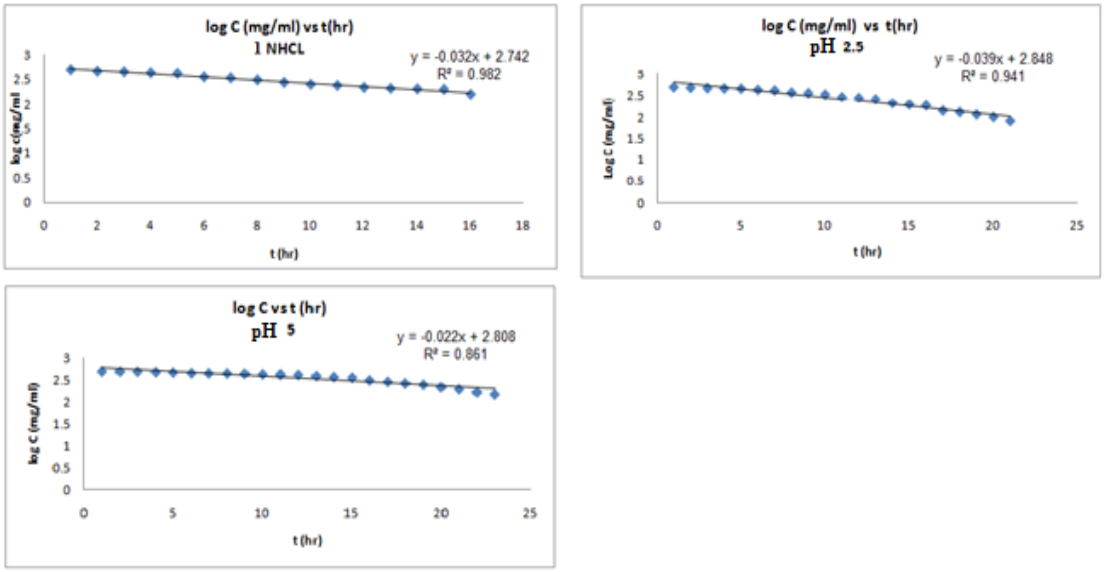


Figure15. First order hydrolysis plot for the intraconversion of 6-aminocaproic acid **ProD1** in (a) 1N HCl, (b) buffer pH 2.5 and (c) buffer pH 5.

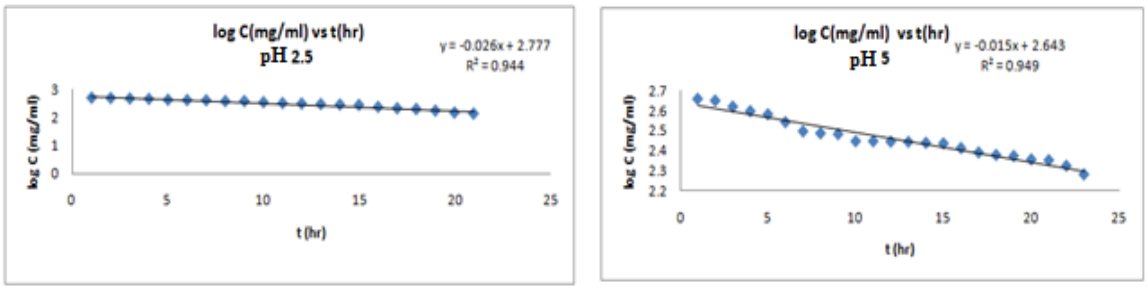


Figure 16. First order hydrolysis plot for the intraconversion of 6-aminocaproic acid **ProD2** in (a) buffer pH 2.5 and (b) buffer pH 5.

Conclusions and Future directions

Chapter Five

5. Conclusions and Future directions

5.1. Conclusions:

Based on Kirby`s enzyme model (Proton transfer in N-alkylmaleamic acids) three different 6-aminocaproic acid prodrugs were synthesized and their acid-catalyzed hydrolysis demonstrated that the rate-limiting step was the collapse of the tetrahedral intermediate. The experimental $t_{1/2}$ value for 6-aminocaproic acid **ProD1** in 1N HCl, buffer pH 2.5 and buffer pH 5 were 11 hours, 20.6 hours and 23.4 hours, respectively. On the other hand, no hydrolysis was observed at pH 7.4. The lack of the hydrolysis at pH 7.4 might be due to the fact that at this pH 6-aminocaproic acid **ProD1** exists mainly in the ionized form (pK_a about 4) taking into consideration that the free acid form is a mandatory requirement for the acid-catalyzed hydrolysis to proceed.

