CHAPTER ONE
INTRODUCTION

1.1. History of chromatography

It can be argued that, high performance chromatography began with the publication of James and Martin [(1)] in 1952. It is certainly true that their publication on the use of gas as a mobile phase in the separation of volatile fatty acid initiated the research that has resulted in the wide spread use and popularity of chromatography. Although chromatography entered a new phase in the early 1950, the Russian botanist Tswett [(2)] is generally referred to as father of chromatography. His work, published in 1906, described the separation of plant pigment by column chromatography. The original paper is of a significant historical interest and serves as an introduction to a discussion of the concept of "chromatography".

Column chromatography developments accelerated in the 1940. Synge and Martin [(3)] published their Nobel Prize winning paper in which they introduced liquid-liquid chromatography and the acompanying theory that became known as the plate theory. The plate theory was further explored by Craig [(4)], who published a paper entitled "Ion Chromatography and Countercurrent Distribution" in 1950.

In 1955 Glueckauf [(5)] published an alternative to the plate theory; the so-called rate theory came into prominence about the same time. The paper that has had the greatest impact was the one published by Dutch workers Van Deemter and Zuiderweg [(6)]. They described the chromatographic process in terms of kinetics and examined diffusion and mass transfer. The popular van Deemter plot resulted a few years later. Giddings [(7)] published

another paper on this topic, and theory has since become the backbone of chromatographic theory. The new technique, gas chromatography (GC), was found to be simple, fast and capable of producing separation of volatile materials that were impossible by distillation. Furthermore, the theories were found to be rather accurate in predicting optimal operating condition. It was natural to apply the successful results from GC to the older technique of liquid chromatography. Some of the credit for that transfer technology belongs to Giddings paper entitled "LC with operating condition analogous to those of paper "LC" [8]. This set off a revolution in LC that brought it to level of efficiency similar to that achieved in GC. The acronym high performance liquid chromatography (HPLC) was born.

1.2. Chromatographic separation technique

The chromatographic separation method is a technique used for separation of mixture of compounds into its components. It enables us to separate trace impurities and major fraction from each other, based on the chemical or physical interaction between the analyzed sample and the stationary and the mobile phase [9].

However, identification usually requires other analytical procedure, depending upon the detector used and other external methods such as infrared (IR) spectroscopy, Nuclear Magnetic Resonance (NMR), or Mass spectroscopy (MS).Quantitative analysis can be carried out by measuring the height or area of the chromatographic peak. Hence chromatography can be used for qualitative as well as quantitative analysis [10-11].

1.3. Liquid Chromatography

Liquid chromatography (LC) is a method for separation of sample components as they pass down a column packed with a stationary phase, due to the distribution of sample components between two phases the liquid mobile phase and the stationary phase. There are two types of (LC); classical and high performance liquid chromatography (HPLC). Classical (LC) uses a long column approximately 20-50 cm packed with large particles 50-250µm diameter. Sample volumes in the milliliters range are often common. The liquid mobile phase is generally a gravity-feed at slow flow rates. Since the deep pores of packing limits mass transfer, the analysis time may be in order of hours. Fraction collection of separated sample components for later spectroscopic and other identification method is a usual practice with this technique [(12)].

The second, HPLC is a technique that separates mixture components more efficiently than classical LC. HPLC uses a column packed with fine particle stationary phase in the µm ranges. Therefore, the use of a pump is necessary to deliver a mobile phase at constant flow rate through the column under pressure that typically reaches 5 to 50 MPa. The most obvious advantage of HPLC over classical LC is that samples can be separated much more quickly with very high efficiency [(13)]. HPLC requires special sample-injection system columns, and pumps to provide uniform flow rates. The stationary phase must be sufficiently rigid so that its dimension does not change with pressure. A further consequence of the reduction in column and particles size was that the volume of the detector cell which has to be small to accommodate the eluted sample components. The importance of HPLC is evidenced by the fast growth in published scientific papers [(14)], which site the technique as the chosen method of

analysis for wide varies of compounds in comparison with GC.

The efficiency of the chromatographic separation is usually described by the number of theoretical plates; **N**. Column with high efficiency has a relatively larger **N**. The value of **N** is related to the high equivalent to theoretical plates, (HETP), **H,** by

$$H = \frac{L}{N} \text{.....(1.1)}$$

Where L is the length of the column. The efficiency of separation in HPLC is higher due to the large number of mass transfer equilibria obtained with small values of H. This can be resulted by using small particle size of the stationary phases as described by Van-Deemter equation [15]. This equation related H with the mobile phase velocity **u**, as well as to other parameters:

$$H = \frac{A + B}{u + Cu} \text{.....(1.2)}$$

First parameter **A** is the coefficient of eddy diffusion, the equation is

$$A = 2\lambda dp \text{.....(1.3)}$$

Where **λ** is the packing constant equals 0.5 for best packed columns, and **dp** is the particle size diameter of stationary phase. So **A** is depend on the particle size of stationary phase, a small value of **A** will be obtained when a small particles are used. The second parameter **B** is coefficient of longitudinal diffusion, its equation is as follows:

$$B = 2\psi Dm \text{.....(1.4)}$$

Where **ψ** is the geometry factor depends on the nature of packing and **Dm** is the coefficient of molecular diffusion in mobile phase. **B** depends on

the temperature and pressure. The last parameter **C** is coefficient of mass transfer, which is equal to:

$$C = Cs + Cm \quad \text{...(1.5), and}$$

$$Cs = 2td\left[\frac{K'}{(\kappa' + 1)}\right] \quad \text{.....(1.6)}$$

Where **Cs** is the mass transfer effects into stationary phase and **td** is the mean adsorption time, and

$$Cm = \Pi \frac{d^2 p}{Dm} \quad \text{.....(1.7)}$$

Where **Cm** is the mass transfer effects into mobile phase and **л** is the function of packing structure, and **Dm** is the diffusion of solute in mobile phase. A close look at Van-Deemter equation indicated that small values of H may be obtained using a small particle size.

The small particle represents a good compromise between efficiency, pressure drop, analysis time, and reproducibility of packing. HPLC is not of limited applicability by component volatility or thermal stability as in GC. This makes it the method of choice for the analysis of almost all known samples including polymer, polar, ionic, and thermally unstable material. Choice of stationary phase and proper control of the composition of mobile phase can lead to better separation. Other advantages of LC methods are that many detectors in use are non-destructive. This facilitates sample recovery and provides the opportunity for subsequent spectro-analytical and other studies [16]. The efficiency of the chromatographic separation is described also by resolution that can be defined as:

$$Rs = \frac{1}{4}(N)^{\frac{1}{2}}\left[\frac{(\alpha - 1)}{\alpha}\right]\left[\frac{(K_2')}{1 + K_2'}\right] \quad \text{.....(1.8)}$$

Where **α** is the selectivity factor which as defined by the ratio of their partition coefficients on a given column,

$$\alpha = \frac{K_B}{K_A} = \frac{(V'_R)_B}{(V'_R)_A} \quad .....(1.9)$$

V'R is the adjusted retention volume or time and **Ḱ** is the capacity factor which is equal to:

$$k' = \frac{t_R - t_o}{t_o} \quad .....(1.10)$$

Where $\mathbf{t_R}$ is the retention time, and $\mathbf{t_{o\ is}}$ the retention time of mean retained component. A final measure of column efficiency is the peak capacity or the number of peaks that can be resolved. Chromatographic separation can be effected by differences in partition coefficient as well as the efficiencies of the column in which they are run. HPLC has been used for analysis of wide variety of pharmaceutical products, body fluids and environmental samples [17].

1.4. Modes of liquid Chromatography

HPLC can be carried out in any of the classical modes such as normal and reversed phase as well as ion exchange (cationic or anionic) chromatography. Separation modes can be chosen for particular application depended on the properties of analytes to be separated, and can be optimized by choosing different combination of mobile phase and stationary phase materials [18].

1.4.1. Normal phase (Adsorption) chromatography

The separation in normal phase chromatography is carried out by using conventionally polar stationary phase, and a non-polar organic mobile phase [(19)]. In this liquid solid adsorption chromatography, the lattice of common porous adsorbent stationary phase (e.g. alumina or silica) is terminated at its surface with polar hydroxy groups. These groups; provided the mean for surface interactions with solute molecules. The sample is applied to the column; molecules with polar functional groups are attracted to the active sites on the column packing.

The eluent (a non-polar solvent), commonly hexane, containing a small amount of polar additives, such as 2-propanol is used. The mechanism of separation involves no partition of the sample solute in the stationary phase; instead the polar groups of each organic solute interact through primarily hydrogen bonding forces at the polar sites of the stationary phase [(20)]. As chromatography is developed, the sample components are passed down the column to be re-adsorbed on the fresh sites of the stationary phase packing. The ease of displacement of solute molecules will depend on their relative polarities. The more polar molecules will be adsorbed more strongly and hence elute more slowly from the column [(21)]. One of the strength of adsorption chromatography is its ability to separate isomers, particularly aromatic functionalized compound with polar groups as reported by Majors[(22)], who was reported the separation of nitro aniline isomers in the retention order ortho> meta> para.

Cyano-and amino-derivatives of silica remained the most popular bonded normal stationary phases, and several studies attempted to define their retention characteristics relative to bare silica. Oestman and Coimsjoe [(23)] have used series of un-substituted and alkylated benzene and polycyclic

aromatic hydrocarbons (PAHs) samples to compare silica with aminopropyl and cyano propyl silica. The authors noted the effect of methyl and mono alkyl substitution on retention with amino phase. Weaker retention of PAHs on the cyano phase relative to silica and differences in the effects of alkyl substitution on retention were noted as an indication that cyano groups of the bonded phases were the primary adsorption sites not the residual silanolols.

Pietrogrande [24] carried out a similar study for the separation of benzodiazepines and pharmaceutically compounds. He monitored the change in retention that occurred with increased amounts of polar solvents (2-propanol and ethyl acetate) added to hexane-based mobile phase.

Ando and et al [25] studied the retention of fat-soluble vitamins using silica aminopropyl and cyonopropyl column using ethyl acetate and tetrahydrofuran (THF) were used as the polar mobile phase modifier. The amino phase was found to be more retentive than the cyano phase. Pharr et al [26, 27] introduced two new normal bonded phases that were used to separate (PAHs) and (PCBs). The two phases were trichloroacetamide and trichloroethoxy. To increase charge transfer and hydrogen bonding characteristics over fluorine containing phases, mobile phase effects in normal phase chiral separations were studied by Akanya and Taylor [28]. They demonstrated that alcoholic modifier greatly reduce the resolution of chiral esters of amino acids and gave that the alcoholic moiety interfered with hydrogen bonding chiral recognition. Aprotic modifier such as dichloroethane and 1, 1, 2-trichlorotrifluoroethane were used in separation of chiral tryptophane [29].

1.4.2. Reversed phase chromatography

In reversed phase mode the interactions and separations are based on a non- polar stationary phase and a relatively polar eluent. The retention of an analyte depends on the degree to which it partitioned into the stationary phase and it is largely determined by the hydrophobic interactions of the analytes with the mobile phase. The mobile phase that is mostly used in a reversed phased system is a mixture of water and methanol [19].

The packing materials have been developed in which the stationary phase is chemically bonded to an insoluble matrix (solid support). The main advantage of this bonded-phase column (BPC) packing is its quite stability and cannot be easily removed or lost during use. The availability of a wide variety of functional groups in BPC allowed both normal and reversed phase chromatography to be carried out in a relatively simple and straight forward manner. BPC involved a relatively non-polar stationary phase used in conjugation with polar mobile phase to separate a wide variety of less polar solutes [20].

BPC packing is prepared by many methods. Solid support can be either silica or synthetic polymer such as polystyrene-divenylbenzene. Functional groups such as hydrocarbons, amino, ethers, and ion exchange groups such as sulfonic acid and quaternary ammonium salts were attached chemically to the support [20]. Tanaka [30] prepared alkylated stationary phases for reversed phase liquid chromatography based on polymer particles with aliphatic backbones, having hydroxyl groups. The chromatographic properties were examined in terms of steric selectivity and its preference towered aromatic and saturated compounds. Polymer support stationary phases were less hydrophobic than silica-based phases, but it showed preferential retention of aromatic compounds. The preference shown by the polymer-based stationary

phases toward rigid, compact molecules over flexible and/or bulky molecules can be explained by the contribution of the polymer network structure, to the retention process. The polymer-based stationary phases showed greater variation of selectivity due to changes in the composition of the mobile phase [20].

1.5. Stationary phase

As mentioned early of the two phases forming a chromatographic system is the stationary phase. It is the part of chromatographic system responsible for retention of the analytes, which are being carried through the system by the mobile phase. It may be a solid, a gel or a liquid. It may be distributed on a solid support. The solid support may or may not contribute to the separation process. The liquid may be chemically bonded to the solid (bonded phase) or immobilized onto it (immobilized phase) [31]. Particularly in gas chromatography, the stationary phase is most often a liquid coated on solid support.

1.5.1. Bonded stationary phase material

Kanazawa et al [32] have used HPLC adsorbents stationary phase by modifying the surfaces of micro particulate silica gel using functional polymers. The thermo responsive copolymer, poly (*N*-isopropylacrylamide-*co*-*n*-butylmethacrylate) (IBc) was used to modify the silica stationary phase surfaces. This polymer-grafted surface exhibits temperature-regulated hydrophilic/hydrophobic property changes in water. PTH-amino acid interactions with this surface were readily modulated by changing the column temperature using an isocratic aqueous mobile phase.

Kobayashi et al [33] have used cross-linked poly [*N*-isopropylacrylamide-*co*-acrylic acid) (Poly (IPAAm-*co*-AAc)]-grafted silica bead surfaces and applied as new column matrix materials that exploit temperature-responsive anionic chromatography to separate basic bioactive compounds.

Meyer et al [34] have used three poly (ethylene-co-acrylic acid) copolymers (–CH2CH2–)x[CH2CH(CO2H)–]$_y$ with different chain lengths and mass fractions of acrylic acid and were covalently immobilized as stationary phases on silica via two variants of spacer molecules namely;(3-aminopropyltriethoxysilaneand-3-glycidoxypropyltrimethoxysilane). Different motilities of the alkyl chains in the stationary phases were observed using ^{13}C solid-state NMR spectroscopy. These stationary phases had better selectivity for geometric-carotene and xanthophylls isomers (provitamin A derivatives). Also, all the separations of the analytes were affected by polar interactions with the stationary phases.

Hayrapetyan and Khachatryan [35] have used silica gel modified with a polymer containing two different functional groups (C-18 and COOH) by the use of a copolymer of octadecyl methacrylate and butylacrylate and introduction of maleic anhydride. The chromatographic properties of these stationary phases in reversed-phase HPLC have been studied over a broad pH range. Introduction of maleic anhydride as the third component of the polymer layer on the surface of micro-spherical silica leads to repartition C-18 groups, which affects the hydrophobicity of the materials obtained. The presence of maleic anhydride secures the availability of carboxyl groups on the surface, and thus the acidic properties of the material.

Akiyama et al [36] have used acryloyl cyclodextrin (CD) and *N*, *N*-methylene bisacrylamide with vinylated silica-gel in the presence of template and water. A thin layer of molecularly imprinted CD polymer was

immobilized on a porous silica-gel support. This mechanically weak polymer was reinforced by the silica-gel and successfully used as a stationary phase in (HPLC). When using L-Phe-L-Phe as template, the polymer/silica-gel conjugate retained L-Phe-L-Phe in the aqueous eluent more strongly than D-Phe-D-Phe. Selective separation of antibiotics was also achieved by the polymer/silica-gel conjugate. Molecularly imprinted cyclodextrin polymer, immobilized on silica-gel, was an eminent stationary phase for HPLC in water.

Yang et al [(37)] have used a molecularly imprinted polymer (MIP) prepared using diniconazole, triazole-based fungicide with a broad antifungal spectrum, as the template and a mixture of methacrylic acid and acrylamide as the functional monomers, and ethylene glycol dimethacrylate as the cross linker. HPLC was used to study the molecular recognition mechanism that regulates the binding behavior, and to evaluate the binding performance of these MIPs for the template and for paclobutrazol. The results showed that the MIP had high affinity and selectivity for diniconazole in water-containing system, and the retention of diniconazole became stronger on increasing the water content. This behavior gave the MIP the potential for use in the enrichment, separation and detection of diniconazole in biological fluids. In addition, a solid-phase extraction column packed with diniconazole-imprinted polymers was used to enrich the diniconazole extraction recovery range from 63.8% to 80.5%.

Kanazawa et al [(38)] have proposed, a thermo-responsive polymer carrying an amino acid ester residue for the stationary phase of HPLC. They have investigated the new concept of chromatography. A temperature-responsive chromatography, using temperature-responsive poly (*N*-isopropyl acrylamide) (PNIPAAm)-modified surface for HPLC with a constant

aqueous media as the mobile phase. In this study, they designed and synthesized thermo-responsive poly (acryloyl-l-proline methyl ester) and its copolymer.

The *N*-isopropylacrylamide (NIPAAm) photopolymers of acryloyl-l-proline methyl ester and copolymer were prepared by the reaction of radical telomerization. Steroids and amino acids with a variety of hydrophobicity were separated using a sole aqueous mobile phase. In contrast to a PNIPAAm-modified surface, a poly (acryloyl-l-proline methyl ester)-modified surface showed a greater affinity for hydrophobic amino acids**.**

1.5.2. Polymeric stationary phase material

Stationary phase materials were based on particles of a cross- linked organic polymeric material. Cross-linked organic polymer was introduced as packing in column liquid chromatography since 1960[(39)]. The first organic polymer based packing was ion exchanger made by condensation polymerization of phenol and formaldehyde. Typical materials of this kind of stationary phase were polystyrene divenylbenzene copolymers (PS-DVB) and modified PS-DVB materials. Other requirements which must be met are insolubility, resistance to oxidation, reduction, a defined, controllable and reproducible size and pore structure [(40)].

Polymerization is performed either by condensation or addition polymerization, depending on the type of starting monomer. For cross-linking, co-monomers such as divenylbenzene, bis-ethylene glycol methacrylate, 2, 3-dibromopropanol are added [(41)]. The cross-linking reagent can be added to amount as much as 70 % (w/w). Macro porous copolymers are synthesized in the presence of an inert solvent, which functions as a volume modifier. Both the cross-linked and the inert solvent have a

substantial impact on the kinetics of the polymerization and the resulting properties of the copolymer. As in the synthesis of silica packing, specific properties must be chosen in polymerization to manufacture polymeric packing with beads of controlled size distribution by technical polymerization described below [41].

1. Emulsion polymerization starts with a solution of a detergent to which the monomers are added. As a result, micelles swollen with the monomer are formed. After water- soluble initiator is added (for styrene as a monomer), polymerization leads to particles of exactly the same size as the swollen micelles. Emulsion polymerization processes generate particles of up to 0.5µm in one step [42].
2. Suspension polymerization is usually designed to prepare larger beads of > 0.5µm mean particle diameter. The monomer or co-monomer solution is vigorously agitated in water in the presence of colloidal suspending agent. The colloidal agent coats the hydrophilic monomer droplets (in the case of, styrene or divinylbenzene). Coalescence of the droplets is prevented by the surface charge of the droplets. Adding a lipophilic catalyst or initiator starts the polymerization in the droplets and this continues until the beads are solidified in bulk. The size of the beads is thus controlled by the size of the droplets via the stirring speed [43].
3. A third variant in polymerization technology is the swollen emulsion polymerization pioneered by Ugelstad [44]. The procedure is performed in two steps. First by adding a swelling agent, which causes the sub-micrometer polymer particle to swell by large volumes of the monomer before starting the polymerization. The increase in volume can reach a

factor of 1000. Second, in a consecutive step the monomer swollen beads of defined size are polymerized.

Having briefly examined the structure of organic polymer packing and the various routes in their manufacture, the most important features may be summarized as follows [45]: -

1. Hydrophilic as well as lipophilic organic polymer pickings are synthesized with a controlled pore and surface structure depending on the type of monomer/co-monomers and the polymerization reaction. The surface structure can be attired by controlled consecutive surface reactions.
2. In accordance with the bulk composition, polymer packing is stable across almost the entire pH range, particularly under strong alkaline conditions.
3. The chemical stability is affected by oxidizing and reducing solutions.
4. Although cross-linking reactions have been optimized in as much as rigid pressure, stable particles can be manufactured, and some remaining swelling property is often noted when changing the solvent composition in HPLC.
5. As in the manufacture of silica, porosity, pore size, and surface area of polymer packing can be adjusted over a wide range, and micro-, meso-, and macro- as well as nonporous beads are synthesized reproducibly.

1.5.3. Literature survey

Kwang-Pill LEE et al [46] have prepared cyclodextrin (CD) polymers from the reaction of native CD with a hexamethylene diisocyanate (HDI) in a dried DMF solution. The obtained CD polymer contained a range of (8-14) % nitrogen due to HDI by elemental analysis. An HPLC column was prepared using the CD polymer stationary phase with a carbamate linker by a slurry method. Separation of the phenol isomers (o-, m-, and p-nitro phenols) was conducted using the CD polymer stationary phase by HPLC.

Sunamoto et al [47] have developed a new HPLC method using packing materials modified with cross-linked poly (N-isopropylacrylamide) (PNIPAAm) hydro gel. A temperature-responsive surface by polymerization of NIPAAm in the presence of a cross-linker on the silica support. The surface properties and functions of the stationary phases change, in response to the external temperature have been studied. It was easy changing the interaction of a solute with the surface and with a constant aqueous mobile phase with temperature. A temperature-responsive elution behavior was observed on the separation of steroid and PTH-amino acids. The method is expected to be applied in the pharmaceutical and biomedical fields [48].

Yokoyama et al [49] has developed a new low-capacity cation exchange column for the separation of amino acids. A highly cross-linked macro porous polystyrene- divinylbenzene copolymer was functionalized by a sulfoacylation reaction. The exchange capacity was controllable at the acylation step. Capacities between 55 and 60 μmol/column were adequate for the practical separations in acceptable retention time. The 5-μm base polymer having average pore diameter smaller than 3nm gave satisfactory results and those of 1.5nm pore were most favorable for several isocratic

elution conditions. At different pH values adjusted by phosphate buffer has provided with a good separation for individual classes of amino acids, i.e., acidic, neutral, hydrophobic, and basic groups. The results provided fundamental data for programming gradient elution system required for the simultaneous separation of protein amino acids [(50)].
The resins as matrix materials through chemical modification can be conveniently changed into various types of HPLC packing with high column efficiency, high permeability and different selectivity. Ion exchanger HPLC packing carrying tertiary amino groups, quaternary ammonium groups, carboxyl groups and sulfonic acid groups. The preliminary tests have shown that most of the modified resins as new HPLC packing possessed excellent chromatographic properties especially for the separation of biochemical substances such as amino acids [(51)].

1.6. HPLC Detection system

The detectors in HPLC are employed for continuously monitoring the column eluent. The detector signal is generally amplified and processed to a potentiometric recorder to obtain a permanent signal record with time in the form of a chromatogram [(52)].

Wide variety of HPLC detectors have been developed with high sensitivity and universal detection requirements. The HPLC detectors can be generally classified as either responsive to change in the property of the mobile phase, when a solute (sample component) is present or to a property of the actual solute itself [(53)]. These include UV-Visible spectrophotometer, refractive index, conductivity, fluorescence, electrochemical detectors and others [(54)].

UV spectrophotometer detector is the most widely used in HPLC. The popularity is due to a wide range of applicability, excellent stability, and

low cost. They are relatively insensitive to temperature change and flow variation. Detection limit at the nanogram level for a certain type of compounds can be easily achieved. This device has a high sensitivity for many solutes but samples must absorb in the UV-Visible region (190-900nm) to be detected. Both fixed and variable wavelength detectors are commercially available with HPLC equipments [55]. These detectors are equipped with a low-volume flow cell (8 µl or less), usually 1 cm in path length. The UV-Visible detector has a detection limit of a bout $1x10^{-9}$ g cm^{-3} for highly absorbing compounds with large extension coefficient [56].

Janssen et al [57] have described a method for amino acid determination that was used phenylisothiocyanate (PITC) to form phenylthiocarbamyl (PTC) derivatives of amino acids which can be separated by reversed phase HPLC and detected by UV at 254 nm.

The second type of the detector is the refractive index (RI). It works by measuring the change in refractive index of the eluent as the solute passes through the sample cell. The RI detector is non destructive and can be used on a continuous basis. It can sense all materials, so it is considered a universal detector. RI cannot be used in gradient elution mode since the baseline is continuously varied as the solvent mixture changes which causes refractive index variation. The detection limit of RI ranges between 10^{-8}-10^{-6} g cm^{-3} [58].

The third type of detection system is the conductivity detector. Electrical conductivity is a universal property of all ionic solutions. After a chromatographic system equilibrates with the eluent ions, the magnitude of the response is proportional to the difference in conductance of the analytes and the eluent ions as well as the concentration of the analytes. In order to detect a small analytes signal it is necessary to employ an eluent, which

gives a relatively low conductance [59]. Response of conductivity detectors is temperature dependent; consequently, temperature must be controlled carefully. This detector has been extremely valuable for analyzing both inorganic and organic ionic substances in aqueous mobile phase.

A fluorescence detector is an important detector used in HPLC. In this detector the solute is excited by UV radiation of a given wavelength (the excitation wavelength) and the fluorescence energy which is emitted at a longer wavelength (the emission wavelength) is detected [60].

