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A new liquid chromatographic method for determination of some statin drugs and activity HMGCR level by different methods.

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Dedication

I would like to express my sincere gratitude to the one whose heart was expanded with love to me and implanted the thirst for science and education in my soul…my father, God bless his soul and may have mercy on him and grant him paradise. To the sea of tenderness and sacrifices icon. To the one who taught me Life. To the one who is credited with what I have achieved…my beloved mother. To my support in life... my brothers and sisters: Omer, Abeer, Olaa, Asim.

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ALI.M. MAHIR

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Summary

This thesis concerns the separation and determination of the three statin drugs (Rosuvastatin, Atorvastatin and Simvastatin) in pharmaceutical dosage forms which play an essential role in treating with hypercholesterolemia diseases and measurement the HMGCR enzyme activity by two methods.

The study starts with an overview of the chromatography technique and how this technique is being introduced firstly as a separation technique. The most famous types of statin drugs have been taken in this chapter to analyze pharmaceuticals using the HPLC technique and how these drugs affect the hypercholesterolemia disease.

Methodology and experimental part have been clarified and reviewed which includes chemicals, tools and devices, and the general procedure for the preparation of standard solutions. The study also covers effect of the ACN content, the buffer concentration and the pH effect on the retention of the three pharmaceuticals. Moreover, serum lipid profile assay, Enzyme-Linked Immunosorbent Assay kit of HMGCR enzyme, the primer of the enzyme are used in this study and the RT-PCR instrument.

*T*he research findings can be interpreted as follows:

- HILIC stationary phases (ZIC-1 and ZIC-4) are investigated for chromatographic Rosuvastatin separation. The eluent's retention behavior in various sodium buffer-containing acetonitrile and pH of the Rosuvastatin was reported. The separation mechanism leads to a mode for Rosuvastatin according to hydrophobic interaction. The calibrating graphs were produced for two exchangers, and linear range (0.1-7µgmL^{-1}), RSD% (0.34± 0.22 and 0.56± 0.08), LOD (0.0094 and 0.0081µg mL^{-1}), LOQ (0.0284 and 0.0245µg mL^{-1}), respectively. For pharmaceutical samples, the methods proposed have been effective. Additionally, the proposed methods' findings are compared to the standard method, demonstrating that their precision and accuracy are equivalent.

- The chromatographic separation and quantification of Atorvastatin, zwitterionic stationary phases with a high capacity were prepared using zwitter-molecules connected to a PS / DVB particle. The retention activity of atorvastatin was determined using eluent at varied

pH values, mobile phase concentrations, and ACN percentages. Separation strategies are based on separating Atorvastatin from hydrophobic interactions. With direct calibration curves, a linearity of 0.1-7 µg mL^{-1} for two columns was obtained, as well as RSD percent (0.705 ± 0.075 and 0.6 ± 0.05), LOD (0.0020 and 0.0013µg mL^{-1}), and LOQ (0.0060 and 0. 0039µg.mL-1). Additionally, the results of the proposed methods are compared to those of the standard method, proving that their precision and accuracy are comparable.

- The chromatographic Simvastatin separation, hydrophilic stationary phases (ZIC-1 and ZIC-4) have been investigated. The retention activity of the eluent in different acetonitrile containing sodium acetate buffer amounts and pH was investigated for Simvastatin. According to hydrophobic interaction, the separation mechanism leads to a mixed-mode for the Simvastatin. The calibrating graphs were produced for two exchangers, and linear range (0.05-4µgmL^{-1}), RSD percent (0.495 ± 0.055 and 0.39 ± 0.03), LOD (0.023 and 0.015µgmL-1), LOQ (0.069 and 0.045µgmL^{-1}), respectively. For pharmaceutical samples, the methods proposed have been effective. And the results of the proposed methods are compared with the comparison method, and their precision and accuracy are comparable.

- The study aslo comprises fifty subjects divided into two groups; **controls group** which involves 20 persons (12 males and 8 females), and the **patients` group** applies 30 patients (14 males and 16 females) before and after treatment. The blood samples have been collected from patients in Baghdad Teaching Hospital during the period from February to June 2021, including the ages from 33-78-years.
- This study shows a significant difference between the control group and the patients `group after statin drugs intake in lipid profile. The mean of **T. Cho (mg/dL)** has appeared to be high significantly increased (P= <0.001) in patients` group (226.166 ± 53.9) compared with control group (149.4 ± 21.84). The mean of **Tg (mg/dL)** has appeared to be significantly increased (P = 0.011) in patients` group (212.633 ± 119.57) compared with control group (141.45 ± 8.75). The mean of **VLDL (mg/dL)** has appeared to be significantly increased (P = 0.011) in patients` group (42.52 ± 23.91) compared with control group (28.29 ± 1.75),

The mean of LDL (mg/dL) has appeared to be significantly increased (P= 0.001) in patients` group (130.46 ± 54.73) compared with control group (67.26 ± 22.99), while the mean of HDL (mg/dL) was appeared to be no significant (P = 0.85) in patients group (53.17 ± 13.98) compared with control group (53.84 ± 8.89).

- Serum levels of HMGCR activity are being estimated by the enzyme-linked immunosorbent assay (ELISA) technique. And whole blood levels of HMGCR activity are evaluated by Real-Time PCR. The first detection method (ELISA kit) in this study is the atorvastatin and rosuvastatin drugs, as shown, inhibited HMG-CoA reductase activity in a concentration-dependent manner which finds a significant decrease in HMG-CoA reductase activity in patients with hypercholesterolemia after statin treatment than before statins treatment (15.7± 7.5; 26.3 ± 11.1) respectively. The Real-Time PCR detection method in this study has inhibited HMG-CoA reductase activity through a copy number gene of this enzyme which conducts a significant decrease in HMG-CoA reductase activity in patients, with hypercholesterolemia, after statin treatment than before statins treatment 348442.4 ± 485652.53; 110.7 ± 20.424) respectively. There were significant differences between RT-PCR and designed ELISA depending on hyperlipidemia patients before and after statin treatment.

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List of Abbreviations:

Abbreviations	Full Name
ACN	Acetonitrile
ATOR	Atorvastatin
ADA	American Diabetes Association
BPC	Bonded phase chromatography
BMI	Body Mass Index
BP	Blood pressure
CVD	Cardio Vascular Disease
DBP	Diastolic blood pressure
ESI-MS	Electrospray ionization mass spectrometry
ELISA	Enzyme-linked immunosorbent
F.S.G	Fasting Serum Glucose
GC	Gas chromatography
GLC	Gas liquid chromatography
GSC	Gas solid chromatography
HILIC	Hydrophilic interaction chromatography
HETP	The height equivalent of an adretical plate
HPLC	High performance liquid chromatography
HDL.C	High Density Lipo protein Cholesterol
HMG-CoA	β-Hydroxy β-methylglutaryl-CoA
ICP-AES	Inductively coupled plasma atomic emission spectrometry
IP	Isoelectric point
LLC	Liquid liquid chromatography
LSC	Liquid solid chromatography
LDL.C	Low Density Lipo protein Cholesterol
NP-LC	Normal phase liquid chromatography
PCR	Polymerase Chain Reaction
PEEK	Polyether ether ketone
PS/DVB	Polystyrene/divinyl benzene
ROSU	Rosuvastatin
RP	Reversed phase
RP-HPLC	Reversed phase-high performance liquid chromatography
RP-LC	Reversed phase liquid chromatography

SDM	Sulfamethoxazole
SEC	Size exclusion chromatography
SBP	Systolic blood pressure
SIMV	Simvastatin
SD	standard deviation
SE	standard error
TLC	Thin layer chromatography
TMA	Trimethylamine
TRM	Trimethoprim
T.Cho	Total Cholesterol
TG	Triglycerides
VLDL	Very Low Density Lipo protein
WHO	World Health Organization
ZIC-1	Sulfobetaine exchangers with one-methylene group between the charged groups
ZIC-4	Sulfobetaine exchangers with four-methylene groups between the charged groups

Chapter One
Introduction and Literature review

1 Introduction

Chromatography is based on the principle where molecules in mixture applied onto the surface or into the solid, and fluid stationary phase (stable phase) is separating from each other while moving with the aid of a mobile phase. The factors effective on this separation process include molecular characteristics related to adsorption (liquid-solid), partition (liquid-solid), and affinity or differences among their molecular weights [1,2]. Because of these differences, some components of the mixture stay longer in the stationary phase, and they move slowly in the chromatography system, while others pass rapidly into mobile phase, and leave the system faster [3]. Based on this approach three components form the basis of the chromatography technique.

- Stationary phase: This phase is always composed of a "solid" phase or "a layer of a liquid adsorbed on the surface a solid support ."
- Mobile phase: This phase is always composed of "liquid" or a "gaseous component".
- Separated molecules

1.1 High performance liquid chromatography (HPLC)

Separating solute molecules which are passing through column because of the various distributions that are related to solutes between stationary phase as well as the liquid mobile phase is an approach that is referred to as liquid chromatography LC. Liquid chromatography has two major types, HPLC and classical liquid chromatography. The first classical liquid chromatography applied columns with about (2cm) of the internal diameter x (50cm) in length. Columns have been filled with the porous particles of diameter (50-250μm), also they are needed sample quantities regarding milliliters. Typically, the mobile phase operates through gravity at low flow rates as well as the separation times could be in 3 hours [4].

In 1970, the HPLC development has been presented as significant approach for the purpose of overcoming high back pressures which are occurring in the case when using small particles for packing. HPLC applies materials have been made of the porous particles of diameter in range of (3-10μm). Such small particles have been packed into the columns with length (100-250mm) and internal diameter of (4-4.6mm). Whereas, LC includes just column and solvent reservoir, HPLC needs complex apparatus [4].

HPLC could be utilized to separate and determine various substances regarding biological, organic, as well as inorganic origin. HPLC types have been classified depending on the type related to the stationary phase to used separation approach: SEC, adsorption, affinity, and ion exchange. The advantages regarding HPLC when compared to traditional LC are elevated efficiency, high sensitivity, short analysis times, more continuous operation, further more effective reproducibility [5].

1.2 Chromatographic Mechanisms in HPLC

The HPLC's separation technique based on the basis of equilibrium created between molecules existing in mobile phase, and they maintained in stationary phase [4,5]. Also, the differences in equal concentrations regarding molecular species in one of the phases and the other is determining if the species are eluted or retained with mobile phase. In a case when the solute's concentration is high in mobile phase in comparison to stationary phase, the solute is eluted fast in comparison to chromatographic column. The opposite will occur in the case when the solute's concentration is considered to be high in stationary phase [6]. With regard to such condition, the solute is retained, also the elution happens following long time period [7].

The major types related to separation mechanisms for the molecular species between two phases including:

1.2.1- Partition separation mechanism

Such type of the separation mechanism happens in the case when the solute's molecules are distributed between the two liquid phases. With regard to HPLC, one of the liquid phases is the stationary on solid materials, while the other is the eluent. Also, the liquid's immobilization for becoming stationary state in the chromatography of the partition has been done, for instance, in the case when the liquid is very polar and might be establishing hydrogen bonds with solid support [7]. An instance has been a water on silica surface. With regard to such condition, the mobile phase must contain liquid not more polar in comparison to the water. Yet, the partition separation mechanism might be utilized for the non-polar stationary phase as well as more polar mobile state. Also, the theory of the separation in the chromatography of the partition is on the basis of liquid/liquid concepts of extraction. Furthermore, the various molecular species which are in constant equilibrium between stationary and mobile phases, is going to be separated on the basis

of tendency for existing in high concentrations in stationary liquid or mobile liquid, based on the affinity for the phases [8]. With regard to the partition chromatography, “immobile liquid” is majorly indicated in “loose” way. For instance, a layer regarding the adsorbed water on silica solid support surface, or the layer which is related to bonded organic chains on the surface of the silica (like C18 columns), or layer regarding the mechanically held polymer on inert core have been all specified as liquid stationary states for the chromatography of the partition. The potential to perform chromatography utilizing two liquid phases without having a single liquid phase immobilized has been exploited in counter-current chromatography [9].

1.2.2 Adsorption separation mechanism .

Such type of the separation mechanism happens in the case when the molecules are exchanged between liquid mobile phase and the solid surface (stationary phase). Suggesting that the stationary phase is extremely polar in comparison to mobile phase, the polar molecules from mobile phase are adsorbed on the surface of the solid stationary phase, whereas molecules with less polarity are mainly maintained in mobile phase. Being in the equilibrium between liquid and solid, molecules with higher polarity elute from chromatographic column, yet later compared to the compounds of less polarity. Adsorption and partition are used majorly as models to describe the equilibrium’s type, yet as difference between the two processes has not been majorly indicated from thermo-dynamic viewpoint [6]. Furthermore, in a lot of cases, the separation might be specified as adsorption or partition; the differentiation is made just for estimating differently the separation parameters, whereas classification is not impacting the real process. Figure (1.1) explains the essential phase distribution mechanisms.

1.2.3- Ion exchange separation mechanism.

The equilibrium between the solution’s ions happens in many chemical reactions. With regard to implementations in the HPLC, one ionic specie should be immobilized, for instance, through connection to solid matrix via covalent bond. An instance for such ion type might be sulfonic group that is connected to polystyrene. The solution’s ions might be bound through ionic interaction types to immobilized counter ion or might stay in the solution. The equilibrium between mobile and solid phases, is based on the bond’s strength to stationary phase, might be providing the ways for separation [10].

1.2.4 -Size exclusion separation mechanism .

The size exclusion applies stationary phase which contains porous structure, where the small molecules might be penetrating and spending time to pass over long channels which are related to solid materials, whereas the larger molecules have no ability for penetrating the pore system regarding the stationary phase, also have not been maintained. Utilized in HPLC, the larger molecules are eluted earlier, whereas small molecules are maintained longer. The equilibrium might be envisioned between the molecules in mobile phase and the ones which are partly trapped in solid matrix [11].

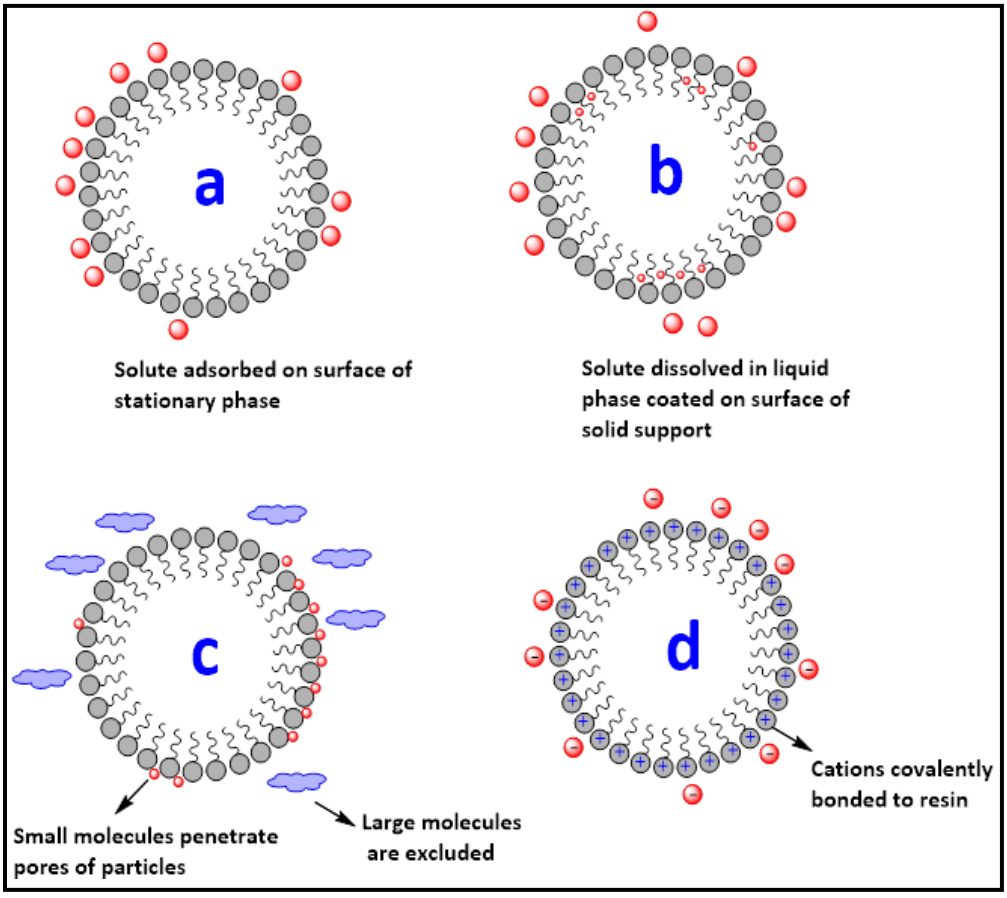


Figure 1.1: Separation mechanisms in chromatography. (a) adsorption (b) partition (c) size exclusion (d) ion exchange.

1.2.5 -Affinity separation mechanism .

Such interaction type is common for the protein binding, also leading to equilibrium which is allowing extremely specific separations. Furthermore, the examples regarding the interactions are avidin-biotin and protein-antibody. The affinity chromatography is majorly utilized at low pressure for the protein purification [12].

1.3 Hydrophilic Interaction Liquid Chromatography (HILIC)

Totally distinctive story in comparison to ZIC is the HILIC. Typically, HILIC and ZIC are different in 3 factors. The first one is that the inorganic ions can't be counted with regard to classical-HILIC analyte such as ZIC, yet of organic (uncharged or charged and small polar or polar) composites. The second one is the mobile phase which is utilized in HILIC that needs high concentration regarding the organic solvents, whereas reverse situation happens in the ZIC. The third factor is that the mechanism of separation utilized in HILIC is different from that of ZIC that is going to be examined in following paragraphs. The solutes' mechanism of separation in HPLC has been studied extensively. Basically, there are some likely models of separation principles which can be used with HPLC: partitioning [13, 14], adsorption [15, 16] as well as retention that consisting of two simultaneous impacts, for example, mixed adsorption as well as partitioning [17]. HILIC can be considered as substitute to the NP-LC.

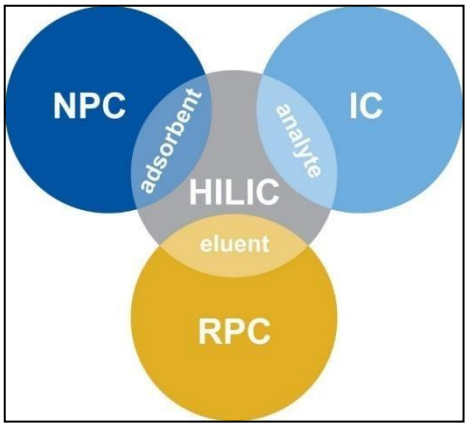


Figure 1.2: HILIC complements other chromatographic liquid areas [18].

Yet, the approaches utilized in HILIC have extra difficulty in comparison to that in NP-LC. With regard to mobile phase in the HILIC, solvents utilized represent typical solvents with regard to RP-LC [18]. Figure (1.2) shows the way that HILIC is located in triangle regarding RP-LC, NP-LC, and IC. In 1990, Alpert initially introduced the term HILIC [19]. The mechanism of separation in HILIC depends on analyte molecules' partitioning between water-enriched layer that is related to the semi-immobilized water on hydrophilic stationary phases as well as solvent-rich mobile phases, as can be seen in Figure (1.3). Separation depends on analyte molecule's hydrophilicity, with the increase in polarity regarding analytes the interactions will be strong with regard to water-rich layer on stationary phases [18].

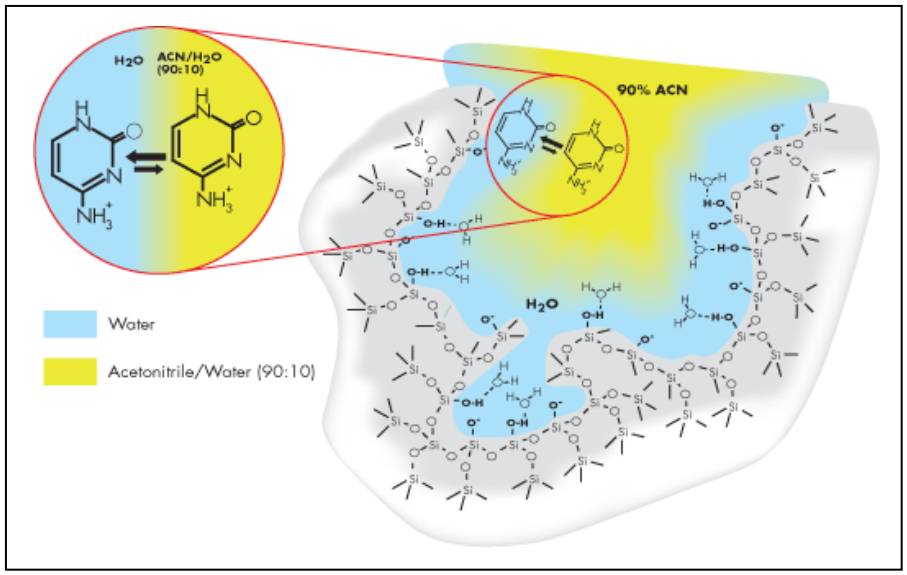


Figure 1.3: Scheme of the HILIC separation system [18].

1.4 ZIC-HILIC Stationary phases

1.4.1 The Zwitterionic stationary phases

Zwitterionic phases are known for distinctive properties as a result of their potential utilization for simultaneously separating cations and anions. Based on the classification that presented by Nesterenko [22], the classification of zwitterionic stationary phases is dependent on their structures and the distributions of the groups of opposite charges in the phase in the following manner: initially, the zwitterionic exchangers with functional groups that are negatively and positively charged, and distributed in a random manner in the entire polymer surface volume as illustrated in

Figure 1.4 (a), for example, a snake-cage resin has been synthesized by “Hatch et al.” [23], where the resin matrix of the ion-exchange comprises a polymer “cage”, which is co-polymerized by an organic counter ion for the constitution of the linear chains of the polymer of negatively and positively charged polymers “snakes”. The most significant benefits of the snake-cage resin are a smaller rate of the affinity concerning the non-electrolytes and, as a result, de-salting the non-ionic solution types [21,22].

Zwitterionic stationary phases can be defined as a new polar stationary phase category, which are utilized for the separations of the HILIC mode. The characteristic property of the zwitterionic phases is the existence of negatively as well as positively charged sites which are joined in one molecule that is attached to the surface of the polymer [20]. The ZIC is a new evolution trend in the stationary phases for a variety of liquid chromatography approaches. Stach has synthesized in 1951 as the first zwitterionic exchanger with the outer sulfonic acid groups and the inner quaternary amines [21]. The number of the publications about the ZIC has witnessed a substantial increase, as defined in outstanding evaluations that published by “Nesterenko et al.” [20, 22].

Secondly, stationary phase with the negative and positive charge functional groups distributed the charges in a separate manner on surface as depicted in Figure 1.4 (b), for example, the multi-functional zwitterionic stationary phase [24, 25]. The simplest of the manners for producing this type is the dynamic coated of the substrates of the reverse phase with a mix of the anionic as well as the cationic surfactants [22]. Macka and Borák, in 1993 were capable of developing a material where a mix of the anionic (octa-nesulfonate) and the cationic (tetra-butylammonium) are modified dynamically to the octa-decylsilica (ODS) [26] for separating anionic, cationic, and uncharged platinum (II) complexes. Third, stationary phases with the zwitterionic molecules exist in one molecule as shown in Figure 1.4 (c). Two ways exist for preparing this type, and those are, the dynamic modification and the covalently attached with the surfactants of the zwitterion.

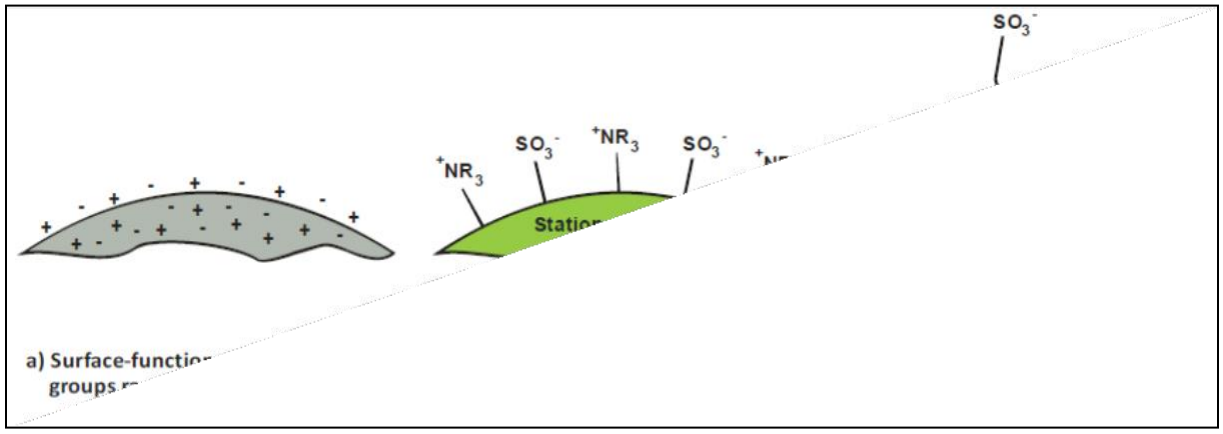


Figure 1.4: The diagram illustrates the many types of ZIC columns used in liquid chromatography [22].

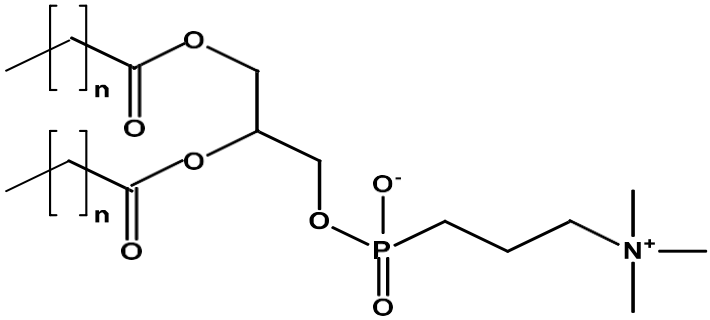
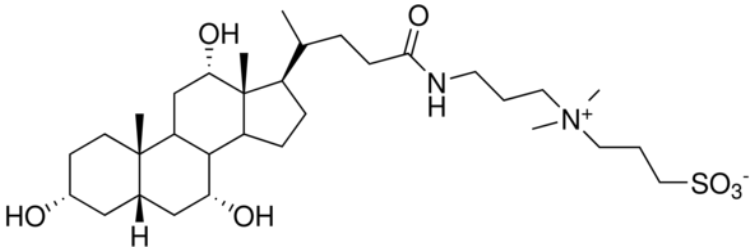
1.4.1.1 Stationary phases which are dynamically coated with the zwitterion molecules

To obtaining the zwitterionic stationary phase, dynamic modification can be considered as one of the very easy way for the creation of a surface of polymer with the zwitterionic molecules. In actual fact, covalent zwitterionic group binding for supporting the materials can be considered as a highly complicated issue [20]. Which is why, primary researches have been carried out through coating RP materials by the zwitterionic surfactants. Thereby, several researches which deal with the stationary phases that are modified dynamically with the zwitterionic surfactants, like the carboxy-betaine-types [27, 28], phospho-choline-types [29, 30] and sulfo-betaine-types [31-35].

Table 1.1 lists the variety of the zwitterionic surfactant structures that are utilized to prepare the dynamically coated zwitterionic stationary phases. Numerous mechanism models of separating the zwitterionic stationary phases were suggested. For the ZIC-mode "Okada et al." [36, 37] have researched approaches which based on the theory of Poisson–Boltzmann, "Hu et al." [38, 39] have presented a approaches of retention based on a model of the binary-EDL, "Cook et al." [40, 41] have presented a mechanism of the Donnan membrane which based upon each of the impact of the chaotropic interaction and the ion exclusion. For the mode of the ZIC-HILIC, analyte is distributed between the stationary layer which is water-enriched and the low water content mobile phase [19] and is impacted as well with the electro-static interaction [42].

The basic disadvantage of the dynamic coated columns is a considerable stability deficiency. Lucy and Glenn [43] researched the conditions of the coating like the acetonitrile content, concentricity of the surfactant, temperature, in addition to the ionic strength. They have discovered the need to add surfactant to the eluent. Based on that, the time of the retention its effectiveness retained their stability for ≥ 3,000 volumes of column. Therefore, it is only reasonable finding potential resolutions to this issue, which include the concept of the attachment of the zwitterionic molecules in a covalent manner to support materials.