6-Aminocaproic acid **ProD2** was readily converted in 1 N HCl and the experimental $t_{1/2}$ value measured in buffer pH 2.5 and pH 5 were 15 hours and 19.5 hours, respectively. The prodrug was entirely stable at pH 7.4.

6-Aminocaproic acid **ProD3** was stable in 1 N HCl, pH 2.5, pH 5 and pH 7.4 and no reaction was observed. This may be due to the long interatomic distance between the nucleophile (OH) and electrophile (C=O) which renders any nucleophile attack. Hence additional 6-aminocaproic acid prodrugs with reasonable intra-conversion rate at pH 6.5 and pH 7.4 can be synthesized

5.2. Future directions:

The study results suggest synthesis of additional 6-aminocaproic acid prodrugs that may be intra-converted to their parent drug, 6-aminocaproic acid, at pH 6.5 (intestine) and pH 7.4 (blood circulation system) in adequate rates. In vivo pharmacokinetic studies will be done in order to determine the bioavailability and the duration of action of the tested prodrugs and those predicted to be intraconverted in the intestine and blood circulation.

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الملخص

(Prodrug) هو مادة كيميائية يتم فيها ربط العقار تساهمياً إلى مجموعة كيميائية، بحيث تؤثر هذه المجموعة المرتبطة مؤقتا على الخصائص الفيزيائية للدواء لزيادة الفائدة أو تقليل سميتها. يجب أن يتم تحويل الـ(Prodrug) إلى شكله النشط من قبل الأيض و/ أو العمليات الكيميائية داخل الجسم، من الممكن أن تتم عملية التحويل بالاعتماد على عملية التمثيل الغذائي أو باستخدام الأنزيمات التي وزعت في جميع أنحاء الجسم. هذه الإنزيمات قد تقلل إما التوافر الحيوي للدواء، أو قد تظهر مشكلة تعدد الأشكال الوراثية مما يؤدي إلى التباين في تفعيل الـ(Prodrug)، وبالتالي تؤثر على فعالية وسلامة الدواء المساعد. في العقود القليلة الماضية استخدمت أساليب الكيمياء الحسابية في حساب الخصائص الفيزيائية والجزيئية للمركبات. باستخدام هذه الأداة من الممكن تصميم (Prodrug) بحيث يتحول الـ(Prodrug) إلى الدواء النشط عن طريق روابط داخل الجزيء نفسه دون أي تدخل من الإنزيمات. بناء على حسابات (DFT) تم تصميم ثلاثة لـ(Prodrug) حمض امينو كابرويك، ومن ثم تمت دراسة تحويل هذه الأدوية إلى الدواء الأصلي ووجد أن ($t_{1/2}$) تتأثر إلى حد كبير بدرجة حموضة الوسط. لـ(**ProD1** 6-aminocaproic acid) كانت النتائج التجريبية لل($t_{1/2}$) في حمض الهيدروكلوريد، درجة حموضة (2.5) ودرجة حموضة (5) كالتالي (11) ساعة، (20.9) ساعة و(23.4) ساعة، على التوالي. (**ProD2** 6-aminocaproic acid) تم تحويله بسرعة في حمض الهيدروكلوريد، بينما على درجة حموضة 2.5 كانت15 ساعة وعلى درجة حموضة (5) كانت (20) ساعة بينما كان مستقر تماما في درجة الحموضة (7.4). من ناحية أخرى، (6-aminocaproic acid **ProD3**) لم يكن هناك أي تحول للدواء النشط على جميع درجات الحموضة المستخدمة في هذه الدراسة.

عمادة الدراسات العليا
جامعة القدس

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اعداد

فاطمه صادق عبدالله ثوابته

رسالة ماجستير

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المشرف الرئيسي: بروفيسور رفيق قرمان

قدمت هذه الأطروحة استكمالا لمتطلبات درجة الماجستير فرع الصناعات الدوائية في التكنولوجيا التطبيقية والصناعية من كلية الدراسات العليا جامعة القدس–فلسطين.

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