Fluorescence detector has been successfully employed to compounds which are naturally fluorescent, or which have been chemically reacted to form fluorescent derivatives [61]. Fluorescent detection system has given a useful selectivity in trace analyses when either the sample size is small or the solute concentration is extremely low [62].

The number of fluorescing species can be enlarged by treatment of samples with reagents that form fluorescent derivatives. A 5-dimethylaminonaphthalene-1-sulphonylchloride (dansylchloride), which reacts with primary and secondary amines, amino acids, and phenols to give fluorescent compounds, has been widely used for the detection of amino acids in protein hydrolysis. Aminoquinoline (AMQ) which reacts with amino acids to form fluorescent derivatives has been used by Cohen and Antonis [63] to study the separation of amino acid derivatives (AMQ-amino acids) using ODS (ocadecylsilannol) column with fluorescence detector at excitation wavelength 250 nm and emission wavelength 395 nm.

Most of the HPLC derivatization chemistry is based on well-known reactions reported in organic or inorganic chemistry literatures [64]. The fluorescent derivatization of primary amines such as amino acids using orthophthalaldehyde (OPA) and mercaptoethanol either in the pre-column or

post-column modes has been well studied by Lindroth and Mopper[65] to separate OPA-amino acids derivatives at the nanogram level on 25 cm C 18 column with fluorescence detector (excitation wavelength 330 nm and emission wavelength 418 nm).

Electrochemical detectors provide useful selectivity for electro-active compounds such as ketons, aldehydes, marcaptans, peroxides, phenols, and aromatic amines. Many electro-reducible and electroxidizable compounds can be detected in column effluents at very low concentrations by selective electrochemical (EC) measurements. With this approach the current between polarizable and reference electrodes is measured as a function of applied voltage [66]. The polarizable electrode may be constructed of a material such as platinum, gold, glassy carbon, or graphite electrode. Any reference electrode can be used. Usually the voltage applied to the polarizable electrode is set, and the current monitored with time. The current-generated at a fixed, pre-set potential is amplified to the output response [67] Developments in cell technology have led to electro-chemical detection with mercury - drop electrode [66].

1.7. Column Packing Methods

The optimum or "best" procedure for packing columns is determined by the nature and size of the packing particles. The goal is to pack a uniform bed with no cracks or channels and without sizing or sorting the particles within the column. Usually rigid solids and hard gels are packed as densely as possible but without fractioning the particles during the packing procedures [66]. The "tap-fill" procedure was recommended for the dry packing of rigid particles with diameter dp >20 μm. The technique for dry-filling high efficiency LC columns is not very different from that used to

prepare high efficiency GC columns [66]. The high-pressure "wet- fill" or slurry-packing techniques were, alternatively, used for packing particles with dp <20 μm. In this technique suitable liquids were used to wet the particles and eliminate particle aggregation during packing [68].

1.7.1. Down-Flow Method

The down-flow method has been most widely used, and it permits the preparation of satisfactory columns of all types of micro particles packing. Rigid particles are required for the best results with this method. An apparatus such as that shown in figure (1-1) is used. The slurry mixture is rapidly forced downward into the column blank with a constant pressure pump [66]. Slurry flow rate is dependent on the pressure used and as the packed bed is formed, the flow rate decreases. Forcing the slurry mixture into the column blank at highest possible velocity generally produce the best column performance. This operation is carried out by pumping the slurry into the column blank at the highest pressure permitted by compression fittings connecting the column to the slurry-packing apparatus. The pump used for packing could be either reciprocating or pneumatic pump [69]. The very high initial velocity as the slurry enters the column blank may fracture weak particles, producing fine particles that tend to plug the column outlet and caused packing structure irregularities [66].

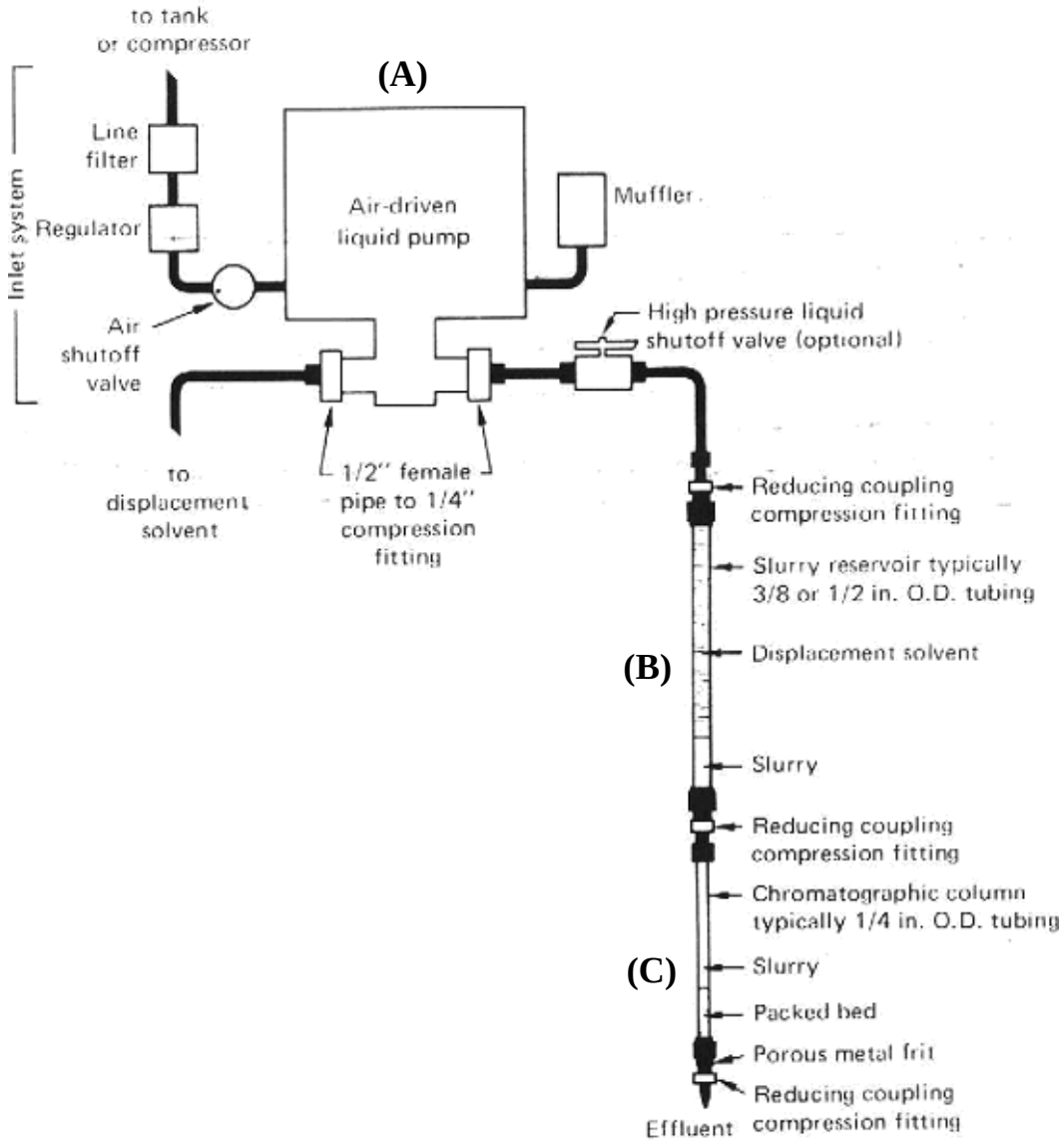
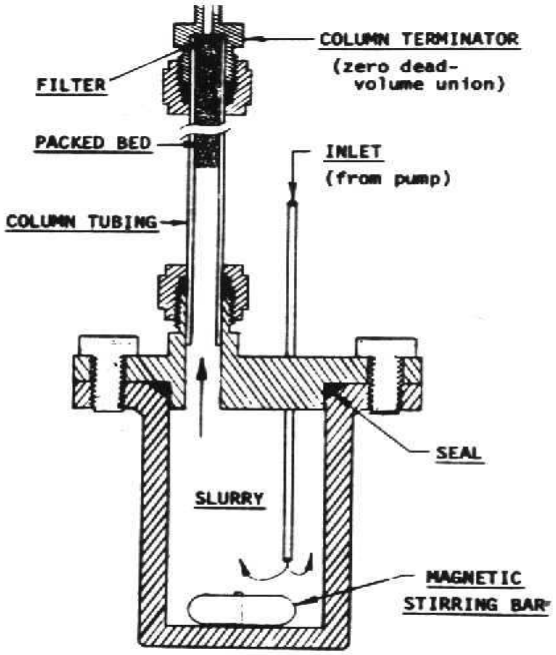


Figure (1-1) High-pressure slurry packing assembly [66].

1.7.2. Up-Flow Method

An alternative technique for wet filling columns is the up-flow approach, which has been described by Bristow [70]. Experience with the up-flow packing method has not extensive, but column performance results have been about equivalent to those for the down-flow procedure. The equipment used for the up-flow packing is shown in figure (1-2). In this case the slurry is pumped up into the column blank from the reservoir whose contents are continuously diluted by incoming pressurized liquid [71].



Figure(1-2)Up-Flow Packing System [71]

In the up-flow approach the velocity of the particles striking the forming bed must be sufficiently high and the liquid sufficiently great to prevent particles bed from falling back into the reservoir [72].

1.8. Analyses of drugs

Luis et al [73] have developed an HPLC determination of atenolol in human plasma samples to compare the bioavailability of 2 atenolol tablet (50 mg) formulations in 24 volunteers of both sexes. Atenolol concentrations were analyzed by a combined reversed phase liquid chromatography with fluorescence detection at wavelength 300 nm.

Santos et al [74] were determine ß-blocker drug in patients with ventricular, atrial and supraventricular arrhythmias. A simple and sensitive method based on HPLC-fluorescence has been described for the quantification of its internal standard. Atenolol was eluted after 5.9min, from a 4-micron C_{18} reverse-phase column using a mobile phase consisting of 80 mM KH_2PO_4, pH 4.6, and acetonitrile (95:5, v/v) at a flow rate of 0.5 ml/min with detection at maximum wavelength 310 nm.

Kumar et al [75] have been reported the determination of atenolol by HPLC in pharmaceutical formulations. The determination was carried out on a reversed phase C18 column using 0.1% orthophosphoric acid (pH=3): acetonitrile (20:80) at a flow rate of 1.0 ml min-1 with UV-detection at 238nm. The statistical evaluation of the methods was examined by determining intra-day and inter-day precision. The methods, when applied to the determination of atenolol in tablets, gave satisfactory results. Accuracy and reliability of the proposed method was further ascertained by parallel determination by reference method and by recovery studies.

Roland et al [76] have determined furosemide in plasma and urine by reverse-phase (HPLC) and fluorescence detection. Zorbax column (SB-C816), 4.6 mm x 15cm17, kept at 40 ºC, and mobile phase consisting of

65% 0.02 M phosphate buffer (pH 2.4) and 35% acetonitrile was used. The mobile phase flow rate was 1.0 ml/min**.**

1.9. Description of the drugs

1.9.1Furosemide

Furosemide, (RS)-4-chloro-n-furfryl-5-sulfamoyanthranilic acid, $C_{12}H_{11}ClN_2O_5S$, (figure (1-3)) is a white powder with molecular weight of 330.7; it melts in the range (145-149c^o). It is sparingly soluble in water; soluble in absolute ethanol and methanol and practically insoluble in ether [77].

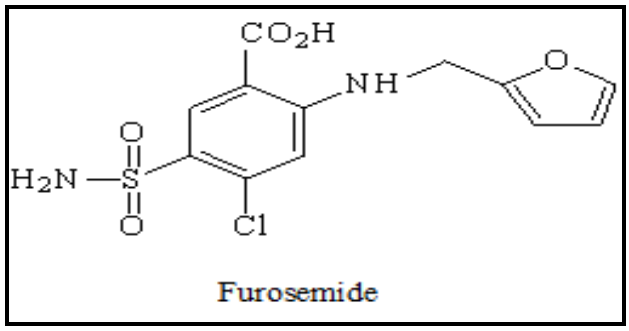


Figure (1-3) structure of furosemide

Furosemide is commonly used in the treatment of arterial hypertension, angina pectoris. Furosmide tablet (Lazix) is manufacture locally by the State Company for Drug Industrial and Medical Appliances (Samarra-IRAQ-SDI). It is the diuretics that have major action on the ascending limb of the loop of Henle. Compared to all other classes of diuretics, this drug has the highest efficacy in mobilizing Na^+ and Cl^- from the body.

Sekikawa et al [78] have been determine furosemide in blood by using 150×65µm Shim-pack CLC-ODS column (Shimaduz), mobile phase gradient consisted of A MeCN : water 20:80 containing 0.3% acetic acid and B MeCN : water 80:20 containing 0.3% acetic acid. A:B 90:10 for 3min, to 60:40 over 7min. maintain at 60:40 for 5min, to 40:60 over 3min, to 60:40 over 2min, to

90:10 over 10min, column temperature 40c°, detector florescent 345nm and 415nm, retention time 15min and detection limit at 5ng/ml.

Matsuura et al [79] have determined furosemide in blood using 35×4.6µm TSK BSA-ODS column, using gradient elution program using of mobile phase A methanol : 2.5mM pH 5 ammonium phosphate 1:50; B MeCN : 2.5mM pH 2.5 ammonium phosphate 31:69; C MeCN : water 50:50. With flow rate of A 1.2; B1; and C1.5 ml/min, detector UV 254nm, retention time 19.7min and detection limit 100ng/ml.

Vree et al [80] have determined furosemide in blood and urine using guard column 75×2.1µm pellicular reversed phase; 250×4.6µm Cp Spherisorb ODS column, mobile phase gradient consisted of MeCN : 0.5% pH 2.1 orthophosphoric acid (98%), from 5:95 to 41:59 over 30min, stay at 41:59 for 5min, return to 5:95 over 5min, equilibrated for 2min before next injection. flow rate 1.2ml/min, detector florescent 345nm and 405nm, retention time was 28.77min and detection limit of 5ng/ml.

1.9.2. Atenolol

Atenolol, (RS)-4-(2-hydroxy-3-[(1-methylethyl)-amino] benzeneacetamide, $C_{14}H_{22}N_2O_3$, (figure (1-4)) is a white powder with molecular weight of 266.3; it melts in the range (152-155c°). It is sparingly soluble in water; soluble in absolute ethanol and methanol and practically insoluble in ether.

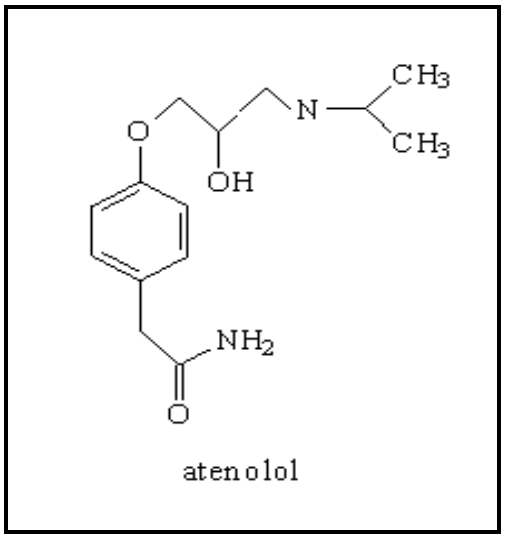


Figure (1-4) structure of atenolol

Atenolol is used commonly in the treatment of arterial hypertension, angina pectoris and cardiac arrhythmias. Tenormine is an atenolol tablet is manufacture locally by the State Company for Drug Industrial and Medical Appliances (Samarra-IRAQ-SDI). This drug also differs in intrinsic sympathomimetic activity, in central nervous system (CNS) effects, and in pharmacokinetics. Although all ß-blockers lower blood pressure in hypertension, they do not induce postural hypotension because the á-adrenoceptors remain functional; therefore, normal sympaththetic control of the vasculature is maintained. ß-blockers are also effective in treating angina, cardiac arrhythmias, myocardial infarction, and glaucoma, as well as serving in the prophylaxis of migraine headaches.

Atenolol preferentially block the ß1 receptors to eliminate the unwanted bronchoconstrictor effect (ß2) of propranolol among asthmatic patient. Cardio-selective ß-blockers, such as acebutolol, atenolol, antagonize ß1 receptors at doses 50 to 100 times less than those required to block ß2 receptor. This cardio-selectivity is thus most pronounced at low doses and is lost at high drug doses [(81)].

These drugs lower blood pressure in hypertension patient and increase exercise tolerance in angina. Atenolol has a very short lifetime due to metabolism of the ester linkage. It is only given intravenously if required during surgery or diagnostic procedure (cystoscopy). In contrast to propranolol, the cardio-specific blockers have relatively little effect on pulmonary function, peripheral resistance, and carbohydrate metabolism. Nevertheless, asthmatics treated with these agents must be carefully monitored to make certain that respiratory activity is not compromised [(82)].

Hartmann et al [(83)] have determined atenolol in blood using 83×4.6µm Pecosphere C18 column, mobile phase was MeCN : Methanol : 10mM pH 4.0 sodium acetate containing 10mM octanesul-fonic acid 17:2:80, detector florescent 228nm and 310nm, retention time 6.0min and detection limit 15ng/ml.

Phelps et al [(84)] have determined atenolol in blood using 250 × 4.6µm Supelcosil LC-8-CB columns, mobile phase was MeCN: 20mm ammonium dihydrogen 6:94 containing 2% triethylamine, pH adjusted to 5.0. Flow rate 1.0ml/min, detector UV 220nm, retention time 8.0min and detection limit 50ng/ml.

Stoschitzky et al [(85)] have determined atenolol in blood and urine using 250×4.0µm LiChrosorb Si 100 modifier with (R, R)-DACH-DNB column, mobile phase was dichloromethane: Methanol 98:2. Flow rate 1.0ml/min, injection volume 20 µl, detector florescent 230nm and 300nm, retention time 8.7min and detection limit 5ng/ml.

1.9.3. Amiloride

Amiloride, (RS)-3, 5-diamino-n-(aminoiminomethyl)-6-chloropyrazine carboxamide, $C_6H_8N_6OCl$, (figure (1-5)) is a white powder with molecular weight of 215.5; it melts in the range (139-142c°). It is sparingly soluble in water; soluble in absolute ethanol and methanol and practically insoluble in ether.

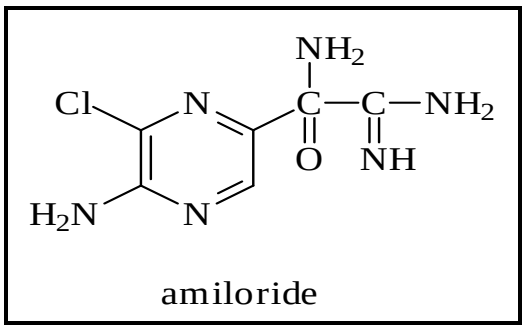


Figure (1-5) structure of amiloride

Amiloride is used commonly in the treatment of hypertension. Amiloride tablet (Saluretic) is manufactured locally by the State Company for Drug Industrial and Medical Appliances (Samarra-IRAQ-SDI). Potassium-sparing diuretic are used primarily when aldosterone present in the excess. It blocks the Na^+ transport channels resulting in increase in Na^+-K^+ exchange in the collecting tubule.

Deleson et al [86] have determined amiloride in blood using 250×4.6µm Supelco C18 column, mobile phase was MeCN : buffer 45:55 (buffer was 44mM KH_2PO_4 containing 1.5 ml/l triethylamine, adjusted at pH 2.5 with phosphoric acid), detector UV 240nm, retention time 3.33min and detection limit 65ng/ml.

El-Sayad et al [87] have determined amiloride in blood using 300×3.9µm Bondapak C18 column, mobile phase was methanol: water 62:38 adjusted to

pH 3.5 with phosphoric acid, temperature 40c°, flow rate 1.2ml/min, detector UV 262nm, retention time 4.0min and detection limit 70ng/ml.

El-Yazigi et al [88] have determined amiloride in blood and urine using 100×8.0µm C8 column, mobile phase was dichloromethane : MeCN : methanol :56 mM ammonium acetate : 1M ammonium hydroxide 73:10:4.4:2.6. Flow rate 2.5ml/min, detector UV 220nm, retention time was 4.7min and detection limit 45ng/ml.

1.10. Vitamin E

Vitamin E, α-Tocopherol, $C_{29}H_{56}O_2$, (figure (1-6)) is a white powder with molecular weight of 436.5; it melts in the range (125-129c°). It is sparingly soluble in water; soluble in absolute ethanol and methanol and practically insoluble in ether.

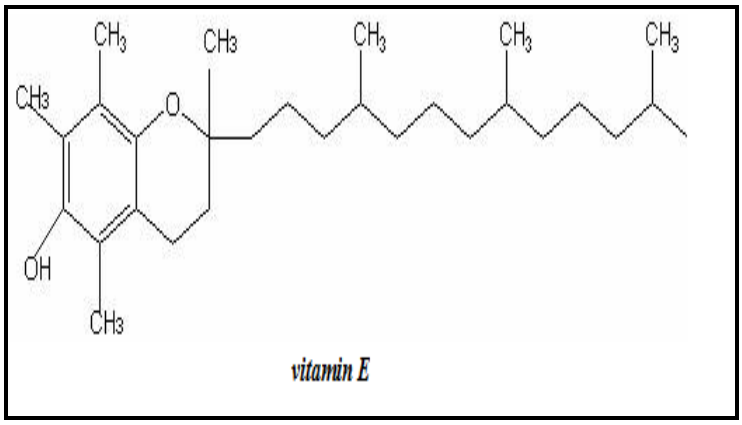


Figure (1-6) structure of vitamin E

Vitamin E is an oil which is present in plants, particularly wheat germ, rice and cotton seeds. Although fish liver oils are rich in vitamins A and D, they are devoid of vitamin E. Vitamin E has the widest natural distribution and the greatest biologic activity as a vitamin. Although there is no reliable evidence that vitamin E is necessary for fertility in human, it is clear that vitamin E-deficient state exists in human with severely impaired intestinal fat absorption. The signs of vitamin E deficiency in humans are muscular weakness,

creatinuria, and fragile erythrocytes. All disappear after the administration of α-Tocopherol. α-Tocopherol is readily absorbed through the small intestine, transported to liver probably in chylomicrons, and delivered by lipoproteins to peripheral tissue. The phospholipids of mitochondria, endoplasmic reticulum, and plasma membranes posses specific affinities for α-Tocopherol, and the vitamin appears to concentrate at these sites. Vitamin E has at least two metabolic roles; it acts as nature most potent fat-soluble antioxidant, it plays specific but incompletely understood role in selenium metabolism [(89)].

Morishige et al [(90)] have determined vitamin E in blood using 75×4.6µm TSK gel ODS-8OTM column, mobile phase was MeCN : 50 mM KH_2PO_4 8:92, detector UV 225nm, retention time was 6.33min and detection limit 650ng/ml.

Miles et al [(91)] have determined vitamin E in blood and serum using C18 column, mobile phase was ethanol: water 95:5, flow rate 1.2ml/min, detector UV 229nm, retention time was 4.50min and detection limit 700ng/ml.

Luksa et al [(92)] have determined vitamin E in blood using 150×4.6µm Nucleosil RP-18 column, mobile phase was MeCN : 5mM pH 2.8 KH_2PO_4 10:90, flow rate 1.0ml/min, detector UV 227nm, retention time was 5.57min and detection limit 45ng/ml

1.11. Analysis of amino acids

Abraham et al [(93)] have determined Tryptophane using 75×4.6µm ODS-8 column, mobile phase was MeCN, detector UV 254nm, retention time was 4.33min and detection limit 80ng/ml.

Anderson et al [(94)] have determined Tryptophane using C18 column, mobile phase was ethanol: THF: water 80:5:15, flow rate 1.3ml/min, detector UV 254nm, retention time was 4.50min and detection limit 44ng/ml.

Baron et al [95] have determined Tryptophane using C-18 column, mobile phase was ethanol : 2mM pH 2.8 KH_2PO_4 95:5, flow rate 1.0ml/min, detector UV 254nm, retention time was 4.57min and detection limit 35ng/ml.

Bennett et al [96] have determined Phenylalanine using ODS column, mobile phase was MeCN: methanol 65:35, detector UV 254nm, retention time was 7.93min and detection limit 55ng/ml.

Berezkin et al [97] have determined Phenylalanine using C-18 column, mobile phase was ethanol: THF 80:20, flow rate 0.8ml/min, detector UV 254nm, retention time was 6.95min and detection limit 70ng/ml.

Beroza et al [98] have determined Phenylalanine using C-8 column, mobile phase was acetonitrile: water 90:10, flow rate 1.0ml/min, detector UV 254nm, retention time was 7.37min and detection limit 100ng/ml

Cantrell et al [99] have determined Tyrosine using C-8 column, mobile phase was ethanol: phosphate buffer pH 2.8 95: 65, detector UV 254nm, retention time was 2.89min and detection limit 190ng/ml.

Ettre et al [100] have determined Tyrosine using ODS column, mobile phase was acetonitrile, flow rate 1.1ml/min, detector UV 254nm, retention time was 3.22min and detection limit 120ng/ml.

Giddings et al [101] have determined Tyrosine using C-18 column, mobile phase was ethanol: water 75:25, flow rate 1.1ml/min, detector UV 254nm, retention time was 3.33min and detection limit 100ng/ml

The aim of the work

The aim of this work was to prepare new cross-linked polymers to be used as HPLC stationary phases. The first polymer was prepared based on condensation reaction of glycerol, triethanolamine, and maleic anhydride, while the second was based on the addition reaction by free radical reaction of acrylamide, bisacrylamide with ammonium persulphate and N,N,N,N-tetramethyldiamine TEMED.