Table 1.1: A description of several zwitterionic surfactants used in dynamic coating [20, 22].

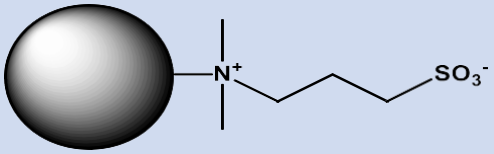
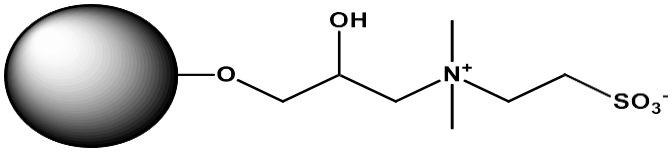
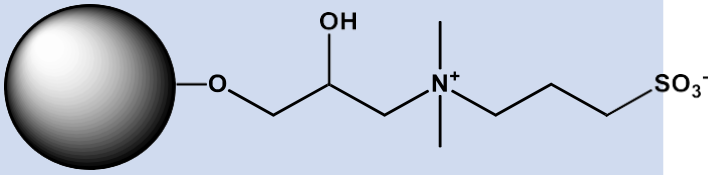
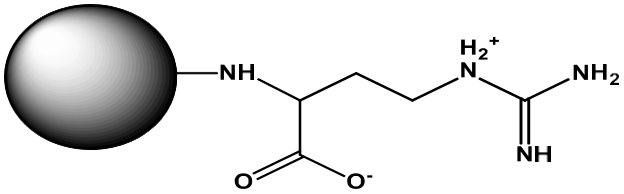
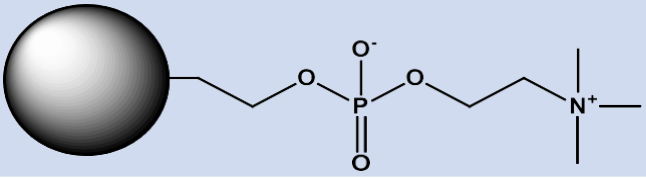
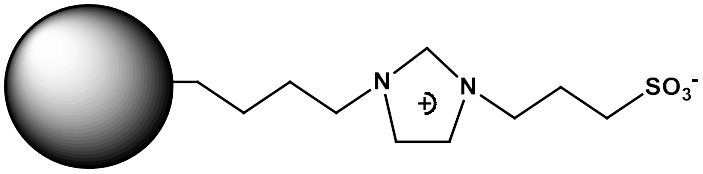
Surfactants zwitterionic	Zwitterionic surfactants: structure
Zwittergent 3-14	
Dimyristoyl-phosphadylcholine	
N-(Dodecyl-N,N dimethylammonio) butyrate	
CHAPS	

1.4.1.2 Stationary phases which are attached covalently to the zwitterion molecules

Firstly, the direct functionalizing [44] according to the activated 2hydroxyethyl methacrylate-ethylene di-methacrylate co-polymer (HEMA-EDMA) with the epichlorohydrin, after that, treating by 2-(di-methylamino) ethane-sulfonic acid (DMAES), which produce the stationary phase of zwitterion with the outer sulfonic acid functional group and the inner quaternary amine (Table 1.3) for simultaneously separating the inorganic anions and cations with the use of the perchlorate and the water as the eluents. two distinct stationary phases of zwitterion based as well on the activated group route of the hydroxyl with the use of the 3-[N,N-di-methyl-N-(metha-cryloyloxyethyl) ammonium] propane-sulfonate and 3-dimethylaminopropanesulfonate as the reagent of the reagent 2nd step, which produces zwitterion stationary phases respectively S-300-ECH-DMA-PS and S-300-TC-DMA-PS (Table 1.3) [45]. Secondly, the graft polymerization [46] functionalizing the particles of the silica is carried out with the graft polymerization of the free radical with 3-[N,N-di-methyl-N-(metha-cryloyloxyethyl) ammonium] propane-sulfonate for producing KS-TC-TBHP-SPE stationary phase of the zwitterion, which is capable of separating the basic and the acidic proteins. One more way for obtaining the stationary phases of the zwitterion is attaching amino acids through the covalent bonding to the polymer surface like the glutamic acid-silica [47], monoliths of the lysine-silica [48], glutamine-HEMA, arginine-HEMA, and histidine-HEMA [49]. This stationary phase type includes some advantages, in regards to the potential of the selective manipulations which result from the different negative and positive site capacities according to the pH of the eluent [20]. Besides the above-mentioned phases, two new zwitterion materials for separating the non-polar and the polar composites presented by Sielc (“Prospect Heights, IL, US”), with trading names Obelisc R and Obelisc N. the former has a character of the reversed-phase (with the exchange group of the inner anion and the exchange group of the outer cation), Obelisc R has shown to be more suitable for RP application separation as a result of ionic sites and hydrophobic chain. Obelisc N is of normal-phase character (with the exchange group of the inner cation and the exchange group of the outer anion), Obelisc N exhibits is more suitable for the applications of separating NP as a result of the ionic groups and the hydrophilic chain. This material has been offered for the applications of the HILIC [50]. Sonnenschein and Seubert [51] have performed the synthesis of 5 new molecules of the sulfo-betaine stationary phases according to the PS/DVB with the covalent bonding with the use of a

grafting reaction that results in the zwitterionic exchangers which differ in the length of chain between charged functional groups; amino acids [52] and inorganic anions [51] were utilized as the analytes. Some widely spread zwitterionic exchangers are listed in Table1.2. It should be noted that limited amounts of covalent bonded stationary phases of zwitterion were researched earlier. Which is why, systematic research on the homologous zwitterionic exchanger set might play a significant role in a better understanding about the ZIC and ZIC-HILIC mode zwitterionic chromatography.

Table 1.2: A survey of zwitterionic exchangers that are covalently linked [20].

Molecules with a column zwitterionic structure	**The stationary phase's structure**
ZIC-pHILIC **ZIC-HILIC**	
HEMA–DMAES	
HEMA-DMAPS	
Arg-HEMA	
ZIC-cHILIC	
SiImPS	

1.5 Preparation of zwitterionic stationary phases

In accordance with the procedure described in the references, the two sulfobetain monomers were synthesized as styrene derivatives with internal quaternary amines and external sulfonic acids [53-55]. The pharmaceutical separation stationary phases (ZIC-HILIC-A and ZIC-HILIC-B) were produced via grafted sulfobetain monomers [74, 75, 56] were produced via grafted sulfobetain monomers. The column ZIC-HILIC-A (4-vinylbenzyl-dimethylammonio methane sulfonate-PS/DVB) and the column ZIC-HILIC-B (4-Vinylbenzyl Dimethyl Ammonia Pentane sulfonate-PS/DVB) onto PEEK columns are mounted on the PS/DVB (100 mm x 4 mm I.D.) as shown in Figures 1.5 and 1.6. Raskop et al. have described the detailed process of the grafting reaction [57].

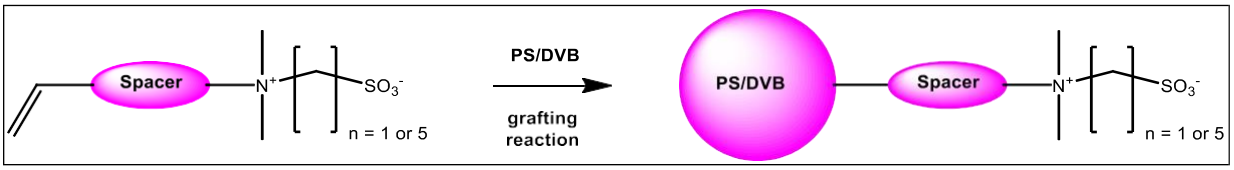


Figure 1.5: Sketch of the method to produce ZIC-HILIC-A and ZIC-HILIC-B stationary phases bonded to PS/ DVB.

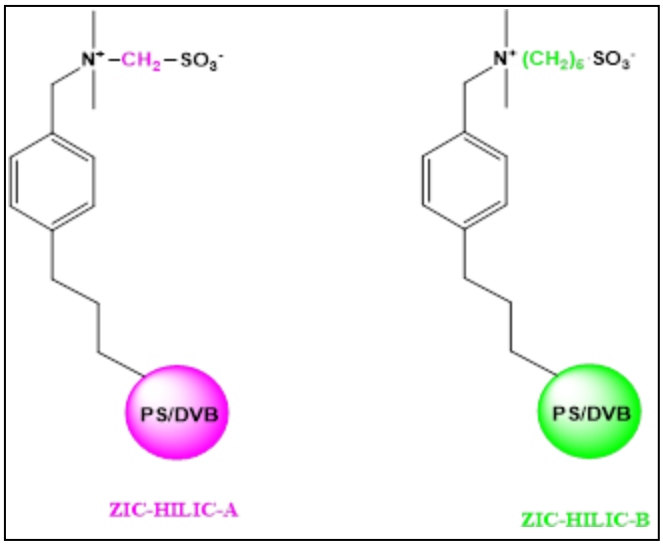


Figure 1.6: Structure of ZIC-HILIC-A and ZIC-HILIC-B stationary phases bonded to PS/DVB.

1.6 Applications of hydrophilic interaction chromatography

The HILIC mode is extensively used to separate some biomolecules, as well as organic and some inorganic compounds, based on their polarity differences [20,58,59].

There are numerous applications of HILIC (Table 1.3) analysis of small polar molecules, such as biomarkers, nucleosides, nucleotides/oligonucleotides, amino acids, peptides, and proteins, saccharides, glycosides, oligosaccharides, hydrophilic drugs, alkaloids, and carbohydrates, as well as other small polar or ionize able compounds that contribute to the fields of pharmaceutical chemistry.

Table 1.3: Applications of HILIC systems.

Columns	Mobile phase	Detection	Application	Ref.
ZIC-HILIC	ACN: acetate buffer	UV	Genistein in herbs and propolis	[60]
HILIC columns	Formic acid or two buffers, 10 mM ammonium formate, pH 3, and 10 mM ammonium acetate, pH 6.	MS	Peptides in bovine-serum-albumin	[61]
X-Bridge BEH Amide	From 80:20 to 15:85 acetonitrile:water (ACN:H_2O)	MS	Amino sugar stereoisomer and muramic acid biomarkers	[62]
Zwitterionic, crosslinked diol and triazole stationary phases	10 mM acetate buffer solution was adjusted to pH 7.0 in one solvent reservoir separately, then it was mixed with acetonitrile	photodiode-array (PDA)	Hydrophilic neutral and ionic analytes antagonists	[63]
ACQUITY UPLC BEH Amide	(0.2% formic acid and 5 mmol/L ammonium acetate in H2O/acetonitrile (95:5, v/v)	MS	40 endogenous amino acids and derivatives in cell	[64]
HILIC column	Water/ methanol/ formic acid (95:5:0.6, v/v/v)	MS/MS	Eremomycin and vancomycin in pre-clinical study	[65]
Waters Acquity UPLC BEH Glycan	500 mL water+75 µL formic acid+300 µL ammonium hydroxide	MS/MS	Paralytic Shellfish Toxins and Tetrodotoxin in Mussels	[66]
zwitterionic stationary phase	Ammonium acetate and acetonitrile	MS/MS	Oligonucleotide	[67]

Shodex HILICpak VN-50	H_2O/acetonitrile/ammonium acetate	MS/MS	Oligonucleotide	[68]

Continued (Table 1.3)

Columns	Mobile phase	Detection	Application	Ref.
ZORBAX NH_2	ammonium acetate, pH adjusted to 4.0 with glacial acetic acid	UV	Amlodipine besylate	[69]
Obelisc N	Ammonium acetate and acetonitrile	MS/MS	Polar pesticides in food matrices	[70]
ZIC_5-HILIC	ACN: Sodium acetate (NaOAc) buffer	UV	Amino Acids	[71]
ZIC_5-HILIC	ACN: NaOAc buffer	ICP-HPLC	Ce (IV)–DFOM complex	[72]
ZIC_{1-4}-HILIC	ACN: NaOAc buffer	UV	Carboxylic Acids	[73]
ZIC_{1-4}-HILIC	ACN: NaOAc buffer	UV	Inorganic ions	[74]
ZIC_5-HILIC	ACN: NaOAc buffer	ICP-HPLC	metal–TFPH complexes	[75]
ZIC_1-HILIC ZIC_5-HILIC	ACN: NaOAc buffer	UV	Deoxyguanosine in Serum	[76]
ZIC_1-HILIC ZIC_5-HILIC	ACN: NaOAc buffer	UV	Deoxyuridine in Human Serum	[77]
ZIC_5-HILIC	ACN: NaOAc buffer	ICP-HPLC	Epinephrine in pharmaceutical dosage	[78]
$ZIC_{1,5}$-HILIC	ACN: NaOAc buffer	UV	Famotidine in pharmaceutical dosage	[79, 80]
$ZIC_{1,5}$-HILIC	ACN: NaOAc buffer	UV	Ranitidine in pharmaceutical dosage	[81]
ZIC_1-HILIC ZIC_5-HILIC	ACN: NaOAc buffer	UV	2-deoxycytidine in serum	[82]
ZIC_1-HILIC ZIC_5-HILIC	ACN: NaOAc buffer	UV	Salbutamol Sulfate in pharmaceutical preparations	[83]
Halo	ACN: NaOAc buffer	UV	Cefixime in pharmaceutical preparations	[84]
ZIC®-HILIC	ACN: acetic buffer	UV	Rutin in teaes	[85]
Halo	ACN: acetate buffer	UV	Ceftriaxone in pharmaceutical preparations	[86]

Halo	ACN: NaOAc buffer	UV	Cefixime in pharmaceutical preparations	[84]
ZIC®-HILIC	ACN: acetic buffer	UV	Rutin in teas	[85]
Halo	ACN: acetate buffer	UV	Ceftriaxone in pharmaceutical preparations	[86]

Continued (Table 1.3)

Columns	Mobile phase	Detection	Application	Ref.
ZIC_1-HILIC ZIC_5-HILIC	ACN: Sodium acetate (NaOAc) buffer	UV	Deoxyadenosine in human serum	[87]
ZIC_2-HILIC ZIC_3-HILIC	ACN: NaOAc buffer	UV	Theophylline in pharmaceutical dosage forms	[88]
ZIC_2-HILIC ZIC_4-HILIC	ACN: NaOAc buffer	UV	Esculin in ointments	[89]
ZIC_1-HILIC ZIC_5-HILIC	ACN: NaOAc buffer	UV	Chlorpheniramine maleate in pharmaceutical preparations	[90]
ZIC_1-HILIC ZIC_5-HILIC	ACN: NaOAc buffer	UV	prednisolone acetate in pharmaceutical dosage forms	[91]

1.7 Column packing and determination of capacities

In PEEK columns (100 mm x 4 mm I.D) the stationary phases were packed using a technique for slurry filling [74]. In acetate buffer (150 mM, pH 4.75), the active content was suspended in slurry water. A constant packing force of 500 bar was applied to dense packaging. The capabilities of the two exchangers were determined by elementary sulfur and nitrogen combustion analysis and sulfur x-ray fluorescence [53-55]. The average capacities of four investigated ZIC-HILIC columns are shown in Table 1.4. The letter A indicates the one methylene group between the charged groups in exchanger, while the letter B indicates the five methylene groups between the charged groups in exchanger as shown in Figure 1.6.

Table 1.4: Capacities of the two ZIC-HILIC columns [92, 93].

	ZIC-HILIC-A	ZIC-HILIC-B
Capacity (μeq g^{-1})	432	488

1.8 Statin drugs

Statins are among the most regularly given medications worldwide. Statins are HMG-CoA reductase inhibitors that work by blocking the enzyme HMG-CoA reductase, which is required for the liver to produce cholesterol (Figure 1.7). HMG-CoA reductase (HMGCR) catalyzes the rate-limiting step in cholesterol production, the conversion of HMG-CoA to mevalonate [94]. Reduced intracellular cholesterol levels enhance LDL receptor expression on the hepatocyte cell surface, resulting in increased LDL-C extraction from the blood and decreased circulating LDL-C concentrations [95]. Statins also have beneficial effects on other lipid indicators, such as a rise in high-density lipoprotein cholesterol (HDL-C) and a decrease in triglyceride levels [96]. Cardiovascular disease has been linked to elevated cholesterol levels, and statins have been demonstrated to protect those at high risk [97]. Statins have also been shown to have positive cardiovascular benefits in the absence of their lipid-modifying capabilities [98]. These pleiotropic effects are due to the fact that mevalonic acid inhibits the formation of nonsteroidal isoprenoid molecules [99, 100].

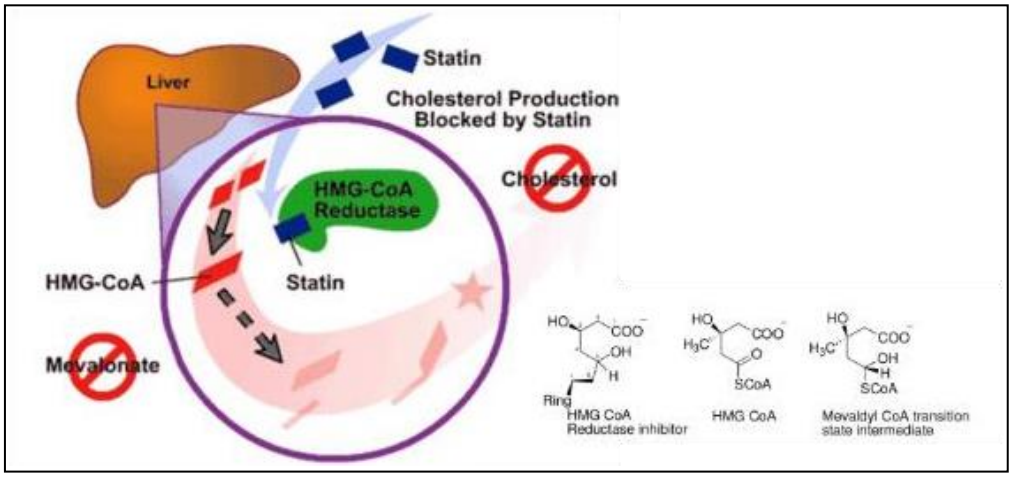


Figure 1.7: Mechanism of action of statins drugs [101].

For type 1 statins such as lovastatin, simvastatin, and pravastatin, the statin pharmacophore is a modified 3,5 dihydroxy glutaric acid (DHGA) moiety, whereas for type 2 statins such as rosuvastatin, Fluvastatin, and atorvastatin, the statin pharmacophore is a 3,5 dihydroxyhept-6-enoic acid derivative (Figure 1.8). The statin pharmacophore (enzyme inhibitor) binds to the same active site as the substrate HMG-CoA, which inhibits the HMG-CoA reductase enzyme. All statins must have the crucial 3R,5R stereochemistry because they are all stereo selective. The type 2 statins have an E-conformation for the 3,5-dihydroxyhept-6-enoic acid side chain around the C6-C7 double bond. All statins have the same pharmacophore, and the clinical and molecular variations result from the connected ring, which can be partially decreased (pitavastatin). Some pharmacological effects of the statins are determined by the substituents on the rings. Statins of type 2, often known as low-density lipoprotein (LDL) receptors, have a common 4-fluorophenyl substituent, although some (such as rosuvastatin) have additional polar substituents (such as the methane sulphonamide group) to improve their ability to bind HMGCR. The hexahydro-naphthalene structure of type 1 statins is shared by these two medications. [102].

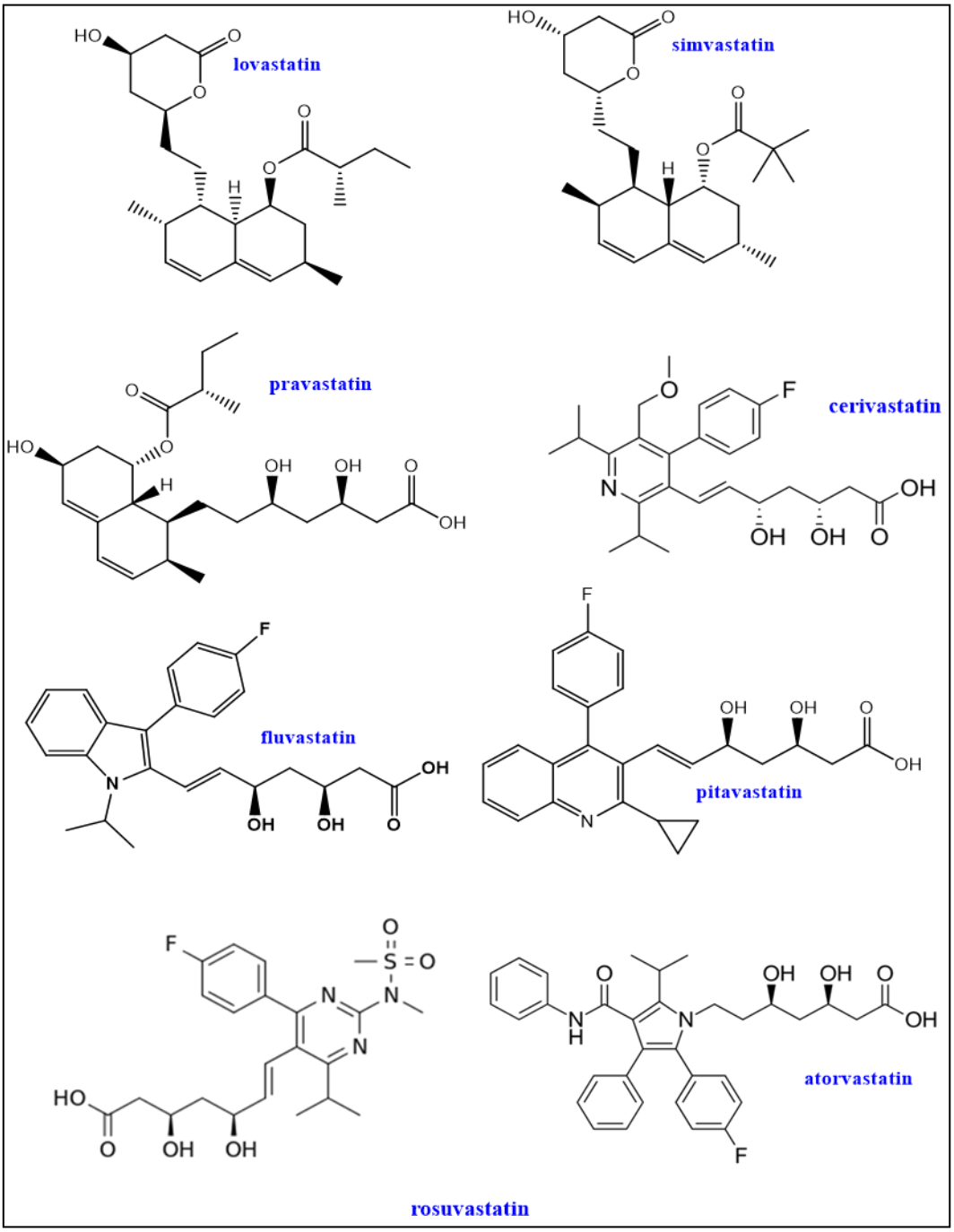


Figure 1.8: Chemical structures of statin drugs [103].

Three are three statin drugs Rosuvastatin, Atorvastatin-3rd generation and Simvastatin-1st generation were used in our study which will be explained as below:

1.8.1 Rosuvastatin

(ROSU) is a statin drug in the United States that Astra-Zeneca first synthesized and received approval in 2003 [104]. Mevalonique coenzyme A also targets the following enzyme: 3-hydroxy-3-methylglutaryl-coenzyme (HMG-CoA) reductase, which catalyzes the breakdown of HMG-CoA to mevalonate. The hydroxymethyl-glyceramino-glutylation of cholesterol is a rate-limiting step in cholesterol biosynthesis [105]. At nearly every single stage of cholesterol biosynthesis, atorvastatin is a selective, competitive inhibitor in the three-carbon (mevalonate) coenzyme (HMG-CoA) in cholesterol synthesis. That is involved in the total synthesis of HMG-CoA (a precursor of sterols, including cholesterol). Cholesterol decreased in the cell membranes causes an increase in the LDL receptor's density on the liver's surface, which causes an increase in the removal of LDL (low-density lipoprotein) from the blood [106]. Table (1.5) shows the abstract of literature survey utilized for estimating ROSU with HPLC.

Table 1.5: Literature survey of ROSU determination using HPLC methods.

Columns	Mobile phase	Detection	Statistical analysis	Application	Ref
C18	A= Water B= acetonitrile 30:70 v/v	UV at 248 nm	R2 = 0.999 Rec% = 98	Drugs	[107]
C18	A= Water (pH 3, adjusted with acetic acid). B= acetonitrile 55:45 v/v	UV at 254 nm	R2 = 0.999 Rec% = 102.89	Plasma	[108]
C18	A= acetonitrile B= MeOH, (pH, adjusted with 0.1M formic acid) 65:35 v/v	UV at 242 nm	Linear range 0.5 - 10 µg/mL	Serum	[109]
C18	A= MeOH. B= Water (pH 3, adjusted with trifluoroacetic acid) (68:32 v/v)	UV at 242 nm	Linear range 2 - 256 ng/mL	Plasma	[110]
C18	A= acetonitrile B= Water (pH 8, adjusted with	UV at 230 nm	Linear range 30 - 90 µg/mL	Drugs	[111]

	0.02M phosphate buffer) (40:10:50 v/v/v)				

Table 1.5 (Continued)

Columns	Mobile phase	Detection	Statistical analysis	Application	Ref
C18	A= acetonitrile B= Water Gradient elution	Fluorescence at 366, 410 nm	Linear range 0.01 - 20 ng/mL	Serum	[112]
C18	A= acetonitrile B= Water (82:18 v/v)	UV at 254 nm	Linear range 5 - 50 µg/mL R2 = 0.9986 Rec% = 99.97	Plasma	[113]
C18	A= Water and 0.1% orthophosphoric acid B= acetonitrile	UV at 240 nm	Linear range 0.5 -200 µg/mL Rec% = 95.9	Drugs	[114]
C18	A= 0.2% acetic acid B= acetonitrile (50:50 v/v)	UV at 248 nm	Linear range 0.01 -1 µg/mL R2 = 0.99997 Rec% > 90	Drugs	[115]
C18	A= 0.1% formic acid in acetonitrile B= 0.1 % formic acid in water (70:30 v/v)	LC–MS/MS	Linear range 0.2 -20 ng/mL R2 = 0.999 Rec% > 95	Plasma	[116]
C18	A= 0.05M formic B= acetonitrile (70:30 v/v)	UV at 240 nm	Linear range 0.02 -10 µg/mL R2 = 0.999 Rec% = 85-110	Plasma	[117]
C18	A= MeOH. B= Water (pH 3, adjusted with orthophosphoric acid) (60:40 v/v)	UV at 238 nm	Linear range 2 - 256 ng/mL R2 = 0.999 Rec% = 98.11-102.75	Plasma	[118]
C18	A= MeOH. B= Acetonitrile C= phosphate buffer (pH 3.5) (60:20:20 v/v/v)	Diode array at 237 nm	Linear range 5 - 22 µg/mL R2 = 0.999 Rec% = 96.97	Rabbit-Plasma	[119]

C18	A= Acetonitrile B=0.05 M phosphate buffer (PH=3) (60:20 v/v)	UV at 230 nm	Linear range 1 - 1500 ng/mL R2 = 0.995 Rec% = 96.97	Plasma, Urine, Wastewater	[120]

1.8.2 Atorvastatin

(ATOR) has an anti-inflammatory effect and helps preventing the development of atherosclerotic plaque accumulation [121]. The medication inhibits vascular smooth muscle cell proliferation, which is a significant pathologic factor in the development of atherosclerosis Atorvastatin has the effect of restricting both further development of atherosclerosis and vascular stenosis [122]. ATOR is used as a drug to lower total cholesterol, LDL, and apolipoprotein B levels in primary hypercholesterolemia. In addition to that, as a booster to improve HDL levels in patients with mixed dyslipidemia patients with elevated serum T.G. levels and those who fail to respond to diet treatment can also benefit from drug treatment. Since it is used in those with homozygous familial hypercholesterolemia, a disease in which both the father and the mother have the same abnormal, elevated levels of LDL-cholesterol and total cholesterol (e.g., LDL apheresis) [123]. Table (1.6) shows the abstract of literature survey utilized for estimating ATOR with HPLC.