The chromatographic performance of columns filled with these polymers was studied for the separation of furosmide, atenolol, amiloride, phenylalanine, tryptophane, tyrosine and vitamin E. The properties of these used columns were compared with imported ODS (C-18) column.

CHAPTER TWO
EXPERIMENTAL

2.1. Chemicals

The materials and chemicals listed Table (2-1) which was obtained from different solvents has been used in this work

Table (2-1) Chemicals and their suppliers

Compounds	***Supplied from***	***Purity***
Acetone	BDH	99.9%
Acetonitrile(analar)	Fluka	99.9%
Acrylamide	Fluka	98.9%
Ammonium chloride	BDH	99.9%
Ammonium persulphate	BDH	99.9%
Amiloride	Samarra	99.9%
Atenolol	Samarra	98.9%
Benzene	BDH	97.9%
Benzoyl peroxide	BDH	99.9%
Chloroform	Hopkins and Williams	98.9%
Carbon tetrachloride	BDH	99.9%
Dioxine	Fluka	96.9%
DMF	Fluka	99.9%
DMSO	Fluka	97.9%
Ethanol(analar)	Fluka	99.9%
Furosmide	Samarra	98.9%
Glycerol	Fluka	98.9%
Hexane	Merck	99.9%
Isopropanol	Merck	97.9%
Maleic anhydride	Fluka	99.9%
Methacrylic acid	BDH	98.9%
Methanol(analar)	Fluka	98.9%
N,N-bisacrylamide	BDH	98.9%
n-heptane	Merck	99.9%
N,N,N,N-tetramethyldiamine (TEMED)	BDH	99.9%
Orthophosphoric acid	BDH	97.9%
Potassium dihydrogen	BDH	98.9%

phosphate		
Phenylalanine	BDH	99.9%
Sodium acetate	BDH	96.9%
5-Sulphosalsalic acid	BDH	99.9%
THF	BDH	98.9%
Toluene	BDH	99.9%
Triethanolamine	BDH	94.9%
Tryptophane	BDH	99.9%
Tyrosine	BDH	98.9%
Vitamin E	Fisher	99.9%

Atenolol, furosmide and amiloride standard and Tenormine, Lazix and Saluretic tablets (100mg) were a gift from state Company for Drag Industries and Medical Appliances (Samarra-IRAQ-SDI) Novaten tablets (100mg) marketed by Ajanta Pharmaceutical Limited Company India. 100mg Egyptian Industrial pharmaceutical Industries Company (EIPICO) was purchased from local markets.

2.2. INSTRUMENTS AND EQUIPMENTS

- High performance liquid chromatograph used in this work was Shimadzu (Kyoto, Japan) which consisted of a system controller model SCL-10 AVP, a degasser model DGU-12A, two liquid delivery pumps model LC-8AVP, UV-Visible detector model SPD-10AVP, and injector model SIL-10A, equipped with 20 µl sample loop. The HPLC system has been interfaced with computer via a Shimadzu class-VP5 chromatography data system program supplied by the manufacturer; Epson LQ-300 printer model P852A (Japan).
- Sonicator Sonerex model Super PK 103H Mandolin (Germany).
- Glass combination electrode was used to measure the pH of polymer solutions (Germany).
- Shimadzu Fourier transforms infrared model FTIR 8300 (Kyoto, Japan) was used to measure the IR spectra for the prepared polymers.

- X-Ray diffractometer, Siemens SRS D500 (Germany).
- Two blank stainless steel columns, dimensions 25x 0.4cm (i.d.) were obtained locally.

2.3. Preparation of co-polymer *(triethanylamine glycerol maleate)*

In a 100ml round-bottom flask placed in a sand-bath and equipped with a thermometer and stirrer, a mixture of 7.45gm (0.05mole) triethanolamine and 4.61gm (0.05mole) glycerol were placed. The mixture was stirred for 15min. and 14.7gm (0.15mole) of maleic anhydride was added to the mixture and the temperature rise gradually to 160 ^{o}C, and maintained for 3 hours. The reaction was performed under vacuum pressure. Continued heating at this temperature causes an increase in viscosity of the solution until crystalline polymer was formed. The final product was washed with warm water and methanol for several times, and then dried in vacuum oven at 50^{o}C over night.

The same reaction was carried out three times by adding a mixture consisting of (100:0), (75:25), (50:50) and (25:75) triethanolamine; glycerol, respectively, instead of glycerol alone [(102)].

The most suitable polymer, as far as its ability for grinding was produced from reaction using (50:50) triethanolamine and glycerol with maleic anhydride.

2.4. Preparation of co-polymer (acrylamide bisacrylamide methacrylic acid)

In order to obtain the best mixture ratio of the reactants that can be used to prepare the polymer, the amount of acrylamide was changed from 2gm to 0gm, while that of methacrylic acid was changed from 0gm to2gm and that of N, N'-methylene-bisacrylamide was changed from 0.12gm to 0.90gm. To all these material 10µl of TEMED and 0.35ml of 1.5% ammonium persulphate were added. The final volume of all reactants was made up to 10ml with distilled water. These proportions are listed in Table (2-2). The solution was filtered and store in the dark in refrigerate 4C°. This polymer was used as gel in electrophoresis [103].

Table (2-2) the proportion of reactants used for preparation of co-polymer (acrylamide-bisacrylamide-methacrylic acid)

Acrylamide	*Merthacrylic acid*	*Bis acrylamide*	*TEMED*	*Amonium persulphate*
2.00gm	0.00 gm	0.12 gm	١٠µl	0.35ml
1.50 gm	0.50 gm	0.12 gm	١٠µl	0.35ml
1.00 gm	1.00 gm	0.12 gm	١٠µl	0.35ml
0.50 gm	1.50 gm	0.12 gm	١٠µl	0.35ml
0.00 gm	2.00 gm	0.12 gm	١٠µl	0.35ml
2.00 gm	0.00 gm	0.24 gm	10µl	0.35ml
1.50 gm	0.50 gm	0.24 gm	10µl	0.35ml
1.00 gm	1.00 gm	0.24 gm	10µl	0.35ml
0.50 gm	1.50 gm	0.24 gm	10µl	0.35ml
0.00 gm	2.00 gm	0.24 gm	10µl	0.35ml
2.00 gm	0.00 gm	0.60 gm	10µl	0.35ml
1.50 gm	0.50 gm	0.60 gm	10µl	0.35ml
1.00 gm	1.00 gm	0.60 gm	10µl	0.35ml
0.50 gm	1.50 gm	0.60 gm	10µl	0.35ml
0.00 gm	2.00 gm	0.60 gm	10µl	0.35ml
2.00 gm	0.00 gm	0.90gm	10µl	0.35ml
1.50 gm	0.50 gm	0.90gm	10µl	0.35ml

1.00 gm	1.00 gm	0.90gm	10µl	0.35ml
0.50 gm	1.50 gm	0.90gm	10µl	0.35ml
0.00 gm	2.00 gm	0.90gm	10µl	0.35ml
2.00 gm	0.00 gm	0.60 gm	20µl	0.75ml
1.50 gm	0.50 gm	0.60 gm	20µl	0.75ml
1.00 gm	1.00 gm	0.60 gm	20µl	0.75ml
0.50 gm	1.50 gm	0.60 gm	20µl	0.75ml
0.00 gm	2.00 gm	0.60 gm	20µl	0.75ml
2.00 gm	0.00 gm	0.90gm	20µl	0.75ml
1.50 gm	0.50 gm	0.90gm	20µl	0.75ml
1.00 gm	1.00 gm	0.90gm	20µl	0.75ml
0.50 gm	1.50 gm	0.90gm	20µl	0.75ml
0.00 gm	2.00gm	0.90gm	20µl	0.75ml

It was found that the most suitable polymer, to meet HPLC requirement was the polymer (acrylamide-bisacrylamide) which produced from reaction of 2gm (0.2 mol.) acrylamide, 0.6gm (0.08mol.) N, N'-methylene-bisacrylamide with 20µl TEMED and 0.75ml amonium persulphate. This polymer was then used to pack HPLC columns

2.5. Stability tests

A- The solubility of poly (triethanylamine-glyceryl maleate) and poly (acrylamide-bisacrylamide-Methacrylic acid) polymers has been examined using different polarity solvents. The polymer was tested against water, methanol, ethanol, dioxane, chloroform, carbon tetrachloride, hexane, and benzene.

B- The stability of the polymers in acidic and alkaline solutions was also examined by treating the polymer with solutions of different pH ranged from 1 to 9 for a period of two weeks.

2.6. Polymer Swelling Determination

Swelling measurements of the polymers was done by placing a small quantity of dry washed polymer sample in solvents such as Ethanol, Methanol, Acetonitrile, Acetone, and Water. The weight of the polymer after (48) hours was measured and the swelling of the polymer was calculated as percentage using the following equation [(104)].

$$\Delta m = \frac{m_t - m_0}{m_0} \times 100$$

Were Δm = swelling
m_o = weight of dry sample
m_t = weight of hydrate sample

2.7. Packing of the column

Two different methods were used to pack HPLC columns:

A- The empty column used was first cleaned with 50% nitric acid and then with methanol after washing with distilled water and drying. The column was packed with appropriate stationary phase as describe bellow.

The column was packed using down-flow packing method. Shimadzu pump (a) as shown in the figure (1-1) was used instead of the air-driven pump for packing. The packing material was dispersed in 100 ml acetonitrile and placed in the slurry reservoir (b). The column (c) was plugged with its appropriate fitting from one side and placed in a beaker for draining; the other side was connected to the slurry reservoir (b). The pump was set at the beginning to deliver 4 ml/min. The back pressure read 5.0Mpa. The flow rate was then increased by 1 ml/min increment at constant period of time until reaching 8 ml/min. The recorded pressure was 6.0Mpa, 7.0Mpa, 8.0Mpa and 9.0Mpa, respectively. After the compression process was completed, the column was then disconnected

from the system and was checked visually and found to be completely packed, and then connected to the HPLC system.

B- The empty column tube used in this work was first cleaned as before. It was than packed with appropriate stationary phase as follow. The column was plugged with column fitting from one side and hanged by a clamp near the open end. It was placed in the sonicator bath filled with water. Slurry of 3grams of the polymer was made with 15ml hexane. A few milliliters (1-2ml) of this slurry were poured into the column using a dropper, and the sonicator turned on. After about 15 minutes, which was found to be enough to lead the polymer to settle down inside the column, another portion was poured in. This process was continued until the column was completely filled with the polymer as was found by visual inspection. The column fitting was re-placed on the open side and connected to the liquid delivery pump of the HPLC. The pump was set at the beginning to deliver 1ml/min, using hexane. The backpressure was monitored until it reached a stable value of 1.5Mpa .The flow rate was then increased by 1ml increments until reaching 5ml/min, at constant period of time. The recorded backpressure was 3.2Mpa, 4.6Mpa, 5.5Mpa, and 6.4Mpa, respectively. After the compression process was completed; one side of the column opened (the one used for packing) was inspected visually for complete packing. More stationary phase was poured into the column when it was found not completely full. These processes, packing and compression were repeated until having a well-packed column. The column was then connected to the HPLC system. The flow rate set at 1ml/min. Hexane was passed through the system for about 2 hours before analysis.

2.8. Sample preparation

2.8.1. Preparation of standard

A stock solution of 50ppm of standard drugs were prepared by dissolving 5mg of furosmide in 0.1M of NaOH and diluted to 100ml with distilled water or dissolving 5mg of furosmide in methanol and then diluted to 100ml with distilled water. The same procedure for amiloride and atenolol was followed in the preparation of their stock solutions. Other standard solutions were prepared by subsequent dilution of the stock solutions. The solvent used to prepare these solutions before injection into HPLC was the buffer that was usually used as the mobile phase employed for their separation.

A stock solution of 50ppm of standard amino acids and vitamin E were prepared by similarly.

2.8.2. Preparation of drug formulation solutions

A standard solution of atenolol obtain from three different tablet manufactures were prepared (Approximately 1000ppm). One atenolol tablet from each brand was grinded and mixed thoroughly. A sample of 50mg was dissolved in a small amount of ethanol and filtered. The filtrate was diluted to 50ml with ethanol [(105)]. Furosmide and amiloride solutions were prepared similarly.

2.8.3. Serum sample preparation

In order to analyze drugs in serum, the samples were prepared by mixing 0.25ml of serum or plasma with 0.25ml of buffer (saturated ammonium chloride at pH 9.5), and 0.50ml of a mixture containing 60% chloroform -14% isopropanol-26% n-heptane. The solution was shaken gently for 10min, centrifuged at 2800rpm for 10min. The organic layer was withdrawn and evaporated under vacuum at 45c^{o}. A 100 ml of

mobile phase was added to residue, and centrifuge at 2800rpm for 5min. A 1ml of superannuated was withdrawn then diluted to 20ml and filtrated using minipore filter paper [106].

In the analysis of vitamin E in serum, the samples prepared by adding 50µl of 15% 5-sulphososalsic to 400µl of deprotenation frozen serum, then mixed and centrifuge at 3000rpm for 10min. The supernated was taken and diluted ten folds with distilled water and filtrated using minipore filter paper [107].

2.9. Analysis of samples

All the prepared samples, standard solutions, and mixtures of then have been chromatographically analyzed with the prepared two new packed columns using different mobile phases. Mobile phase such as methanol, ethanol, acetonitrile, and buffer or mixture of some of them at optimum flow rate. UV-VIS detection at wavelength 233nm, 245nm and 229nm for drugs, amino acids and vitamin E, respectively.

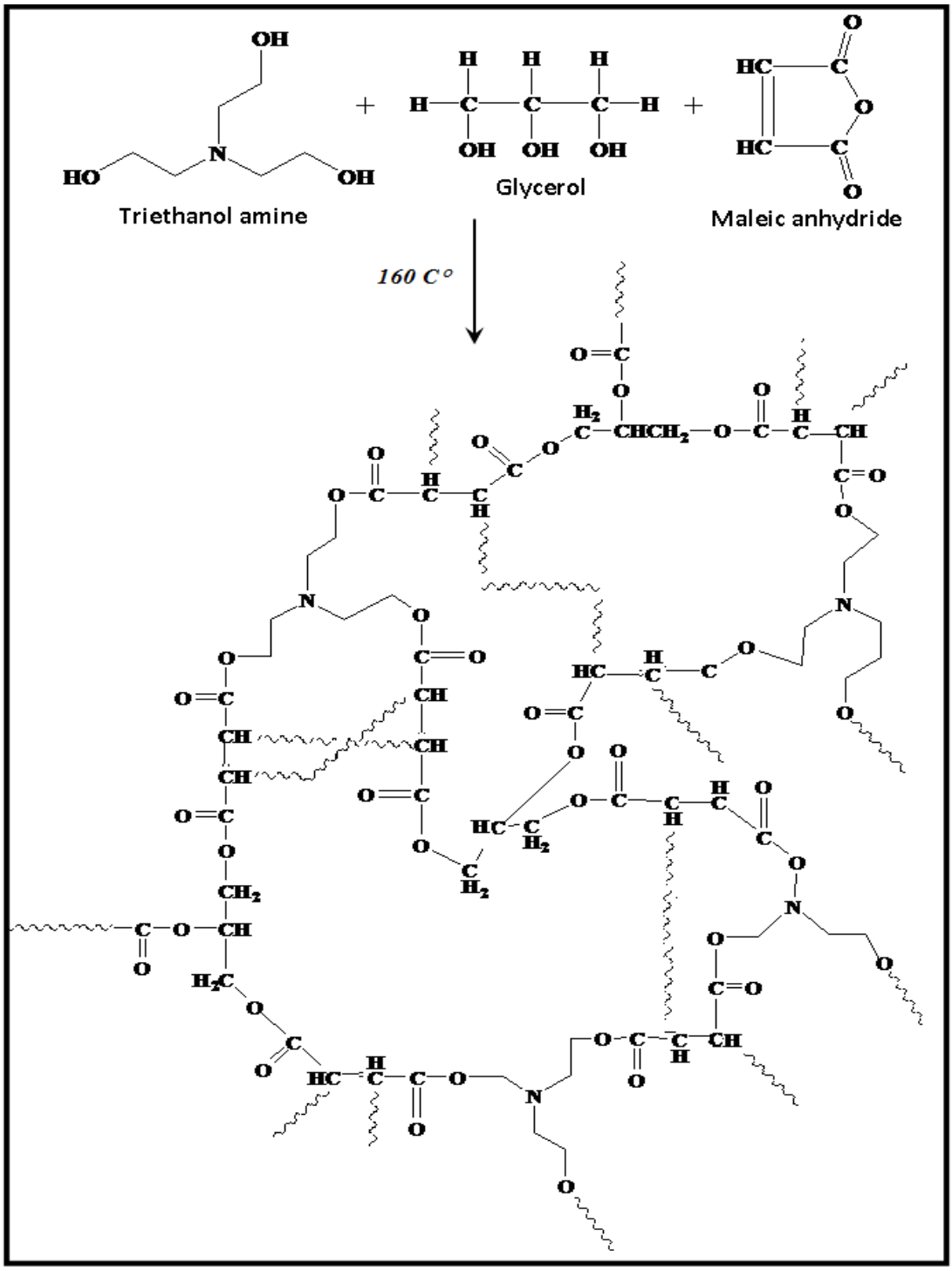
The same samples were analyzed with C-18 column using the literate parameter.

CHAPTER THREE
RESULTS AND DISCUSIONS

3.1. Synthesis of co-polymer (triethanolamine-glycerol-maleate)

Synthesis of co-polymers was done via condensation polymerization process by addition-elimination mechanism on the carbonyl group of the carboxylic acid [(108)].

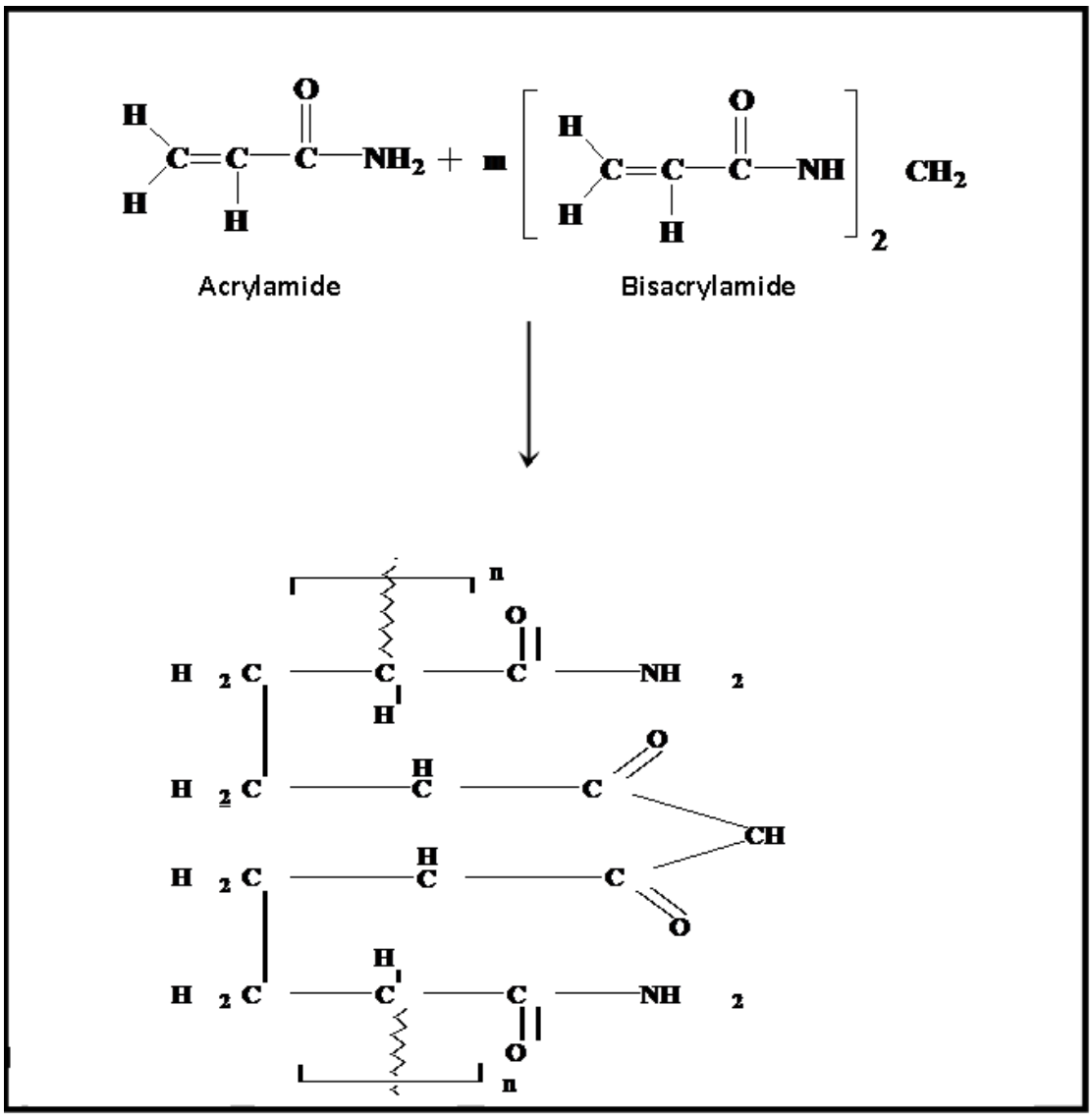
It is known that condensation reaction of glycerol with maleic anhydride produce a hard and rigid co-polymer [(109).] On the other hand, it was found that condensation of triethanolamine and glycerol with maleic anhydride produced less hard and rigid co-polymers (polymer I, II, and III). While the condensation of triethanolamine with maleic anhydride produced co-polymer tend to be elastic. The chemical reaction for the suggested co-polymer is shown in scheme (3-1).



Scheme (3-1) Suggested structure of co-polymer (triethanolamine-glycerol-maleate).

3.2. Synthesis of co-polymer (acrylamide-bisacrylamide)

Synthesis of the co-polymer was done via addition polymerization by free radical mechanism and the suggested structure of the co-polymer is shown in scheme (3-2):-



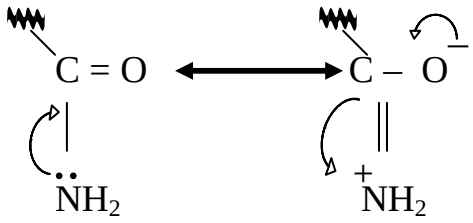
Scheme (3-2) Suggested structure of co-polymer (acrylamide-bisacrylamide)

3.3. Polymer characterization

All prepared co-polymers were characterized by their solubility in different solvents. The high degree of cross linking of these co-polymers prevented the solvation process, and for this reason it was defiant to determine the molecular weight and the degree of polymerization.

However, the co-polymer was identified by FTIR spectrum as is showed in figure (3-1). The spectrum showed the appearance of the characteristic absorption bands at 1732 cm^{-1} due to the stretching vibration of the (C=O) of the formed ester. A band at 1161 cm^{-1} was due to the (C-O) stretching of ester and appearance of (C-N) band at 1296 cm^{-1}.

The FTIR spectrum for acrylamide-bisacrylamide co-polymer was also obtained and it as showed in figure (3-2). It is noticed that the stretching vibration of the (NH_2) group with it is two symmetric parts 3200 cm^{1} and with asymmetric at about 3370 cm^{-1}. These two peaks were present cases of the hydrogen bonding.



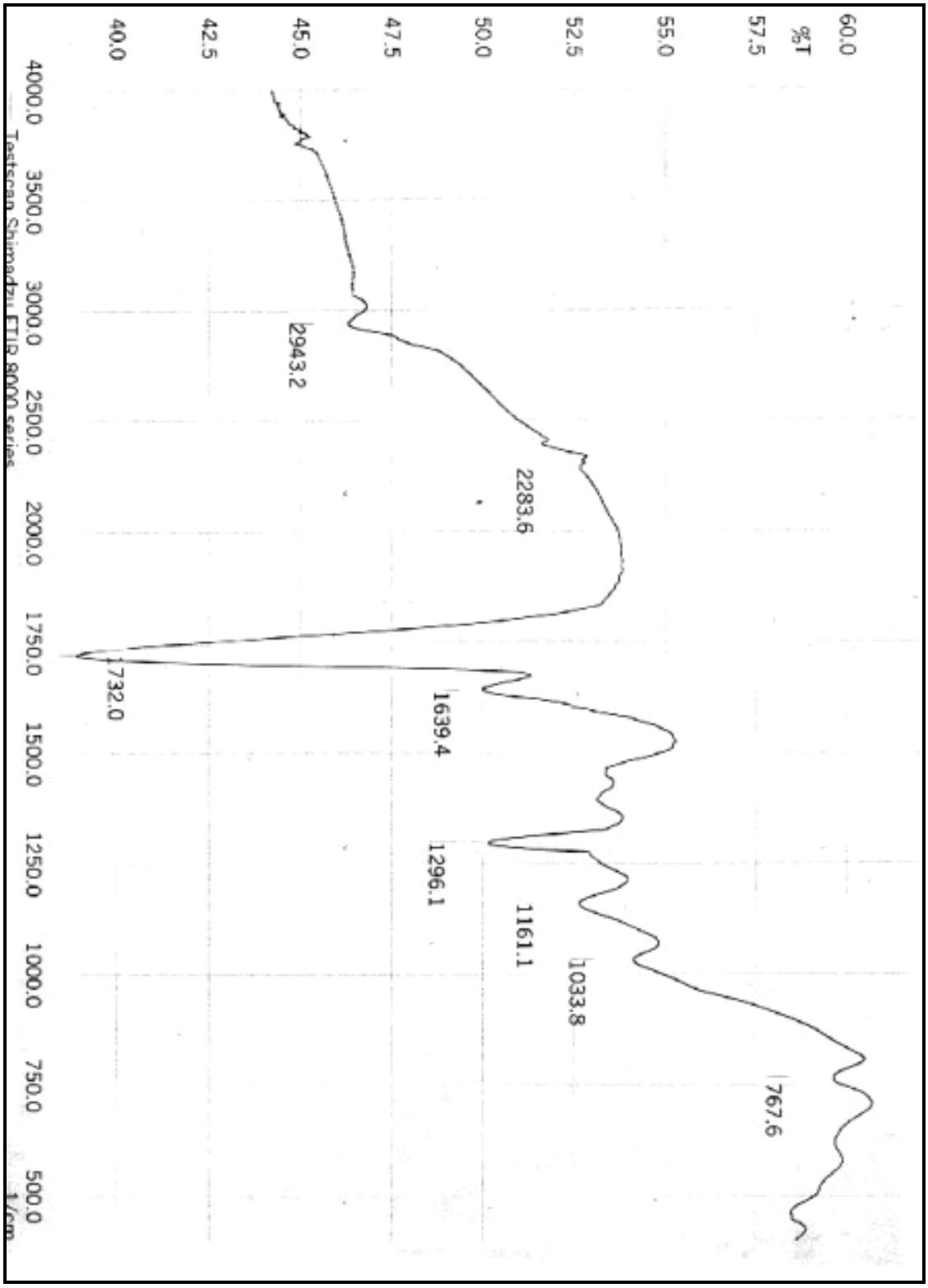


Figure (3-1) FTIR spectrum of the co-polymer(triethanolamine-glycerol-maleate)

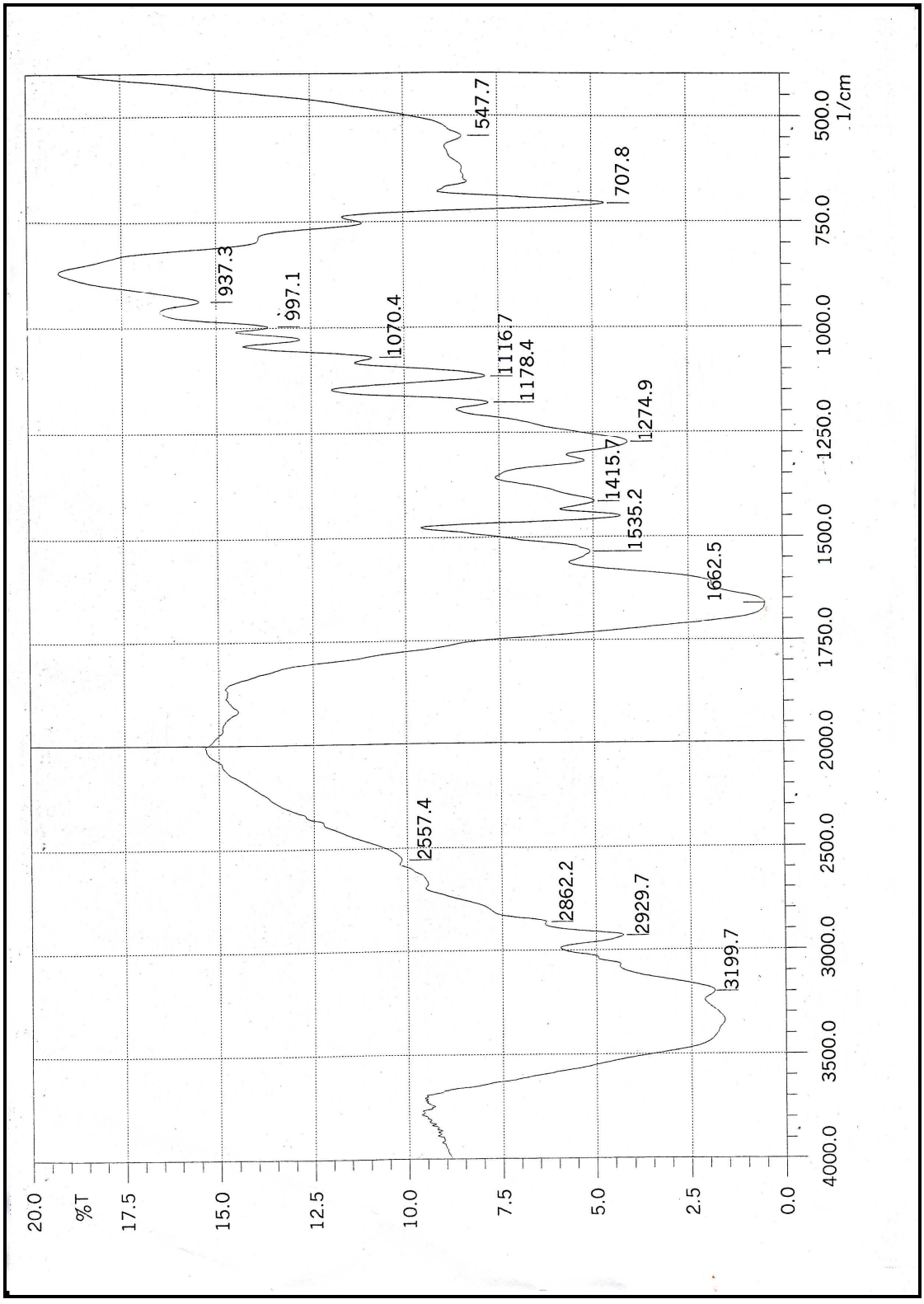


Figure (3-2) FTIR spectrum of the co-polymer (acrylamide-bisacrylamide)

All the above mentioned cases, the peak at 1535 cm^{-1} retain to the group of $C=^{+}NH_2$ and the streaching of the peak at 1275 cm^{-1} which belongs to the bond of $C - O^{-}$ which consists during the resonance.

X-ray diffraction was also used to identify the nature of the polymers whether it is a crystalline or not. The results showed that the polymers have different crystillanity forms in which the polymers revealed crystalline nature greater than acrylamide-bisacrylamide polymer as it is showed in figures (3-3) and (3-4).

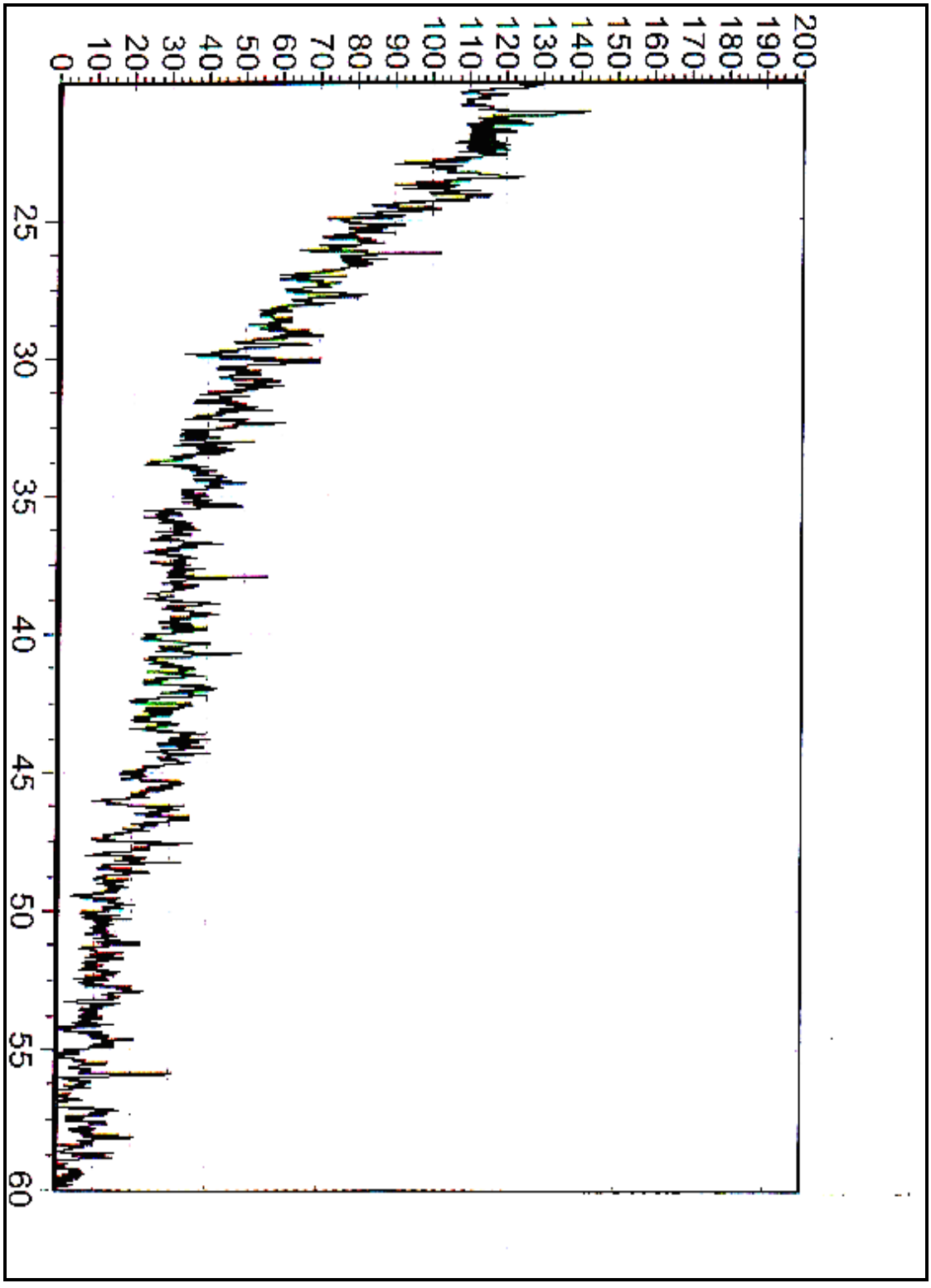


Figure (3-3) X-ray diffraction of the co-polymer (triethanolamine-glycerol-maleate)

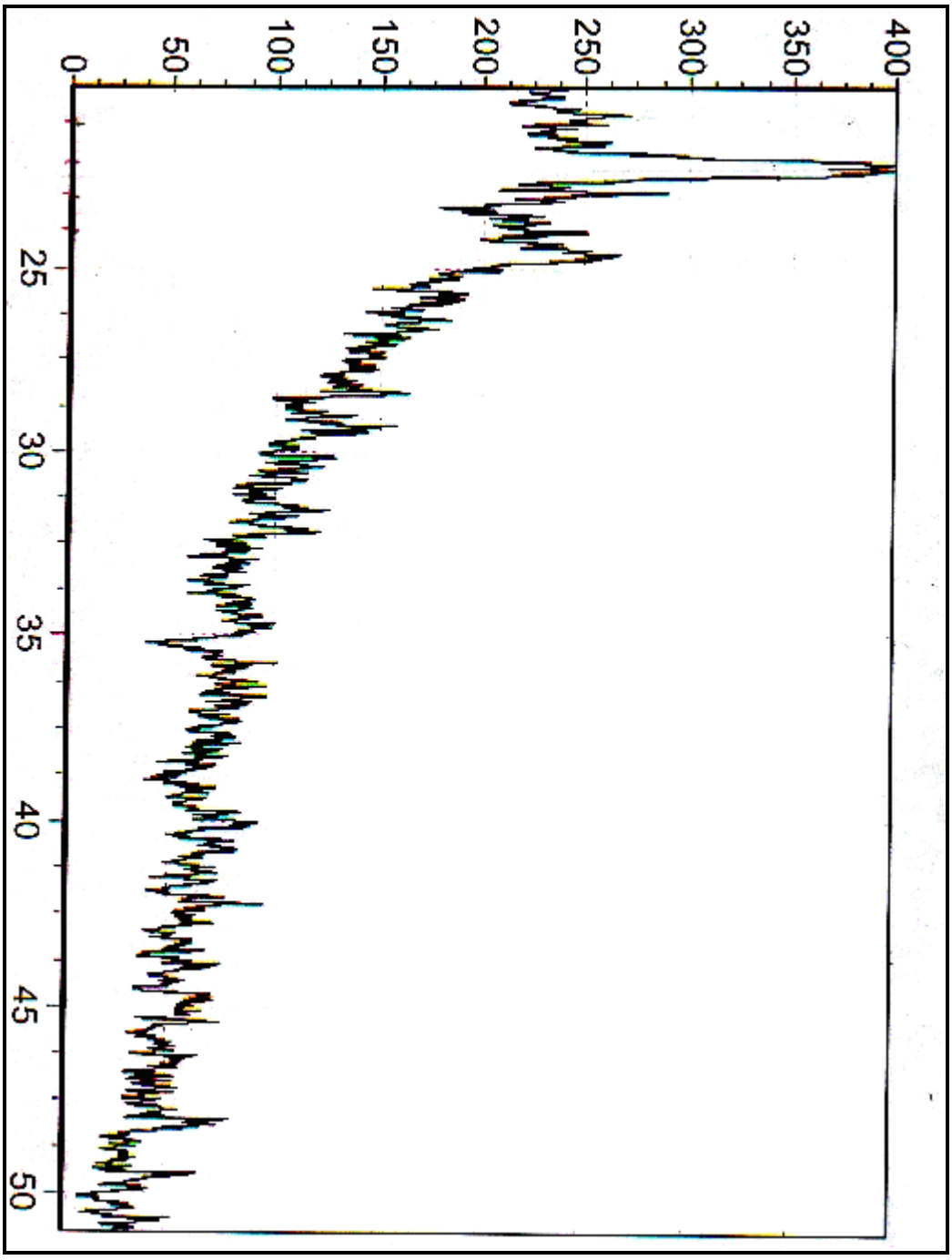


Figure (3-4) X-ray diffraction of the co-polymer (acrylamide-bisacrylamide)

3.4. Swelling of the modified co-polymers

Swelling of the modified co-polymer to be used as stationary phase and its performance when packed in an HPLC column is of a considerable practical importance. In HPLC applications, polymer particles are packed into a column; swelling will cause the packed bed to be tightened and subsequently will affect the flow of the mobile phase through the column producing excessive back pressure, and will cause polymer bed to fracture. The reverse, or polymer bead contraction, can be equally undesirable in column operation. In this case, the flow rate is sharply changed due to shrinking of the polymer particles, and channeling, particularly at the column walls. This has also a significant effect on how the mobile phase flows through the column [(110)].

Swelling test for prepared polymeric stationary phases was performed according to ASTM procedure [(111)]. The degree of cross-linking has been measured using polar, moderately polar and non polar that are usually used in HPLC such as solvents water, acetone, acetonitrile, and hexane. The results of the swelling tests are listed in Table (3-1) which indicates the swelling value. These results were theoretically expected; the values were ranged from 1.1-3.4% compared with the expected swelling of 3.6%**,** but that in water was unexpected because it was over 3.6% which could be attributed to the presence of hydrogen bonding forming moiety as the polymer surfaces.

Table (3-1): Swelling of polymeric stationary phase.

The co-polymers	***solvents***	***Swelling percentage (%)***
Triethanolamine-glycerol-maleate co-polymers	Water	4.1
	Acetone	2.4
	Hexane	1.6
	Acetonitrile	1.1
Acrylamide-bisacrylamide co-polymer	Water	5.6
	Acetone	3.4
	Hexane	1.2
	Acetonitrile	2.8

3.5. Solubility tests for cross-linked polymers

The solubility has been examined using different solvents such as acetonitrile, benzene, chloroform dioxane, DMF, DMSO, hexane, methanol and water. It is found that the polymers were insoluble and undecompose in all these solvents. It is found that these polymers were stable and remained unchanged. These results were attributed to the high cross-linking of the prepared polymers. However, at pH solutions greater than 10.0 the polymer (triethanolamine-glycerol-maleate) begins to decompose (dissolve). This may be due to the hydrolysis of ester bond in the structure of polymer.

3.6. Column Packing

Column packing is not the only critical factor, but it represents one of the most important aspects affecting the quality of the chromatographic system. They all agreed [112], however, in that stationary phase must be suspended in a liquid to give slurry. A high–pressure pump then conveys the slurry into the column at a great speed. This process prevents sedimentation, which separates the stationary phase according to size and thus impairs the separation performance. In this work at the prepared polymer was first grinded to a fine powder and sieved to obtain a (36µm) average mesh size.

The slurry was then produced by mixing of triethanolamine-glycerol-maleate polymer powder 100 ml acetonitrile and homogenized in an ultrasonic bath and placed in the slurry reservoir. The column was packed using down-flow packing system. HPLC pump was used instead of the air-driven pump that as showed in figure (1-1). The column was plugged with its appropriate fitting from one side and placed in a beaker for draining; the other side was connected to the slurry reservoir .The pump was set to deliver 4 ml/min acetonitrile. The back pressure at read 5 Mpa at this flow rate. The flow rate was then increased by 1 ml/min increment until reaching 8 ml/min at constant period of time and the pressure read 9Mpa at final flow rate.

Slurry of acrylamide-bisacrylamide polymer powder in hexane was homogenized in an ultrasonic bath before and during packing. After the packing was completed, the column was operated under pressure many times greater than the subsequent operating level, as recommended by several coworkers [103]. The packed column was operated at flow rate of 5ml/min, which has produced a backpressure of 6.4 MPa. About 500ml of

distilled water was pumped through the column at this maximum pressure level to prevent a subsequent collapse of the packing. The recorded pressure was sustained (unchanged) for long period of times, which indicating consistent and uniform packing. The column was then connected to the chromatograph and flow rate was set at 1 ml/min, with methanol for an hour.

3.7. Column Evaluation

Since this work includes packing of a new column, its performance should be tested. This has been done by evaluation the plate number**(N)**, height equivalent to theoretical plates , **HETP(H)** , and capacity factor, **K'** , for different chromatographic runs. The plate number of column (**N**) was calculated using the well-known equation [17];

$$N = 16\left(\frac{t_r}{w}\right)^2 \quad \textbf{or} \quad N = 5.54\left(\frac{t_r}{W_{1/2}}\right)^2$$

Where $\mathbf{t_r}$ is retention time, (**W**) and ($\mathbf{W_{1/2}}$) are the peak width at baseline and half height, respectively. The $\mathbf{t_r}$, **W**, $\mathbf{W_{1/2}}$ and other subsequent parameters were calculated using the optimum flow rate. The optimum flow rate was measured by plotting (**H**) versus different flow rates. The **H** values were calculated from **H=L/N** (where **L** is the length of the column). Figures (3-5) and (3-6) show the plots of **H** versus the mobile phase flow rate for both acrylamide-bisacrylamide and triethanolamine-glycerol-maleate columns, respectively.

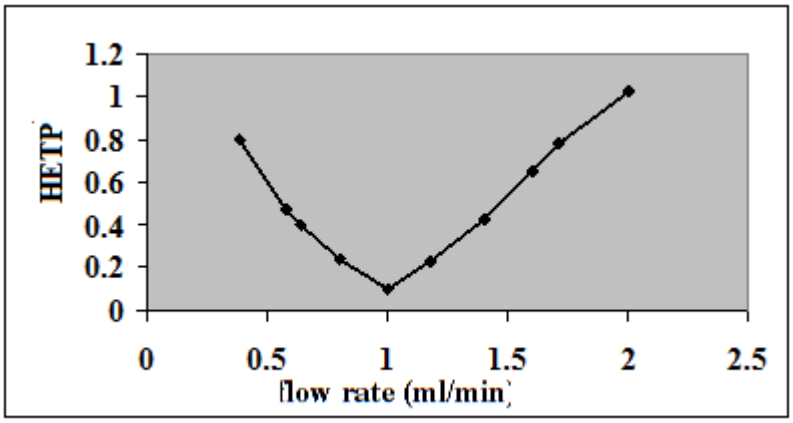


Figure (3-5) Plot of plate height H versus flow rate, using triethanol amine-glycerol-maleate column (25×0.4 cm (id)). Eluent gradient 20% phosphate buffer-80% MeOH, detection wavelength 233nm 10ppm of furosemide.

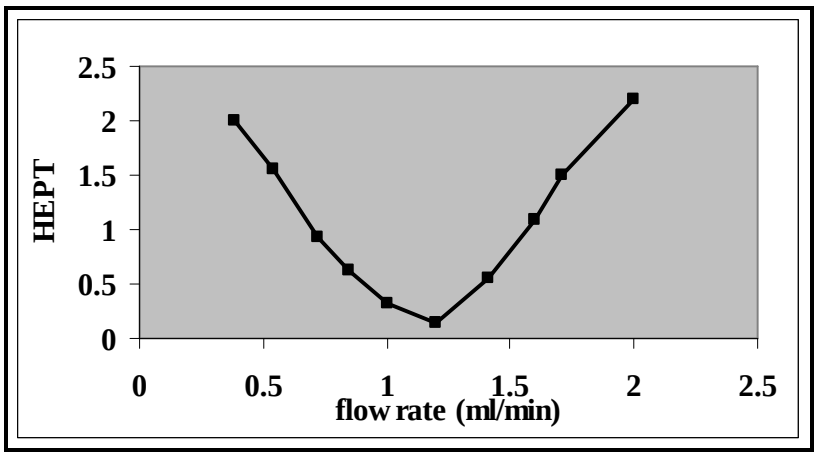


Figure (3-6) Plot of plate height versus flow rate, using acrylamide-bisacrylamide column (25×0.4 cm (id)). Eluent 10% phosphate buffer-90% EtOH, detection wavelength 233nm10ppm of furosemide.

The optimum flow rates were measured by analyzing furosmide at different mobile phase flow rate ranging from 0.10-2.50ml/min. A plot of **H** versus flow rate has given minimum **H** near 1ml/min for the triethanolamine-glycerol-maleate column and 1.2ml/min for acrylamide-bisacrylamide column. Some of the measured chromatographic parameters such as plate numbers **N**, plate heights **H**, capacity factor **Ќ**, separation factor **α**, and peak asymmetry for newly packed columns were then calculated using this optimum flow rate. These parameters were measured by chromatographic analysis of furosemide, atenolol, amiloride, phenylalanine, tryptophane, tyrosine and vitamin E.

3.8. Chromatographic analysis

Vitamin E was analyzed with triethanolamine glycerol-maleate column, of the similarly of the functional groups on both vitamin E and stationary phase, using 20µl sample loop, with flow rate of 1ml/min. Mobile phase was 100% acetonitrile, the resulting chromatogram is showed in Figure(3-7).

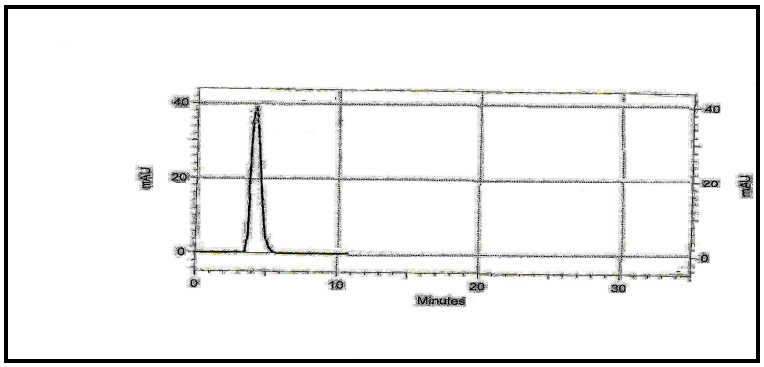
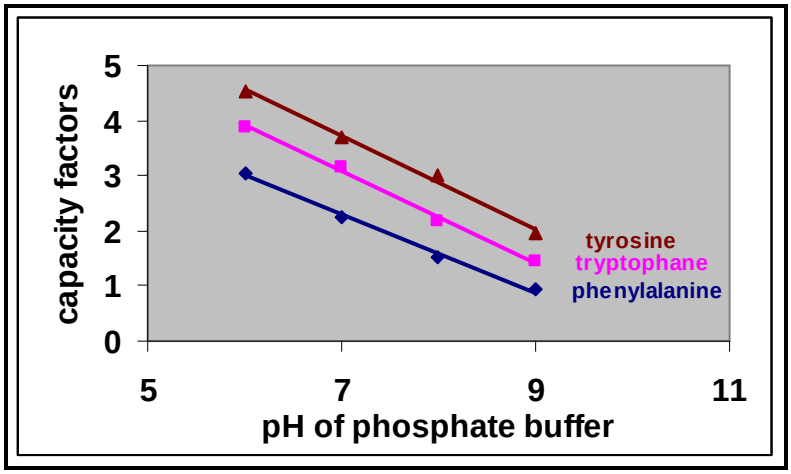


Figure (3-7) Chromatogram of 5.0 ppm solution, mobile phase 100%acetonitrile, flow rate 1ml/min and detection wavelength 229nm.

The retention times of vitamin E gave a good sharp peak using acetonitrile eluent the retention times for analyzed vitamin E was 4.68min. The capacity factor was 1.39 and peak asymmetry was 1.11.