Table 1.6: Literature survey of ATOR determination using HPLC methods.

Columns	Mobile phase	Detection	Statistical analysis	Application	Ref
C18	A=ammonium acetate buffer (pH= 4). B= acetonitrile (40:60) (v/v)	UV at 220 nm	R2 = 0.999 µg/mL Rec% = 100 ± 2%.	Tablet	[124]
C8	A=sodium dihydrogen phosphate buffer (pH= 5.5) B= acetonitrile (60:40) (v/v)	UV at 245 nm	R2 = 0.999 Rec% = 100.2 ± 7%.	Plasma	[125]
C18	A= MeCN,B= MeOH, C= 20 mM K2HPO4 (pH3.0) solution (34.27:20:45.73) (v/v/v)	UV at 239 nm	R2 = 0.998 Rec% = 100 ± 2%.	Tablet	[126]

C18	A= sodium phosphate buffer (0.05 M, pH 4.0). B= methanol (33:67) (v/v)	UV at 247 nm	R2 = 0.9965. Rec% = 95%.	Serum	[127]
C18	Methanol in 0.05 M sodium phosphate buffer at pH 3.5	UV at 247 nm	R2 = 0.999.	Myalgia syndrome	[128]

Table 1.6 (Continued)

Columns	Mobile phase	Detection	Statistical analysis	Application	Ref
C18	A=acetonitrile B=sodium dihydrogen phosphate buffer (pH= 5) (45:55)(v/v)	PDA at 246nm	R2 = 0.9995 Rec% = 99.37	Nano crystals	[129]
C18	A= Methanol. B= Water (containing 0.05 % glacial acetic acid) (pH 3.0) (70:30) (v/v)	UV at 248 nm	R2 = 0.9999 Rec%= 94.76 ± 0.56	Plasma	[130]
C18	A= mixture THF in ACN 10% (v/v), B= 0.025M phosphate buffer solution (pH = 3.5)	diode array detector at 248 nm	R2 = 0.9997 Rec%=99.9-101.4	Tablet	[131]
C18	A= MeOH., B =phosphate buffer with a (pH of 4.5) (30/70) (v/v)	diode array detector at 248 nm	R2 = 0.998 Rec% > 61.2	Biological fluids	[132]
C8 and DSC-18	A= ACN, B= water, C= formic acid (50-30-20) (v/v/v)	LC–MS/MS	R2 = 0.99 Rec% = 50 and 68	Plasma	[133]
C18	A= water, B= methanol C= acetonitrile buffered with triethylamine at pH8 (1.58:1:1) (v/v/v)	UV at 237 nm	R2 = 0.99 Rec% = 98- 102	Plasma	[134]
C18	A= ACN, B= Water (pH 2.8) (75:25) (v/v)	UV at 235 nm	R2 = 0.999 Rec% = 96- 108	Serum	[135]
C18	A= Methanol, B= Water	UV at 250 nm	R2 = 0.9944 Linear range	Tablet	[136]

	(95:5) (v/v)		0.39 - 50 µg/mL Rec% = 95 – 102		
C18	A= pH 4.7 ammonium acetate , B= acetonitrile	UV at 240 nm	R2 = 0.9999 Rec% = 100 – 101	Tablet	[137]
C18	A= ACN: B= 0.1 M sodium perchlorate (pH 2.5) (70:30) (v/v)	UV at 210 nm	R2 = 0.999 Rec% = 98.89–103.5	Tablet	[138]
C18	A= ACN, B= phosphate buffer (0.01 M, pH 2.0)	PDA detector at 247 nm	R2 = 0.9996 Rec% = 103.7	Tablet	[139]

1.8.3 Simvastatin

(SIMV), a methylated derivative of lovastatin, has become the most commonly prescribed medication worldwide for the treatment of hypercholesterolemia, a significant risk factor for the development of atherosclerosis [140]. SIMV is used in conjunction with a healthy diet, weight loss, and exercise to help minimize the risk of heart attack and stroke and the likelihood of needing heart surgery in patients who already have or are at risk of developing heart disease [140]. SIMV is also used to lower the levels of fatty substances in the blood, such as low-density lipoprotein (LDL) cholesterol ("bad cholesterol") and triglycerides, and to raise the levels of high-density lipoprotein (HDL) cholesterol ("good cholesterol") [141]. SIMV may also be used to lower cholesterol and other fatty substances in the blood of children and adolescents aged 10 to 17 years who have familial heterozygous hypercholesterolemia (an inherited condition in which cholesterol cannot be removed from the body normally). SIMV belongs to a class of drugs known as HMG-CoA reductase inhibitors (statins) [141]. It works by reducing the amount of cholesterol produced in the body, hence reducing the amount of cholesterol that can build up on the walls of arteries, obstructing blood flow to the heart, brain, and other vital organs [142, 143]. Table (1.7) shows the abstract of literature survey utilized for estimating SIMV with HPLC.

Table 1.7: Literature survey of SIMV determination using HPLC methods.

Columns	Mobile phase	Detection	Statistical analysis	Application	Ref
C18	A= ammonium acetate buffer, (PH= 4). B= acetonitrile (40:60) (v/v)	UV at 220 nm	R2 = 0.999 Rec% = 100 ± 2%.	Tablet	[124]
C18	A= Water and 0.1 % Orthphosphoric acid. B= acetonitrile (55:45)(v/v)	UV at 240 nm	R2 = 0.9998 Rec% = 100.6	skin penetration (in vitro)	[114]
C18	A= acetonitrile B= Water and 0.1 % Orthphosphoric acid (PH= 2.8) (65:35) (v/v)	UV at 235 nm	R2 = 0.995 Conc. Range = 0.1 to 50 µg/mL Rec% = 97 to 98.86	Human Plasma	[144]
C8	A= Methanol B= 0.1 % phosphoric acid (PH= 3.25) (80:20) (v/v)	UV-DAD at 238 nm	R2 > 0.98 Rec% = 95 to 105.0	Human Plasma	[145]

Table 1.7 (Continued)

Columns	Mobile phase	Detection	Statistical analysis	Application	Ref
C8	A= Methanol B= 0.1 % phosphoric acid (PH= 3.25) (80:20) (v/v)	UV at 238 nm	R2 = 0.999 Rec% = 100 ± 5	lecithin/chitosan nanoparticles	[146]
C8	A= methanol B= acetonitrile C= tetrahydrofuran D= phosphate buffer pH 5.5 (35:35:9:21) (v/v/v/v)	UV at 240 nm	R2 = 0.999 Rec% = 97.42±0.54 to 99.67±0.21	Tablet human urine	[147]
C18	A= acetonitrile B= 0.1 % glacial acetic acid (80:20) (v/v)	UV at 240 nm	R2 = 0.999 Rec% = 92.95 to 97.77	Tablet	[148]
C18	A= acetonitrile B= 0.1M ammonium acetate buffer PH (5.0) (70:30) (v/v)	UV at 240 nm	R2 = 0.9992 Rec% = 99.12 to 101.5	Tablet	[149]
C18	A= Methanol	UV at 243 nm	R2 = 0.999 Rec% = 100.12	Tablet	[150]

	B= acetonitrile C= 0.1% Orthophosphoric acid PH (5.6) (75:20:05) (v/v/v)				
C18	A= 10 mM phosphate buffer, B= Methanol C= acetonitrile (45:35:20) (v/v/v)	UV at 255 nm	R2 = 0.9999 Rec% = 99.55±0.32	Tablet	[151]
C18	A= acetonitrile B= ammonium acetate PH (5.0) (65:35) (v/v)	UV at 240 nm	R2 = 0.999 Rec%=98.82 to 101.6	Tablet	[152]
C18	A= acetonitrile B= Methanol C= buffer (trimethylamine) (pH-3) (15:45:40) (v/v/v)	UV at 240 nm	R2 = 0.9999 Linearity= 0.5 to 20 µg/mL Rec% =99.41 to 101.34	Tablet	[153]
C8	A= Acetonitrile B= 0.05 M Phosphate buffer (pH-2.5) (70:30) (v/v)	UV at 232 nm	R2= 0.999 Rec% =99.61	Tablet	[154]
C8	A= acetonitrile B= Methanol C= buffer (0.1 M ammonium acetate) (pH-3)(62:10:28)(v/v/v)	UV at 250 nm	R2 = 0.999 Linearity= 0.1 to 16 µg/mL Rec% =88.8	Human Plasma	[155]
C18	A= acetonitrile B= Water (51:49) (v/v)	UV at 238 nm	R2 = 0.9996 Rec% = 97.52 to 104.80	Human Plasma	[156]

Table 1.8: Physical and chemical characteristics of the three statin compounds studied [157].

Pharmaceuticals	**pI***	**pK_a**	**logP_{ow}****
Atorvastatin	-	4.31	4.92
Rosuvastatin	1.12	4.00	1.23
Simvastatin	-	12.76	4.46

***pI:** Isoelectric point**.**

**** logPo/w:** The partition coefficient is a ratio of concentrations of an un-ionized compound in the two phases of immiscible solvents (water and n-octanol) at equilibrium. logPo/w is the 10-base logarithmic measure of the coefficient.

$$\log P_{oct/wat} = log\ (\frac{solute_{octanol}}{solute_{un-ionized\ \ water}}) \text{ ..... (1)}$$

1.9 The influence of ACN fraction on behavior of pharmaceuticals

The mobile phase in HILIC is similar to those in the RP-LC, but requires significantly higher organic content in order to provide effective hydrophilic activity [18]. HILIC separation mechanism is built upon the distribution of the polar analyte from the water-enhancing surface over to the HILIC column to the mobile phase ACN-rich, the organic solvent fraction has the greatest effect on polar and non-polar compounds retention in the mobile phase [19].

1.10 The influence of buffer concentration on behavior of pharmaceuticals

For ZIC-HILIC separations of test compounds with homologous sequence of prepared exchanger, the mixed mode separation mechanisms have been shown in most columns, a change in buffer intensity is the next factor in the eluent composition [74]. The retention factor improved with a constant ACN fraction (80%) when sodium acetate buffer strength has been increased from 10 to 80 mM (pH 4.75). In all HILIC stationary phases this effect was observed [56].

1.11 The influence of pH on behavior of pharmaceuticals

The eluent pH must be varied (3-5.5) in order for us to get a full picture of the pharmaceutical separation under HILIC state. The pH effect was evaluated at constant ACN Fraction (80%) with a constant buffer capacity of 40 mM [54].

1.12 Hyperlipidemias

Coronary heart disease (CHD) is the leading cause of death in the United States, accounting for approximately half of all deaths. The incidence of coronary heart disease (CHD) is associated with elevated levels of low-density lipoprotein (LDL) cholesterol and triacylglycerol and a deficiency of high-density lipoprotein (HDL) cholesterol. Cigarette smoking, hypertension, obesity, and diabetes are additional risk factors for CHD. A person's cholesterol levels may be increased as a result of his or her lifestyle (for example, by lack of exercise and consumption of a diet containing excess saturated fatty acids). Hyperlipidemias can also be caused by a single inherited lipoprotein metabolism gene deficiency or, more typically, by a mix of genetic and lifestyle variables. Appropriate lifestyle adjustments combined with pharmacological therapy can

result in a slowing of the progression of coronary plaque, regression of preexisting lesions, and a 30% to 40% reduction in coronary heart disease mortality. Antihyperlipidemic medications must be continued indefinitely, as once therapy is discontinued, plasma lipid levels revert to their pretreatment levels. The normal metabolism of serum lipoproteins is depicted in Figure 1.9, as are the characteristics of the major hereditary hyperlipidemias [157].

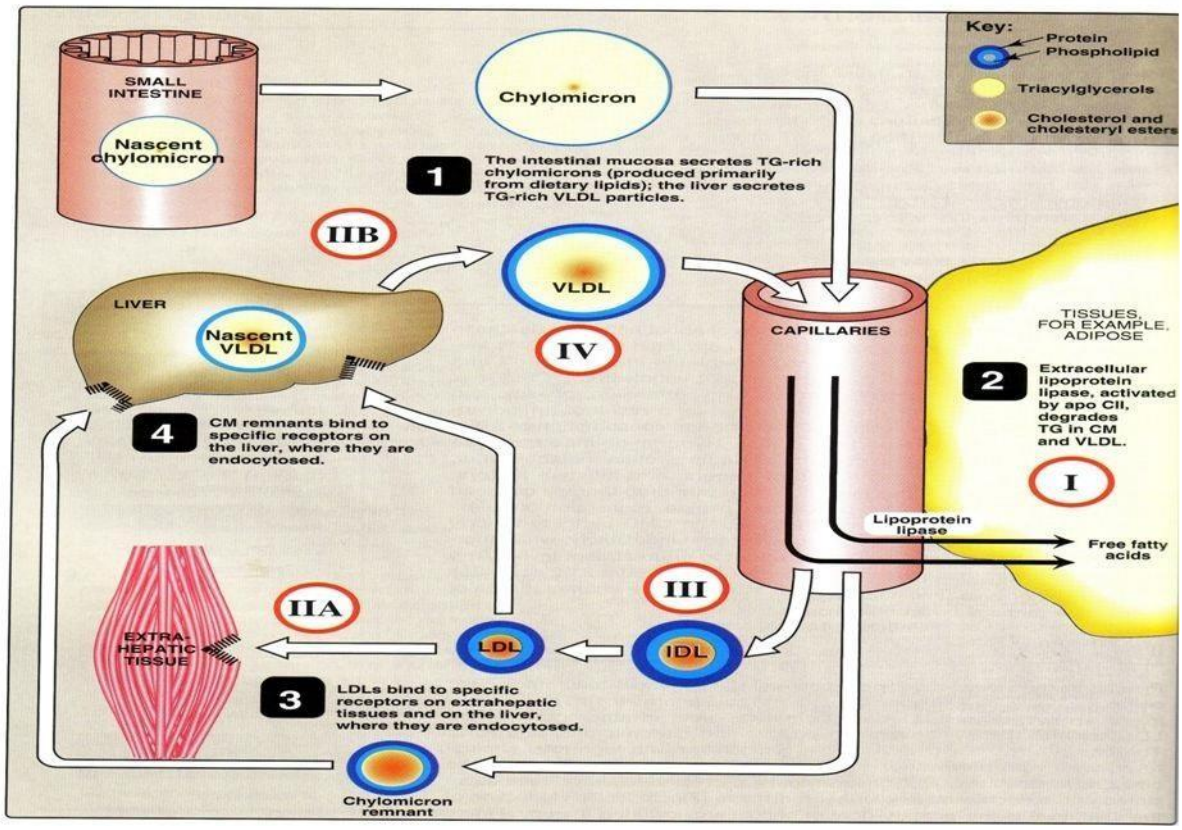


Figure 1.9: Metabolism of plasma lipoproteins and related genetic disease [157].

1.13 Treatment Goals

Lipoproteins, which are spherical macromolecular complexes of lipids and particular proteins, make up the majority of plasma lipids (apolipoproteins). The clinically significant lipoproteins are LDL; very-low density lipoprotein (VLDL) and chylomicrons; and HDL. CHD are highly related with higher total cholesterol and even more strongly with elevated LDL cholesterol. In comparison to LDL cholesterol, high HDL cholesterol levels have been linked to a lower risk of heart disease. The basic goal of cholesterol–lowering therapy is to decrease the LDL level [157].

1.14 Cholesterol

Cholesterol is a critical cellular component that plays a role not only in maintaining membrane permeability and fluidity, but also in modulating trans membrane signaling pathways and in the manufacture of steroid hormones, bile acids, and vitamin D [158-160]. Although cholesterol plays a fundamental role in a plethora of intracellular mechanisms. It is well established that persons with elevated plasma cholesterol levels are at an increased risk of developing atherosclerotic heart disease [161]. According to the American Heart Association approximately, 31.7 percent of persons in the United States have elevated LDL-C readings. Due to the strong correlation between plasma cholesterol levels and the risk of developing cardiovascular disease, numerous cellular, experimental, and clinical studies have been conducted over the last decades to elucidate the pathways regulating cholesterol homeostasis and their effect on atherosclerosis development [162]. As shows in figure (1.10) cholesterol synthesis.

1.14.1 Cholesterol Absorption

Although most cells create cholesterol, the majority of it is produced by hepatocytes and enterocytes. It is worth noting that the size of the intestinal cholesterol pool is mostly determined by endogenous sources (800–1000 mg), since diet provides only 300–400 mg of cholesterol per day [163]. Numerous mechanisms contribute to cholesterol absorption, including the solubility and release of sterols from micelles [164], as well as a regulatory network of proteins that respond to sterol balance. The Niemann–Pick–C1-like-1 (NPC1L1) protein regulates net cholesterol absorption in the upper small intestine [165]. Once inside the enterocyte, the cholesterol molecule is esterified with a fatty acid via acyl-cholesterol acyl transferase (ACAT2), an endoplasmic reticulum (ER) membrane-localized enzyme [166]. The resultant cholesteryl ester is integrated into chylomicrons and transported to the Golgi apparatus for additional processing before being secreted into the circulation via the thoracic duct [167]. Apical transporters, such as ATP-binding cassette transporters subfamily G member 5 (ABCG5) and subfamily G member 8 (ABCG8), excrete a tiny amount of free cholesterol back into the intestinal lumen [168].

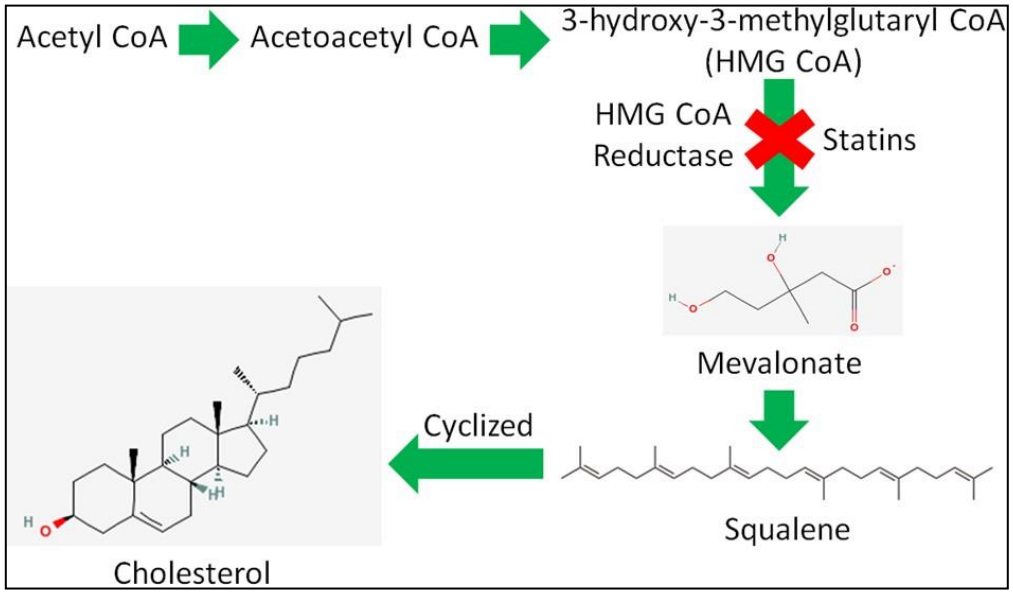


Figure 1.10: illustrate the pathway of cholesterol synthesis. [169]

1.14.2 Cholesterol Biosynthesis and Uptake

The biosynthesis and uptake of cholesterol are strictly regulated by a negative feedback mechanism that detects cholesterol and oxysterols. The transcription factor sterol regulatory element-binding protein-2 (SREBP-2; Srebf2) is a critical regulator of cholesterol synthesis genes such as HMG-CoA reductase (HMGCR), HMG-CoA synthase (HMGCS), and mevalonate kinase (MVK), as well as the LDL receptor (LDLR), which is responsible for cholesterol uptake [170, 171].

1.14.3 Cholesterol Synthesis and Enzymatic Control

Cholesterol production takes place in the ER and involves around 30 chemical processes, with acetate serving as a precursor [172]. The first two reversible processes, performed by thiolase and HMG-CoA synthase, result in the condensation of two acetate molecules to generate acetoatetyl-CoA, which is then condensed with a third acetate molecule to form 3-hydroxy-3methylglutaryl-CoA coenzyme A. (HMG-CoA). The subsequent reaction is critical for cholesterol synthesis regulation because it is mediated by the enzyme HMG-CoA reductase, a transmembrane protein of the ER that lowers HMG-CoA to mevalonate [173]. Mevalonate is transformed to isopentenyl pyrophosphate, an activated isoprene, in a series of processes. The following phase stimulates the

condensation of six isopentenyl pyrophosphate molecules to generate squalene, which is then cyclized and transformed to cholesterol in a series of processes [174].

1.14.4 Cholesterol Balance

There is a close relationship between cholesterol absorption and synthesis, which is required to maintain normal cholesterol levels throughout the body. Compensatory mechanisms reverse the pathways, so that as synthesis increases, absorption decreases, and vice versa. As a proof of concept, treatment with a cholesterol synthesis inhibitor (Atorvastatin) resulted in a 76 percent decrease in lathosterol (a marker of endogenous cholesterol synthesis) and a 70% increase in sitosterol in hyperlipidemic men (marker of cholesterol absorption) [175].

1.15 Triglyceride

Since 1959, epidemiological research has established a link between excessive triglyceride levels and an increased risk of coronary heart disease (CHD). In univariate analysis, the majority of research indicate a positive correlation between blood triglyceride levels and coronary heart disease. When high-density lipoprotein (HDL), low-density lipoprotein (LDL), and/or total cholesterol levels are included in a multifactor analysis, the triglyceride level frequently loses predictive value for coronary heart disease (CHD) [176, 177]. Although the discrepancy between single- and multiple-factor results in these studies has been used to argue that triglyceride levels are not an independent risk factor for coronary heart disease, this conclusion may be unwarranted for a variety of reasons. To be specific, the high intra- and interpatient variability of plasma triglyceride levels can impair the capacity to detect connections, particularly when compared to other less variable lipid measures [178]. Additionally, there is mounting evidence that disorders characterized by elevated levels of smaller, cholesterol-enriched very low-density lipoprotein (VLDL) and/or hypoalphalipoproteinemia, such as familial dysbetalipoproteinemia [179] and familial combined hyperlipidemia [180], are associated with an increased risk of CHD. Similarly, a number of disorders linked with hypertriglyceridemia, including diabetes mellitus, chronic renal disease, and some primary hyperlipidemias, are associated with an increased risk of cardiovascular disease [181].

1.16 Gene Expression

Gene expression is the process through which genetic information contained in gene transcripts is converted into a functional gene product (protein, non-protein peptide, or functional RNA) [182]. In another sense, gene expression is the assessment of changes (increase or decrease) in the expression of a gene by the quantitative real-time polymerase chain reaction technique (qRT-PCR). This technique is used to monitor a gene's reaction to a given set of conditions [183].

1.16.1 Real-time PCR

Real-time PCR (RT-PCR) or quantitative PCR (qPCR) is a frequently used technology in gene analysis. This approach is used in a variety of applications, including the discovery of biomarkers, pathogen detection, RNA interference measurement, and quantitative gene expression. The notion of real-time PCR is the detection of amplified DNA at the moment of amplification (real-time amplification), which enables estimation of the amplicon's beginning concentration. Unlike traditional PCR, real-time PCR requires the use of fluorescent reporter molecules (TaqMan probe or SYBR Green dye) to identify the buildup of the PCR product as the fluorescent reporter's fluorescence emission grows in lockstep with the amplification progress [184]. To quantify gene expression, mRNA from the examined gene is reverse transcriptase converted to complementary DNA (cDNA) and then used in qPCR; this is referred to as reverse transcription-qPCR (RT-qPCR). RT-qPCR can be performed in two ways [185]. The first is a two-step reaction, while the second is a one-step reaction. The real-time PCR device creates an amplification plot, which depicts the product accumulation during the course of the PCR operation.

1.16.1.1 One step RT-qPCR

The reverse transcription reaction and qPCR are carried out in the same tube in this protocol. One-step RT-qPCR is expected to reduce experimental variability and the likelihood of contamination, as well as simplifying the reaction steps, as both reactions occur in the same tube [186]. Because one-step RT-qPCR employs the mRNA strand as a template, and because RNA degrades rapidly, this reaction is not recommended when the same samples must be analyzed on multiple times over a period of time [185].

1.16.1.2 Two steps RT-qPCR

The reverse transcription reaction and the quantitative polymerase chain reaction (qPCR) are carried out in separate tubes in this methodology. The first step is to synthesize cDNA from mRNA templets using reverse transcriptase, and the second step is to transfer the produced cDNA to another tube for qPCR [192]. A diluent for the cDNA generated in the first step can be utilized in additional qPCR tests. The downside of this approach is that it increases the possibility of DNA contamination during quantitative PCR [191].

1.17 The aims of the present work

1. Development of new chromatographic methods for the separation and determination three statin drugs (Rosuvastatin, Atorvastatin and Simvastatin) in pharmaceutical dosage forms by using new HPLC-developed columns for achieving the goals.
2. Investigate the effect of the chain length between ionic sites in sulfobetain (one methylene group ZIC-1 and four methylene groups ZIC-4) on retention of Rosuvastatin, Atorvastatin and Simvastatin.
3. Although the use of HILIC has increased widely over the past decade, parts of its mechanism remain unresolved to this day and this is what motivated us to investigate the separation mechanism of pharmaceuticals using HILIC columns.
4. Study the effect of Statin drugs on lipid profile.
5. Determination of HMGCR activity by Copy Number Gene, ELISA and comparison between them.

Chapter Two
Materials and Methods

2 Materials and Methods

2.1 Materials

The chemicals, reagents, and applications used are listed in the following Tables 2.1-2.3.

Table 2.1: Chemicals and reagents for the preparation of eluents and stock solutions of pharmaceutical compounds.

Name	M.wt (g/mol)	Quality	Manufacturer
Rosuvastatin calcium	500.57	≥98% (HPLC)	Sigma-Aldrich
Simvastatin	418.57	≥97% (HPLC)	Sigma-Aldrich
Atorvastatin Calcium	1209.39	≥99% (HPLC)	Sigma-Aldrich
Acetic acid	60.05	p. a., 100%	Carl Roth
Sodium acetate	136.08	≥ 99.4%	J. T. Baker
Acetonitrile	41.05	gradient grade, ≥ 99.9%	Sigma-Aldrich

Table 2.2: Pharmaceutical applications samples using in this study.

Pharmaceutical	Manufacturer	Origin
1- Atorvastatin	Accord	England
2- Lipitor	Pfizer	USA
3- Siros	PHARMA development	France
4-Rosuvastatin Denk	Denk pharma	Germany
5- CRESTOR	Astra Zeneca	Belgaum
6- Lodal	PHARMA development	France
7- Simvastatin	Crescent Pharma	UK
8- Simvastatin	Pharma International	Jordan
9- Simvastatin	Mylan	New Zealand

Table 2.3: Chemicals and reagents used in ELISA and RT-PCR analysis.

Materials	Company	Origin
HMGCR ELISA Kit	Oxford Epigene (OE-E5517HM)	UK
Total Cholesterol Kit	HUMAN ETI 1210021	Germany
Triglyceride Kit	HUMAN ETI 1210021	Germany
HDL- Cholesterol Kit	HUMAN ETI 1210031	Germany
Master Mix (SYBR green)	Wiz-bio	Korea
Blood extraction Kit	Wizbio	Korea
Blood DNA extraction kit *200*	MagPurix	Taiwan
Loading dye	Geneaid	Korea
TBE buffer (10x)	Bioneer	Korea
Absolute ethanol	Wiz-bio solution	Korea
Fluorescent loading dye	Wiz-bio solution	Korea

2.2 Specifications for the instruments and system for HPLC, ELISA, and RT-PCR

The research was performed with a modular HPLC system (Merck-Hitachi, Germany-Japan). The system specifications with certain devices are summed up in the Table 2.4. In addition to shows the devices and tools used in ELISA and RT-PCR analysis.