Also the study was carried out for analysis of amino acids, phenylalanine, tryptophane and tyrosine using triethanolamine-glycerol-maleate column, because it also has the same functional groups that can be interacted with the functional groups of stationary phase at 20µl sample loop, with flow rate 1ml/min. Mobile phase that is used in the first polymer was acetonitrile but it gave a bad separation of mixture of amino acids, because all amino acids have the same retention time so it cannot be separated them. So, to improve separation of amino acids mixture phosphate buffer at different pH was prepared and the best pH was chosen. The results are showed in figure (3-8) and gave in Table (3-2)



Figure(3-8) Plot of Capacity factor versus pH, using triethanolamine-glycerol-maleate column (25×0.4 cm (id)), flow rate 1ml/min, detection wavelength 233nm, 10ppm of phenylalanine, tryptophane and tyrosine as a samples, respectively.

The results listed in Table (3-2) indicate that at low pH < 6 and at higher pH > 9 the amino acids, phenylalanine, tryptophane and tyrosine were not eluted and detected.

Table (3-2): Capacity factor Ќ and separation factors α of amino acids at different pH in acetonitrile as mobile phase, using triethanolamine-glycerol-maleate column (25×0.4 cm (id)).

Compounds	*Changing in pH of phosphate buffer*									
	6		*7*		*8*		*9*		*>9*	
	Ќ	**α**	**Ќ**	**α**	**Ќ**	**α**	**Ќ**	**α**	**Ќ**	**α**
Phenylalanine	3.79		2.66		1.86		1.25		--	--
Tryptophane	4.28	1.13	3.55	1.33	2.51	1.35	1.98	1.58	--	--
Tyrosine	4.53	1.07	4.40	1.24	2.99	1.19	2.59	1.31	--	--

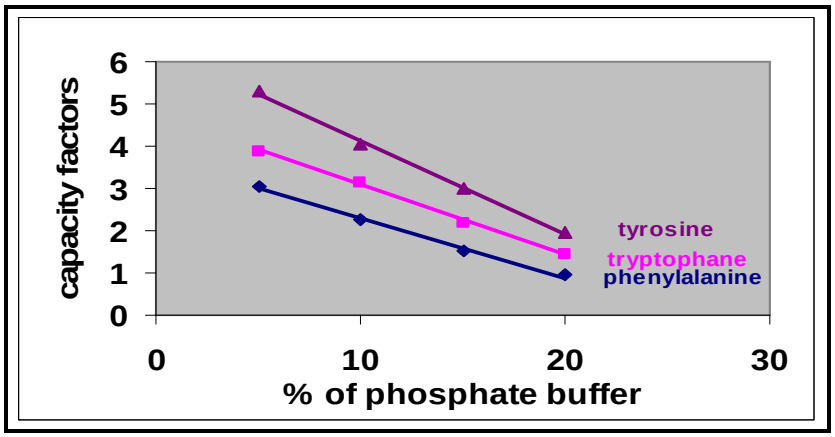
--Not detected

The ion ratio, which is commonly called capacity factor **Ќ**, is an important term that describes the interactions between the stationary phase and mobile phase. **Ќ** is independent on the column length, mobile phase flow rate and represents the molar ratio of the compound in the stationary phase, to that in the mobile phase. Capacity factor between 1.5-5.0 are preferred [(92)].Lower **Ќ** value indicates no stationary phase. Interaction occurs and hence no chromatography. However, large **Ќ** value is accompanied by long analysis times. The capacity factor for the samples chromatographic on triethanolamine-glycerol-maleate column with acetonitrile as eluent buffered at different pH was calculated. Phosphate buffer content has been found to affect the capacity factor **Ќ.** The value of **Ќ** at pH 6.0 was ranged from 3.79-4.53, at pH 7.0 was ranged from 2.66-4.40, at pH 8.0 was ranged from 1.86-2.99 and at pH 9.0 was ranged from 1.25-2.59 as it is showed in figure (3-8). These results are summarized in Table (3-2).

The values of selectivity α for phenylalanine, tryptophane, and tyrosine were ranged from 1.13-1.07 at pH 6.0, 1.24-1.33 at pH 7.0, 1.35-1.19 and 1.58-1.31, at pH 6.0, 7.0, 8.0 and 9.0 respectively. These variations in

capacity and separation factors of these analytes indicate that pH 6.0 is the best buffer that can be used for their separation.

Changing the percentage of phosphate buffer concentration in acetonitrile as a mobile phase from ≤5% to ≥25% has a great effect on k and consequently on α when using triethanolamine-glycerol-maleate as separating column as it is shown in figure (3-9) and listed in Table (3-3).



Figure(3-9) Plot of Capacity factor, versus present of phosphate buffer, using triethanolamine-glycerol-maleate column (25×0.4 cm (id)), flow rate 1ml/min, detection wavelength 233nm and 10ppm of phenylalanine, tryptophane and tyrosine as samples.

Table (3-3): Capacity factor Ќ and separation factors α variation with changing the composition of mobile phase for amino acids using triethanolamine-glycerol-maleate column (25×0.4 cm (id)).

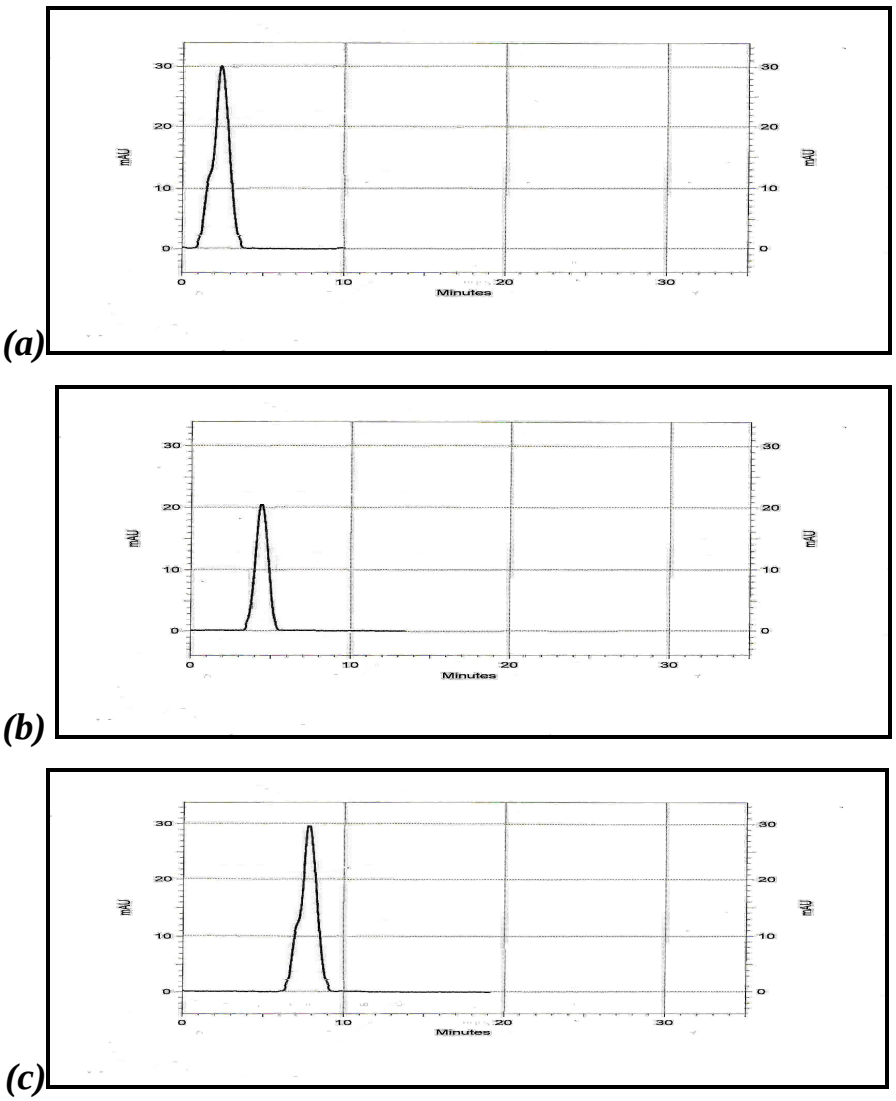
Compounds	*Percentage of phosphate buffer in mobile phase*									
	5%(0.01m) of buffer		*10%(0.02m) of buffer*		*15%(0.04m) of buffer*		*20%(0.05m) of buffer*		*≥25%(0.06m) of buffer*	
	Ќ	α	Ќ	α	Ќ	α	Ќ	α	Ќ	α
Phenylalanine	1.66		2.02		3.01		3.54		--	--
Tryptophane	2.29	1.44	2.47	1.24	3.05	1.08	4.68	1.34	--	--
Tyrosine	3.46	1.57	3.58	1.49	3.66	1.13	5.56	1.20	--	--

--Not detected

Capacity factor **Ќ** was ranged from 1.66-3.46, 2.02-3.58, 3.01-3.66 and 3.54-5.56 at 5%, 10%, 15% and 20% in acetonitrile. These results indicate that the best indicated competitive interaction was between these compounds in the stationary phase and the mobile phase at percentage 15% of phosphate buffer.

Separation factor **α** at 5% was ranged from 1.44-1.57, 1.24-1.49, 1.08-1.13 and 1.34-1.20 at 5%, 10%, 15% and 20% phosphate buffer in the mobile phase, respectively. At 20% phosphate buffer gave good separation factors were obtained, however, it may be possible to achieve a better separation at percentage 15% of phosphate buffer as it is showed in figure (3-9). These results are summarized in Table (3-3).

The chromatograms of phenylalanine, tryptophane and tyrosine with the triethanolamine-glycerol-maleate column, using 10µl sample loop, with flow rate 1ml/min are showed in figure (3-10). The mobile phase was a mixture of 15% phosphate buffer and 85% acetonitrile at pH 6.0. These are analyzed in to amino acids and had given well shaped peaks as well as good detector responses. As it is showed in figure (3-10), the more polar amino acid tyrosine has a retention time of 8.23min and the nonpolar phenylalanine has lower retention time at 3.12min. This mean that the mechanism of interaction between amino acids and stationary phase depended on the polarity, and because appearance of nonpolar first means that the stationary phase was relatively polar and interaction depended on the hydrogen bonding of N-H and O-H groups of amino acids with O-H groups of triethanolamine-glycerol-maleate of polymeric column.



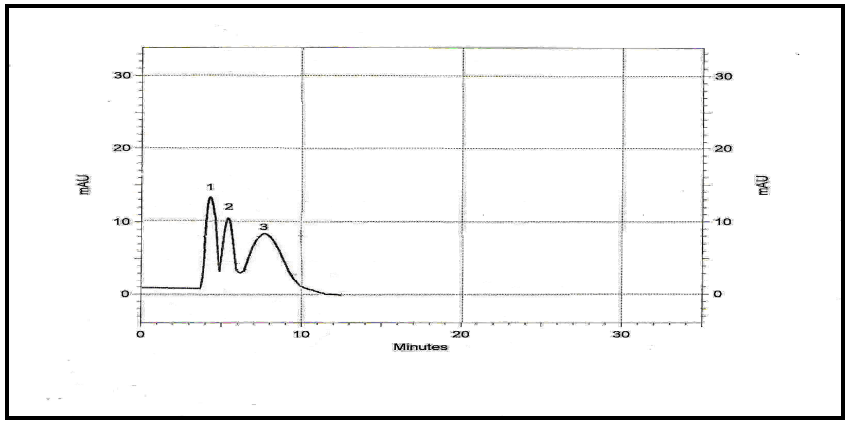
(a)

(b)

(c)

Figure (3-10) Chromatograms of a standard of 2.0 ppm (a) phenylalanine, (b) tryptophane and (c) tyrosine, flow rate 1ml/min using 15%phosphatebuffer-85%acetonitrile as mobile phase .

The separation of a mixture of phenylalanine, tryptophane, and tyrosine using above is showed in figure (3-11).



Figure(3-11) Chromatogram of standard mixture of 0.2ppm phenylalanine (1), 0.1ppm of tryptophane (2)and 0.5 ppm of tyrosine (3), using triethanolamine-glycerol-maleate column (25×0.4 cm (id)). Eluent 15% phosphate buffer-85% acetonitrile, flow rate 1ml/min and detection wavelength 245nm.

So to improve this separation of a programmer of gradient eluention has been designed based as changing both mobile phase component with time. The best program was achieved by using the results listed in Table (3-4) and shown in figure (3-12).

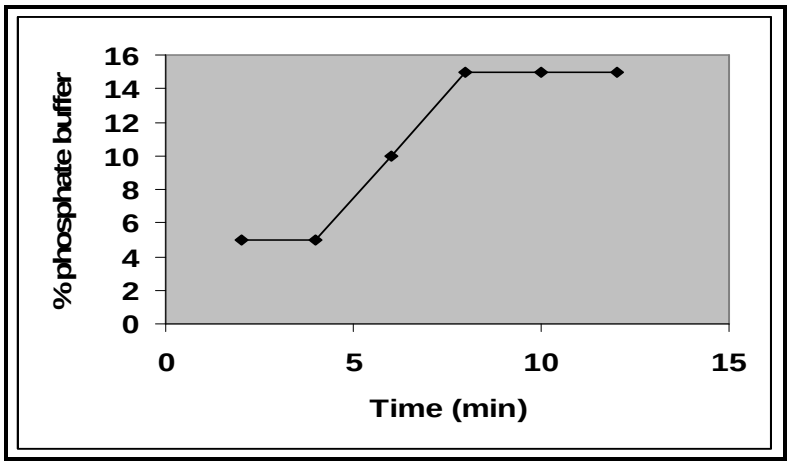


Figure (3-12) Time programming of gradient eluention.

Table (3-4): Time programming of gradient eluention for amino acid separation used triethanolamine-glycerol-maleate column (25×0.4 cm (id)).

Time duration, min	*% flow rate of pump B (phosphate buffer)*	*% flow rate of pump A (acetonitrile)*
2	5	95
2	5	٩٥
2	10	٩٠
٢	15	85
٢	15	85
٢	15	85

The separation of a mixture of phenylalanine, tryptophane, and tyrosine is showed in figure (3-13) using the above gradient eluention program. The difference between the retention times of these amino acids gave good separation factors previously in Table (3-5). When using gradient eluention, has showed to give a good resolution for amino acids than isocratic eluention.

Figure (3-13) shows a chromatogram of three amino acids, 0.2ppm phenylalanine, 0.1ppm of tryptophane, and 0.5 ppm of tyrosine using gradient eluent (0-15) % phosphate buffer in acetonitrile at pH 6.0, flow rate was1ml/min, and detection wavelength at 245nm.

The following results were obtained from chromatograms of amino acids, phenylalanine, tryptophane and tyrosine analyzed with the triethanolamine-glycerol-malate column at optimum flow rate of the mobile phase.

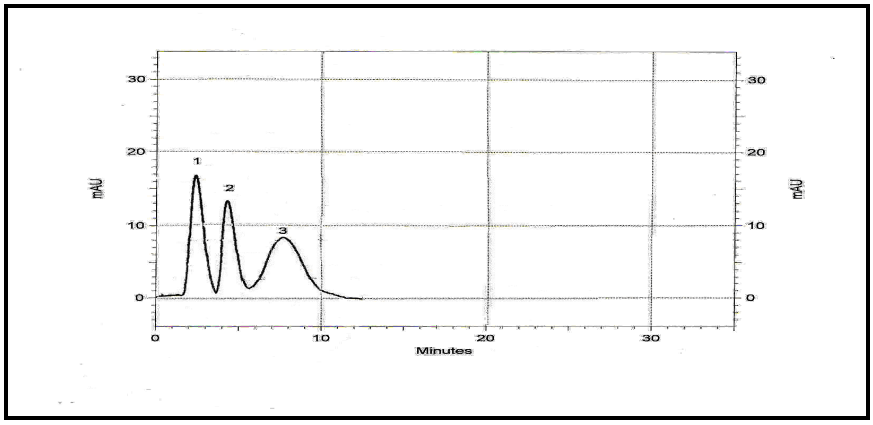


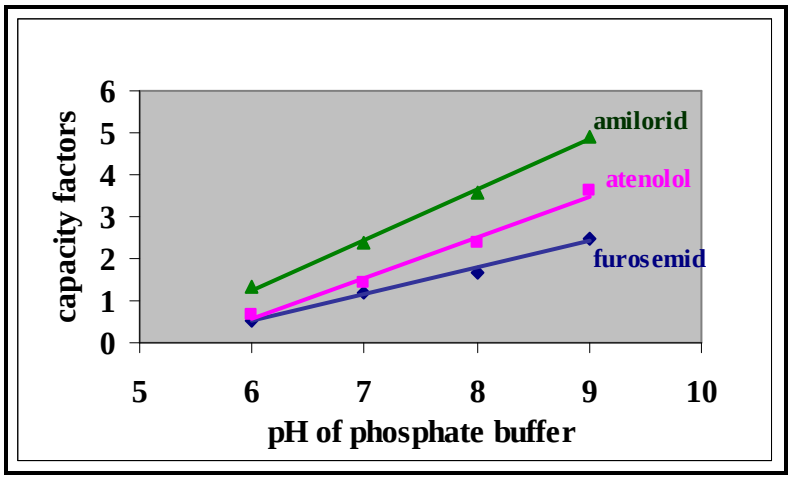
Figure (3-13) Chromatogram of separation of standard mixture of 0.2ppm phenylalanine(1), 0.1ppm of tryptophane(2) and 0.5 ppm of tyrosine (3), using triethanolamine-glycerol-maleate column (25×0.4 cm (id)). Eluent gradient (0-15) % phosphate buffer in acetonitrile, flow rate 1ml/min and detection wavelength 245nm.

Table (3-5): Retention time tr, capacity factor Ḱ , separation factor α, resolution and peak asymmetry for amino acids using triethanolamine-glycerol-maleate column (25×0.4 cm (id)). Eluent gradient 15% phosphate buffer-85% acetonitrile, flow rate 1ml/min and detection wavelength 245nm.

Compounds	*Retention time tr*	*Capacity factor Ḱ*	*Separation factor α*	*Resolution*	*Peak Asymmetry*
Phenylalanine	3.12	1.73			1.08
Tryptophane	4.85	1.94	1.12	1.56	1.04
Tyrosine	8.23	2.13	1.10	1.89	1.32

Table (3-5) listed the retention times for; phenylalanine, tryptophane, and tyrosine which were reproducible. The RSD % was ranged from 0.543-0.601 with an average value 0.594, capacity factor was ranged from 1.73-2.13 with an average of 1.93, selectivity factor was ranged from 1.10-1.12 with an average value 1.11 and peak asymmetry was ranged from 1.08-1.32 with an average 1.1.

Also the study was carried out for the effect of phosphate buffer concentration and pH for analysis of some drugs, such as furosmide, atenolol and amiloride using triethanolamine-glycerol-maleate column.



Figure(3-14) Plot of Capacity factor, versus pH, using triethanolamine-glycerol-maleate column (25×0.4 cm (id)), flow rate 1ml/min, detection wavelength 233nm and 10ppm of furosmide, atenolol and amiloride as a samples, respectively.

The results in Table (3-6) showed that at low pH <6 and at higher pH >٩furosmide, atenolol, and amiloride cannot detected. The capacity factors using triethanolamine-glycerol-maleate column with eluent MeOH and phosphate buffer at different pH were calculated. Phosphate buffer content has been found to affect the capacity factor **Ќ**. The values of **Ќ** was ranged from 0.70-1.13, 1.18-1.79, 2.30-2.79 and

4.93-5.27 at pH 6.0, 7.0, 8.0 and 9.0, respectively as it is showed in figure (3-14) and the results are summarized in Table(3-6).

Table (3-6): Capacity factor Ḱ of drugs at different pH in methanol as mobile phase, using triethanolamine-glycerol-maleate column (25×0.4 cm (id)).

Compounds	***Changing in pH of phosphate buffer***									
	6		***7***		***8***		***9***		**>9**	
	Ḱ	**α**	**Ḱ**	**α**	**Ḱ**	**α**	**Ḱ**	**α**	**Ḱ**	**α**
Furosmide	0.70		1.88		2.31		4.93		--	--
Atenolol	0.88	1.25	1.44	1.44	2.51	1.09	4.15	1.19	--	--
Amiloride	1.13	1.13	1.79	1.24	2.76	1.11	5.27	1.27	--	--

--Not detected

The column selectivity is originally called the separation factor **α** is defined as the ratio of the capacity factors of two adjacent peaks **α = Ḱ2/Ḱ1**. Minimum value of 1.1 is required to achieve separation of two adjacent peaks at any given experimental conditions. The **α** values for furosmide, atenolol, and amiloride were ranged from 1.25-1.28, 1.22-1.24, 1.09-1.11 and 1.19-1.27 at pH 6.0, 7.0, 8.0 and 9.0 respectively. These variations in capacity factors and separation factors of these analytes may indicate that the pH 8.0 is the best pH buffer that can used for separating these analytes.

Table (3-7): Capacity factor Ḱ and separation factors α variation with changing of composition of mobile phase for drugs using triethanolamine-glycerol-maleate column (25×0.4 cm (id)).

Compounds	Percentage of phosphate buffer in mobile phase									
	5%(0.01m) of buffer		10%(0.02m) of buffer		15%(0.04m) of buffer		20%(0.05m) of buffer		≥25%(0.06m) of buffer	
	Ḱ	α	Ḱ	α	Ḱ	α	Ḱ	α	Ḱ	α
Furosmide	2.95		1.98		1.86		1.52		--	--
Atenolol	3.14	1.06	2.49	1.26	2.15	1.16	1.85	1.22	--	--
Amiloride	4.98	1.59	3.75	1.51	2.74	1.28	2.34	1.27	--	--

–Not detected

The variation of the capacity factor and separation factor for furosemide, atenolol, and amiloride with different percentage of phosphate buffer (percentage in methanol) ranged from <5% to ≥25% are listed in Table (3-7) using column triethanolamine-glycerol-maleate and shown in figure (3-15). Capacity factor **Ḱ** was ranged from 2.95-4.98, 1.98-3.75, 1.86-2.74 and 1.52-2.34 at 5%, 10%, 15% and 20% phosphate buffer. This indicated that a good competitive interaction of these compounds with the stationary phase and the mobile phase at percentage of 20% phosphate buffer.

The separation factor **α** was ranged from 1.06-1.59, 1.26-1.51, 1.16-1.28 and 1.22-1.27 at 5%, 10%, 15% and 20% phosphate buffer as it is showed in figure (3-15) and Table(3-7).

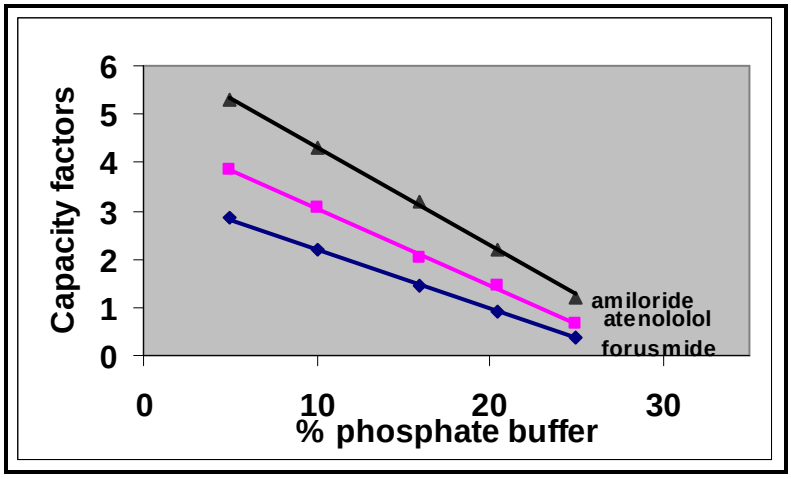


Figure (3-15) Plot of Capacity factor, versus present of phosphate buffer in MeOH as eluent, using triethanolamine-glycerol-maleate column (25×0.4 cm (id))., flow rate 1ml/min, detection wavelength 233nm and 10ppm of furosmide, atenolol and amiloride as a sample.

The analysis of amiloride, furosmide and atenolol were performed on triethanolamine-glycerol-maleate column, using 10µl sample loop, at flow rate 1ml/min, mobile phase was a mixture of 20% phosphate buffer and 80% methanol. The chromatograms of these compounds are showed in figure (3-16). The more polar amiloride appeared at high retention time of 9.16min and the nonpolar drug furosmide eluted at shorter retention time of 4.65min. This means that the interaction between these drugs and stationary phase depended on the polarity. The stationary, as a consequence was relatively polar (normal phase) and interaction depended on the hydrogen bonding between N-H groups of drugs and O-H groups of triethanolamine-glycerol-maleate co-polymer. On the other hand, these analyzed drugs have given well resolved peaks as well as a good detector response.

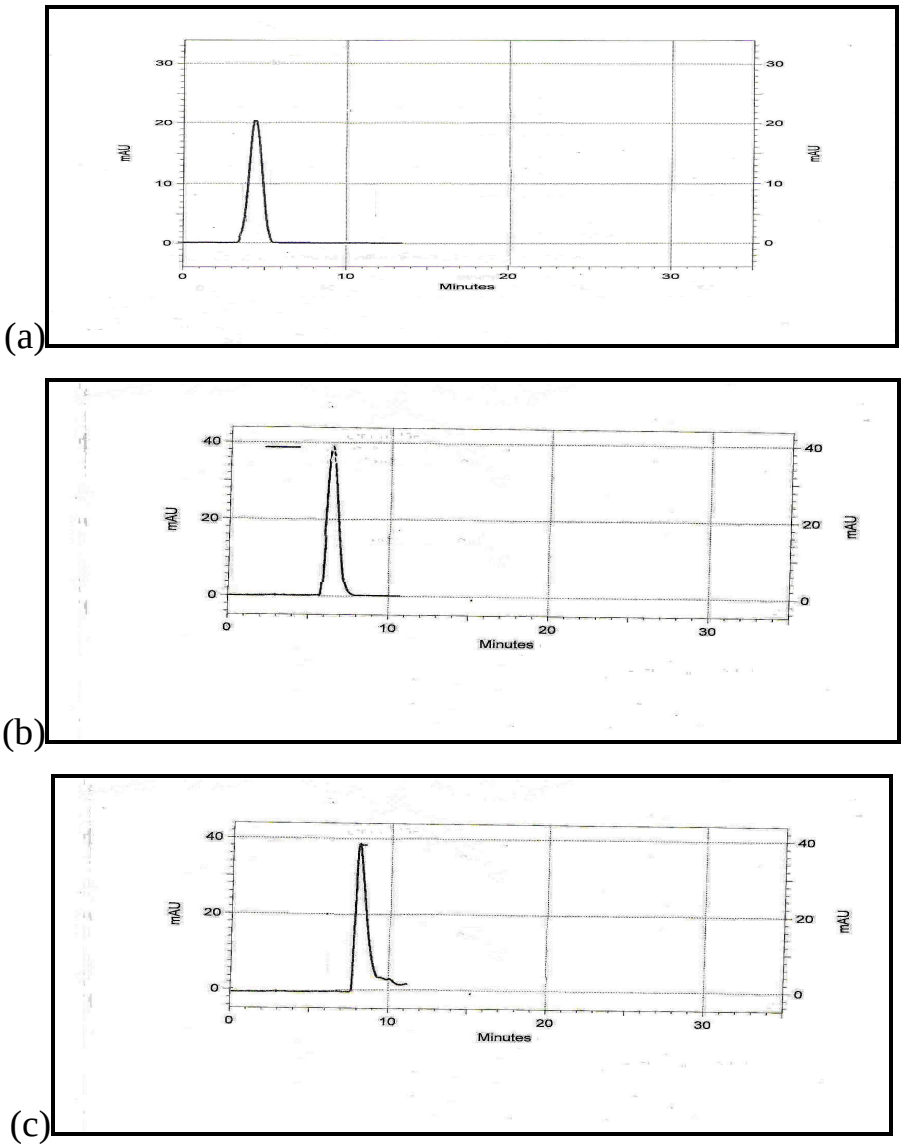


Figure (3-16) Chromatogram of a standard of2.5 ppm (a) furosmide, (b) atenolol and (c) amiloride, flow rate 1ml/min using 20% phosphate buffer and 80% methanol as mobile phase.

The separation of a mixture of furosmide, atenolol and amiloride is showed in figure (3-17) using 20% phosphate buffer and 80% methanol phase.

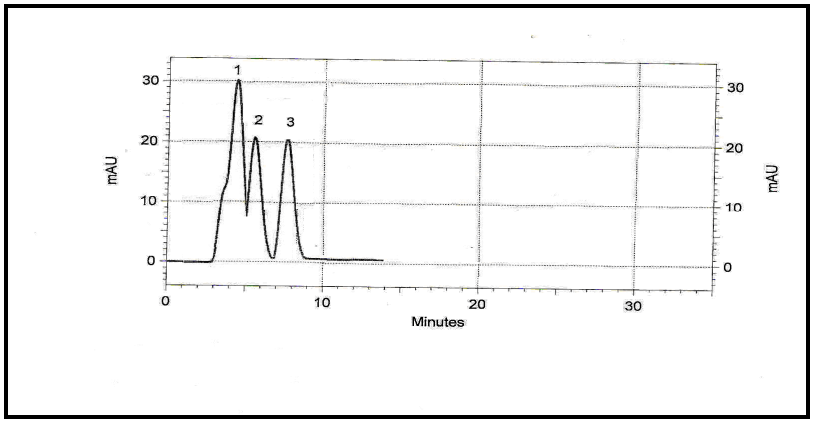


Figure (3-17) Chromatogram of standard mixture of 0.2 amiloride(1), 0.1ppm of furosmide(2) and 0.5 ppm of atenolol(3), using triethanolamine-glycerol-maleate column (25×0.4 cm (id)). Eluent 20% phosphate buffer-80% MeOH, flow rate 1ml/min, and detection wavelength 233nm.

The separation of these drugs in mixture was improved using gradient eluention programming. The best gradient elution program was listed in Table (3-8) and it is showed in graphically in figure (3-18), to improve the separation.

Table (3-8): Time programming of gradient eluention for drug separation used triethanolamine-glycerol-malate column (25×0.4 cm (id)).

Time duration, min	*Percent of flowed pump B (phosphate buffer)*	*%flow rate pump A (MeOH)*
2	5	95
2	5	95
3	10	90
3	15	85
2	20	80
2	20	80

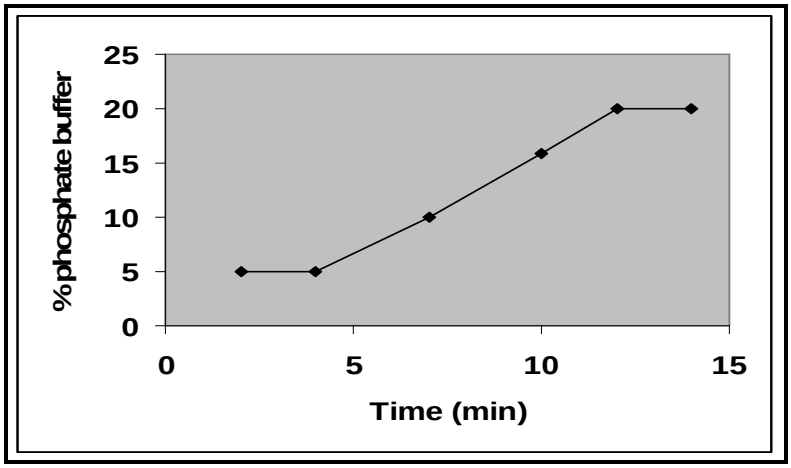


Figure (3-18) Time programming of gradient eluention.

The chromatographic separation of these drugs mixture is shown in figure (3-19) using the designed gradient eluention. The difference between the retention times of these drugs is given a good separation factor compared to that given previously in Table (3-9). Gradient eluention gave a good resolution for drugs than isocratic eluention.

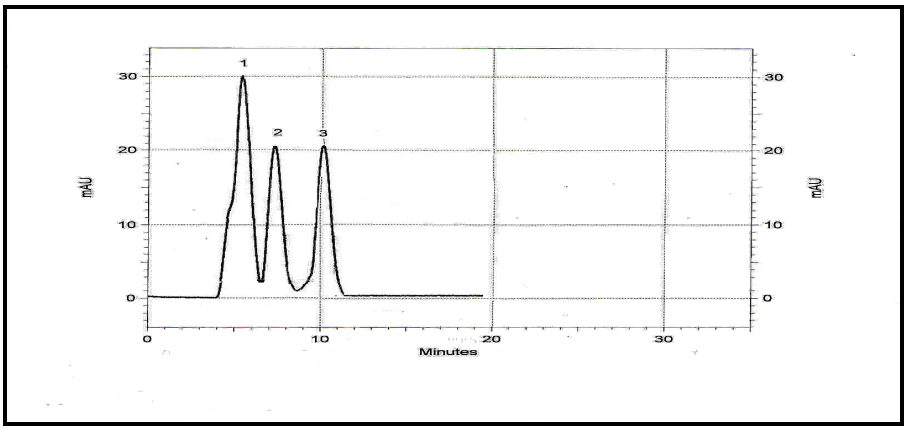


Figure (3-19) Chromatogram of Separation of standard mixture of 0.2ppm furosmide (1), 0.1ppm of atenolol (2) and 0.5 ppm of amiloride (3), using triethanolamine-glycerol-maleate column (25×0.4 cm (id)). Eluent gradient (0-20) % phosphate buffer in MeOH, flow rate 1ml/min and detection wavelength 233nm.

The chromatogram of three drugs is 0.2 furosmide, 0.1ppm of atenolol, and 0.5 ppm of amiloride using gradient eluent (0-20)% phosphate buffer in MeOH at pH 8.0, flow rate was 1ml/min, and detection wavelength at 233nm as it is showed in figure (3-19).The average (**N**) was found to be 779.3, and (**H**) was calculated and found to be equal to 0.02cm. Figure (3-17) shows chromatograph for the separation of three drugs; 0.2 furosmide, 0.1ppm of atenolol, and 0.5 ppm of amiloride, using isocratic eluent 20% phosphate buffer and 80% MeOH at pH 8.0, flow rate 1ml/min, and detection wavelength at 233nm. Table (3-9) list the retention times for furosmide, atenolol, and amiloride which are reproducible. The RSD% ranged from 0.567-0.627 with average value 0.588. Capacity factor ranged from 1.51-2.13 with average of 1.83, selectivity factor ranged from 1.22-1.27 with average value 1.25 and peak asymmetry ranged from 0.99-1.14 with an average 1.50.

Table (3-9): Retention time tr, capacity factor Ḱ , separation factor α, resolution and peak asymmetry for drugs using triethanolamine-glycerol-maleate column (25×0.4 cm (id)). Eluent gradient 20% phosphate buffer-80% MeOH, flow rate 1ml/min and detection wavelength 233nm.

Compounds	*Retention time tr*	*Capacity factor Ḱ*	*Separation factor α*	*Resolution*	*Peak Asymmetry*
Furosmide	4.65	1.52	--	--	1.14
Atenolol	7.05	1.85	1.22	1.22	0.99
Amiloride	9.16	2.13	1.27	1.45	1.02

The day-day, week-week and month-month retention time reproducibility expressed in relative standard deviation (RSD) for all analytes analyzed on the new column are listed in Table (3-10).

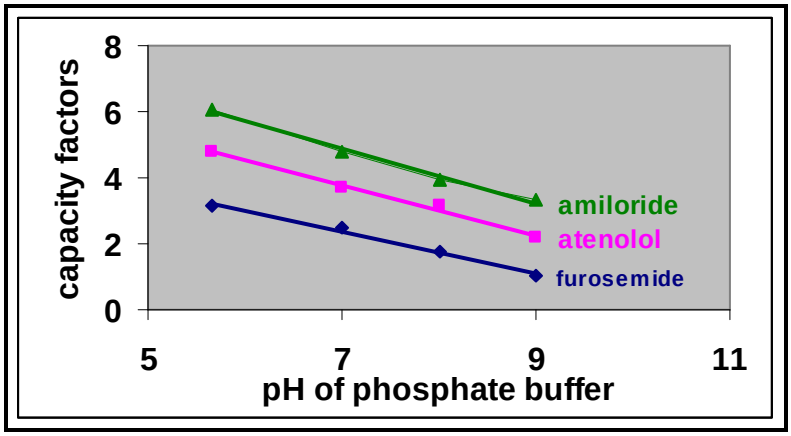
The day-day retention time reproducibility ranged from 0.759-1.027% with an average value 0.840%. The week-week RSD ranged from 0.792-1.114% with an average value 0.913%. While that of month-month RSD ranged from 1.607-2.317% with an average value 1.954%

Table (3-10): Reproducibility in retention time

Compounds	***RSD%* same day***	***RSD%* day-day***	***RSD%* weak-weak***	***RSD% * month-month***
Furosmide	0.594	0.759	0.792	1.607
Atenolol	0.423	0.652	0.676	1.689
Amiloride	0.522	0.709	0.736	1.758
Phenylalanine	0.756	0.988	0.907	1.928
Tryptophane	0.648	0.857	0.895	1.937
Tyrosine	0.712	0.891	0.931	1.942
Vitamin E	0.899	1.027	1.114	2.317

***each result was calculated for at less 5 runs**

The same study analyzed furosmide, atenolol and amiloride using acrylamide-bisacrylamide column. It has the same functional groups that can be interacted with the functional groups of stationary phase 20µl sample loop, with flow rate 1ml/min. Mobile phase used in the first was ethanol, but it gave a bad separation of mixture drugs, because all drugs have the same retention time so at cannot separated. Therefore, phosphate buffer at different pH was used in order to choose the best pH to improve the separation of the mixture. The results are listed in Table (3-11) and showed in figure (3-20).



Figure(3-20) Plot of Capacity factor versus pH, using acrylamide-bisacrylamide column (25×0.4 cm (id)), flow rate 1.2ml/min, detection wavelength 233nm, 10ppm of furosmide, atenolol and as amiloride samples, respectively.

The result listed in Table (3-11) for capacity factors and separation factors can be calculated in the range of pH between 6-9. Above pH 9 and less 6 cannot be detected the peak of furosmide, atenolol, and amiloride.

Table (3-11): Capacity factor Ќ and separation factors α of drugs at different pH in ethanol as mobile phase, used acrylamide-bisacrylamide column (25×0.4 cm (id)).

Compounds	*Changing in pH of phosphate buffer*									
	6		٧		٨		٩		>9	
	Ќ	α	Ќ	α	Ќ	α	Ќ	α	Ќ	α
Furosmide	3.93		2.98		2.06		1.57		--	--
Atenolol	4.15	1.05	3.67	1.16	2.51	1.21	1.71	1.09	--	--
Amiloride	4.60	1.11	4.29	1.23	3.59	1.43	1.79	1.15	--	--

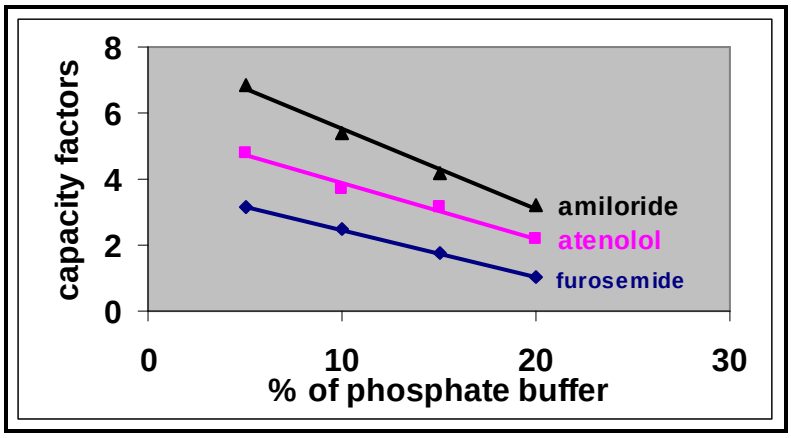
--Not detected

The capacity factor for furosmide, atenolol and amiloride analysis on acrylamide-bisacrylamide column with EtOH as eluent has been determined at different pH. Phosphate buffer has been found to affect the

capacity factor **Ќ**, the **Ќ** value ranged from 3.93-4.60, 2.98-4.29, 2.06-3.59 and 1.57-1.79 at pH 6.0, 7.0, 8.0, 9.0 respectively. These are represented in figure (3-20) and summarized in Table (3-11).

The column selectivity **α** values for furosmide, atenolol and amiloride ranged from 1.05-1.11, 1.16-1.23, 1.21-1.43 and 1.09-1.15 at pH 6.0, 7.0, 8.0 and 9.0, respectively. These variations in capacity and separation factor of these analytes indicate that pH 6.0 is the best buffer that can be used for their separation.

Variation of the capacity factor and separation factor for drugs, with different percentage of phosphate buffer composition (in ethanol) as mobile phase from ≤5% to ≥25% a great effect on k and consequently on α when using triethanolamine-glycerol-maleate as separating column as it is showed in figure (3-21) and listed in Table (3-12).



Figure(3-21) Plot of Capacity factor, versus present of phosphate buffer in ethanol as eluent, using acrylamide-bisacrylamide column (25×0.4 cm (id))., flow rate 1.2ml/min, detection wavelength 233nm and 10ppm of, furosmide, atenolol and amiloride as a samples.

Table (3-12) Capacity Ḱ and separation factors α variation with changing the composition of mobile phase using acrylamide-bisacrylamide column (25×0.4 cm (id)).

Compounds	Percentage of phosphate buffer in mobile phase									
	5%(0.01m) of buffer		10%(0.02m) of buffer		15%(0.04m) of buffer		20%(0.05m) of buffer		≥25%(0.06m) of buffer	
	Ḱ	α	Ḱ	α	Ḱ	α	Ḱ	α	Ḱ	α
Furosmide	4.06		3.30		1.68		1.27		--	
Atenolol	4.98	1.22	3.43	1.04	2.27	1.35	2.03	1.59	--	--
Amiloride	5.79	1.16	3.81	1.11	3.27	1.44	2.33	1.15	--	--

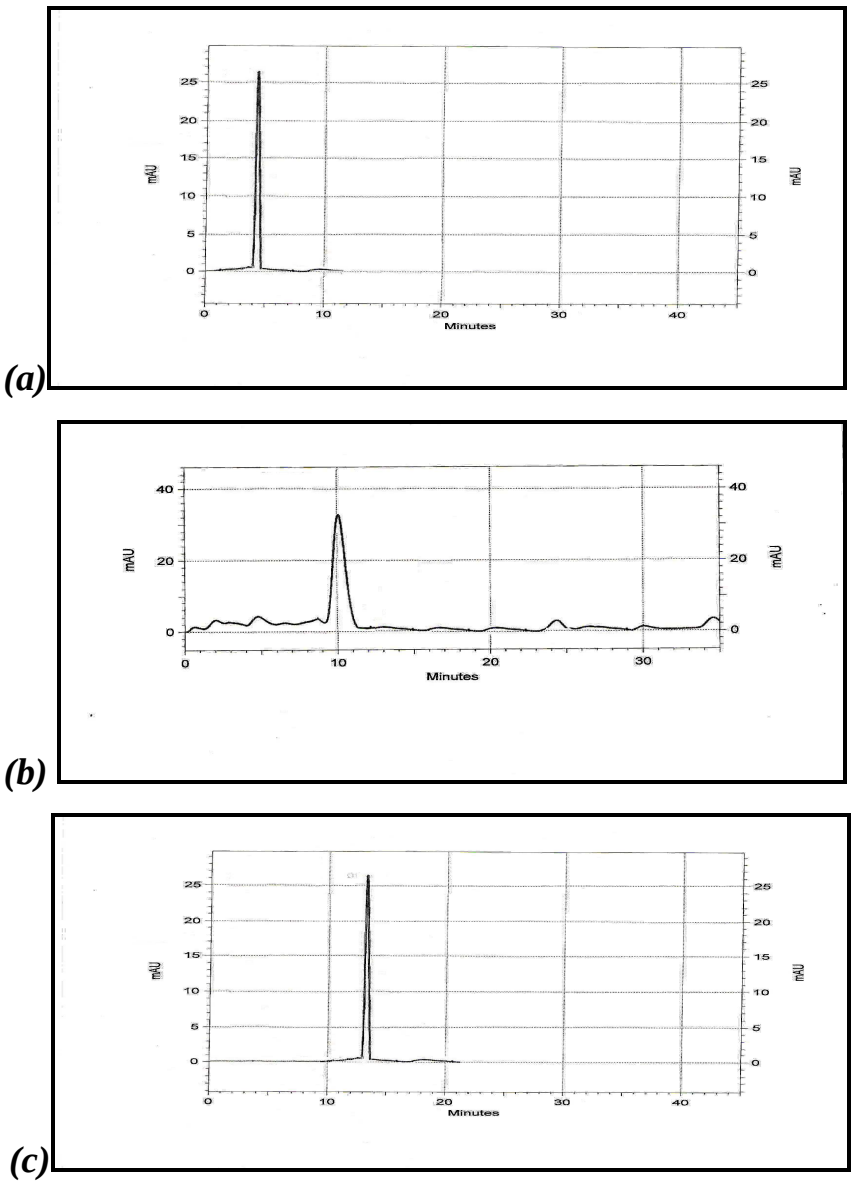
--Not detected

Capacity factors **Ḱ** ranged from 4.06-5.79, 3.30-3.81, 1.68-3.27 and 1.27-2.33 at 5%, 10%, 15% and 20% buffer in ethanol, respectively. This indicated that a good competitive interaction between these compounds in the stationary phase and the mobile phase at 10% of buffer.

Separation factor **α** ranged from 1.16-1.22, 1.04-1.11, 1.35-1.44 and 1.15-1.59 at 5%, 10%, 15% and 20% buffer in ethanol, respectively. Good separation factors may be possible to achieve better separation at 10% of phosphate buffer as it is showed in figure (3-21) and the result are summarized in Table (3-12).

The drugs were analyzed with the acrylamide-bisacrylamide column, using 20µl sample loop, with flow rate was 1.2ml/min as it is showed in figure (3-22). The mobile phase was a mixture of 10% phosphate buffer and 90% ethanol. The chromatograms of these compounds are showed in figure (3-22). The more polar amiloride has a retention time of 13.24min and the nonpolar furosmide has lower retention time of 4.91min. This means that the mechanism of interaction between these drugs and stationary phase depends on the polarity. The stationary phase, as a consequence was relatively polar and interaction depended on the

hydrogen bonding between N-H groups of drugs with N-H and O-H groups of acrylamide-bisacrylamide of polymeric column.



(a)

(b)

(c)

Figure (3-22) Chromatograms of a standard of 3.0 ppm (a) furosmide, (b) atenolol and (c) amiloride, flow rate 1ml/min using 10% phosphate buffer and 90% ethanol as mobile phase.

These analyzed drugs have given well resolved peaks as well as a good detector response. The separation of a mixture of furosmide, atenolol, and amiloride is showed in figure (3-23) using 10% phosphate buffer and 90% ethanol mobile phase. The difference between the retention times of these drugs, furosmide, atenolol and amiloride gave good separation factors as given previously in Table (3-13) when using 10% phosphate buffer and 90% ethanol mobile phase.

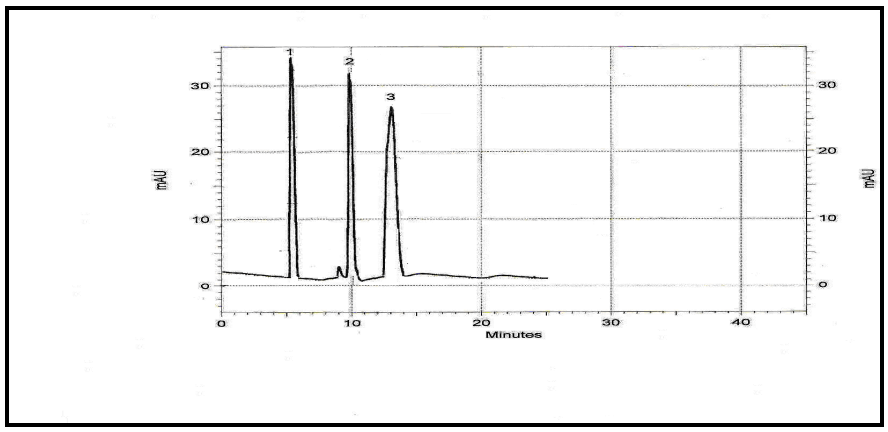


Figure (3-23) Chromatogram of standard mixture 2.0ppm of furosmide (1) , 2.0ppm of atenolol (2) and 3.0 ppm of amiloride (3), drugs using acrylamide-bisacrylamide column (25×0.4 cm (id)). Eluent 10% phosphate buffer -90% EtOH, flow rate 1.2ml/mim and detection wavelength 233nm.

Figure (3-23) shows a chromatogram of three drugs, 2.0ppm furosmide, 2.0ppm of atenolol and 3.0 ppm of amiloride using isocratic eluention 10% phosphate buffer -90% EtOH at pH 6.0, flow rate was 1ml/min, and detection wavelength of 233nm. The following results were obtained from chromatograms of furosmide, atenolol and amiloride analyzed with -bisacrylamide column at optimum flow rate of mobile phase. The average (**N**) was calculated and was found to be 1589.5, the (**H**) was calculated and found to be equal to 0.01cm.

Table (3-13) lists the retention times for all analyzed drugs, furosmide, atenolol and amiloride which were reproducible RSD 0.423-0.721% with

an average value 0.576%. The capacity factor ranged from 2.30-3.02with an average of 2.66, selectivity factor ranged from 1.33-1.51 with an average value 1.14 and peak asymmetry ranged from 1.01-1.05 with an average 1.07.

Table (3-13) Retention time tr, capacity factor Ḱ , separation factor α, resolution and peak asymmetry for drugs using acrylamide-bisacrylamide column (25×0.4 cm (id)). Eluent isocratic 10% buffer in 90% EtOH, flow rate 1.2ml/min and detection wavelength 233nm.

Compounds	*Retention time tr*	*Capacity factor Ḱ*	*Separation factor α*	*Resolution*	*Peak Asymmetry*
Furosmide	4.91	2.30			1.04
Atenolol	9.97	2.65	1.51	1.56	1.05
Amiloride	13.24	3.02	1.33	1.62	1.01

Phenylalanine, tryptophane and tyrosine can be analysed by using acrylamide-bisacrylamide column with phosphate buffer at different pH and concentration as it is showed in figure (3-24) and listed in Table (3-14).

The results in Table (3-14) show that capacity factor cannot be detected at low pH 6 and at high pH 9 for the amino acids, phenylalanine, tryptophane and tyrosine.