Table 2.4: Specifications for the instrument

Device	Specification	Origin
Pump	L-6200 gradient pump	Merck-Hitachi
Injection valves and Column Oven	Separation Center T-6300	Merck-Hitachi
Detectors	L-4200Ultraviolet–Visible	Merck-Hitachi
Data analysis	Photographic Data Workstation software	Metrohm
UV-Vis spectrophotometer	Shimadzu double beam UV-1800	Japan
Ultrasonic water bath	Fisherbrand-CPXH	USA
Electronic balance	Kern	Germany
Centrifuge	Hermle	Germany
pH meter	Thermo	USA
Spectrophotometer SEMI	human	Germany
Blood pressure monitor	Rossmax	Germany

ELISA	Micro ELISA system (Human Reader HS)	Germany
Deep freeze	Sanyo	Japan
Water bath	Memmert	Germany
Centrifuge	Hattich	Germany
Elisa reader: microplate reader and washer	Human	Germany
Blood Collection Plane tubes		china
Micropipettes	human	Germany
Laboratory timer	Memmert	Germany
Automatic multi-channel pipette (0-250 µl)	Salmed	Germany
Micro Centrifuge tubes (Sterile) - 1.5ml	Bio-base	China
Slides and cove slides		China
Nano-drop	LabTech	UK
Magpurix	Zinexts life science corp.	Taiwan
Incubator	Dra-well	China
Micropipette 1.0-10µl Micropipette 10 – 100µl Micropipette 100-1000 µl	Dragon-lab	China
RT-PCR-quantitative real time polymerase chain reaction	Wizbio	Korea
Vortex mixer	Bionex	Korea
Work station	Yuesen Med	China
Eppendorf tube (1.0 ml) PCR tube (0.2 µl)	Genome	China
EDTA tube K3		China

2.3 Chromatographic

The chromatographic systems follow the principle of detection and the eluent mixture in their construction. The devices used are divided into the following chapter by detection methods and illustrated by the respective system sketches. A column oven and gradient pump were used for analysis with UV /VIS detection. The use of a gradient pump allows a high-pressure mixing of the aqueous phase and organic solvent. The pump and the organic part are made of a steel pump head for pumping the acoustic cycle. The eluents were passed through degasser device to remove any gases before going through the pump. Subsequently both aqueous and organic modification passes into a T-formed connecting component individually. In assure a homogeneous mixing of the eluent was a capillary steel loop. In order to protect the column material from injector pressure decreases, a pulsation damper between the pump and the injection valve was used. Therefore, fast gradient

separations and rapid variations in eluent are possible without the components having been mixed. In ZIC-HILIC mode this method was used to determination pharmaceutical compounds.

2.4 Preparation of the measurement solutions

All reagent solutions were prepared with 0.054 μs/cm conductivity of Millipore water (Millipore system-USA). Statin drugs, rosuvastatin, simvastatin, and atorvastatin stock solutions were dissolved into eluent and all the solutions were stored at cooling in the dark, using opaque bottles to protect against light or sunlight. A stock solution of 1000 μg.mL-1 for each of the three drugs were prepared by dissolving 0.1 g for each of these three drugs in eluent solution and completed to 100 mL in a volumetric flask. The solutions were sonicated and filtered with 0.45 μm membrane filters before injection and it is noticeable to have placed all solutions in the dark bottles. By subsequent dilution of the stock solution, other standard solutions were prepared **.**

2.5 Eluents

Different compositions were used as eluents for ZIC-HILIC functional mode. Dissolving pure substances in Millipore water was used in the preparation of eluents. Mixtures of acetate buffer and acetonitrile were used as eluent in ZIC-HILIC mode. ZIC-HILC eluents were produced by means of a pump with a high-pressure gradient using the organic modifier mode. In order to prevent pump related interference and a lower detector range, the mobile phase should preferably be degassed daily.

2.5.1 Preparation of the different buffer pH

The proportion of the individual components of an acid/base pair was determined in buffered systems according to the Henderson-Hasselbalch equation [187] via the pH value, the pKa value of the buffer, and the concentrations of the base [A−] and the corresponding acid [HA]. Different buffer was prepared in 1000 mL at pH (3-5.5) from mixing of acetic acid and sodium acetate. Table (2.5) lists buffer (pH), volume of acetic acid and weight of sodium acetate.

Table 2.5: Preparation buffer range (3-5.5) [188].

Buffer (pH)	Acetic acid (µL) 17.3 M	Weight of Sodium acetate (g)
3.00	2247.6	0.095
4.00	1941.9	0.822
4.50	1463.8	1.959
4.75	1143.8	2.722
5.00	823.8	3.484
5.50	345.7	4.621

2.5.2 Preparation of the different buffer concentration

Different buffer was prepared in 1000 mL at concentration (10-80 mM) from mixing of acetic acid and sodium acetate. As shown in Table (2.6) list buffer concentration, volume of acetic acid and weight of sodium acetate.

Table 2.6: Preparation buffer range (10-80 mM) at pH 4.75 [188].

Buffer concentration (mM)	Acetic acid (µL) 17.3 M	Weight of Sodium acetate(g)
10	285.7	0.680
20	572.4	1.361
30	858.1	2.041
40	1143.8	2.722
50	1429.5	3.402
60	1715.2	4.082
70	2001.9	4.763
80	2287.6	5.443

2.6 Preparation of pharmaceutical samples

2.6.1 Statin drugs

2.6.1.1 Rosuvastatin samples

Thirteen tablets for each of the rosuvastatin samples of three companies were weighed the equivalent to about 10-20 mg of rosuvastatin was dissolved an adequate size of eluent and transferred into a 100 mL volumetric flask to the mark (100-200 µg ml-1). Subsequently, the solution was filtered by Millex® Syringe filters (0.45 µm). Other standard solutions were prepared by subsequent dilution of the stock solution.

2.6.1.2 Atorvastatin samples

10 mg of the atorvastatin was weighed after mixing a thirteen of tablets of three companies, dissolved in the eluent and transferred to a volumetric flask of 100 ml and completed to the mark (100 µg ml-1). Next, the solution was filtered by Millex® Syringe filters (0.45 µm). Other standard solutions were prepared by the subsequent dilution of the stock solution.

2.6.1.3 Simvastatin samples

10-20 mg of the simvastatin was weighed after mixing a group of tablets of three companies, dissolved in the eluent and transferred to a volumetric flask of 100 ml and completed to the mark (100-200 µg ml-1). Next, the solution was filtered by Millex® Syringe filters (0.45 µm). Other standard solutions were prepared by the subsequent dilution of the stock solution.

2.7 Methodology

2.7.1 Choosing the optimal wavelength detection to separate the selected drugs

Based on the American Pharmacopoeia, the optimal wavelength was chosen for the estimation and separation of Statin drugs, rosuvastatin, simvastatin, and atorvastatin. A number of experiments were conducted to choose the optimal wavelength to obtain the optimum separation of drugs depending on the optimal conditions of the mobile phase by increasing and decreasing 10-50 nm from the wavelengths in the American Pharmacopoeia.

2.7.2 Study separation mechanism of pharmaceuticals under HILIC condition

Separations of Statin drugs, rosuvastatin, simvastatin, and atorvastatin-using acetate buffer/acetonitrile eluents are accomplished for the characterization in the ZIC-HILIC mode. The Statin drugs were detected by UV at 240 nm. Retention of these pharmaceuticals can be accomplished, with all two HILIC exchangers. To get a clue about the separation properties of the individual stationary phases and thus about the separation mechanism of Statin drugs, rosuvastatin, simvastatin, and atorvastatin, mobile phase compositions were changed methodically by variation of ACN content, eluent concentration, and eluent pH.

2.7.2.1 Statin pharmaceuticals

2.7.2.1.1 Effect of ACN content on retention of pharmaceuticals

The effect of the organic modifier was accomplished on retention of Statin drugs while keeping NaOAc/HAc buffer constant at 40 mM and the pH at 4.75 while varying the ACN volume fraction (60-95%) in the mobile phase.

2.7.2.1.2 Effect of eluent concentration on retention of pharmaceuticals

The effect of the NaOAc/HAc buffer concentration was accomplished on retention of Statin drugs while keeping ACN% constant at 80% and the pH at 4.75 while varying the NaOAc/HAc buffer concentration (10-80 mM) in the mobile phase according to the paragraph .)2.4.2(

2.7.2.1.3 Effect of eluent pH on retention of pharmaceuticals

The effect of the eluent pH was accomplished on retention of Statin drugs while keeping ACN% constant at 80% and the 40 mM buffer concentration while varying the NaOAc/HAc buffer pH (3-5.5) in the mobile phase according to the paragraph .)2.4.1(

2.7.3 Calibration graph

2.7.3.1 Statin pharmaceuticals

2.7.3.1.1 Rosuvastatin, Atorvastatin, and Simvastatin

Aliquots (10-7000 μL), (50-5500 μL), and (30-7000 μL), of rosuvastatin, atorvastatin, and simvastatin respectively (10 μg mL−1) of stock standard solutions were transferred into 10 mL volumetric flasks. The chromatography analysis was successfully accomplished on a ZIC-1, ZIC-4 columns with eluent of 20% NaOAc/HAc buffer (40 mM-pH 4.75) and 80% ACN at a flow rate of 0.5 mL/min and ultraviolet detection at 240 nm. The calibration curves of rosuvastatin, atorvastatin, and simvastatin were plotted of the peak area against the rosuvastatin, atorvastatin, and simvastatin concentrations, and the corresponding linear regression equation was used to convert the peak area into rosuvastatin, atorvastatin, and simvastatin concentrations for all the pharmaceutical samples.

2.8 Hypercholesterolemia Patients

The present study was conducted at the department of chemistry, college of science, university of Anbar and Baghdad. Thirty patients (16 females and 14 male) with Hypercholesterolemia were subjected in this study before and after statins treatment totally sixty serum blood samples, where their ages ranged from 33 to 78 years. The mean age in years (50.06 ± 12.76). However, the examined sample was randomly chosen from those attending Baghdad teaching hospital during February to June 2021. Questionnaire sheet was completed for each patient (Appendix I) after full clinical exanimation by the consultant physicians, Patients were free of severe diseases or infection at time of study and with known illness, while the patients with disorders. The diagnosis of hypercholesterolemia was made on the foundation of the recommended standards by WHO [189]. Patients were excluded from study if they had a history of renal impairment, thyroid gland disorder and heart disease.

2.8.1 Controls

Twenty healthy individuals (8 females and 12 males) with ages ranged from 35 to 60 years. the age means were (45.45 ± 7.265). Were examined are in this study as a control. The control sample was healthy in terms of non-hypertensive, non-hypercholesterolemia, no other endocrine disorders or kidney diseases metabolic and free of acute illness or infection at time of testing.

2.8.2 Characteristics of Patients and Subjects

Control samples and patients were described in the terms of gender, age, disease history, body mass index (BMI), and fasting serum glucose (FSG), Total cholesterol, triglyceride, HDL, LDL, and VLDL.

2.9 Blood Sampling

Five mL of blood was collected by venipuncture after 12–16 hours of fasting from healthy control and patients with hypercholesterolemia before and after statins drugs. The blood was placed in the plain tube and left to coagulate for 30 min at room temperature. Serum was collected by centrifuging at 4000 rpm for 10 min. The serum was divided into three parts. The first one was used for the biochemical assay lipid profile, second one for Elisa kit, the third one for Stains drug,

whereas the second and third one was frozen at (-20 c°) by (250µl) Eppendorf tubes until use, while 2 ml whole blood was also collected from previous subjects for RT-PCR technique.

2.10 Blood Pressure Records

Systolic and diastolic blood pressure (SBP & DBP) (mmHg) and rate of pulse (Min^{-1}) were measured by mercury sphygmomanometer. For people over 60 years, SBP and DBP should be less than 150 mmHg 90 mmHg respectively while for people under 60 years, SBP and DBP should be less than 140 mmHg 90 mmHg respectively [190].

2.11 Determination of Body Mass Index (BMI)

The BMI has been proposed as an alternative to the traditionally used height-weight tables in assessing obesity. BMI measures weight corrected for height and is significantly correlated with total body fat content. BMI was calculated as weight (in kilograms) divided by height (in meters) squared [191].

2.12 Serum Lipid Profile Assay

2.12.1 Determination of Serum Total Cholesterol

In the sample, cholesterol originates a colored complex according to the interaction below:

$$\text{Cholesterol esters} + H_2O \xrightarrow[\text{Acids}]{\text{Cholesterol Esterase}} \text{Cholesterol} + \text{Fatty} \ldots\ldots\ldots 2$$

$$\text{Cholesterol} + O_2 \xrightarrow[4H_2O]{\text{Cholesterol oxidase}} \text{4-Cholestenone} + H_2O_2 \ldots\ldots\ldots\ldots 3$$

$$2\,H_2O_2 + \text{Phenol} + \text{4-aminophenozone} \xrightarrow{\text{Peroxidase}} \underset{\text{Colored Complex}}{\text{Quinonimine} + 4H_2O} \ldots\ldots 4$$

The color intensity is proportional to the concentration of cholesterol in the sample [192].

➢ Kit Content

Reagent Types	Materials	Conc.
R1	Pipes pH 6.9	90mmol/L
	Phenol	26mmol/L
R2	Cholesterol esterase(CHE)	300U/ L
	Cholesterol oxidase(CHOD)	300U/L
	Peroxidase(POD)	1250U/L
	4-Aminophenazone(4-AP)	0.4mmol/L
Calibrator	Cholesterol stander	200mg /dL

➢ Assay Procedure

1 .The working reagent was prepared by adding the content of R2 in R1and mixed with the dissolved contents.

2 .The spectrophotometer was set to zero with D.W.

3. Three tubes were prepared as shown below:

Tubes solution	Blank	Standard	Sample
Working reagent (ml)	1	1	1
Standard (µl)	-	10	-
Sample (µl)	-	-	10

4 .The tubes were incubated for 5 min in water bath at 37˚C.

5. The absorbance was measured at 505 nm, and calculated according to the following equation:

$$\text{Conc. of cholesterol in sample mg/dL} = \frac{Abs\ Sample}{Abs\ Standard}\ x\ 200\ \text{(standard conc)} \quad \ldots\ldots\ldots\ldots 5$$

➢ Normal value :

Cholesterol = < 200 mg/dL

2.12.2 Determination of Serum Triglycerides.

Sample was incubated with enzyme of lipoprotein lipase (LPL) and triglycerides transform to glycerol and free fatty acids.

$$\text{Triglycerides} + H_2O \xrightarrow{\text{Lipase}} \text{glycerol + free fatty acids} \quad \ldots\ldots\ldots\ldots\ldots 6$$

By adding adenosine triphosphate and glycerol kinase the glycerol will convert into glycerol-3-phosphate (G3P) and ADP.

$$\text{Glycerol + ATP} \xrightarrow{\text{Glycerol}} \text{glycerol -3- phosphate + ADP} \quad \ldots\ldots 7$$

G3P is converted into dihydroxyacetone phosphate and H_2O_2 by glycerol phosphate dehydrogenase (GPO).

$$\text{G3P + O2} \xrightarrow{\text{Glycerol 3- phosphate oxidase (POD)}} \text{dihydroxy acetone phosphate + } H_2O_2 \quad \ldots 8$$

Red color formed when H_2O_2 reacts with 4-aminophenazone (4-AP), p-chlorophenol in the participation of POD.

$$H_2O_2 + \text{p-chlorophenol + 4-aminophenazone} \xrightarrow{\text{Peroxidase}} \text{Quinone + } H_2 \quad \ldots\ldots 9$$

The quantity of red dye quinone formed is proportional to the cholesterol concentration [193].

➢ Kit Content

Reagent Types	Materials	Conc.
R1	GOOD pH 7.5 p-chlorophenol	50mmol/L, 2mmol/L
R2	Lipoprotein lipase(LPL), Glycerol kinase(GK), Glycerol – 3 –P Oxidase(GPO) , Peroxidase(POD) 4-Aminophenazone(4-AP) , ATP	150000U/L 500U/L 2500U/L 440U/L 0.1mmol/L 0.1mmol/L
Calibrator	Triglyceride Stander	200mg /dL

➢ Assay Procedure

1 .The working reagent was prepared by adding the content of R2 in R1and mixed to dissolved contents.

2 .The spectrophotometer was set to zero with D.W.

3. Three tubes were prepared as shown below:

Tubes solution	Blank	Standard	Sample
Working reagent (ml)	1ml	1	1
Standard (µl)	-	10	-
Sample (µl)	-	-	10

4. The tubes were incubated for 5 min in water bath at 37˚C.

5. The absorbance was measured at 500±10 nm, and calculated according to the following equation:

$$\text{Conc. of triglyceride in sample mg/dl} = \frac{Abs\ Sample}{Abs\ Standard}\ x\ 200(\text{ standard conc}).....10$$

- **Normal value :**

Triglycerides < 150 mg/Dl

2.12.3 Determination of Serum HDL–Cholesterol.

Precipitation of VLDL and LDL chylomicrons and lipoproteins was accomplished using a 4% phosphotungstic acid solution containing 10% magnesium chloride at a pH of 6.2. The supernatant contains the HDL concentration following centrifugation. The cholesterol level can be measured using the companion kit described above [194].

- **Assay Procedure**

1. Reagents and samples were left at temperatures between 23-27 ˚C.

2. Five hundred µL of serum was added to 50 µL of R1 and mixed after that left it for 10 min at temperatures between 23-27 ˚C.

Precipitating Reagent (µl)	50
Sample (µl)	500

3. The samples were centrifuged for 2 min to obtain clear supernatant.

4. Three tubes were prepared as shown in the table below:

Solutions	Blank	Standard	Sample
Reagent (cholesterol) ml	1	1	1
Standard (µl)	-	10	-
supernatant (µl)	-	-	10

5 .Incubated for 5 minutes at 37°C in water bathe.

6 .The spectrophotometer was set to zero with Millipore water.

7. The absorbance was measured at 505 nm.

$$\text{Conc. of HDL} - \text{C in sample mg/dl} = \frac{Abs\ Sample}{Abs\ Standard}\ \text{x}\ 50 \ldots\ldots\ldots\ldots\ldots 11$$

- **Normal value :**

HDL = 40-60 mg/dl

2.12.4 .Calculation of Low-Density Lipoprotein cholesterol

Low density Lipoprotein cholesterol (LDL–C) can be calculated mathematically from the total cholesterol, by using Friedwald's formula as described below [195]:

LDL-C = T. Cholesterol - (T.G)/5- HDL-cholesterol… 12

LDLc = TC – HDLc – VLDL c= TC – (HDLc + VLDLc)

The formula is only valid at TG concentration less than (5.32 mmol/L) (400 mg/dL).

Optimal LDL-C value = 100 –129 mg/dL.

2.12.5 .Calculation of Very Low-Density Lipoprotein.

Very low-density lipoprotein (VLDL) concentration was calculated as one – fifth of the serum TG [196].

$$\text{VLDL mg/dl} = \frac{\text{T. G}}{5} \ldots\ldots\ldots 13$$

- **Normal value :**

VLDL = 20 - 40 mg/dl

2.13 HMG-CR ELISA Kit

2.13.1 Principle of assay:

The kit used enzyme-linked immune sorbent assay (ELISA) based on biotin double antibody sandwich technology to assay Human 3-Hydroxy-3methylglutaryl CoA Reductase (HMGCR). Added 3-Hydroxy-3-methylglutaryl CoA Reductase (HMGCR) to wells that are pre-coated with 3-Hydroxy-3methylglutaryl CoA Reductase (HMGCR) monoclonal antibody and then incubated. Following incubation, biotin-tagged anti-3Hydroxy-3-methylglutaryl CoA Reductase (HMGCR) antibodies were added to form the immunological complex with Streptavidin-HRP. any unbound enzymes were removed and added substrates A and B following incubation and washing. Due to the acid's impact, the solution was turned blue and eventually yellow. The color of the solution was inversely proportional to the concentration of human 3-Hydroxy-3-methylglutryl CoA Reductase (HMGCR).

2.13.2 Materials Supplied in the Test Kit

Configuration	96 wells	48 wells	Preservation
Instruction	1	1	
Seal plate membrane	2	2	
Hermetic bag	1	1	
Coated ELISA plate	12-well * 8 Tubes	12-well * 4 Tubes	2-8°C
Standard solution (80 ng/ml)	0.5ml ×1	0.5ml×1	2-8°C
Streptavidin-HRP	6ml ×1	6ml×1	2-8°C
Stop solution	6ml ×1	6ml×1	2-8°C
Substrate solution A	6ml ×1	6ml×1	2-8°C
Substrate solution B	6ml ×1	6ml×1	2-8°C
Anti HMGCR antibodies labeled with biotin	1ml ×1	1ml×1	2-8°C
Standard dilution	3ml ×1	3ml×1	2-8 °C
Washing concentrate	(20ml×25)×1	(20ml×25)×1	2-8 °C

2.13.3 Serum sample:

Allow 10-20 minutes for the serum to coagulate at room temperature (25°C) before centrifuging for approximately 20 minutes (at 2000-3000 RPM). The supernatants were collected with care.

2.13.4 Procedure for the assay

1. Standard solution dilution: (this lamp supplies one standard solution at its original concentration).

Users may dilute individually in small tubes according to the chart below:

40ng/ml	StandardNo.5	120µl original Standard+120µlStandard diluent
20ng/ml	StandardNo.4	120µl StandardNo.5+120µlStandard diluents
10ng/ml	StandardNo.3	120µl StandardNo.4+120µlStandard diluents
5ng/ml	StandardNo.2	120µl StandardNo.3+120µlStandard diluents
2.5ng/ml	StandardNo.1	120µl StandardNo.2+120µlStandard diluents

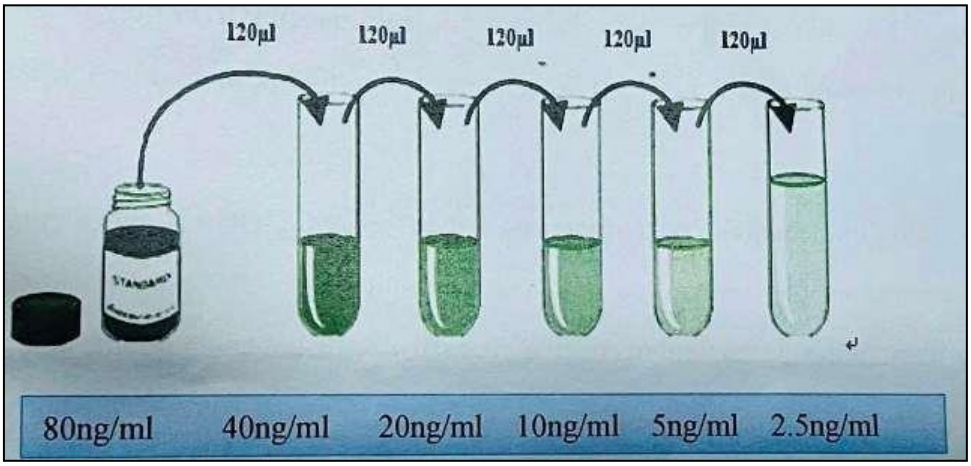


Figure 2.1: Standard original concentration dilutes in small tubes.

2 .The required number of stripes was determined by the number of samples to be tested plus the standard. It was advised that as many wells as feasible be used for each standard solution and blank well.

3 .Injection of the sample: A) No sample was added to the blank well; instead, an anti-HMGCR antibody tagged with biotin or streptavidin-HRP was added to the comparative blank. To begin, chromogen solutions A&B are added and stop solution; the remaining steps were identical. B) a standard solution was prepared by adding 50 l standard to 50 l streptavidin-HRP (biotin antibodies

had united in advance in the standard so no biotin antibodies were added). C) the sample was prepared well for testing by adding 40l sample, followed by 10l HMGCR antibodies and 50l Streptavidin-HRP. A seal plate membrane was covered, gently shacked to combine. Then, Incubated for 60 minutes at 37 °C.

4 .Preparation of washing solutions: For later use, the washing concentration was diluted (25X) with Millipore water.

5 .Cleaning: the membrane from the seal plate was carefully removed, drained any excess liquid, and shacked off the remaining. Each well should be filled with washing solution and allowed to stand for 30 seconds before draining. Five times more, then blot the plate.

6 .Color development: 50l chromogen solution was added A to each well first, followed by 50l chromogen solution B. Gently shacked to combine. For color development, incubated for 10 minutes at 37 °C away from light.

7 .To halt the reaction, 50l Stop solution was added to each well (color changes from blue to yellow immediately at the moment).

8 .Assay: a blank well was used as a reference, determine the absorbance (OD) of each well individually at 450nm, which should be done within 10 minutes of adding stop solution.

9. The standard curve's linear regression was calculated by equation using the standard concentration and related OD values. Then, using the OD values of the samples, determined the concentration of each sample. Additionally, statistical software might be used.

2.14 Real Time PCR

2.14.1 Primer

Table 2-9: Specific designed primers were used in this study:

Target Gene	HMGCR	Length in bp	Tm	GC%
F	CTCACAGGATGAAGTAAGG	19	60.8	47.4
R	CTGACATGGTACCAACTC	18	60.8	50
	Product size	113	76.7	-

2.14.2 Methods

2.14.2.1 Clinical Data

Every participant was interviewed and asked to answer information including age, sex (male and female), family history, smoker, married, any genetic disease.

2.14.2.2 Blood Samples collection

Amount of two to three ml of venous blood was withdrawn from each subject under aseptic conditions. The blood was placed in EDTA tube (1.5 mg/ml) and kept at -20 C^{o} to be used in molecular study.

2.14.2.3 Genomic DNA extraction

Genomic DNA was extracted from the whole blood samples by using two methods, manual and automated.

2.14.2.4 Automated extraction of genomic DNA

Genomic DNA was extracted from the whole blood samples by using Blood DNA extraction Kit 200 depending on the MagPurix technology in figure 2,2, a fully automated, highly innovative system for quick molecular biology sample which is a state-of-the-art platform that uses magnetic beads to extract nucleic acids from samples. The platform commits truly walk-away automation in nucleic acid purification from samples to results. The MagPurix technology ensures top quality results in just 3 easy steps: load samples, run pre-programmed protocol, and collect results. The purification process contains steps of lysis, binding, washing, and elution.

* Procedure of Automated Genomic DNA extraction

The protocol of automated genomic DNA extraction was done as follow:

1. Frozen blood was thawed completely at room temperature. The blood sample was thoroughly mixed for at least 10 minutes in a shaker at room temperature (18-22 C) .
2. Before the work, starting the auto UV sterilization was done for 2 min.
3. The power was switched on and waits until the LCM screen displays “MagPurix® System Stand-By .”

4. The “Start” button was Pressed (The system will process a self-testing, and then go to steady mode). The reagent drawer was opened and the sample rack was removed out of the instrument.
5. Reagent cartridges and all plastic disposables (Polygon reaction chamber, tip holder, piercing pin, and filter tip) were loaded as numbered respectively.
6. Sample tube and elute tube was loaded into sample rack within the device.
7. The samples were loaded to the sample tube.
8. The Sample rack was placed on the instrument platform and the reagent drawer was closed.
9. For 200 µl of blood sample and 200 µl of elution, the protocol barcodes were scanned to select the purification protocol.
10. The instructions were followed on the LCM screen to double-check the operating steps before program running .
11. “Enter” was pressed so the instrument started to run the protocol program automatically until whole processes were completed .
12. At the end of the run, the instrument alarms briefly and the LCM screen indicated, “Protocol completed .”
13. The instrument door was opened. The elute tubes containing the purified nucleic acid was removed .
14. The used cartridges were discarded because it cannot be reuses, and all plastic consumables were discarded into biohazard waste.
15. Finally, the extracted DNA was stored at a temperature of -20 ^{o}C until used.

Figure 2.2: The Mag Purix technology system [200].

2.14.2.5 Agarose Gel Electrophoresis

After genomic DNA has been extracted, agarose gel electrophoresis was adopted to confirm the presence and integrity of the extracted DNA [198].

* Preparation of 1X TBE Buffer

Amount of 100 ml of TBE buffer 10X was diluted to 1X by added 900 ml of Millipore water, and stored at room temperature (18-22 oC).

*Agarose Gel Preparation (1%)

The steps of agarose gel preparation are as the following:

1. Amount of 60 ml (1X) TBE was put into a beaker.
2. Agarose powder (1 gm) was added to the buffer.
3. The solution was heated to boiling point using microwave until all gel particles were dissolved; care was taken to avoid evaporation of the solution, which would lead to the change in the concentrations of the components.
4. Amount of 30 µl of fluorescent loading dye was added to the agarose solution, and mixed gently to avoid the bubbles.
5. The mixture was left to cool down at 50 – 60 ^{o}C.

* Casting of the horizontal electrophoresis agarose gel.