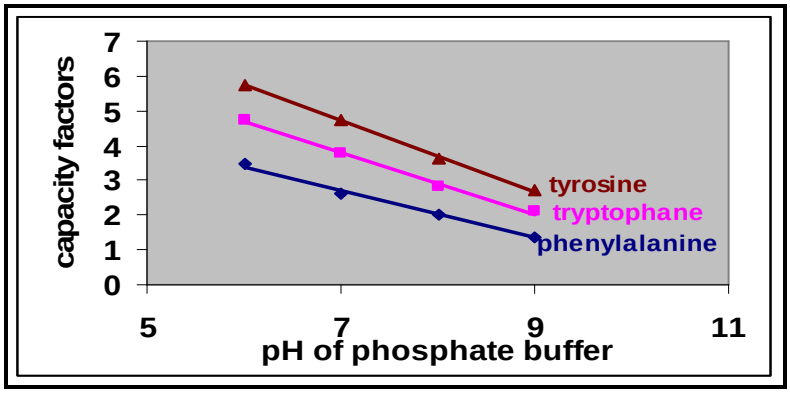


Figure (3-٢٤) Plot of Capacity factor verses pH, using acrylamide-bisacrylamide column (25×0.4 cm (id)), flow rate 1.2ml/min, detection wavelength 233nm and 10 ppm of phenylalanine tryptophane and tyrosine as samples, respectively.

The capacity factors for phenylalanine, tryptophane and tyrosine chromatographed on acrylamide-bisacrylamide column with eluent EtOH and phosphate buffer at different pH can be determined. Phosphate buffer content has been found to affect the capacity factor **Ќ**.

Table (3-14) Capacity factor Ќ and Separation factor α of amino acids at different pH in ethanol as mobile phase, using acrylamide-bisacrylamide column (25×0.4 cm (id)).

Compounds	*Changing in pH of phosphate buffer*									
	6		*7*		*8*		*9*		>9	
	Ќ	**α**	**Ќ**	**α**	**Ќ**	**α**	**Ќ**	**α**	**Ќ**	**α**
Phenylalanine	4.02		2.87		2.01		1.37		--	--
Tryptophane	4.51	1.12	3.76	1.32	2.65	1.34	2.10	1.57	--	--
Tyrosine	4.76	1.06	4.41	1.23	3.13	1.18	2.71	1.30	--	--

--Not detected

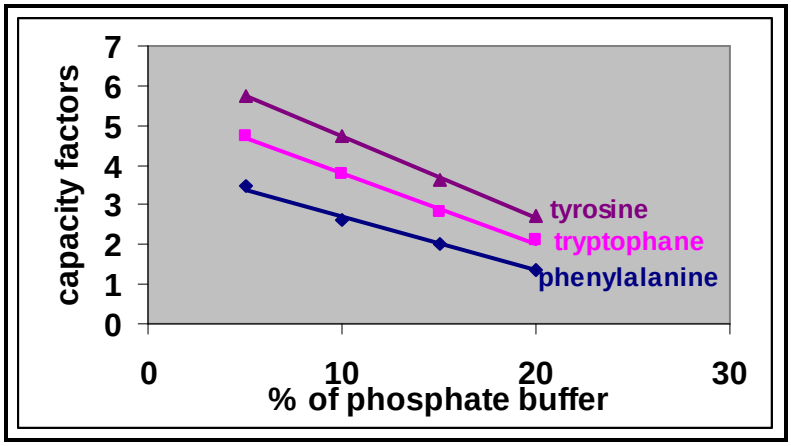
The values of **Ќ** ranged from 4.04-4.76, 2.87-4.40, 2.01-3.13 and 1.37-2.71 at pH 6.0, 7.0, 8.0 and 9.0, respectively as it is showed in figure (3-24) and summarized in Table (3-14).

The column selectivity α values for amino acids, phenylalanine, tryptophane and tyrosine ranged from 1.12-1.06, 1.23-1.32, 1.18-1.34 and 1.30-1.57 at pH 6.0, 7.0, 8.0 and 9.0, respectively. This variation in capacity factor and separation factor of these analytes may indicate that the pH 6.0 is the best pH for phosphate buffer that used for separating these amino acids. The variation of the capacity factor and separation factor for amino acids, phenylalanine, tryptophane, and tyrosine with different percentage of phosphate buffer percentage in ethanol ranged from ≤5% to ≥25% are listed in Table (3-15) using column acrylamide-bisacrylamide and showed in figure (3-25).

Table (3-15): Capacity factor Ќ and separation factors α variation with changing the composition of mobile phase for amino acids using acrylamide-bisacrylamide column (25×0.4 cm (id)).

Compounds	***Percentage of phosphate buffer in mobile phase***									
	5%(0.01m) of buffer		***10%(0.02m) of buffer***		***15%(0.04m) of buffer***		***20%(0.05m) of buffer***		***≥25%(0.06m) of buffer***	
	Ќ	**α**	**Ќ**	**α**	**Ќ**	**α**	**Ќ**	**α**	**Ќ**	**α**
Phenylalanine	3.76		3.30		1.63		1.41		--	
Tryptophane	3.87	1.03	3.76	1.14	2.15	1.32	2.24	1.59	--	--
Tyrosine	4.22	1.09	4.06	1.08	3.09	1.44	3.25	1.45	--	--

--Not detected



Figure(3-25) Plot of Capacity factor versus present of phosphate buffer in EtOH, using acrylamide-bisacrylamide column (25×0.4 cm (id)), flow rate 1.2ml/min, detection wavelength 233nm and 10ppm of phenylalanine tryptophane and tyrosine as samples.

Capacity factors **Ќ** ranged from 3.76-4.22, 3.30-4.06, 1.63-3.09 and 1.41-3.25 at 5%, 10%, 15% and 20% phosphate buffer. This indicates a good competitive interaction of these compounds and the stationary phase and the mobile phase at percentage of 15% phosphate buffer.

The separation factors **α** ranged from 1.03-1.09, 1.14-1.08, 1.32-1.44 and 1.45-1.59 at 5%, 10%, 15% and 20% phosphate buffer. This indicated that good separation factor, however, it may be possible to achieve better separation at percentage of 15% phosphate buffer as showed in figure (3-25) and summarized in Table (3-15).

These amino acids were chromatographed with the acrylamide-bisacrylamide column, using 20µl sample loop, with flow rate of 1.2ml/min, mobile phase was a mixture of 15% phosphate buffer and 85% ethanol at pH 6.0. The chromatogram of these compounds is showed in figure (3-٢6). The more polar tyrosine appeared at high retention time of 7.58min and the nonpolar phenylalanine eluted at shorter retention time of 2.72min. This means that the of interaction between amino acids and stationary phase depended on the polarity. The stationary phase, was

relatively polar and interaction depended on the hydrogen bonding between N-H and O-H groups of amino acids with N-H and O-H groups of acrylamide-bisacrylamide polymer.

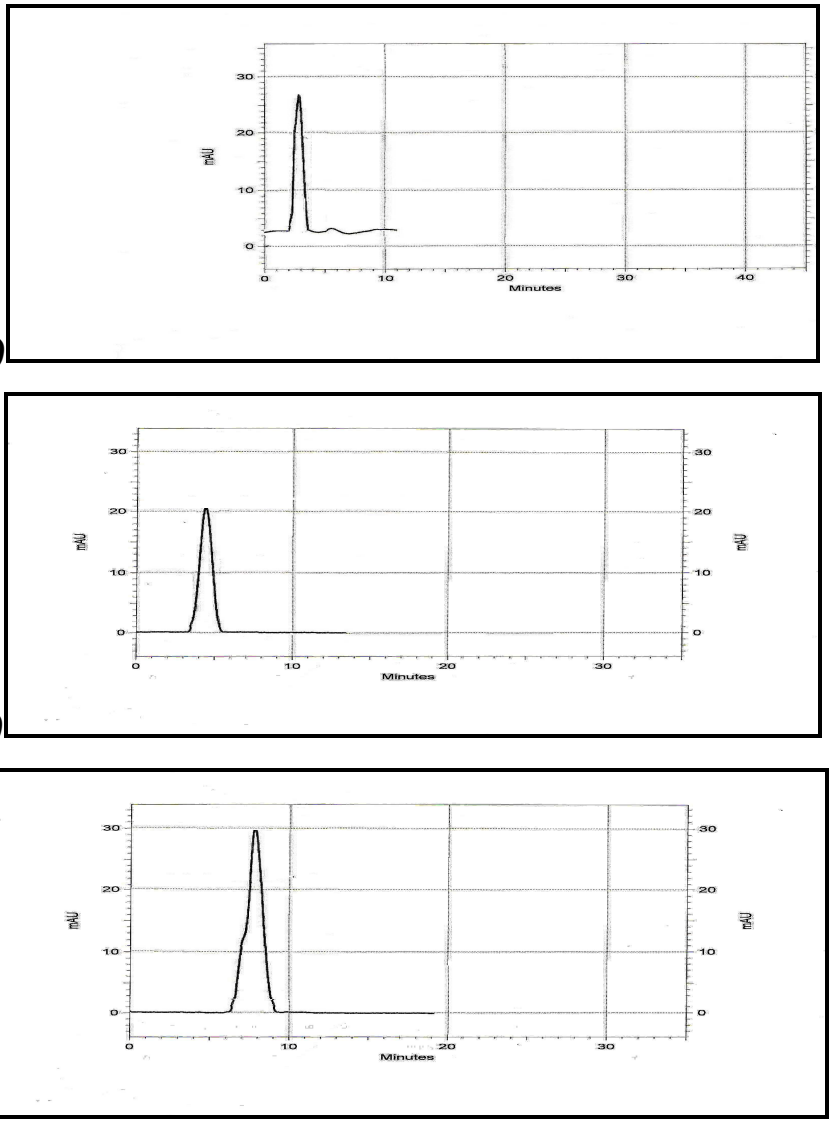
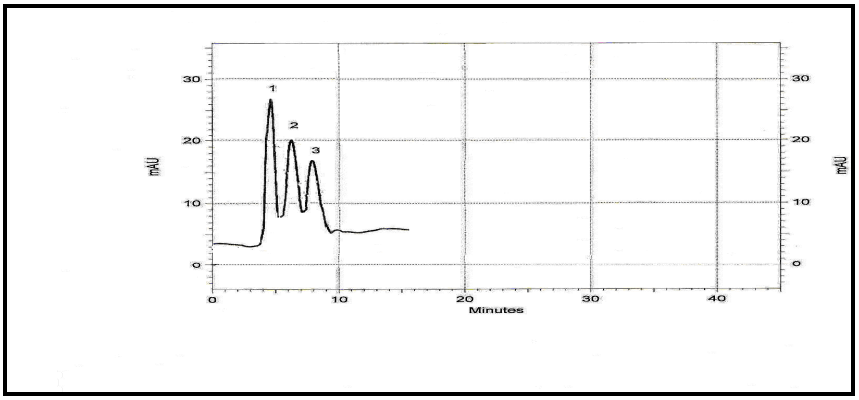


Figure (3-26) Chromatogram of a standard of 2.0 ppm (a) phenylalanine, (b) tryptophane and (c) tyrosine, flow rate 1.2ml/min using 15% phosphate buffer-85% ethanol as mobile.

The separation of a mixture of amino acids is showed in figure (3-27) using 15% phosphate buffer-85% ethanol mobile phase.



Figure(3-27) Chromatogram of standard mixture of 0.2ppm phenylalanine(1), 0.1ppm of tryptophane(2) and 0.5 ppm of tyrosine(3), using acrylamide-bisacrylamide column (25×0.4 cm (id)). Eluent 15% phosphate buffer-85% ethanol, flow rate 1.2ml/min and detection wavelength 245nm.

The separation of this amino acids mixture was improved by using gradient eluention programming. The best gradient program was found to be and listed in Table (3-16) and showed in figure (3-28).

Table (3-16): Time programming of gradient eluention for amino acid separation used acrylamide-bisacrylamide column (25×0.4 cm (id)).

Time Duration, min	*% flow rate of pump B (phosphate buffer)*	*% flow rate of pump A (ethanol)*
2	5	95
2	10	90
2	15	85
2	15	85
2	15	85
2	20	90

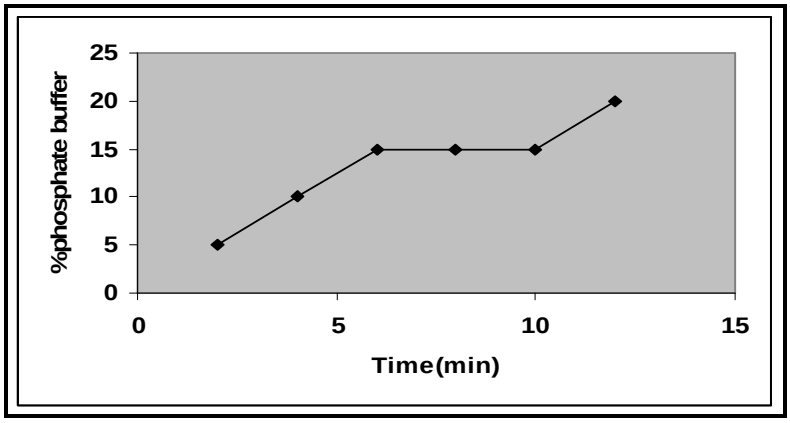
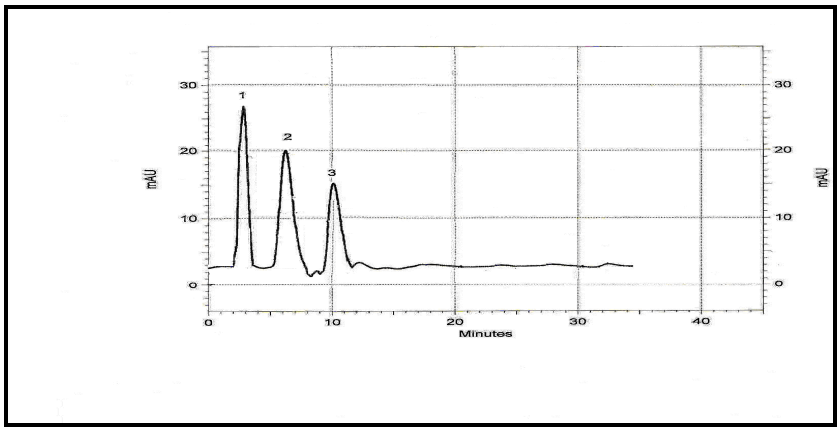


Figure (3-28) Time programming of gradient eluention.

These analyzed amino acids compounds have given well resolved peaks as well as a good detector response. The chromatographic separation of this mixture is showed in figure (3-29) using gradient eluention. Gradient eluention gave a good resolution for amino acids than isocratic eluention.



Figure(3-29) Chromatogram of separation of standard mixture of 0.2ppm phenylalanine(1), 0.1ppm of tryptophane(2) and 0.5 ppm of tyrosine(3), using acrylamide-bisacrylamide column (25×0.4 cm (id)). Eluent gradient (0-15) % phosphate buffer in ethanol, flow rate 1.2ml/min and detection wavelength 245nm.

0.2ppm phenylalanine, 0.1ppm of tryptophane and 0.5 ppm of tyrosine using gradient eluent (0-15) % phosphate buffer in EtOH at pH 6.0, flow rate was 1.2ml/min, and detection wavelength of 245nm. The average (**N**) was found to be equal to 1154.5. The (**H**) was found to be equal to

0.02cm; other chromatographic variations are listed in Table (3-17)

Table (3-17) Retention time tr, capacity factor Ḱ , separation factor α , resolution, and peaks asymmetry for amino acids, using acrylamide-bisacrylamide column (25×0.4 cm (id)). Eluent gradient (0-15) % phosphate buffer in ethanol, flow rate 1.2ml/min and wavelength245nm.

Compounds	*Retntion time tr*	*Capacity factor Ḱ*	*Separation factor α*	*Resolution*	*Peak Asymmetry*
Phenylalanine	2.72	1.73			0.99
Tryptophane	3.22	1.84	1.06	1.02	1.15
Tyrosine	10.11	2.11	1.15	1.03	1.23

Table (3-17) listed the retention times for all analyzed amino acids, which were reproducible. The RSD% ranged from 0.591-0.632% with an average value 0.622%. The capacity factors ranged from 1.73-2.11 with an average value 1.92, selectivity factors ranged from 1.06-1.15 with an average value 1.11 and peak asymmetries ranged from 0.99-1.23 with an average 1.12.

The day-day, week-week and month-month retention time reproducibility expressed in (RSD) for all analytes analyzed on the new column are list Table in (3-18).

The day-day retention time reproducibility's ranged from 0.568-0.856% with an average value 0.648%. The week-week RSD ranged from 0.688-0.946% with an average value 0.793%. While that of month-month RSD ranged from 0.963-1.572% with an average value 1.256%.

Table (3-18): RSD in retention time

compounds	*RSD%* Same day*	*RSD%* day-day*	*RSD%* weak-weak*	*RSD% * month-month*
Furosmide	0.423	0.568	0.688	0.963
Atenolol	0.389	0.467	0.566	0.897
Amiloride	0.452	0.559	0.652	0.941
Phenylalanine	0.591	0.763	0.814	1.025
Tryptophane	0.632	0.785	0.895	1.078
Tyrosine	0.582	0.687	0.831	0.985

***each result was calculated for at less 5 runs**

The results that given in Table (3-10) and Table (3-18) indicate that acrylamide-bisacrylamide was better than triethanolamine-glycerol-maleate column in term of retention time reproducibility's.

Table (3-19) showed the comparison between the triethanolamine-glycerol-maleate and acrylamide-bisacrylamide columns in term of effective plate number, plate height, retention time, capacity factor and separation factor. F-test was also calculated at 95% C.L. and the values were ranged from 6.41-6.54. The calculated values greater than F values (6.39) This is due to the number of functional groups in acrylamide-bisacrylamide polymer is more than the functional groups in triethanolamine-glycerol-maleate polymer, this mean that the interaction of analytes with polymer was better.

Table (3-19) separation factor, capacity factors, and peak asymmetry for amino acids and drugs using, (I) triethanolamine-glycerol-maleate and (II) acrylamide-bisacrylamide column (25×0.4cm (i.d)).

Compounds			*N*	*H*	*tr ±RSD%*	*Ḱ*	*A*	*Peak asymmetry*
#	*Name*	*Col*						
1	*Phenylalanin*	*I*	**1233.5**	**0.02**	**3.12±0.756**	**1.73**		**1.08**
		II	**1154.5**	**0.02**	**2.72±0.591**	**1.62**		**0.99**
2	*Tryptophane*	*I*	**1233.5**	**0.02**	**4.85±0.648**	**1.94**	**1.12**	**1.04**
		II	**1154.5**	**0.02**	**3.22±0.632**	**1.84**	**1.06**	**1.15**
3	*Tyrosine*	*I*	**1233.5**	**0.02**	**8.23±0.712**	**2.13**	**1.11**	**1.32**
		II	**1154.5**	**0.02**	**10.11±0.58**	**2.11**	**1.15**	**1.23**
4	*Furosmide*	*I*	**779.3**	**0.03**	**4.65±0.594**	**1.52**		**1.14**
		II	**1589.5**	**0.01**	**4.91±0.423**	**2.30**		**1.04**
5	*Atenolol*	*I*	**779.3**	**0.03**	**7.05±0.423**	**1.85**	**1.22**	**0.99**
		II	**1589.5**	**0.01**	**9.97±0.389**	**2.65**	**1.33**	**1.05**
6	*Amiloride*	*I*	**779.3**	**0.03**	**9.10±0.522**	**2.13**	**1.45**	**1.02**
		II	**1589.5**	**0.01**	**13.24±0.42**	**3.02**	**1.51**	**1.01**

Amiloride, atenolol and furosmide samples were chromatographed using commercial C-18 column, with flow rate of 0.8ml/min. The mobile phase used to analyze amiloride was a mixture of 60% phosphate buffer and 40% methanol at pH 2.6. Furosmide was chromatographed using a mixture of 46% methanol and 54% phosphate buffer at pH 2.0 (orthophosphoric acid). However atenolol was chromatographed using mixture of 65% methanol - 5% THF - 30% buffer phosphate at pH

2.6[(101)]as it is showed in figures (3-30), (3-31) and (3-32), respectively These analyzed drugs have given symmetrical peaks as well as good detector response. The separation of a mixture cannot be performed because of the difference mobile phase composition that used to analyze the drugs.

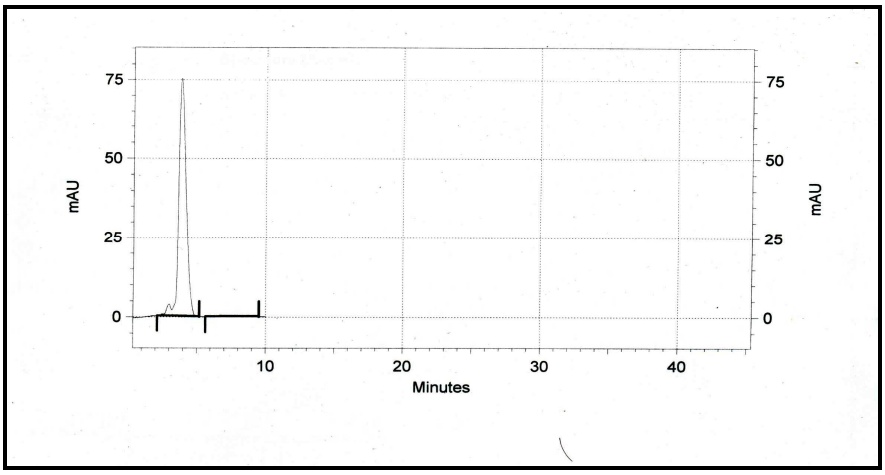


Figure (3-30) Chromatogram of a standard of amiloride 5.0 ppm, using C-18 column (25×0.4 cm (id)). Eluent 60% phosphate buffer and 40% methanol at pH 2.6, flow rate 0.8ml/min, retention time 3.35min and detection wavelength 233nm.

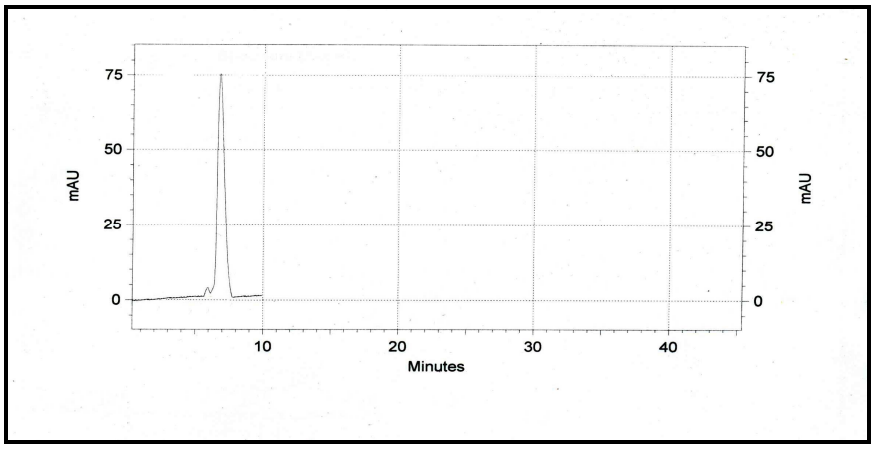


Figure (3-31) Chromatogram of a standard of atenolol5.0 ppm, using C-18 column (25×0.4 cm (id)). Eluent 65% methanol – 5%THF – 30% phosphate buffer at pH 2.6, flow rate 0.8ml/min, retention time 7.55min and detection wavelength 233nm.

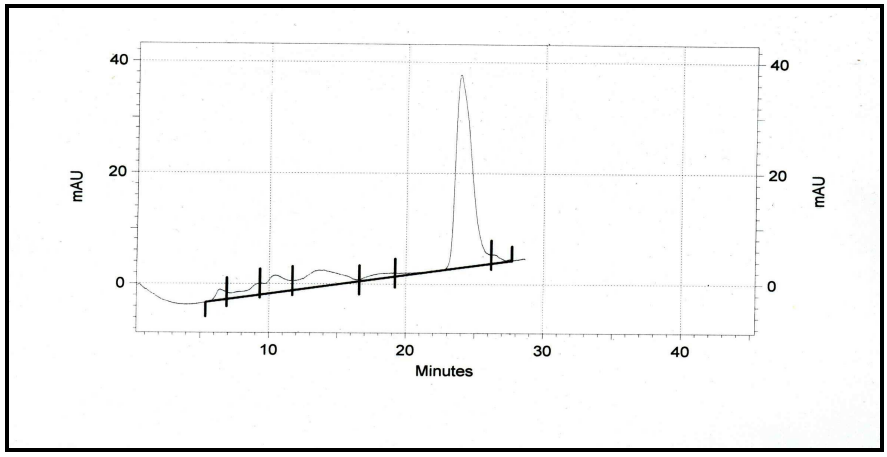
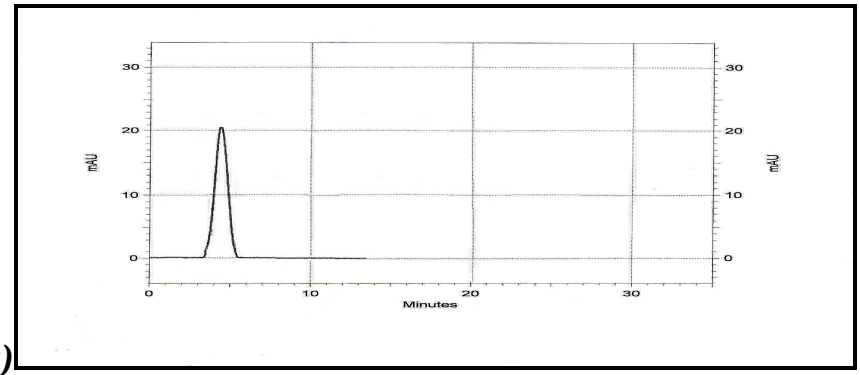


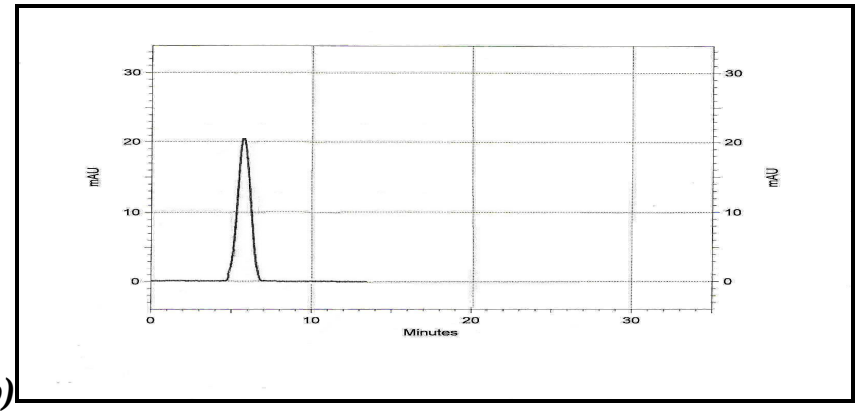
Figure (3-32) Chromatogram of a standard of furosmide5.0 ppm, using C-18 column (25×0.4 cm (id)). Eluent 40% methanol – 60% phosphate buffer at pH 2.6, flow rate 0.8ml/min, retention time 23.35min and detection wavelength 233nm.