After close the edges of the tray with adhesive tape and fixing the comb in 1 cm away from one edge of the tray, the agarose solution is poured into the gel tray and left to solidify at room temperature (18-22o C) for 30 minutes, after that the comb is removed gently and the gel tray was placed in the gel tank which is filled with 1x TBE buffer until the gel tray is fully immersed [197].

* DNA loading and electrophoresis

Amount equal to 7µl of DNA was mixed with 3µl of 6X loading dye. Samples were loaded carefully into the individual wells of the gel, and then electrical power was turned on at 70 volt for 30 minutes afterwards, the DNA moved from cathode (-) to anode (+) poles.

The ethidium bromide-stained bands in the gel were visualized using UV transilluminator and photographed.

2.14.2.6 Estimation of DNA concentration and purity

Nano-drop NAS-99 spectrophotometer was used for evaluation the concentration and purity of DNA in samples. The Nano-drop was first blanked with 2 µl of DNA rehydration solution (same elution material), then the DNA samples were measured one by one .

- Estimating DNA concentration: reading A at 260nm.
- Indicating DNA purity: reading the ratio of A at 260/280nm. DNA purity ratio = A260/A280 = 1.8-2.0
- The final unit of concentration was in ng/µl.

Note: Absorbency at 260nm of equal to 50 ng/µl of pure DNA

2.14.3 Detection of HMGCR gene.

2.14.3.1 Preparation of primers

The primers were used to amplify specific regions of HMGCR gene. These primers were designed by (Deacon Designer V: 8.21). Primers (table 16) were supplied by Alpha DNA Company as a lyophilized product of different Pico moles concentrations. According to the instructions of the manufacturing company, the primers were dissolved in a nuclease free water to give a final concentration of 100 pmol /µl, which was used as a stock solution, 10 µl of this solution

was added to 90 µl of free nuclease water to obtain 10 pmol/µl of primer solutions final concentration that was utilized in PCR technique.

2.14.3.2 Polymerase chain Reaction components and programs

Polymerase–chain reaction components and programs Polymerase Chain reaction was carried out after several attempts of optimization to detect the best temperature for annealing with a total volume of 20 µl. The reaction component is described in Table 2-10 and PCR amplification program is described in Table 2-11.

Table 2-10: PCR reaction components for amplifying the targeted fragments

Component	Quantity
PCR Re Mix (Ready to use) SYBR Green	10 µL
Forward primer	0.75 µL
Reverse primer	0.75 µL
DNA template	3.5 µL
D.W.	5 µL
Final volume	20 µL

Table 2-11: PCR Amplification Program

Steps	Temperature	Time	Cycles
Hold	95	15 min.	1 cycle
Denaturation	95	15 sec.	40 cycles
Annealing	60	20 sec.	
Extension	72	20 sec.	

2.14.3.3 Real-time PCR run

DNA samples from hypercholesterolemia patients (n=60) and apparently healthy subjects (n=20) were genotyped for the HMGCR gene. assay using real time thermo cycler according to the protocol recommended by the manufacturer.

Chapter Three
Results and Discussions

3 Results and discussion

3.1 Study separation mechanism of pharmaceuticals

The eluent composition is systematically varied according to variations in the acetonitrile content, buffer concentration and pH of the eluent following an investigation of the Chromatograms for model compounds, so that a closer look on the mechanisms for the separation of model compounds in relation to spacer chain length is obtained. Applying a mobile phase NaOAc/HAc buffer with varying ACN contents to the two columns of the HILIC, Atorvastatin (ATOR), Rosuvastatin (ROSU) and Simvastatin (SIMV) were chosen as pharmaceutical samples for an analysis of its HILIC mode retention system. The mobile phase compositions of eluent from 20 to 100 mM and their pH from 3 to 5.5 were amended by variation of their content of ACN from 60% to 95%, to get an idea of the separation features and thus of the separation mechanism for each column. Table 3.1 summarizes some of the physical-chemical properties of the three drugs.

3.1.1 The influence of ACN fraction on behavior of (ATOR)

During the current study, the effects of the ACN content were evaluated by increasing the amount of ACN in the mobile phase and keeping the concentration of buffer at 40 mM constant (pH 4.75). The retention of the three-drug separation has increased or decreased in previous works [74, 75, 199], as the ACN content has been increased in the ZIC-HILIC mode. This difference is due to the pharmaceutical hydrophilicity of the three-drug models when the ACN content differs within the eluent, as is apparent in the pharmaceutical log Pow values. Consequently, two conducts of hydrophilicity (HILIC) and hydrophobicity (RP) with increased eluent ACN content, are exhibited in pharmaceuticals. The hydrophilicity of the pharmaceuticals causes this variation in behavior. For both the ZIC-1 and the ZIC-4, the ATOR exhibits RP behavior Figure (3.1) This is due to the log Po/w (4.92) [199], which is the ATOR value. It must be noted that the retention of ATOR in column ZIC-4 indicates optimum retention due to the length of space between packed classes.

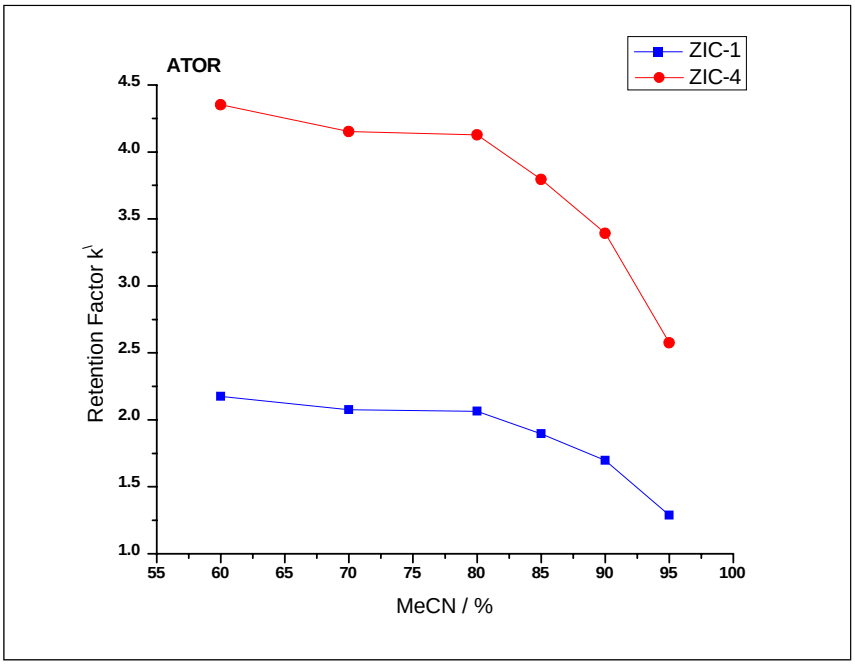


Figure 3.1: Effect of variation of the ACN fraction on behavior of ATOR using ZIC-1 & ZIC-4 columns.

3.1.2 The influence of ACN fraction on behavior of (ROSU)

The drug hydrophilicity value plays a central role in this behavioral disparity. The log $P_{o/w}$ value of ROSU is (1.23) [201, 202] and, therefore, ROSU exhibits RP behavior for both ZIC-1 and ZIC-4 exchangers Figures (3.2).

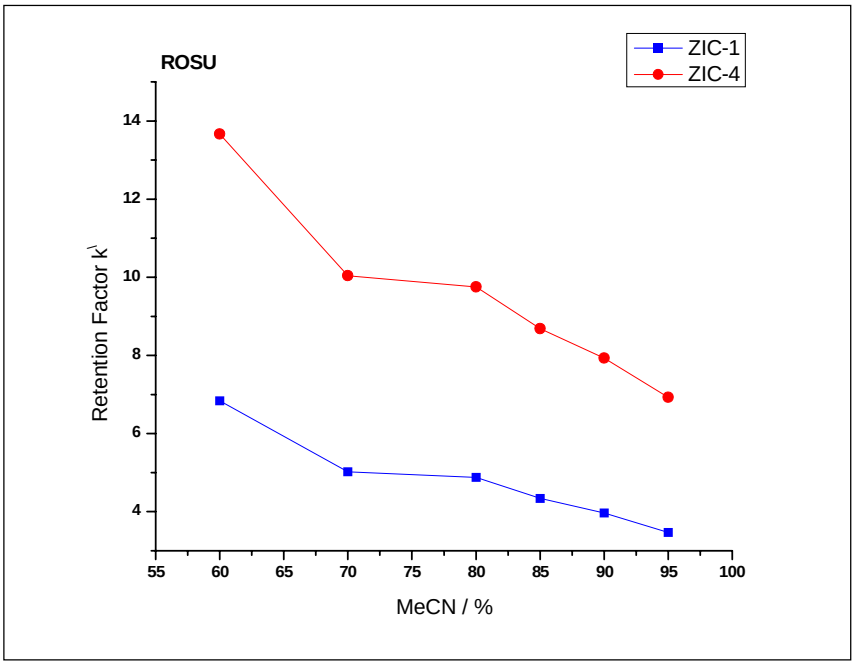


Figure 3.2: Effect of variation of the ACN fraction on behavior of ROSU using ZIC-1 & ZIC-4 columns.

3.1.3 The influence of ACN fraction on behavior of (SIMVA)

SIMV demonstrates RP activity in the mobile phase with growing retention factors with increased acetonitrile content Figure (3.3). The explanation for this disparity in the behavior of the template compounds is due to the hydrophilicity of the compounds in varying ACN fraction in the mobile phase as shown in the log Pow values of the drugs Table 3.1. Similar to ranitidine hydrochloride, the ATOR, ROSU and SIMV have significantly lower hydrophilicity [76]. Due to its low hydrophilicity, log Po/w values indicate that ATOR, ROSU and SIMV have observed a strong RP interaction.

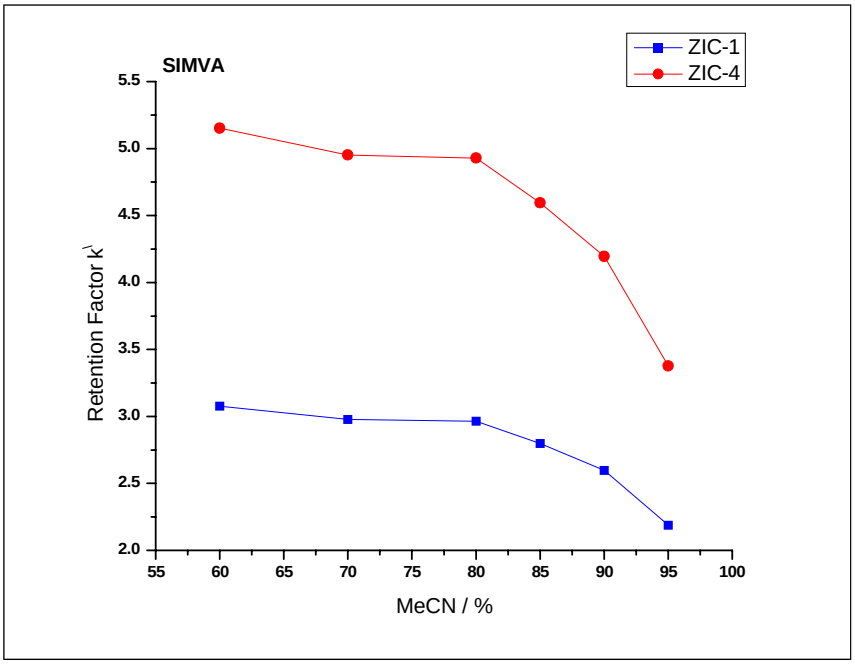


Figure 3.3: Effect of variation of the ACN fraction on behavior of SIMVA using ZIC-1 & ZIC-4 columns.

3.2.1 The influence of buffer concentration on behavior of ATOR

Basically, solute behavior has increased in hydrophilic mode with an increasing buffer concentration, leading to intramolecular ion pairs deactivation. In this way, the linearization of the functional column groups is improved, even though ACN exists [56]. The compounds retention time of the columns HILIC increases or decreases as NaOAc /HAc buffer levels increase [203]. Remarkably, ATOR shows decrease retention factors when the holding ACN at 80% and pH at 4.75 whilst NaOAc / HAc buffer from 10 to 80 mM is increased Figure (3.4). The effect of the acetate buffer on the eluent retention behavior of ATOR was reported in the 10-80 mM (pH 4.75) at 80% ACN in the eluent. Increasing buffer concentrations in the eluent

of acetate decrease the ATOR retention factor in the columns. The reason for ATOR behavior is going to the hydrophilicity of ATOR. ATOR take a different approach to decreasing retention; hence, we advise for two factors. The first factor is ATOR hydrophilicity value, and the second factor is the core material (PS/DVB) for the stationary phases. Subsequently, ATOR separation depended on anion exchange with ZIC-1 and ZIC-4 stationary phases [76].

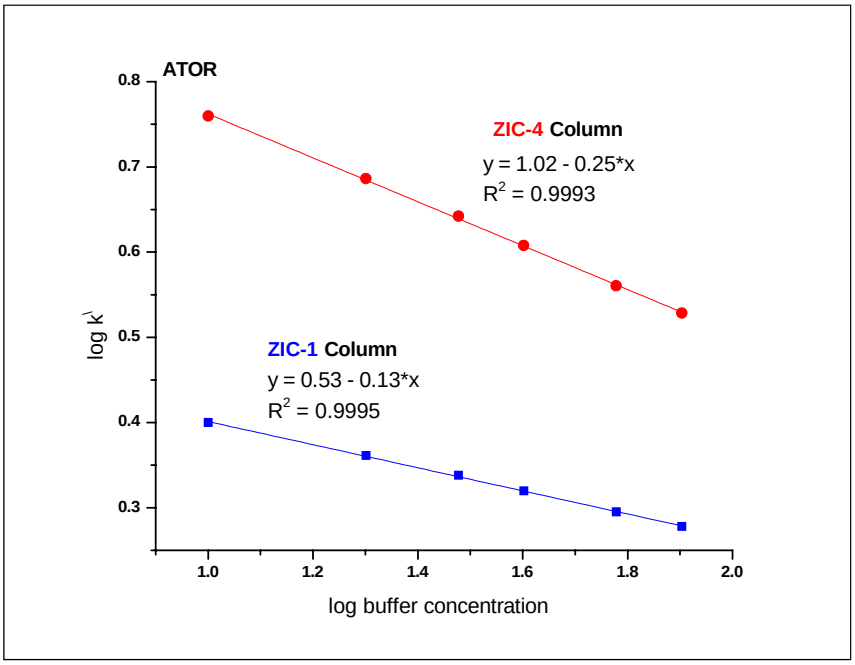


Figure 3.4: Effect of buffer concentration on behavior of ATOR using ZIC-1 & ZIC-4 columns

3.2.2 The influence of buffer concentration on behavior of ROSU

In ZIC-HILIC stationary phases the drug retention decreased or increased with increased buffer level [203]. This is because of the anion and cation interactions. Figure (3.5) indicate and decrease in ROSU retention factor when the NaOAc buffer was increased from 10 to 80 mM with 4.75 pH and 80% ACN. ROUS takes a unique approach to retention reduction; as a result, we recommend two things. The first component is the ROUS hydrophilicity value, and the second factor is the stationary phase core material (PS/DVB). Following that, the separation of ROUS was dependent on anion exchange using the ZIC-1 and ZIC-4 stationary phases [76].

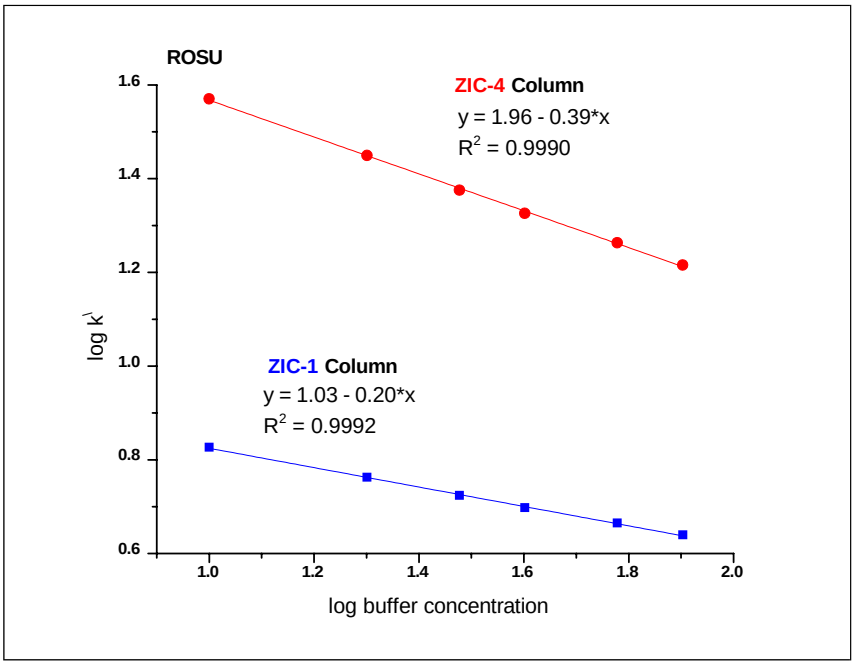


Figure 3.5: Effect of buffer concentration on behavior of ROSU using ZIC-1 & ZIC-4 columns.

3.2.3 The influence of buffer concentration on behavior of Simvastatin

Basically, solute behavior has increased in hydrophilic mode with an increasing buffer concentration, leading to intramolecular ion pairs deactivation. In this way, the linearization of the functional column groups is improved, even though ACN exists [56]. The compounds retention time of the columns ZIC-HILIC increases or decreases as NaOAc /HAc buffer levels increase [203]. Remarkably, SIMV showed almost constant retention factors when the holding ACN at 80% and pH at 4.75 whilst NaOAc / HAc buffer from 10 to 80 mM was increased. Thus, the uncharged nature of SIMV can be attributed Figures (3.6). The properties of the analytes should be taken into account in order to better understand the retention behavior of the compounds. The value of pka (12.59) of SIMV. The SIMV tested should, therefore, be an uncharged nature. Ironically, no SIMV effects on cation and anion columns and two ZIC columns were observed because of the fact that SIMV acts like a neutral molecule. Due to the identical SIMV behavior on two columns and the persistence may be only due to hydrophobic activity, the electrostatic interaction with functional groups in ZIC columns cannot clarify SIMV behavior.

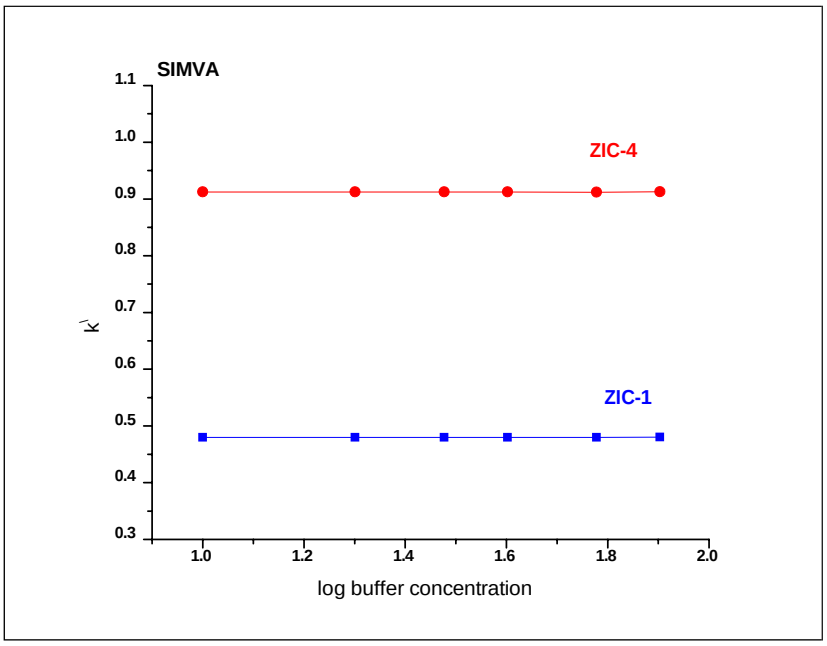


Figure 3.6: Effect of buffer concentration on behavior of SIMV using ZIC-1 & ZIC-4 columns.

3.3.1 The influence of pH on behavior of ATOR

The eluent pH can vary in order to provide a full indication of the ATOR separation in HILIC mode. As eluent pH has risen from 3 to 5.5, The ATOR retention factor decreased while buffer concentration has been preserved in 40 mM with ACN 80%, as shown in Figure (3.7). This is because the ATOR carboxyl group is deprotonated. This reflects the anticipated knowledge of ATOR physicochemical. The ATOR pKa is 4.31. Consequently, the ATOR is anionic form.

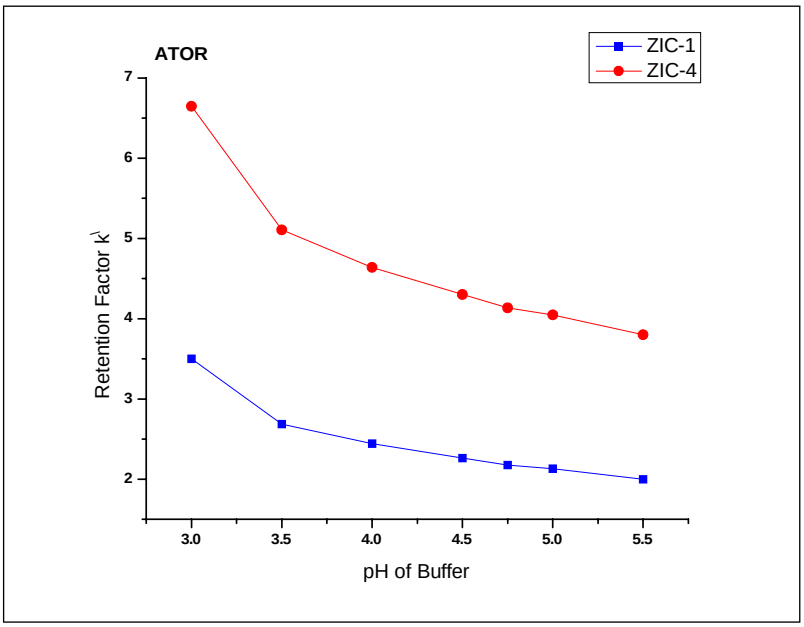


Figure 3.7: Effect of pH on behavior on behavior of ATOR using ZIC-1 & ZIC-4 columns.

3.3.2 The influence of pH on behavior of ROSU

The next improved composition of the eluent can be applied with a change in eluent pH. To complete ROSU separation in HILIC mode, the eluent pH must be changed. The pH improved from 3 to 5.5 at a steady buffer concentration of 40 mM and 80% ACN. As shown in Figure (3.8), ROSU retention factor decreases. This is because the carboxyl group is deprotonated in ROSU. This represents the physicochemical data of ROSU that are predicted. The pKa value 4 and isoelectric point 1.12 of ROSU. Hence the ROSU is anionic form.

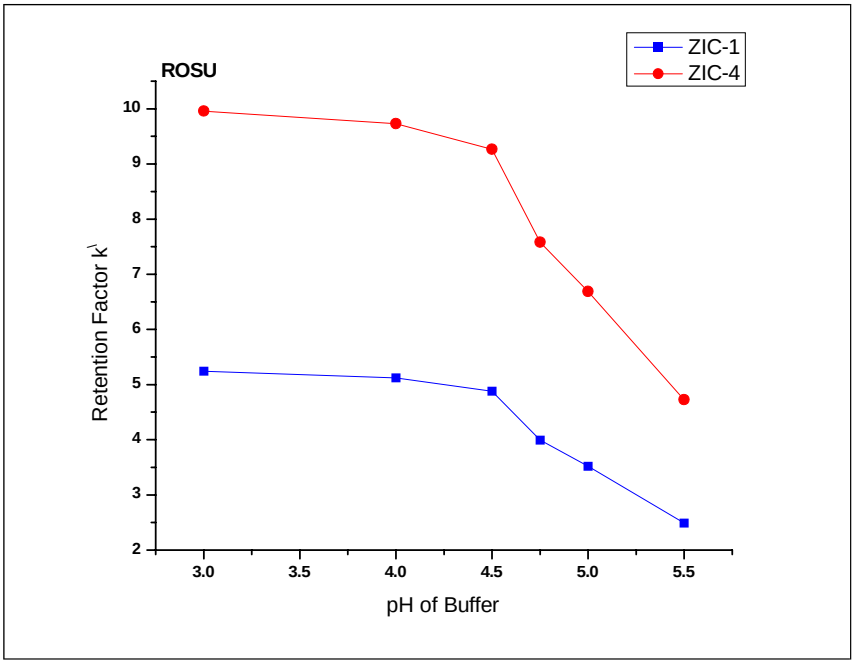


Figure 3.8: Effect of pH on behavior on behavior of ROSU using ZIC-1 & ZIC-4 columns.

3.3.3 The influence of pH on behavior of SIMVA

The eluent pH can vary in order to provide a full indication of the SIMV separation in HILIC mode. As eluent pH has risen from 3 to 5.5, The SIMV slight retention factor decreased while buffer concentration has been preserved in 40 mM with ACN 80%, as shown in Figure (3.9). Due to the charging unchanged (neutral state) of SIMV, the SIMV demonstrates a very slight decrease in interaction in the pH range (3-5.5).

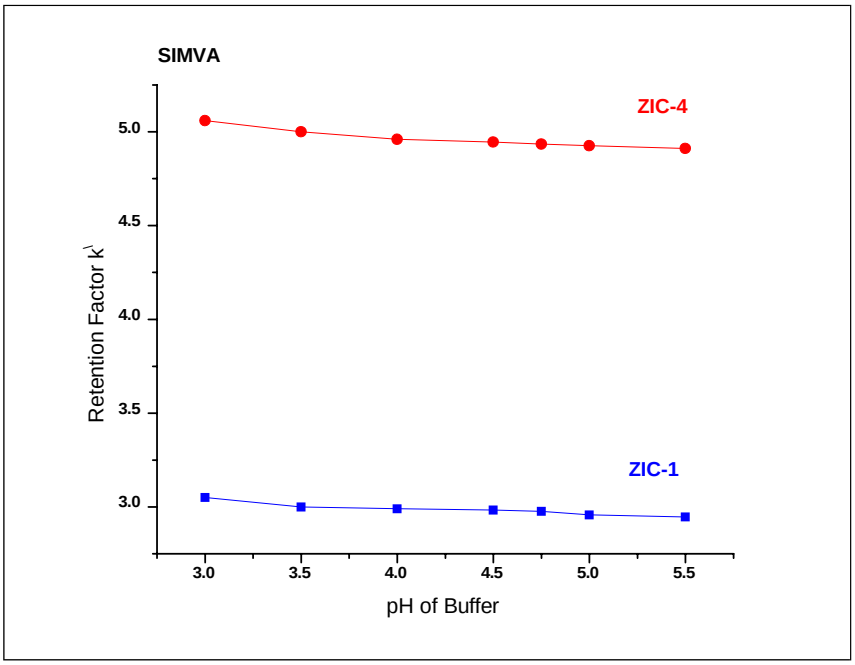


Figure 3.9: Effect of pH on behavior on behavior of SIMV using ZIC-1 & ZIC-4 columns.

3.4 Optimizing the separation of pharmaceuticals:

The retention in ZIC-4 column for ATOR, ROSU and SIMV shows the highest retention compared to three pharmaceuticals in the ZIC-1. The unavoidable explanation for this is the methylene group in ZIC-HILIH columns between charged groups [204, 205]. In ZIC-4 exchanger the strongest pharmaceutical retention tends to be a geometrical arrangement of the sulfo betaine groups. Such interactions occur because the sulfo betaine chains have different flexibilities that affect their ability to form intra- and intermolecular ion pairs. Therefore, the spacers between the charges of stationary phases should have an impact on pharmaceutical retention. ATOR, ROSU and SIMV were chosen as test pharmaceuticals for a study on their retention mechanism in HILIC mode by applying a NaOAc/HAc buffer mobile phase with varying ACN content on the two of ZIC-HILIC columns. The chromatograms Figures (3.10-3.11) were generated at a NaOAc / HAc buffer of 80% ACN and 40 mM (pH 4.75). Table 3.2 includes a description of the chromatographic conditions.

Table 3.1: The chromatographic conditions of the suggested methods.

Chromatographic conditions	
UV-Detector	240 nm
Injection volume	10 µL
Flow rate	0.5 mL/min
Temperature	25 ^{o}C
Mobile phase	ACN/ (NaOAc-HAc Buffer)
Buffer, ACN %, PH	80% / 20% (40 Mm-pH 4.75)

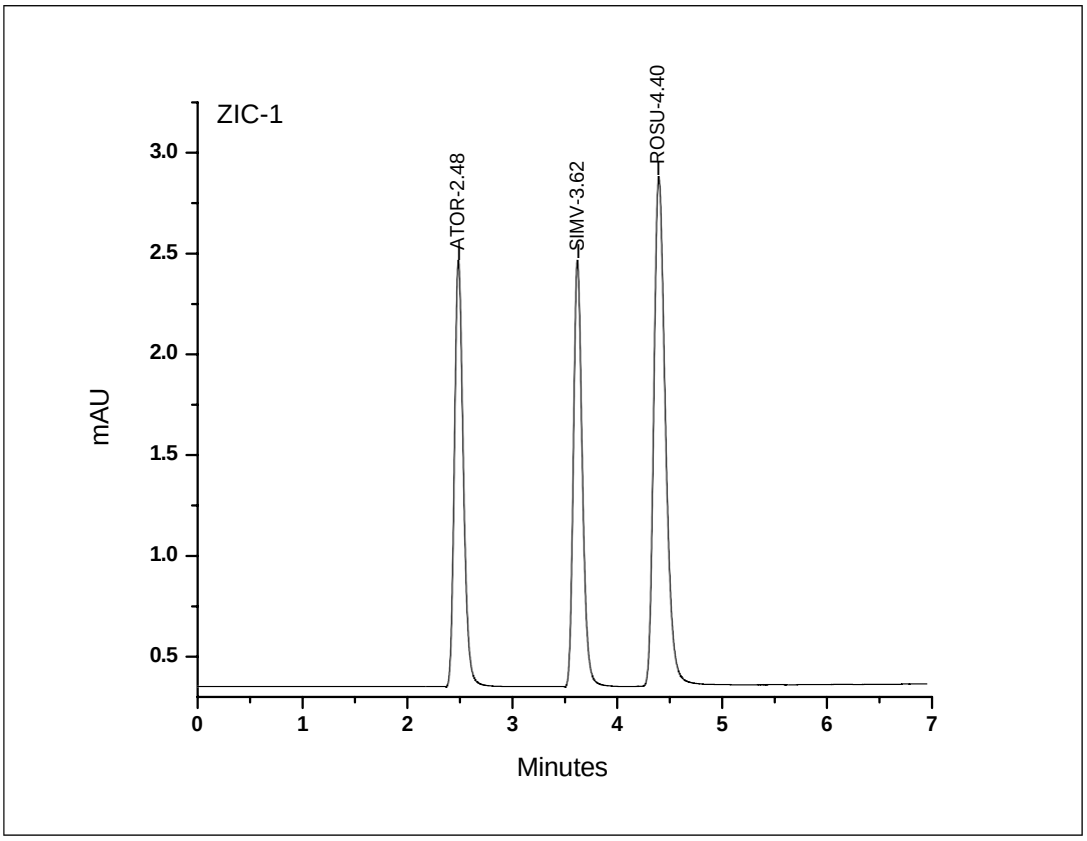


Figure 3.10: Chromatogram for the separations of 0.1 mg/kg (ATOR, ROSU and SIMV) in ZIC-1 column.

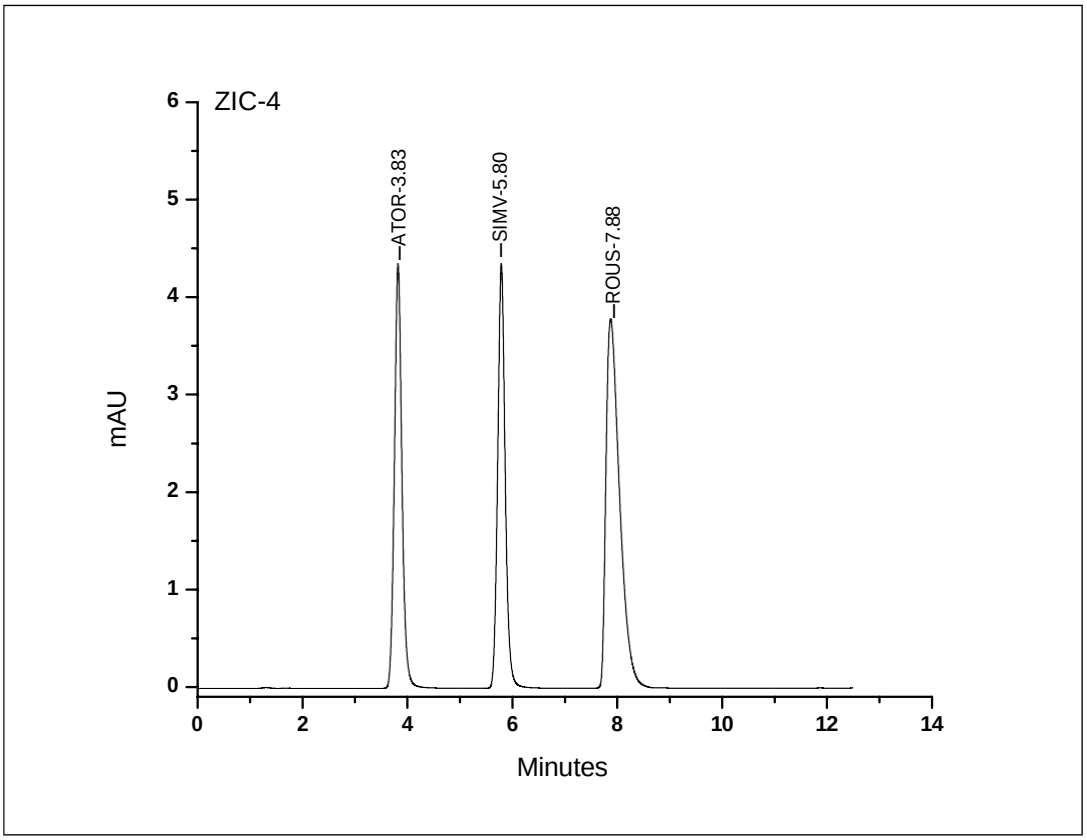


Figure 3.11: Chromatogram for the separations of 0.1 mg/kg (ATOR, ROSU and SIMV) in ZIC-4 column.

3.5 Method Validation

3.5.1 Linearity and calibration of ATOR

An atorvastatin calibration graphs showing the range of concentration (0.1-7 and 0.1-7 µg mL-1) respectively of ZIC-1, ZIC-4 columns, defined by plotted area versus atorvastatin concentrations Figures (3.12-3.13).

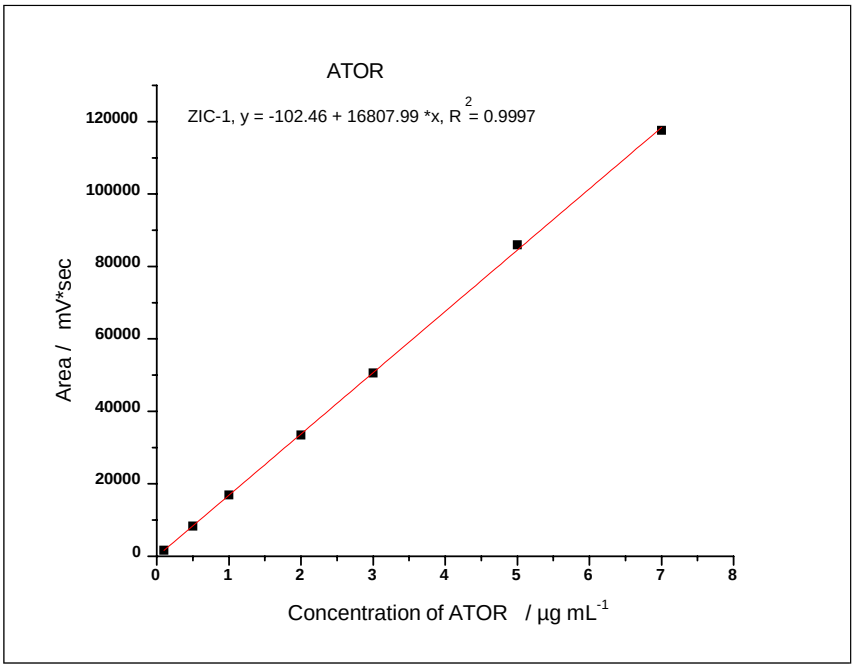


Figure 3.12: Calibration graph for ATOR using ZIC-1 column.

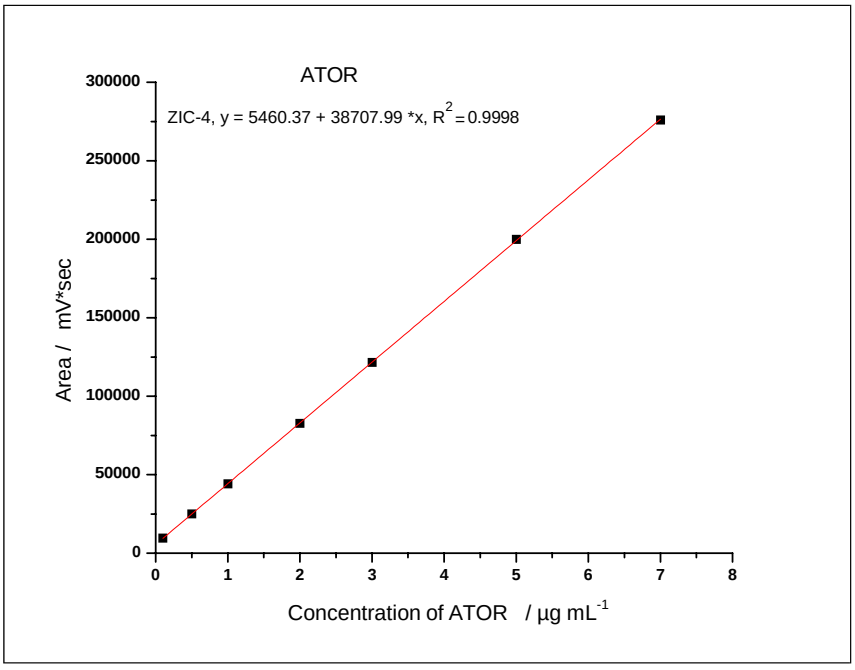


Figure 3.13: Calibration graph for ATOR using ZIC-4 column.

3.5.2 Statistical data analysis of ATOR

The direct calibration graphs were developed and the statistical results shown in Table 3.3 to specifically evaluate ATOR under ZIC-HILIC conditions. Accuracy and precision were tested on the same day and daily basis by percent recover and RSD% measurement,

respectively. The relative deviations from the low standard and the high recovery values suggest that the procedure proposed is accurate Table 3.4.

Table 3.2: Result analytical features- ATOR.

Parameter	ZIC-1	ZIC-4
Conc. Range (µg ml^{-1})	0.1-7.0	0.1-7.0
Linearity R^2	0.9997	0.9998
LOD (µg mL^{-1})	0.0094	0.0081
LOQ (µg mL^{-1})	0.0284	0.0245

Table 3.3: The suggested methods precision and accuracy- ATOR on the same day and on daily.

Same-Day Analysis (n=5)				Day-to-Day Analysis (n=5)		
ZIC-1 stationary phase						
ATOR Added (µg mL^{-1})	**ATOR Obtained (µg mL^{-1})**	**Rec. %**	**RSD (%)**	**ATOR Obtaine (µg mL^{-1})**	**Rec.%**	**RSD (%)**
1.00	0.995	99.50	0.56	0.994	99.40	0.93
2.00	1.980	99.00	0.12	1.98	99.00	0.13
ZIC-4 stationary phase						
ATOR Added (µg mL^{-1})	**ATOR Obtained (µg mL^{-1})**	**Rec.%**	**RSD (%)**	**ATOR Obtained (µg mL^{-1})**	**Rec. %**	**RSD (%)**
1.00	0.993	99.70	0.48	0.993	99.30	0.35
2.00	1.986	99.30	0.64	1.988	99.90	0.28

3.5.3 Determination of Atorvastatin in pharmaceutical formulations

In three of the pharmaceutical products containing ATOR (tablets) at a specified concentration of 10, 20 and 10 mg per unit the proposed procedure was successfully applied, the results obtained are shown in Table 3.5.

Table 3.4: Application in the pharmaceutical preparation of the proposed ATOR methods.

Trade Name	Started conc. (mg)	Get it (mg)	%Rec.	%RSD n=5
	ZIC-1 stationary phase			
Atorvastatin	20	19.91	99.55	0.32
Lipitor	20	20.07	100.35	0.64
Siros	10	9.97	99.70	0.40
	ZIC-4 stationary phase			
	20	19.93	99.65	0.43
	20	20.04	100.20	0.56
	10	9.93	99.30	0.28

The achieved results were contrasted with the results obtained using United States Pharmacopeia protocol [206] to determine the competence and performance of the proposed ZIC-1 and ZIC-4 methods. The results obtained using the two methods, t-test and variance ratio F-test at 95% confidence level Table 3.6, were statistically compared. The determined t and F values did not exceed the theoretical values, suggesting that the accuracy of both methods in the determination of ATOR in pharmaceutical samples does not differ significantly.

Table 3.5: The comparison of the proposed methods ZIC-1 and ZIC-4 with standard method for ATOR analysis by examining t- and F-statistical tests.

Name of drug	**ZIC-1 method**	**ZIC-4 method**	Standard **method [202]**	**t-Test (theor.)**	**F-Test (theor.)**
Atorvastatin	99.55	99.65	99.85	0.76701* (2.7764)	1.0038* (19.000)
Lipitor	100.35	100.20	100.45	0.5086** (2.7764)	0.46670** (19.000)
Siros	99.70	99.30	99.63		

*For ZIC-1 proposed method

**For ZIC-4 proposed method

- Average of five determinations.

$$^{*}\mathrm{t-test} = \frac{|\bar{x}_1 - \bar{x}_2|}{s\sqrt{(\frac{1}{n_1}+\frac{1}{n_2})}}$$

$$s = \frac{\sqrt{(n_1-1)s_1^2-(n_2-1)s_2^2}}{n_1+n_2-2}, \qquad s^2_1 = \frac{\sum(x_i-\bar{x})_1^2}{n_1-1}, \qquad s^2_2 = \frac{\sum(x_i-\bar{x})_2^2}{n_2-1},$$

(n1 + n2 – 2) = number of degrees of freedom.

(n1 – 1) and (n2 – 1) = number of degrees of freedom of proposed method and standard method respectively.

$$^{***}\boldsymbol{F} = \frac{s_1^2}{s_2^2}$$

3.5.4 Linearity and calibration of ROSU

A Rosuvastatin calibration curves that show the linear range (0.1-7 and 0.1-7 µg mL-1) of Rosuvastatin using ZIC-1, ZIC-4 columns respectively, by the peak area (mV*sec) plotting against the concentration of ROSU Figures (3.14-3.15).

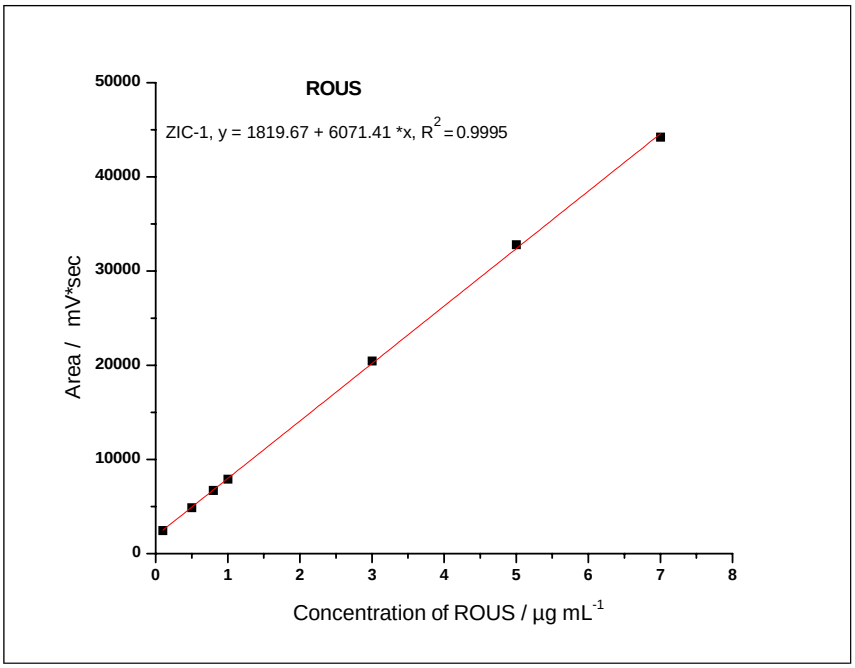


Figure 3.14: Calibration graph for ROSU using ZIC-1 column.

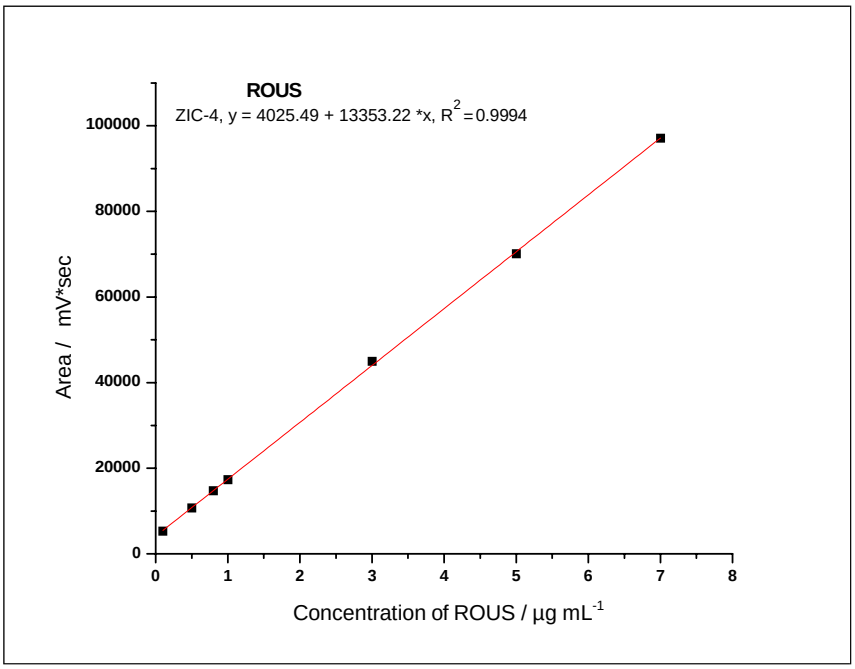


Figure 3.15: Calibration graph for ROSU using ZIC-4 column.

3.5.5 Statistical data analysis of ROSU

The direct calibration graphs were established and statistical results outlined in Table 3.7 for direct determination of ROSU in HILIC conditions. The accuracy and precision of the same day as well as of the day were analyzed and calculated in percentage and RSD% respectively. The low relative standard deviation values and the high recovery values refer. That the proposed method is precise, Table 3.8

Table 3.6: Result analytical features- ROSU.

Parameter	ZIC-1	ZIC-4
Conc. Range (µg ml^{-1})	0.1-7.0	0.1-7.0
Linearity R^2	0.9994	0.9995
LOD (µg ml^{-1})	0.0020	0.0013
LOQ (µg ml^{-1})	0.0060	0.0039

Table 3.7: The suggested methods precision and accuracy-ROSU on the same day and on daily.

Same-Day Analysis (n=5)				Day-to-Day Analysis (n=5)		
ZIC-1 stationary phase						
ROUS Added (µg mL^{-1})	**ROUS Obtained (µg mL^{-1})**	**Rec. %**	**RSD (%)**	**ROUS Obtained (µg mL^{-1})**	**Rec. %**	**RSD (%)**
1.00	1.01	101.00	0.63	1.00	100.00	0.74
2.00	2.02	101.00	0.78	2.01	100.50	0.84
ZIC-4 stationary phase						
ROUS Added (µg mL^{-1})	**ROUS Obtained (µg mL^{-1})**	**Recovery %**	**RSD (%)**	**ROUS Obtained (µg mL^{-1})**	**Recovery %**	**RSD (%)**
1.00	1.00	100.00	0.55	1.00	100.00	0.91
2.00	2.01	100.5	0.65	2.01	100.5	0.14

3.5.6 Determination of ROSU in pharmaceutical formulations

In three of the pharmaceutical products containing ROSU (tablets) at a specified concentration of 10 mg per unit the proposed procedures were successfully applied, the results obtained are shown in Table 3.9.

Table 3.8: Application in the pharmaceutical preparation of the proposed ROSU methods.

Trade Name	Started conc. (mg)	Get it (mg)	%Rec.	%RSD n=5
ZIC-1 stationary phase				
Rosuvastatin Denk	10	10.08	100.80	0.51
CRESTOR	10	9.99	99.80	1.16
Lodal	10	9.93	99.30	0.26
ZIC-4 stationary phase				
	10	10.12	101.20	0.34
	10	10.00	100.00	1.19
	10	9.95	99.50	0.66

The achieved results were contrasted with the results obtained using United States Pharmacopeia protocol [206] to determine the competence and performance of the proposed ZIC-1 and ZIC-4 methods. The results obtained using the two methods, t-test and variance ratio F-test at 95 percent confidence level Table 3.10, were statistically compared. The determined t and F values did not exceed the theoretical values, suggesting that the accuracy of both methods in the determination of ROSU in pharmaceutical samples does not differ significantly.

Table 3.9: The comparison of the proposed methods ZIC-1 and ZIC-4 with standard method for ROSU analysis by examining t- and F-statistical tests.

Name of drug	ZIC-1 method	ZIC-4 method	Standard method [202]	t-Test (theor.)	F-Test (theor.)
Rosuvastatin Denk	100.80	101.20	99.95	0.6007* (2.7764)	3.9619* (19.000)
CRESTOR	99.80	100.00	100.68	0.9818** (2.7764)	5.1845** (19.000)
Lodal	99.30	99.50	100.11		

3.5.7 Linearity and calibration of SIMVA

A Simvastatin calibration curves that show the linear range (0.05-4 and 0.05-4 µg mL-1) of Simvastatin using ZIC-1 and ZIC-4 columns respectively, by the peak area (mV*sec) plotting against the concentration of Simvastatin Figures (3.16 and 3.17).

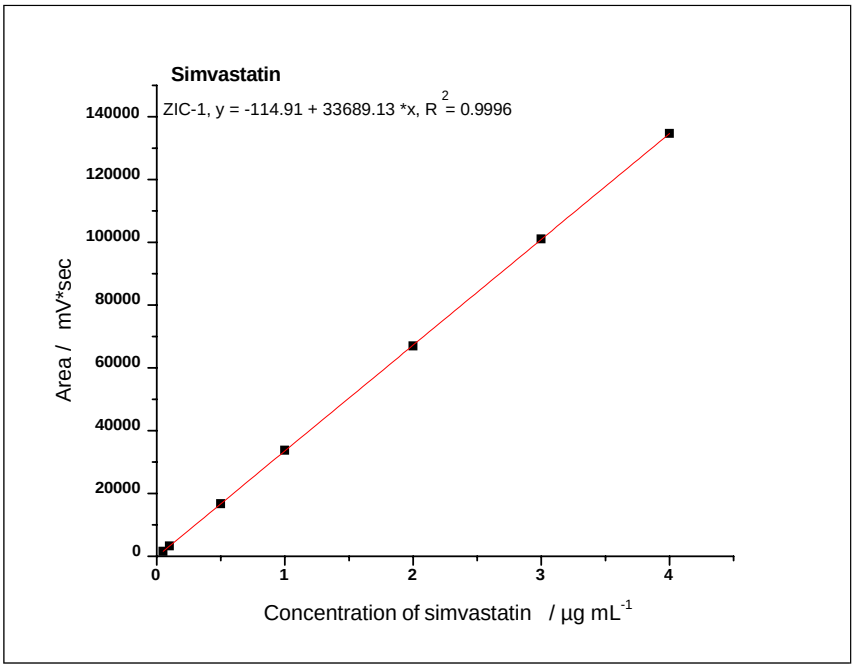


Figure 3.16: Calibration graph for SIMVA using ZIC-1 column.

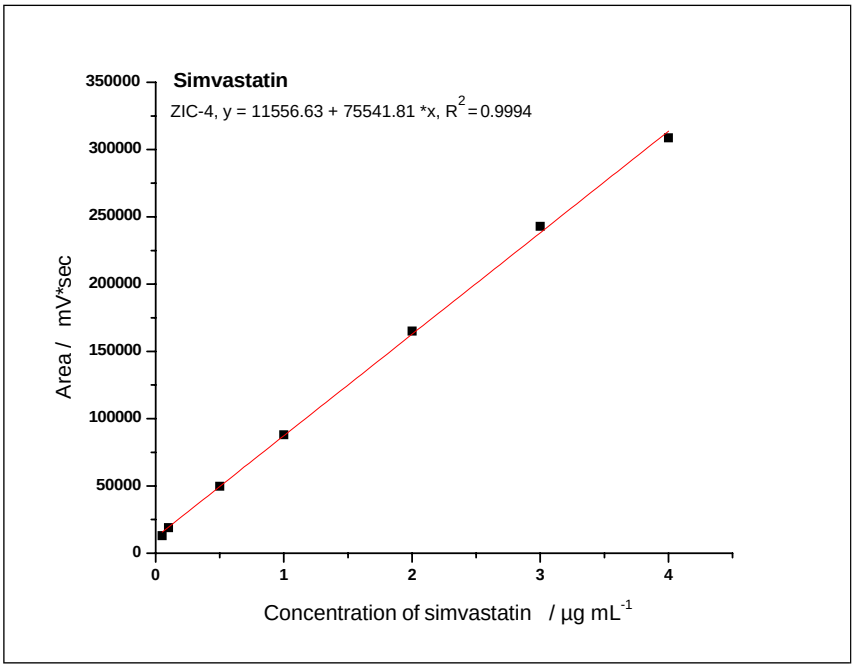


Figure 3.17: Calibration graph for SIMVA using ZIC-4 column.

3.5.8 Statistical data analysis of SIMVA

The direct calibration curves were established and statistical results outlined in Table 3.11 for direct determination of SIMV in HILIC conditions. The accuracy and precision of the same day as well as of the day were analyzed and calculated in percentage and RSD percent respectively. The low relative standard deviation values and the high recovery values refer that the proposed method is precise, Table 3.12.

Table 3.10: Result analytical features- SIMV.

Parameter	ZIC-1	ZIC-4
Conc. Range (µg mL^{-1})	0.05-4	0.05-4
Linearity R^2	0.9996	0.9994
LOD (µg mL^{-1})	0.023	0.015
LOQ (µg mL^{-1})	0.069	0.045

Table 3.11: The suggested methods precision and accuracy-SIMV on the same day and on daily.

Same-Day Analysis (n=5)				Day-to-Day Analysis (n=5)		
ZIC-1 stationary phase						
SIMV Added (µg mL^{-1})	**SIMV Obtained (µg mL^{-1})**	**Rec. %**	**RSD (%)**	**SIMV Obtained (µg mL^{-1})**	**Rec. %**	**RSD (%)**
1.00	0.991	99.10	0.55	0.991	99.10	0.59
3.00	2.990	99.66	0.44	2.992	99.73	0.65
ZIC-4 stationary phase						
SIMV Added (µg mL^{-1})	**SIMV Obtained (µg mL^{-1})**	**Recovery %**	**RSD (%)**	**SIMV Obtained (µg mL^{-1})**	**Recovery %**	**RSD (%)**
1.00	1.010	101.00	0.36	1.020	102.00	0.47
3.00	2.995	99.83	0.42	2.995	99.83	0.68

3.5.9 Determination of SIMVA in pharmaceutical formulations

In three of the pharmaceutical products containing SIMV (tablets) at a specified concentration of 20 and 10 mg per unit the proposed procedures were successfully applied, the results obtained are shown in Table 3.13.

Table 3.12: Application in the pharmaceutical preparation of the proposed SIMV methods.

Trade Name	Started conc. (mg)	Get it (mg)	%Rec.	%RSD n=5
ZIC-1 stationary phase				
Simvastatin	20	20.05	100.25	0.36
Simvastatin	20	19.90	99.50	0.56
Simvastatin	10	9.94	99.40	0.50
ZIC-4 stationary phase				
	20	20.07	100.35	0.55
	20	19.93	99.65	0.43
	10	9.97	99.70	0.27

The achieved results were contrasted with the results obtained using United States Pharmacopeia protocol [206] to determine the competence and performance of the proposed ZIC-1 and ZIC-4 methods. The results obtained using the two methods, t-test and variance ratio F-test at 95 percent confidence level Table 3.14, were statistically compared. The determined t

and F values did not exceed the theoretical values, suggesting that the accuracy of both methods in the determination of SIMV in pharmaceutical samples does not differ significantly.

Table 3.13: The comparison of the proposed methods ZIC-1 and ZIC-4 with standard method for SIMV analysis by examining t- and F-statistical tests.

Name of drug	ZIC-1 method	ZIC-4 method	Standard method [202]	t-Test (theor.)	F-Test (theor.)
Crescent Pharma-UK	100.25	100.35	99.34	0.8362*	0.6709* (19.000)
Pharma International-Jordan	99.50	99.65	100.44	0.8320** (2.7764)	0.4740** (19.000)
Mylan-New Zealand	99.40	99.70	99.65		

3.6 Anthropometric Analysis

50 persons were examined and divided into two groups where 20 healthy persons aged from (35 to 60 years and a control group consists of 12 males and 8 females). Patients group consisting of 30 patients (Before and After) treatment who visited Baghdad Teaching Hospital, included 14 males and 16 females with the age ranging from 33 to 78 years. The obtained results of this study and presented in **Appendix 2.**

3.6.1 Age

Atherosclerotic cardiovascular disease (ASCVD) continues to be the largest cause of death worldwide, accounting for the majority of fatalities among older individuals (aged ≥60 years) [207, 208]. Additionally, the worldwide population of elderly adults is predicted to more than double by 2050 [208]. Elderly persons frequently have a number of comorbid conditions, including hypertension, diabetes, obesity, and hyperlipidemia, all of which enhance the risk of ASCVD events. Additionally, the majority of ASCVD occurrences occur after the age of 65 and are more disabling in this demographic than in younger individuals [209]. The results of the current study in Table 3.15 showed that there were no significant differences in age means between the two groups of healthy controls and Hyperlipidemia patients (P= 0.150) where the age means were (45.45 ± 7.265) and (50.06 ± 12.76) for control and patients respectively.

Table 3.14: Descriptive of age and compare between control and patient.

Parameter	Status	N	Mean	SD	t-test	p-value	Sig
Age (year)	Control	20	45.4500	±7.26546	1.464	0.150	NS
	Patient	30	50.0667	±12.76021			

*P>0.05 Non significant

In comparison to younger patients, elderly dyslipidemia patients are at a higher risk of clinically significant drug-drug interactions (PDDIs), mostly due to the increased number of medications prescribed. Additionally, individuals aged ≥75 years were prescribed a greater number of medicines with a high risk of developing DDIs, particularly those used to treat arrhythmias and heart failure. The risk of adverse responses linked with the possibility of drug-drug interactions (PDDIs) can frequently be decreased through dose adjustment, attentive monitoring, or substitution of an alternate medication [210]. Giral et al. [211] reported that statin discontinuation was associated with a 33% increased risk of admission for cardiovascular event in 75-year-old primary prevention patients.

3.6.2 Body mass index

As shown in Table 3.16, the mean of BMI (kg/m2) has highly significant differences (P= <0.001) between group control group and patient group which indicate a significant increase in patients' group (28.796 ± 4.1) compared to control group (23.07 ± 1.24). These results are backed by those of previous study [191].

Table 3.15: Descriptive of Body Mass Index and compare between control and patient.

Parameter	Status	N	Mean	SD	t-test	p-value	Sig
BMl (Kg/m^2)	Control	20	23.0700	±1.24016	6.035	0.001	HS
	Patient	30	28.7967	±4.10496			

*P< 0.001 High significant

Our findings, along with those of Wenwang Rao et al. [212], indicated a strong connection between various BMI ranges and hyperlipidemia (p<0.001). After adjustment for confounding variables, overweight (OR = 1.651, 95% CI: 1.520–1.793), and obesity (OR = 1.714, 95% CI: 1.457–2.017) were independently related with an elevated risk of hyperlipidemia (p<0.001). Additionally, this finding indicates a large and progressive risk of hyperlipidemia as BMI increases.

3.6.3 Blood Pressure

The mean of blood pressure (mmHg) was recorded a highly significant increasing in both systolic (P= 0.001) and diastolic blood pressures (P= 0.001) as shown in Table 3.17 and Figure 3.18. The level of DBP was increased in patients' group (96.8 ± 17.71) compared with control

group (79.65 ± 1.69). Also, the level of SBP was significantly increased in patients' group (147.76 ± 26.6) compared with control group (119.45 ± 0.759) and at same time. These finding are in agreement with Sung and colleagues reported that cholesterol reduction achieved by lovastatin 20 mg/day for 6 weeks was associated with a marginal decrease in systolic BP levels (by 3 mmHg) in 37 normotensive hypercholesterolemic patients [213]. Systolic BP response to mental stress significantly decreased (by 8 mmHg, $p < 0.05$) following lovastatin treatment in these patients [214]. Likewise, 20-week atorvastatin treatment decreased both systolic and diastolic BP (mean reductions by 6 and 3 mmHg, respectively) in patients with severe hypercholesterolemia (LDL-C levels > 170 mg/dl) [215].

This benefit was also evident even in patients with normal baseline BP levels, and was associated with a statin-related increase in the small artery wall elasticity by 21%. A large-scale clinical study assessed the effect of two different statins (pravastatin 40 mg/day and simvastatin 20 mg/day) on BP levels of 973 patients with mild-to moderate hypercholesterolemia within the first month and at 6 months in the course of treatment [216]. A drop in systolic and diastolic BP measurements (by 2.2 and 2.4 mmHg; by 1.5 and 2.3 mmHg for pravastatin; by 2.9 and 3.0 mmHg for simvastatin, respectively) was evident with both statins as compared with placebo after 6 months of treatment [217]

Nevertheless, BP reductions with statins were suggestive but not significant after 1 month of treatment. Of note, in this study the antihypertensive effect of statin treatment was not limited to patients with high baseline BP levels, but was also observed in patients who exhibited 'prehypertension' as well as in those with low-normal BP. The BP-lowering potency of the lipophilic simvastatin was similar to this of hydrophilic pravastatin. The Brisighella Heart Study was a large-scale randomized clinical trial assessing the impact that various lipid-lowering strategies may have on BP in 1356 subjects with hypercholesterolemia (total cholesterol ≥ 239 mg/dl) [217].

Table 3.16: Compare Blood pressure between control with patient (Before and after) treatment.

Parameter	Status	N	Mean	SD	t-test	P-value	Sig
SBP (mmHg)	Before	30	147.7667	±26.60915	4.741	0.000	HS
	After	30	126.4333	±7.49567	4.138	0.00	HS
	Control	20	119.4500	±.75915			
DBP (mmHg)	Before	30	96.8333	±17.71242	4.311	0.000	HS
	After	30	83.8667	±5.18442	3.502	0.01	S
	Control	20	79.6500	±1.69442			

*P<0.05 Significant

**P<0.001 High significant

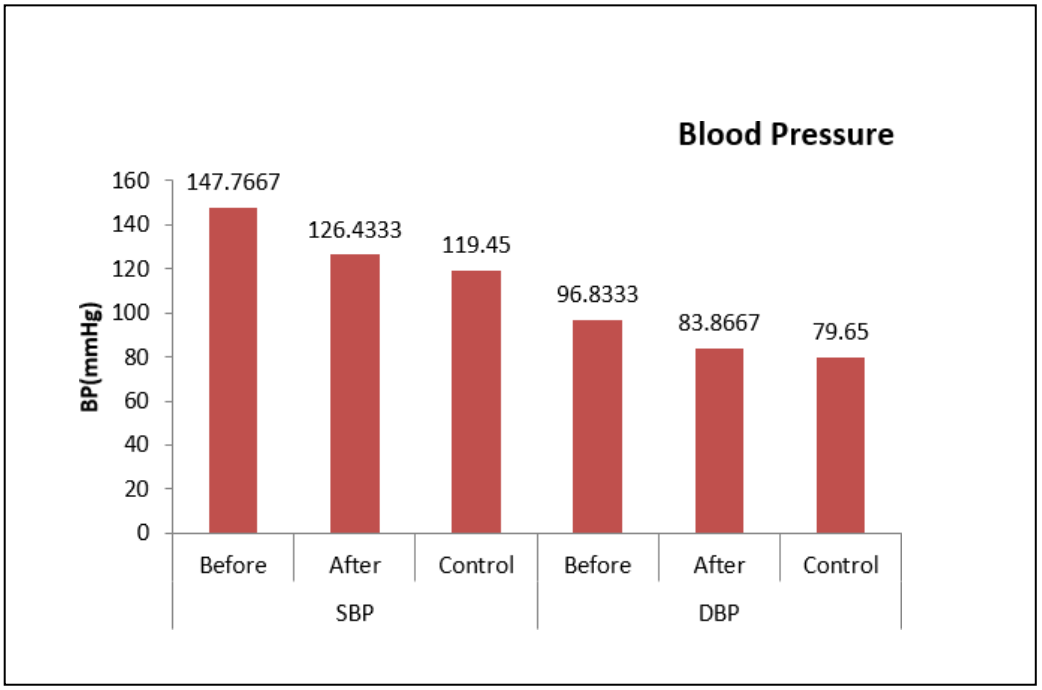


Figure 3.18: Compare Blood pressure between control with patient (Before and after) treatment.

Furthermore, statin treatment for 6 months had no effect on BP and heart rate in 59 patients with hypercholesterolemia irrespective of any cholesterol lowering effect of this statin [218]. In a subgroup of patients who experienced a sustained cholesterol-lowering effect (over a 5-year follow-up), BP and heart rate remained unchanged [218]. Finally, several statins (simvastatin 10 -- 20 mg/day, and atorvastatin 5 -- 10 mg/day) failed to produce significant

changes in ambulatory BP levels of 10 normotensive patients with hyperlipidemia after 2 months of treatment [219].

3.7 Biochemical Variables

3.7.1 Lipid Profile

3.7.1.1 Total Cholesterol (T.Cho), Triglyceride (T.g), Low Density Lipoprotein (LDL), Very Low Density Lipoprotein (VLDL), High Density Lipoprotein (HDL).

Typically, combined hyperlipidemia is defined by an increase in total cholesterol and triglycerides and a reduction in high-density lipoprotein (HDL) cholesterol. This prevalent condition is caused by an excess of apolipoprotein B generated in the liver in very low-density lipoproteins. Increased levels of lipoproteins containing apolipoprotein B, most notably low-density lipoprotein (LDL) particles, are a significant risk factor for coronary heart disease (CHD). Statins are quite successful at lowering both total and LDL cholesterol [220-222].

Table 3.18 and Figure 3.19 show a significant difference between control group and patients group. The mean of T. Cho (mg/dL) was appeared to be high significantly increased (P= <0.001) in patients group (226.166 ± 53.9) compared with control group (149.4 ± 21.84), the mean of Tg (mg/dL) was appeared to be significantly increased (P = 0.011) in patients group (212.633 ± 119.57) compared with control group (141.45 ± 8.75), The mean of VLDL (mg/dL) was appeared to be significantly increased (P = 0.011) in patients group (42.52 ± 23.91) compared with control group (28.29 ± 1.75), The mean of LDL (mg/dL) was appeared to be significantly increased (P= 0.001) in patients group (130.46 ± 54.73) compared with control group (67.26 ± 22.99), The mean of HDL (mg/dL) was appeared to be no significantly increased (P = 0.85) in patients group (53.17 ± 13.98) compared with control group (53.84 ± 8.89). may be because the small dose they used, according to (the CURVES study, 1998) reported When HDL-C was elevated, meaningful results were detected only at a dose of 40 mg. This effect, however, was larger with simvastatin than with atorvastatin .

On the other hand, Vodonos et al. [223] found no correlation between an increase in compliance and an increase in HDL-C levels. This is likely due to the inclusion of simvastatin and lovastatin rather than atorvastatin and rosuvastatin, which are associated with a greater reduction in LDL-C and TG, resulting in a greater increase in HDL-C levels. Vodonos et al. [223] reported a plateau in LDL-C levels when compliance above 80%, but did not estimate HMGCoA-R levels in this scenario [223].

Table 3.17: Mean ± SD of lipid profile healthycontrol with patients before and after treatment.

Parameter	Status	N	Mean	SD	t-test	P-value	Sig
Cholesterol (mg/dl)	Before	30	226.16	53.90	6.030	0.001	HS
	After	30	161.70	44.53	1.114	0.251	NS
	Control	20	149.40	21.84			
Triglyceride (mg/dl)	Before	30	212.63	119.57	2.648	0.011	S
	After	30	173.20	93.78	1.505	0.139	NS
	Control	20	141.45	8.75			
VLDL (mg/dl)	Before	30	42.52	23.91	2.648	0.011	S
	After	30	34.64	18.75	1.505	0.139	NS
	Control	20	28.29	1.75			
LDL (mg/dl)	Before	30	130.46	54.73	4.872	0.001	HS
	After	30	65.03	37.96	0.235	0.815	NS
	Control	20	67.26	22.99			
HDL (mg/dl)	Before	30	53.17	13.98	0.190	0.850	NS
	After	30	62.17	19.78	1.763	0.084	NS
	Control	20	53.84	8.89			

According to the "Comparative Dose Efficacy Study of Atorvastatin Versus Simvastatin in Patients with Hypercholesterolemia" (the CURVES study), atorvastatin 10 mg reduced LDL-C better than simvastatin 10 mg (in descending order of efficacy) [224]. The same effect was observed for TC, where atorvastatin 10 mg, 20 mg, and 40 mg had a greater lowering impact than simvastatin at milligram-equivalent doses (descending order of efficacy). However, substantial variations in TG reduction were observed between atorvastatin and other statins at the 40 mg dose. When atorvastatin was used instead of simvastatin, the drop in TG was higher (descending order of efficacy) [225].

The CURVES study's findings were comparable to prior research on statins' lipid-lowering effects [226]. Simvastatin (10 mg - 40 mg) was observed to reduce LDL-C by 18% - 27%, whereas atorvastatin reduced LDL-C by 35% to 61%, establishing atorvastatin as the most efficient statin [224]. This conclusion corroborated previous comparative research including atorvastatin [227,228].

Jones et al. established data based on dose-to-dose comparisons of atorvastatin and rosuvastatin efficacy in decreasing LDL-C in the STLLAR study [229]. The results indicated that rosuvastatin 40 mg was the most effective, reducing LDL-C by 55.0 percent (-55 percent), followed by rosuvastatin 20 mg (-52.4 percent), atorvastatin 80 mg, atorvastatin 40 mg (-47.8 percent), rosuvastatin 10 mg (-45.8 percent), atorvastatin 20 mg (-42.6 percent), and atorvastatin 10 mg (-36.8 percent). Abhinav Grover et al. [230] found that rosuvastatin 10 mg,

20 mg, and 40 mg significantly reduced LDL-C more than simvastatin in all 14 pairwise comparisons.

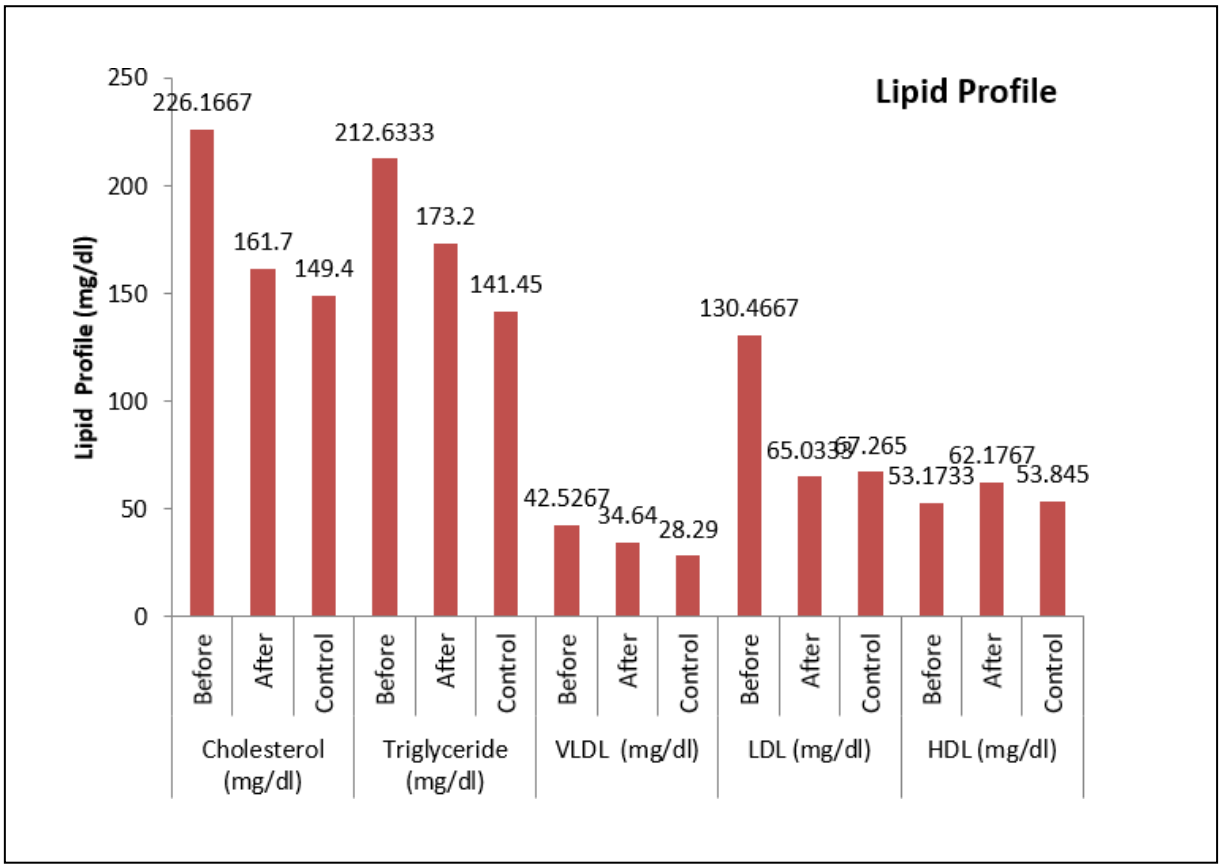


Figure 3.19: Mean ± SD of lipid profile healthy control with patients before and after treatment.

A previous study shown that there are considerable differences in the relative impact of various types of statins on lipid profile improvement. Rosuvastatin is widely regarded as the most effective statin for the treatment of dyslipidemia. Although atorvastatin has a considerable influence on the non-HDL-C profile, it has the least effect on HDL-C of any statin. To balance the benefits and hazards of statins in patients, two things should be emphasized: statins that are very effective at reducing cardiovascular and cerebrovascular events must be identified, as well as those that are well tolerated. There is still a lack of conclusive evidence about the association between statin type and statin efficacy and intolerance in Asians. As a result, additional large prospective trials should be done to elucidate specific relationship [225].

Other studies were conducted to examine the safety and efficacy of atorvastatin and simvastatin in patients with hyperlipidemia, taking into account prior results on the efficacy and safety of various statins. The results indicated that while all three medicines (atorvastatin,

simvastatin, and pravastatin) reduced cholesterol levels (LDLC, TC, TG, and VLDL), atorvastatin reduced them much more than simvastatin in both the first and second reviews. Our findings corroborated those of earlier writers [223, 230, 232]. Additionally, our findings indicate that atorvastatin considerably raises HDL-C levels when compared to simvastatin in a second review.

To summarize, our study found that atorvastatin is a more effective medicine in patients with hyperlipidemia than simvastatin or pravastatin. On this basis, if patients skip a few doses of atorvastatin or rosuvastatin in between, their cardiovascular benefits may not be jeopardized. The optimal therapeutic benefit of statins such as atorvastatin and rosuvastatin, despite their low compliance, may be a result of their pharmacokinetic (longer half-life) and pharmacodynamic (active metabolite) features, which may be sufficient to inhibit the HMGCoA-R enzyme effectively [233]. Additionally, this finding supports the use of statins every other day to treat dyslipidemia [234-236].

The previous study established a relationship and correlation between TC, TG, HDL-C, LDL-C, ApoA1, ApoB, and HMGCoA-R levels and statin compliance. These findings suggest that estimating HMGCoA-R levels in dyslipidemic patients taking statins should be investigated further as a tool for optimizing statin therapy and guiding physicians in dose modification decisions and patient counseling for improved compliance [230].

3.7.2 Measurement Activity of 3-hydroxy-3-methylglutaryl coenzyme-A (HMG-CoA) reductase by ELISA KIT.

Hypercholesterolemia is primarily caused by elevated plasma cholesterol levels, notably low-density lipoprotein (LDL) and triglyceride levels, which can also result in other disorders such as obesity, diabetes, and cancer [237, 238]. The enzyme 3-hydroxy-3-methylglutaryl-

This study was unique to estimate the activity of 3-hydroxy-3-methylglutaryl coenzyme-A (HMG-CoA) reductase in human by ELISA kit because no previous study estimated reductase enzyme by ELISA KIT. The rate-limiting enzyme in the MVA to cholesterol biotransformation pathway is 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase. As a result, it is critical for cholesterol homeostasis [239]. While methods for determining HMG-CoA reductase mRNA and protein expression in diverse regulatory systems are well established [240], a sensitive and cost-effective test for HMG-CoA reductase activity has not been developed.

Conventional methods for determining HMG-CoA reductase activity include elaborate radiochemical approaches [241], chromatographic techniques combined with mass spectrometry (LC/MS) [242], or spectrophotometric monitoring of the decrease in the cofactor NADPH absorbance at 340 nm [243].

In this study the statin drug as showed in Table 3.19 and Figure 3.20, atorvastatin and rosuvastatin, inhibited HMG-CoA reductase activity in a concentration-dependent manner, which found significant decreased in HMG-CoA reductase activity in patients with hypercholesterolemia after statin treatment than before statins treatment (15.7± 7.5; 26.3± 11.1) respectively, which agreement with Gai Liang et al. [244] who found significant decreased in rat HMG-COA activity after and before statins drugs .

Freeman et al. [245] found significant decreased in the activity of HMG-COA in hyperlipidemic patients after statins treatment than patients before statins administration (7.7 +/- 2.6 to 3.6 +/- 0.5 (P less than 0.05) that finding consistent with present study. But disagree with freeman who found the Mean reductase activity was 5.5 +/- 1.0 (n = 21) in hypercholesterolemic subjects versus 11.3 +/- 1.0 in 52 normal subjects (P less than 0.01) while our study revealed mean reductase activity in patients and control was (26.3± 11.1; 6.5 ± 2.6) respectively. This is consistent with the concept that the fundamental abnormality in these patients is impaired low-density lipoprotein (LDL) clearance rather than increased cholesterol production. This means that elevated LDL levels inhibit gut reductase function.

Table 3.18: Mean ± SD of HMGCR Enzyme Activity Between Control and Patient (Before and After) Treatment.

Parameter	Status	N	Mean	SD	t-test	p-value	Sig
HMG CoA Elisa	Before	30	26.39	±11.13	7.814	0.000	HS
	After	30	15.71	±7.50	5.255	0.00	HS
	control	20	6.50	±2.65			

**P<0.001 High significant

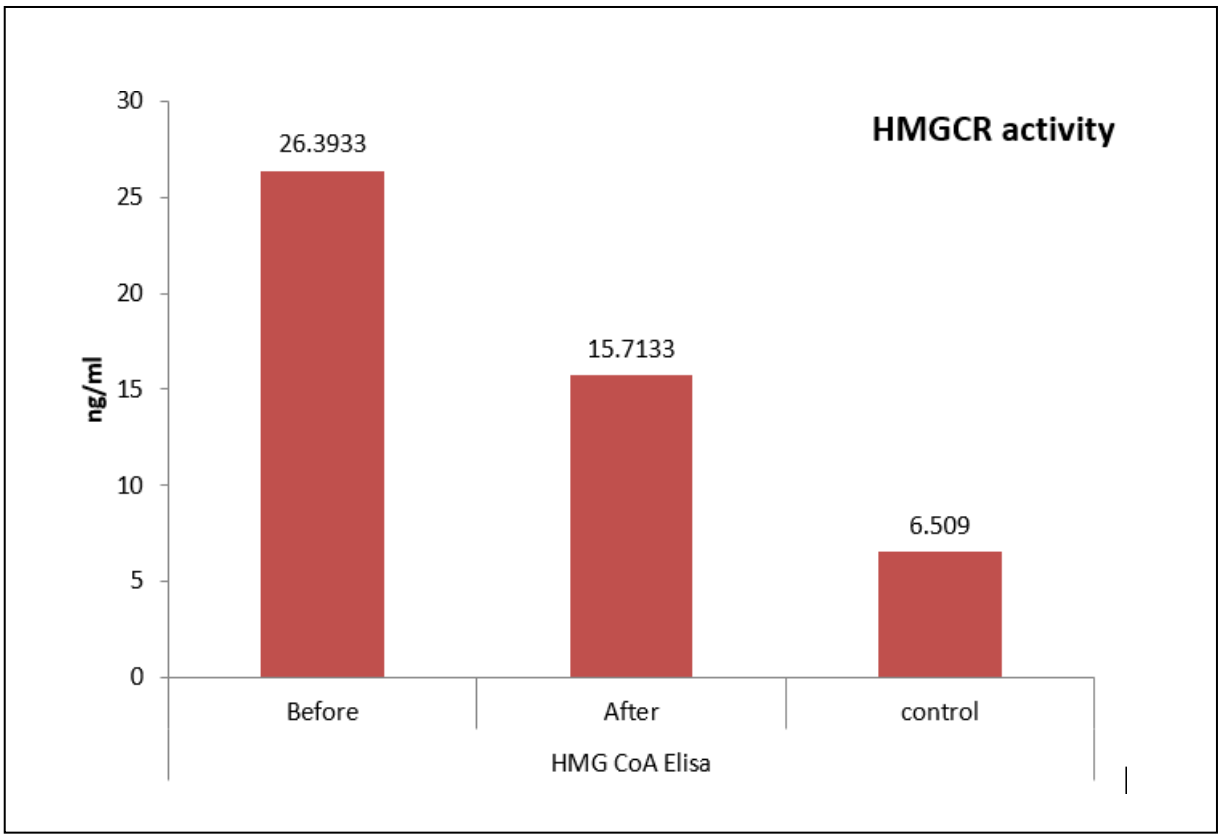


Figure 3.20: HMG-CoA Enzyme Activity by ELISA Between Control and Patient (Before and After) Treatment

3.7.3 A new modified method for determining 3-hydroxy-3-methylglutaryl coenzyme-A (HMG-CoA) reductase activity.

Dyslipidemia (often high serum TC, TG, and LDL-C levels, with decreased HDL-C levels) is a metabolic abnormality that is a significant risk factor for atherosclerosis and cardiovascular disease [246, 247]. To determine the activity of HMG-CoA reductase, a new modified method has created in this study based on genomic DNA extraction from the whole blood samples by using two methods, manual and automated. The activity of HMGCR enzyme was treated with statins drug for two weeks, and measured the activity of enzyme in hyperlipidemia patient and compared with healthy control. This study has shown in Table 3.20 and Figure 3.21, a significant difference between patients group and control group. The mean of HMGCR activity (copy no./µl) was appeared to be significantly increased (P= 0.002) in patients group before treatment (348442.4 ± 485652.53) compared with control group (110.7 ± 20.424).

While there was no significant difference observed neither between patient group after treatment (PA) and Healthy Control Group (C), the mean was (copy no./µl) (P= 0.683) in patients' group after treatment (248.06 ± 896.7) compared with control group (110.7 ± 20.42). This study established that statins directly decrease HMG-CoA reductase function by affecting HMG-CoA reductase gene expression in the entire blood of hyperlipidemic individuals.

Simvastatin reduces the activity of HMG-CoA reductase directly, but has no effect on the expression of HMG-CoA reductase in the livers of hyperlipidemic rats. The negligible difference in HMG-CoA reductase level and activity between control and Simvastatin-treated rats may be due to the high fat diet's indirect suppression of HMG-CoA reductase and cholesterol de novo synthesis [248]. Numerous approaches have been employed to measure the HMGCR activity, including molecular docking for the purpose of determining statins' inhibitory potential against HMG-CoA reductase [251]. It was feasible to predict the mechanism of station inhibition on HMG-CoA reductase using molecular docking [105]. Florescu et al. [252] employed computational docking to predict the bound conformations and binding free energies of statin molecule ligands to macromolecular targets.

Simvastatin-HMG CoA reductase has the lowest energy value (-14.23 kcal/mol), followed by mevastatin (-6.41 kcal/mol) and lovastatin (-5.08 kcal/mol), according to the data presented. The docking investigation revealed that simvastatin is the most effective inhibitor of HMG-Co A reductase, with the complex simvastatin-enzyme having the lowest energy value.

Inhibiting HMG-CoA reductase expression or activity results in decreased cholesterol de novo synthesis in the liver, lowering blood cholesterol. Statins are critical for lowering the elevated serum TC level associated with hyperlipidemia by reducing the activity of HMG-CoA reductase and thereby inhibiting cholesterol production [253].

The expression of HMG-CoA reductase was slightly suppressed in hyperlipidemic rats fed a high-fat diet. Jiao Guoa et al. [254] reported that administration of Fufang-Zhenzhu-Tiaozhi (FTZ) could significantly inhibit the expression and activity of HMG-CoA reductase in hyperlipidemic rats fed a high-fat diet, resulting in a significant reduction in cholesterol These findings suggest that FTZ's cholesterol-lowering action is mediated by the down-regulation of HMG-CoA reductase in the hyperlipidemic rats' livers.

Radioisotope assays were used to evaluate the activity of HMG-CoA reductase using [3-14C] HMG-CoA (PerkinElmer) as a substrate [255, 256]. Despite increased dietary fat consumption, cholesterol production was significantly boosted in the livers of mice fed a high fat diet. The current work found a novel mechanism by which a high fat diet consumed for 5 weeks activates HMG-CoA reductase in the mouse liver. Our data demonstrate unequivocally that enhanced SREBP-2 expression mediated by Sp1 upregulates HMG-CoA reductase, resulting in increased cholesterol production in the liver [257].

In the present study, mice fed a high fat diet for 5 weeks demonstrated a large increase in HMG-CoA reductase activity in the liver, which was associated with considerable increases in HMG-CoA reductase mRNA and protein levels. To determine if enhanced de novo cholesterol synthesis contributed to the accumulation of cholesterol in the liver, tests were performed in HepG2 cells [257].

The current study's findings indicated that enhanced de novo cholesterol production may contribute to hepatic lipid buildup in response to a high fat diet [257]. SREBP-2 regulates the transcription of the HMG-CoA reductase gene [258]. De novo cholesterol production has long been recognized as a prime example of end-product feedback inhibition [259]. According to Medina et al. [250], based on available data, HMGCRv 1 expression accounts for only a fraction of the variation in LDL-C, apoB, and triglyceride response to statins (9%, 15%, and 6%, respectively).

Additionally, when age, race, smoking status, and body mass index are included in the model, they account for 24% of the variation in LDL-C response, 29% of the variation in apoB response, and 8% of the variation in triglyceride response, with HMGCRv-1 gene expression being the most significant predictor of response in all three. Due to the fact that prior investigations of genetic variation linked with statin response accounted between 2% and 7% of the variation in LDL-C and HDL-C [260].

This is the first time that a biological process has been shown to contribute significantly to variation in statin efficacy. Additionally, because HMGCR is directly implicated in cholesterol homeostasis, these findings suggest that variation in pharmacodynamics, in addition to classic pharmacokinetic factors, might affect medication efficacy [248].

Table 3.19: Mean ± SD of HMG-CoA Enzyme Activity Between Control and Patient (Before and After) Treatment.

Parameter	Status	No.	Mean	SD	t-test	P-value	Sig
HMG CoA	Before	30	348442.4	485652.536	3.197	0.002	S
PCR	After	30	248.0667	896.706	0.683	0.498	NS
(Copy number/µl)	Control	20	110.7000	20.424			

*P<0.05 Significant

**P>0.05 Non significant

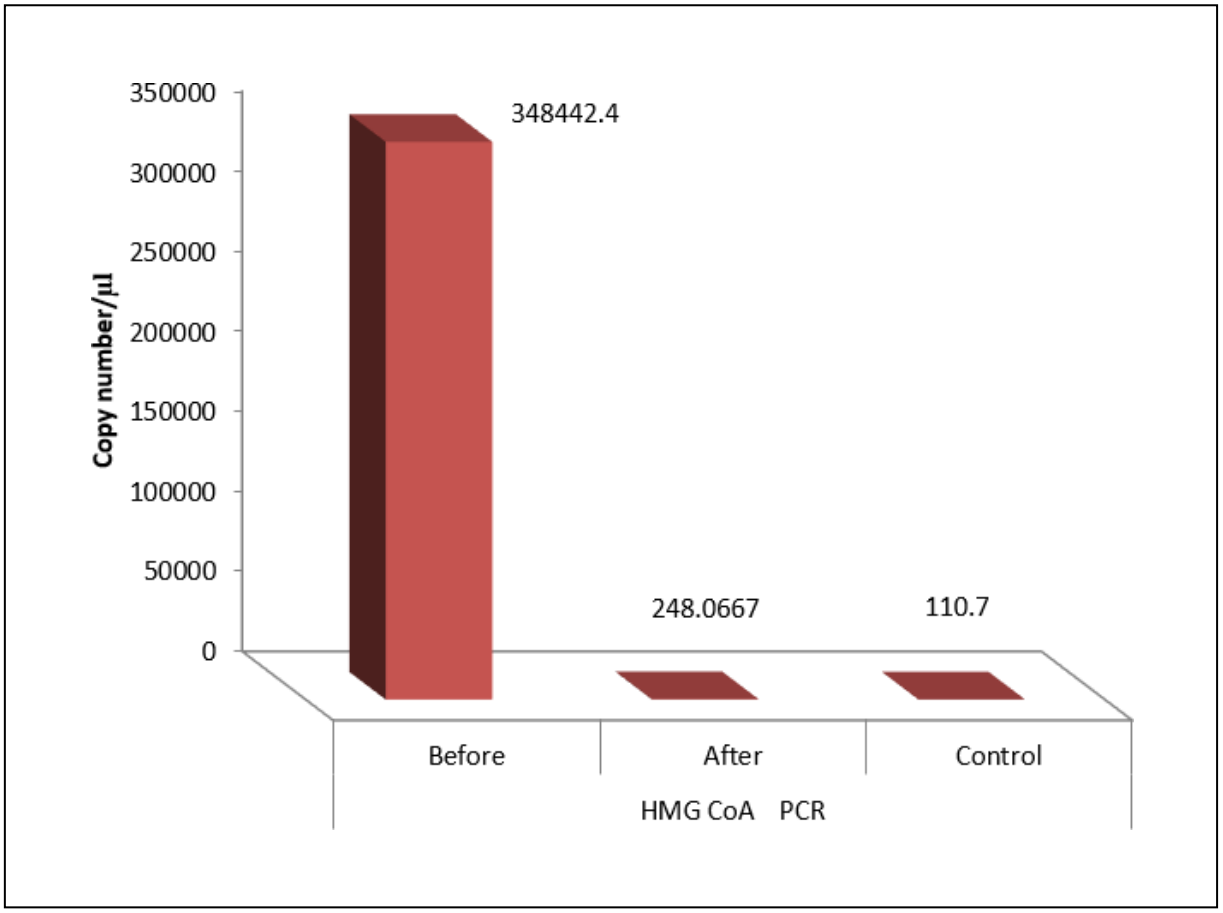


Figure 3.21: HMG-CoA Activity by PCR Between Control and Patient (Before and After) Treatment.

3.7.4 Comparison Between Classic (ELISA) And Modern (Real Time-PCR) Methods to Determine the HMGCR Enzyme Activity .

The real-time polymerase chain reaction (PCR), a DNA-based method, and the enzyme-linked immunosorbent test (ELISA), a protein-based method, are the two most often employed methodologies for species detection in processed meat products. The sensitivity, specificity, agreement between duplicate samples, cost, duration, and ease of use of a real-time PCR assay were compared in this study to a commercial ELISA kit. The present study the results are shown significant a little decreased in HMG-CoA reductase activity in patients with hypercholesterolemia after statin treatment than before statins treatment (15.7± 7.5; 26.3± 11.1) respectively in ELISA technique, while in other hand for Real time-PCR The mean of HMGCR activity (copy no./µl) was appeared to be significantly increased (P= 0.002) in patients group before treatment (348442.4 ± 485652.53) compared with control group (110.7 ± 20.424). While there was no significant difference observed neither between patient group after treatment (PA) and Healthy Control Group (C), the mean was (copy no./µl) (P= 0.683) in patients group after treatment (248.06 ± 896.7) compared with control group (110.7 ± 20.42). The comparison of QPCR and ELISA indicated that QPCR performs better than ELISA although these differences reach statistical significance as shown in Table 3.21 and Figure 3.22.

Table 3.20: Compare two methods to Measure HMG-CoA Enzyme Activity between control and patient (Before and after) treatment

Parameter	Status	No.	Mean	SD	t-test	P-value	Sig
HMG CoA ELISA (ng/ml)	Before	30	26.39	11.135	7.814	0.000	HS
	After	30	15.71	7.50	5.255	0.000	HS
	Control	20	6.50	2.65			
HMG CoA PCR (Copy number/µl)	Before	30	348442.40	485652.53	3.197	0.002	S
	After	30	248.06	896.70	0.683	0.498	NS
	Control	20	110.70	20.42			

*P<0.05 Significant

**P<0.001 High significant

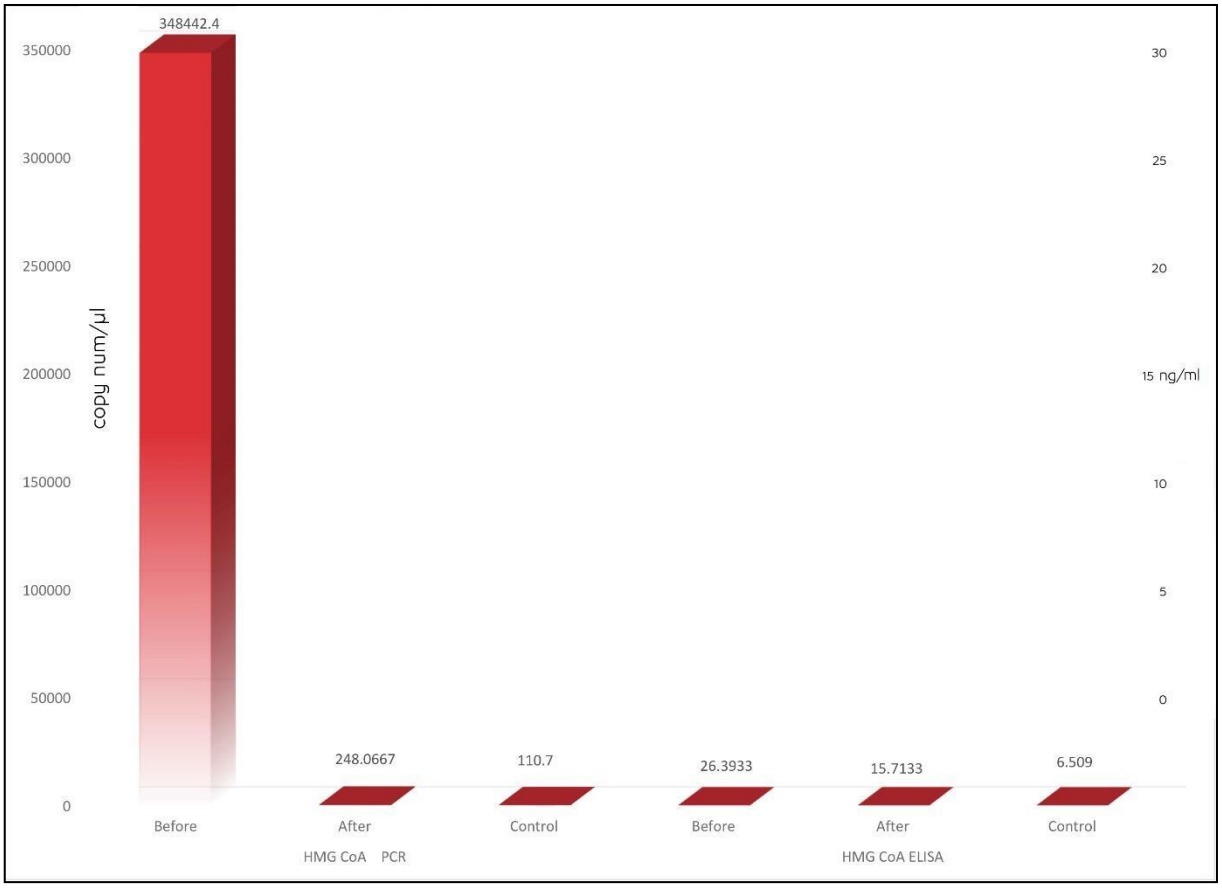


Figure 3.22: Compare two methods to Measure HMG-CoA Enzyme Activity between control and patient (Before and after) treatment.

These results agreement with previous studies [261, 262]. This study demonstrated that the real time polymerase chain reaction test is more accurate than serological ELISA test in the diagnosis of cytomegalovirus infection among pregnant women. The enzyme linked immunosorbent (ELISA) assay was used for the detection of CMV IgG and IgM and real time PCR for detection of CMV DNA. The real time PCR was used as the gold standard for infection diagnosis. Finally, QPCR is suitable alternative method for the determination of HER2 status in the blood of breast cancer patients. Overall QPCR performs better than ELISA in terms of sensitivity in discriminating HER2 IHC positive tumours from both healthy individuals and IHC negative breast cancers. QPCR could be used as diagnostic tool when primary tumour samples are unavailable or to monitor the outcome of the disease and the response to therapy during follow up of breast cancer patients [263].

3.8 Conclusions

Finally, there were a series of conclusions to conclude this thesis:

1. The study of a two of stationary phases for ZIC-HILIC gives more insight into the separation mechanism. For ZIC-HILIC materials, ZIC-1 and ZIC-4 columns appear to be as expected, or different.

2. In comparison with ZIC-1 column it has been found that the ZIC-4 column has a longer retention time with three statin drugs (Rosuvastatin, Atorvastatin and Simvastatin).

3. The difference in the geometrical alignment of sulfo betaine molecules may lead to different comportments with three statin drugs (Rosuvastatin, Atorvastatin and Simvastatin). of ZIC-1 and ZIC-4 columns.

4. The experimental data of three statin drugs (Rosuvastatin, Atorvastatin and Simvastatin) showed that hydrophobic behavior is active as a retention mechanism .

5. The retention characteristics are based on hydrophobic interactions for the three statin drugs (Rosuvastatin, Atorvastatin and Simvastatin). The reason for this behavior hydrophilic interaction can be attributed to the hydrophilicity of statin tested pharmaceuticals.

6. In this study we see the molecular methods high sensitive than other technique (ELISA).

7. The quantity methods using Real time- PCR in our study results high accuracy and sensitivity.

3.9 Recommendations

1. These two stationary phases are a new tool which can be used to investigate the retention mechanisms of multifunctional compounds such as pharmaceuticals. Moreover, it can help to choose the proper stationary for complex analytical problems encountered in pharmaceutical, biological and environmental samples .

2. The PS/DVB used as core material can play a key role in the retention of these pharmaceuticals in comparison to silica or methacrylate core materials. The interesting findings on the separation mode of non-polar compounds and the separation mechanism of three pharmaceutical compounds are a motivation for further investigations on new pharmaceuticals to increase knowledge about the ZIC-HILIC separation mechanism and to create optimized separation applications.

3. In spite of numerous publications in the literature which confirm that zwitterionic type materials are usually well suited for anion separation, and only poorly to separate cationic organic compounds. Nevertheless, more experiments are needed to extend the mechanistic knowledge in ZIC-HILIC mode for such compounds.

4. Synthesis of new zwitterionic exchangers by grafting reaction from monomeric precursors with different inner and outer the charged functional groups.

5. Can be study more statins familly and invastigate the behavior in theses columns (ZIC-1 & ZIC-4).

6. Should be take more patients and healthy control samples then monitore the evaluate the HMGCR activity.

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Appendix 1

Questionnaires for Hypercholesterolemia Disease	
Name	
Age	
Gender	
Disease History	
Date	
Phone number	
Address:	
Smoking	Yes ☐ No☐
Drug intake	ATOR ☐ ROSU ☐ SIMVA ☐
Blood Pressure	
Systolic:-	(mmHg)
Diastolic:-	(mmHg)

Test	Before Treatment	After Treatment
Cholesterol		
Triglyceride		
HLDL		
LDL		
VLDL		

Appendix II

Clinical and Anthropometric Characteristics of Hypercholesterolemia Patients and Controls

Parameter	Statues	N	Mean	SD	t-test	P-value	Sig
SBP	Before	30	147.7	26.6	4.741	0.001	HS
	After	30	126.4	7.49	4.138	0.001	HS
	Control	20	119.4	1.75			
DBP	Before	30	96.8	17.71	4.311	0.001	HS
	After	30	83.8	5.18	3.502	0.01	S
	Control	20	79.6	1.69			

Parameter	Statues	N	Mean	SD	t-test	P-value	Sig
Cholesterol (mg/dl)	Before	30	226.16	53.9	6.030	0.001	HS
	After	30	161.7	44.53	1.114	0.251	NS
	Control	20	149.4	21.84			
Triglyceride (mg/dl)	Before	30	212.63	119.57	2.648	0.011	S
	After	30	173.2	93.78	1.505	0.139	NS
	Control	20	141.45	8.75			
VLDL (mg/dl)	Before	30	42.52	23.91	2.648	0.011	S
	After	30	34.64	18.75	1.505	0.139	NS
	Control	20	28.29	1.75			
LDL (mg/dl)	Before	30	130.46	54.73	4.872	0.001	HS
	After	30	65.03	37.96	0.235	0.815	NS
	Control	20	67.26	22.99			
HDL (mg/dl)	Before	30	53.1733	13.98203	0.190	0.850	NS
	After	30	62.1767	19.78841	1.763	0.084	NS
	Control	20	53.8450	8.89077			

الخالصة

تمت دراسة فصل وتحديد أدوية الستاتين الثالثة)Rosuvastatin و Atorvastatin و Simvastatin(في أشكال الجرعات الصيدالنية التي لعبت دو ″را أساس ″يا في عالج أمراض فرط كوليسترول الدم. يتميز عسر شحميات الدم بارتفاع مستوى الكوليسترول الكلي)TC(، والدهون الثالثية)TG(، ومستويات ارتفاع كوليسترول البروتين الدهني منخفض الكثافة)LDL-C(وانخفاض مستويات كوليسترول البروتين الدهني عالي الكثافة (HDL-C) . ترتبط حاالت عسر شحميات الدم بزيادة خطر اإلصابة بأمراض القلب واألوعية الدموية. تشمل خيارات عالج خلل شحميات الدم التغييرات الغذائية واألدوية التي تؤثر على التمثيل الغذائي للدهون من خالل عدة طرق. العقاقير المخفضة للكوليسترول هي العالج ألول لخفض مستويات LDL-C في الدم. ومع ذلك ، فإن الستاتينات لها العديد من آلثار الضارة ، بما في ذلك االعتالل العضلي واضطراب وظائف الكبد.

تضمنت الدراسة ايضا″ نظرة عامة على تقنية الكروماتوغرافيا وكيف تم تقديم هذه التقنية ألول مرة كأسلوب فصل وشرح آلية التصنيف ألكثر أهمية لهذه التقنية. تم استخدام ودراسة أعمدة HILIC في هذا الفصل ، وكيف بدأت هذه ألعمدة ، وصعوبة حل هذه ألعمدة. ناقش الفصل أي ضا كيفية حل أعمدة HILIC للمشكالت التي يواجهها الباحثون عند استخدام ألعمدة الكالسيكية. تمت مناقشة أنواع HILIC وتطبيقات تلك ألعمدة للمواد المختلفة في عمليات الفصل. بعد ذلك ينتقل الحديث عن المستحضرات الصيدالنية ودورها في عالج أمراض فرط كوليسترول الدم. تم تناول أشهر أنواع عقاقير الستاتين ، وتم تحليل المستحضرات الصيدالنية باستخدام تقنية كروموتوغرافيا السائل عالية الكفاءة (HPLC) .

ويستعرض الجزء التجريبي الذي يشمل المواد الكيميائية واألدوات واألجهزة ، واإلجراء العام إلعداد المحاليل القياسية. باإلضافة إلى منهجية دراسة آلية الفصل للمستحضرات الصيدالنية الثالثة تحت ظروف HILIC والتي تم كشفها من خالل دراسة تأثير محتوى ACN , وتأثير تركيز محلول البفر, وتأثير ألس الهيدروجيني على زمن االحتجازلالدوية الثالثة .

نتائج البحث اظهرت ايضا عدة محاور واستنتاجات

- تم فحص الطور الثابت HILIC لـ)ZIC-1 و ZIC-4(لفصل كروماتوجرافيا لـ Rosuvastatin. تمت دراسة زمن االحتجاز للطور السائل في مختلف نسب الـ acetonitrial , تركيز محلول البفر، ودرجة ألس الهيدروجيني لـ Rosuvastatin. تم إستنتاج الرسوم البيانية للمحاليل القياسية وضمن نطاق خطي)من 0.1 الى 7 مايكروغرام/مل(، RSD٪)0.34 ± 0.22

و 0.56 ± 0.08(LOD ،)0.0094 و 0.0081 مايكروغرام/مل(، LOQ)0.0284 و 0.0245 مايكروغرام/مل(، على التوالي. بالنسبة للعينات الصيدالنية ، كانت الطرق المقترحة فعالة.

- الفصل الكروماتوجرافي وتقدير كمية Atorvastatin ، تم تحضير ألطوار الثابتة zwitterionic ذات السعة العالية باستخدام جزيئات zwitter المتصلة بجسيم PS / DVB. تم قياس زمن االحتجاز لـ Atorvastatin باستخدام نسب مختلفة من acetonitrial ، تراكيز مختلفة لمحلول البفر، وقيم ألس الهيدروجيني المتنوعة . أظهر فصل عقار Atorvastatin من التفاعالت الكارهة للماء. تم الحصول على خطية من 0.1-7 ميكروغرام مل/1 لعمودين ، وكذلك نسبة RSD)0.705 ± 0.075 و 0.6 ± 0.05(LOD ،)0.0020 و 0.0013 مايكروغرام مل/1(، و LOQ) 0.0060 و 0.0039 ميكروغرام مل -1(. باإلضافة إلى ذلك ، تتم مقارنة نتائج الطرق المقترحة مع تلك الخاصة بالطريقة القياسية ، مما يثبت أن دقتها قابلة للمقارنة.

- أظهر فصل Simvastatin لالعمدة لل)ZIC-1 و ZIC-4(حيث تم قياس زمن االحتجاز لـ Atorvastatin باستخدام نسب مختلفة من acetonitrial ، تراكيز مختلفة لمحلول البفر، وقيم ألس الهيدروجيني المتنوعة الـ Simvastatin. أظهرت وضع مختلط للعقار حيث تم إنتاج الرسوم البيانية للمحاليل القياسية ، ونطاق خطي)0.05-4 مايكروغرام/لتر(، نسبة RSD)0.495 ± 0.055 و 0.39 ± 0.03(LOD ،)0.023 و 0.015 مايكروغرام/لتر(، LOQ)0.069 و 0.045 مايكروغرام/لتر(، على التوالى. بالنسبة للعينات الصيدالنية ، كانت الطرق المقترحة فعالة. ويتم مقارنة نتائج الطرق المقترحة مع طريقة المقارنة ودقتها قابلة للمقارنة.
- النتائج إلحصائية لمقارنة الطرق المقترحة لـ ZIC-1 و ZIC-4 لتحديد Rosuvastatin و Atorvastatin و Simvastatin مع تلك التي تم الحصول عليها من خالل طرق المقارنة باستخدام اختبار الطالب ونسبة التباين F-test تُظهر توافق"ا جيد"ا وتشير إلى عدم فرق كبير في الدقة والدقة عند 95٪ من حدود الثقة.
- اشتملت الدراسة على خمسين عينة من المرضى تم تضمينها في هذه الدراسة. تم تقسيمهم إلهًط)12 مجموعتين؛ المجموعة الضابطة ضمت 20 شخ ذكور و 8 إناث(، وضمت مجموعة المرضى 30 مري ضا)14 ذكور و 16 إناث(قبل وبعد العالج. تم جمع العينات من المرضى في

مستشفى بغداد التعليمي خالل الفترة من شباط إلى حزيران 2021 ، وتراوحت أعمار جميع ااألشخاص بين 33-78 سنة. تقدير مستويات المصل من جلوكوز مصل الصيام)FSG(والكوليسترول الكلي)T.Cho(والدهون الثالثية)TG(وبروتين الدهون عالي الكثافة)HDL(وبروتين الدهون منخفض الكثافة)LDL(وبروتين الدهون منخفض الكثافة جدًا)VLDL(تم إجراؤها بواسطة طرق التحليل شبه.

- أظهرت هذه الدراسة فرقًا كبيرًا بين مجموعة االصحاء ومجموعة المرضى بعد تناول عقاقير الستاتين، حيث أظهر أن متوسط T. Cho (mg / dL) مرتفع بشكل ملحوظ)P = <0.001(في **مجموعة المرضى**)226.166 ± 53.9(مقارنة **بمجموعة اَلصحاء**)149.4 ± 21.84(، متوسط Tg (mg / dL) (ظهر زيادة ملحوظة)P = 0.011(في مجموعة المرضى)212.633 ± 119.57(مقارنة **بمجموعة اَلصحاء**)141.45 ± 8.75(، كما ظهر أن متوسط VLDL) mg / dL(قد زاد بشكل ملحوظ)P = 0.011(في مجموعة المرضى)42.52 ± 23.91(مقارنة مع **مجموعة اَلصحاء**)28.29 ± 1.75(، ظهر أن متوسط LDL) mg / dL(قد زاد بشكل ملحوظ)P = <0.001(في **مجموعة المرضى**)130.46 ± 54.73(مقارنة مع **مجموعة اَلصحاء**)67.26 ± 22.99(، بينما لم يظهر متوسط HDL (mg / dL) فروق معنوية)P = 0.85(في **مجموعة المرضى**)53.17 ± 13.98(مقارنة مع **مجموعة اَلصحاء**)53.84 ± 8.89(.

- تم تقدير مستويات نشاط انزيم HMGCR في المصل بواسطة تقنية مقايسة الممتز المناعي المرتبط باإلنزيم)ELISA(. وتم تقييم مستويات نشاط HMGCR في الدم بالكامل بواسطة تفاعل البوليميراز المتسلسل في الوقت الحقيقي. طريقة الكشف اأولى)ELISA kit(في هذه الدراسة ، عقاقير Rosuvastatin و Atorvastatin و Simvastatin ، أعاقت نشاط اختزال HMG-CoA بطريقة تعتمد على التركيز ، والتي وجدت انخفاضا كبيرًا في نشاط إنزيم HMG-CoA في المرضى الذين يعانون من فرط كوليسترول الدم بعد معاملة الستاتين أكثر من قبل عالج الستاتين)15.7 ± SD 7.5 ؛ 26.3 ± SD 11.1(على التوالي. طريقة اكتشاف تفاعل البوليميراز المتسلسل في الوقت الحقيقي في هذه الدراسة ، عقار الستاتين كما هو موضح ، يثبط نشاط اختزال HMG-CoA في عدد نسخ الجين من هذا اإلنزيم ، والذي وجد انخفاضا ملحوظًا في نشاط اختزال HMG-CoA في المرضى الذين يعانون من فرط كوليسترول الدم بعد عالج الستاتين من قبل عالج الستاتين 348442.4 ± 485652.53 20.424 ± 110.7 SD(على التوالي. كانت هناك فروق ذات داللة إحصائية بين RT-PCR و ELISA المصمم اعتمادًا على مرضى فرط شحميات الدم قبل وبعد عالج الستاتين.

جمهورية العراق
وزارة التعليم العالي و البحث العلمي
جامعة ألأنبار
كلية العلوم قسم
الكيمياء

طريقة جديدة لكروماتوغرافيا السائل لتقدير بعض عقاقير الستاتين ومستوى فعالية انزيم HMGCR بطرق مختلفة

رسالة

مقدمة إلى كلية العلوم- جامعة ألأنبار

وهي جزء من متطلبات نيل شهادة الماجستير في علوم الكيمياء

من قبل الطالب

علي محمد ماهر فهد

بكلوريوس كيمياء/ جامعة بغداد ٢٠٠٥ م

بأشراف

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