Phenylalanine, tryptophane, and tyrosine were chromatographed using C-18, with flow rate 1.0ml/min. Mobile phase was a mixture of 18% methanol-2.5%THF- 79.5%sodium acetate (0.2M) at pH 5.9. On the other hand, these analyzed amino acids have given symmetrical peaks as well as a good detector response. The separation of a mixture of amino acids is showed in Figure (3-34). The difference in retention times of these amino acids may indicate a good separation as given in Table (3-20).

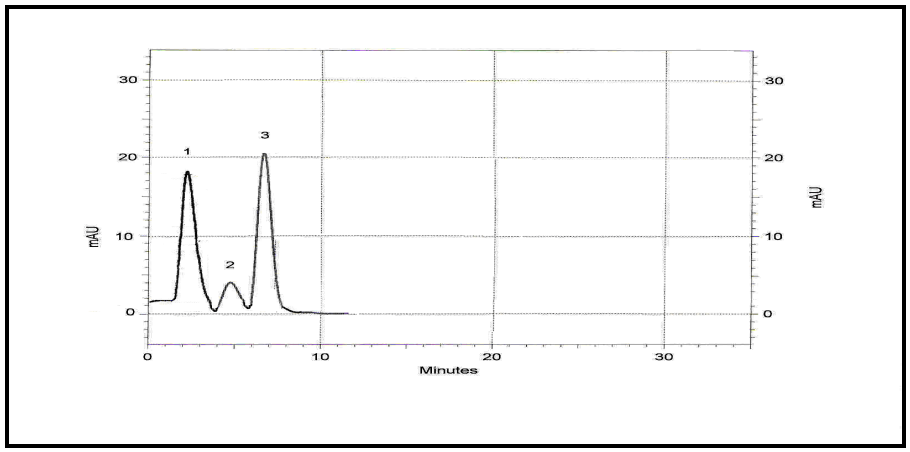
(a)



(b)



Figure(3-33) Chromatogram of a standard of (a)phenylalanine, (b) tyrosine and (c)tryptophane 2.0 ppm, flow rate 1.2ml/min. Used 18% methanol-2.5% THF-79.5% sodium acetate(0.2m) at pH 5.9



Figure(3-34) Chromatogram of separation of standard mixture of 0.2ppm phenylalanine(1), 0.1ppm of tyrosine (2) and 0.5 ppm of tryptophane (3), using C-18column (25×0.4 cm (id)). Eluent gradient 18% methanol-2.5% THF-79.5% sodium acetate (0.2m) at pH 5.9, flow rate 1.0ml/min and detection wavelength 245nm.

Table (3-20) Retention time tr, capacity factor Ḱ , separation factor α, resolution and peak asymmetry for amino acids, using C-18 column (25×0.4 cm (id)). Eluent gradient 18% methanol-2.5% THF-79.5% sodium acetate (0.2m) at pH 5.9, flow rate 1.0ml/min and detection wavelength 245nm.

Compounds	*Retntion time tr*	*Capacity factor Ḱ*	*Separation factor α*	*Resolution*	*Peak Asymmetry*
Phenylalanine	3.53	1.99			1.24
Tryptophane	4.66	2.44	1.23	1.28	1.05
Tyrosine	6.32	3.36	1.38	1.31	1.31

Figure (3-34) shows a chromatogram of 2ppm of phenylalanine, 3ppm of tyrosine, and 5 ppm of tryptophane using 18%methanol-2.5%THF-79.5% sodium acetate (0.2cm) at pH 5.9, flow rate 1.0ml/min, and detection wavelength at 245nm. The average (**N**) was found to be 1235, and the (**H**) was calculated and found to be equal to 0.02cm. Table (3-20) lists the retention times for the analyzed amino acids, RSD% ranged from 0.698-0.743% with an average value 0.685%. The capacity factors ranged from 1.99-3.36 with an average of 2.68, selectivity factors ranged from 1.23-1.38 with an average value 1.31 and peak asymmetries ranged from 1.05-1.31 with an average 1.18. Tables (3-12), (3-17), and (3-19) gave that the chromatographed of phenylalanine, tyrosine and tryptophane using acrylamide-bisacrylamide column which show a better than chromatographed of amino acids using triethanolamine-glycerol-maleate column and C-18 column.

Vitamin E was chromatographed using C-18 column, with flow at 1.2ml/min and mobile phase was a mixture of 5% distilled water-95% ethanol. This analysis has given a well shaped symmetrical peak as well

as a good detector response, as it is showed in figure (3-35).

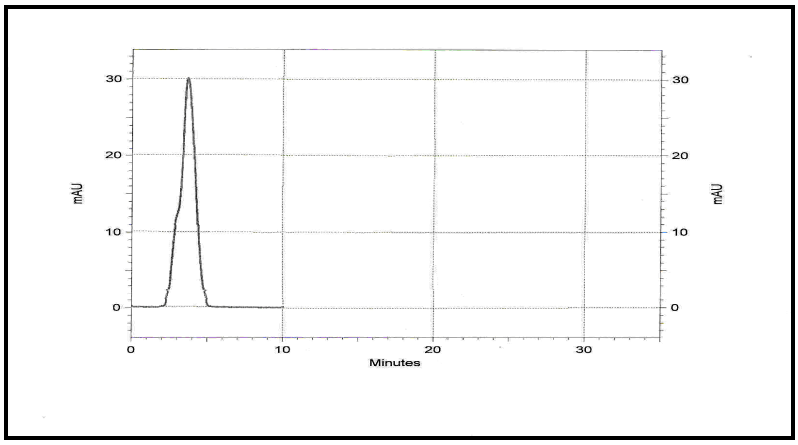


Figure (3-35) Chromatogram of a standard of vitamin E 5.0 ppm, using C-18 column (25×0.4 cm (id)). Eluent 5% distilled water and 95% ethanol, flow rate 1.2ml/min, retention time 4.65min and detection wavelength 229nm.

Table (3-21) shows the comparison between the triethanolamine-glycerol-maleate (I), acrylamide-bisacrylamide (II) and commercial C-18 (III) columns with respect to an effective plate number, a plate height, a retention time, a capacity factor and a separation factor. F-test was, also calculated at 95% C.L. between C-18 column and triethanolamine-glycerol-malate column, the values was ranged from 4.33-4.78. The calculated values were lower than F table (6.39) values in the F-test was also calculated at 95% C.L. between C-18 column and acrylamide-bisacrylamide column, the values were ranged from 4.97-5.23 so the calculated values lower than F table values.

Table (3-21) separation factor, capacity factors and peak asymmetry for amino acids and drugs using, (I) triethanolamine-glycerol-maleate and (II) acrylamide-bisacrylamide column and C-18 column (25×0.4cm (i.d)).

Compounds			N	H	t_r ±RSD%	Ḱ	A	Peak asymmetry
#	Name	Col						
1	Phenylalanin	I	1233.5	0.02	3.12±0.756	1.73		1.08
		II	1154.5	0.02	2.72±0.591	1.62		0.99
		III	1235.0	0.02	3.53±0.662	1.99		1.24
2	Tryptophane	I	1233.5	0.02	4.85±0.648	1.94	1.12	1.04
		II	1154.5	0.02	3.22±0.632	1.84	1.06	1.15
		III	1235.0	0.02	6.32±0.644	3.36	1.23	1.31
3	Tyrosine	I	1233.5	0.02	8.23±0.712	2.13	1.11	1.32
		II	1154.5	0.02	10.11±0.58	2.11	1.15	1.23
		III	1235.0	0.02	2.44±0.671	4.66	1.38	1.05
4	Furosmide	I	779.3	0.03	4.65±0.594	1.52		1.14
		II	1589.5	0.01	4.91±0.423	2.30		1.04
		III	1601.0	0.01	23.4±0.372	1.88		1.08
5	Atenolol	I	779.3	0.03	7.05±0.423	1.85	1.22	0.99
		II	1589.5	0.01	9.97±0.389	2.65	1.33	1.05
		III	1601.0	0.01	7.55±0.321	2.61		1.16
6	Amiloride	I	779.3	0.03	9.10±0.522	2.13	1.45	1.02
		II	1589.5	0.01	13.24±0.42	3.02	1.51	1.01
		III	1601.0	0.01	3.35±0.321	2.79		1.09

3.9.Quantitative analysis

Calibration curves for drugs, amino acids, and vitamin E analyzed on triethanolamine-glycerol-maleate column using gradient eluention program. Figure (3-36) show the calibration curve for Vitamin E. A linear equation dependence of the peak area and the amount of the sample injected was evidence down to the detection limit.

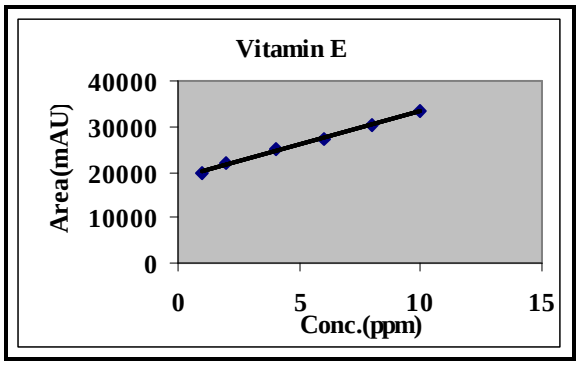


Figure (3-36) Calibration curve of Vitamin E using triethanolamine-glycerol-maleate column (25×0.4 cm (id)). Eluent 100% acetonitrile, flow rate 1ml/min and detection wavelength 229nm.

The slope of the linear calibration curve for vitamin E was 11229.1, the correlation coefficient was averaged 0.9998, and detection limit was 0.1ppm. Standard solutions of vitamin E were injected for at least three times under the same condition, and the measured area was employed to calculate their concentration using the linear equation. The recovery was 90.95% and a relative error was 0.6% as listed in Table (3-23).

Different amino acids were analyzed (at least 3 times for each one) starting from 10ppm to the concentration was the peak area was difficult to calculate due to the high background signal. This concentration was considered as detection limit for that samples peak of each amino acid

was plotted against its concentration. These calibration curves are shown in figure (3-37).

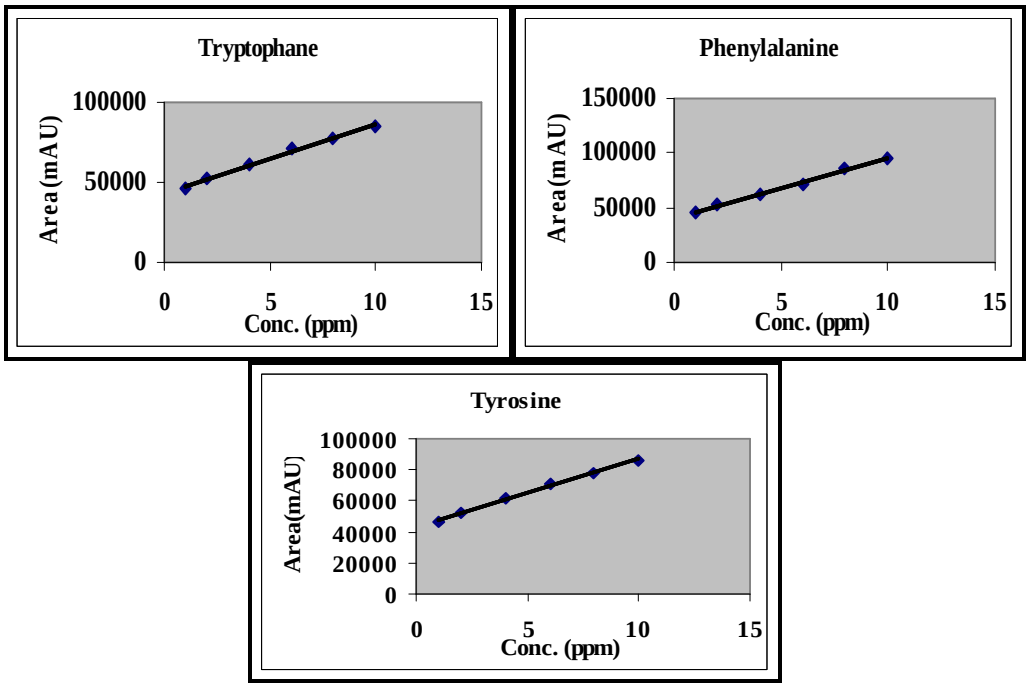


Figure (3-37) Calibration curve of amino acids using triethanolamine-glycerol-maleate column (25×0.4 cm (id)). Eluent gradient (0-15) % buffer in acetonitrile, flow rate 1ml/min and detection wavelength 245nm.

The slopes for the linear calibration curves of amino acid were ranged from 55561.6-59316.9 depended upon kind of amino acid and type of mobile phase. The correlation coefficients ranged from 0.9996-0.9998 and detection limits ranged from 0.05-0.1ppm, using gradient eluention, these values are summarized in Table (3-22).

Table(3-22) Linear equation, correlation coefficients R, and detection limit of amino acids using triethanolamine-glycerol-maleate column (25×0.4 cm (id)). Eluent 15% buffer- 85% acetonitrile, flow rate 1ml/min and detection wavelength 245nm.

Compounds	*Linear Equation Y*=mx*+b*	*Conc. range*	*R*	*Detection Limit (ppm)*
Phenylalanine	Y=55561.6x-41453.4	0.1-10	0.9996	0.10
Tryptophane	Y=57002.1x-45944.6	0.05-10	0.9997	0.05
Tyrosine	Y=59316.9x-46122.2	0.1-10	0.9998	0.10

***y represent peak area, * x represent concentration**

Standard solutions of amino acids were injected for at least three times under the same condition and their concentration was calculated using their linear equation. The recoveries ranged from of 90.00-97.00% with an average value 93.53% and relative errors ranged from 3.00-4.00% with an average value 3.33% as listed in Table (3-23).

Table (3-23) Recovery and percentage relative error of amino acids using triethanolamine-glycerol-maleate column (25×0.4 cm (id)). Eluent 15% buffer- 85% acetonitrile, flow rate 1ml/min and detection wavelength 245nm.

Amino acids	*Conc. Injected (ppm)*	*Conc. Found(calculated*) (ppm)*	*Recovery %*	*Relative error%*
Phenylalanine	1.00	0.96	96.00	4.00
Tryptophane	1.00	0.97	97.00	3.00
Tyrosine	0.50	0.45	90.00	4.00

*** using the linear equation for each amino acids**

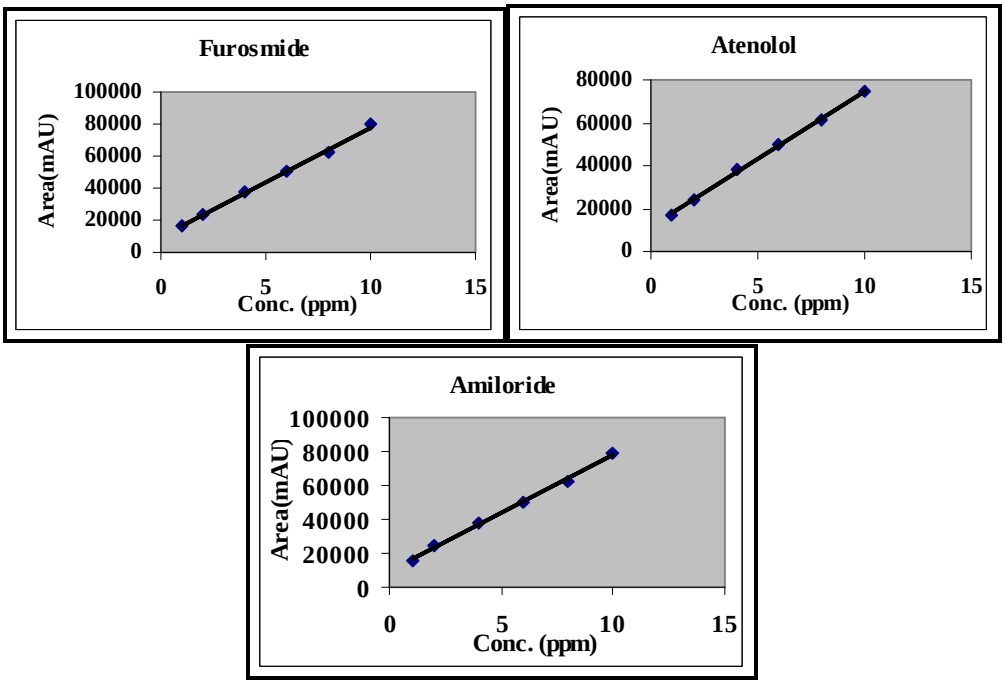


Figure (3-38) Calibration curve of drugs using triethanolamine-glycerol-maleate column (25×0.4 cm (id)). Eluent gradient (0-20) % phosphate in MeOH, flow rate 1ml/min and detection wavelength233nm.

The same procedure was used to construct the calibration curve for the studied medical drugs. The linear calibration curve is shown in figure (3-38). The slopes for the linear calibration curves ranged from 56221.6-59495.9 depended upon kind of drugs. The correlation coefficients ranged from 0.9997-0.9998 and detection limits ranged from 0.05-0.1ppm, using gradient eluention, these values are summarized in Table (3-24).

Table (3-24) Linear equation, correlation coefficients R, and detection limit of drugs using triethanolamine-glycerol-maleate column (25×0.4 cm (id)). Eluent gradient (0-20) % phosphate buffer in MeOH, flow rate 1ml/min and detection wavelength 233nm.

Compounds	*Linear Equation Y*=mx*+b*	*Conc. range*	*R*	*Detection Limit (ppm)*
Furosmide	Y=56221.6x-35273.4	0.1-10	0.9998	0.10
Atenolol	Y=58962.1x-37301.6	0.05-10	0.9997	0.05
Amiloride	Y=59495.9x-38576.2	0.05-10	0.9998	0.05

***y represent peak area, * x represent concentration**

Standard solutions were injected for at least three times under the same condition mentioned earlier using the linear equation. The recovery ranged from 95.00-98.00% with an average value of 96.54% and relative errors ranged from 2.00-5.00% with an average value of 3.5% as listed in Table (3-25).

Table (3-25) Recovery and percentage relative error of drugs using triethanolamine-glycerol-maleate column (25×0.4 cm (id)). Eluent gradient (0-20) % phosphate buffer in MeOH, flow rate 1ml/min and detection wavelength 233nm.

Drugs	*Conc. Injected (ppm)*	*Conc. Found(calculated*) (ppm)*	*Recovery %*	*Relative error%*
Furosmide	3.00	2.94	98.00	2.00
Atenolol	1.50	1.46	97.37	2.67
Amiloride	1.00	0.95	95.00	5.00

*** using the linear equation for each drugs**

Calibration run of the drugs and amino acids analyzed on acrylamide-bisacrylamide column using gradient eluention are showed in figure (3-39) and figure (3-40). A linearity dependence of the peak area of each analytes and on the amount injected in evident for all compounds down to the detection limit.

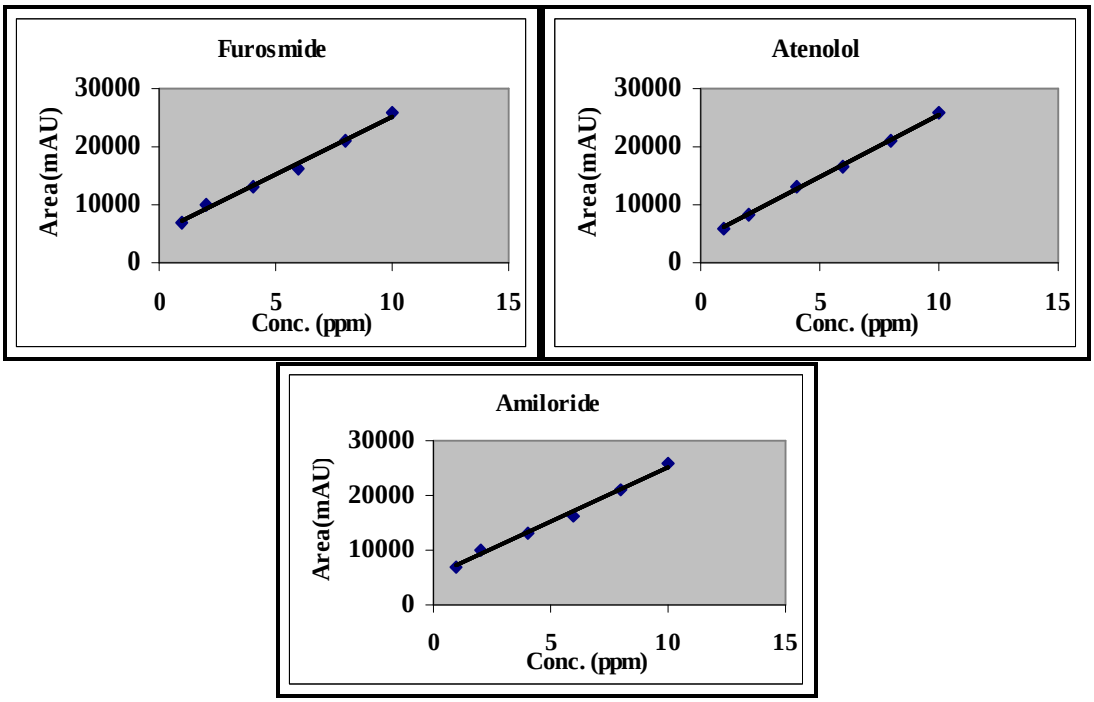


Figure (3-39) Calibration curve of drugs using acrylamide-bisacrylamide column (25×0.4 cm (id)). Eluent isocratic 10% phosphate buffer-90% EtOH, flow rate 1.2ml/min and detection wavelength 233nm.

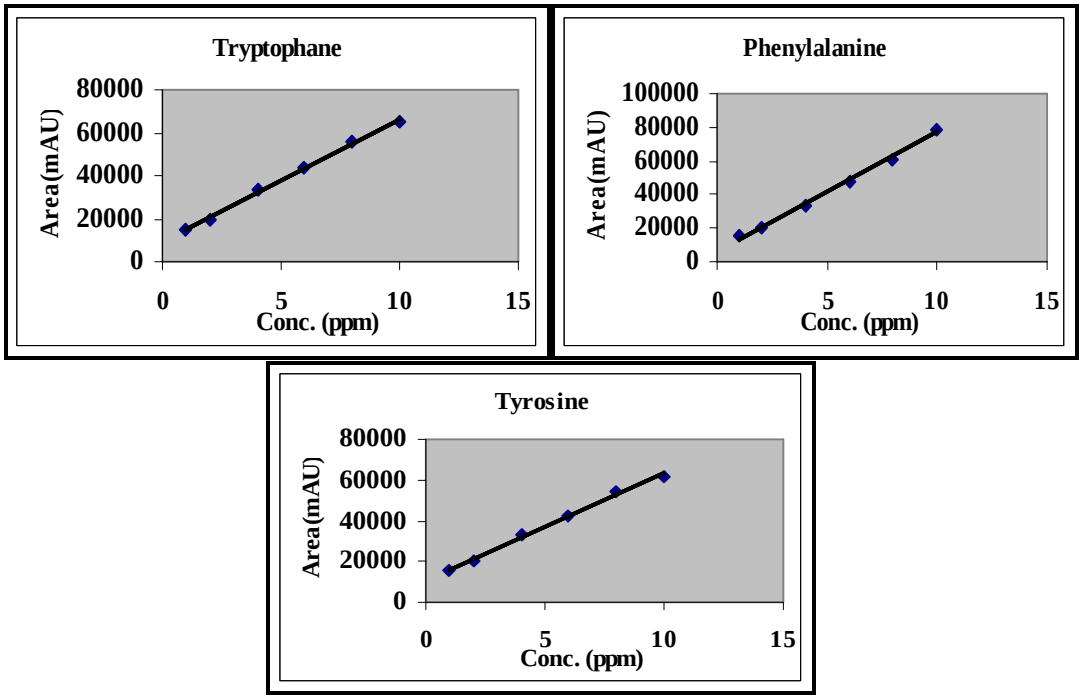


Figure (3-40) Calibration curve of amino acids using acrylamide-bisacrylamide column (25×0.4 cm (id)). Flow rate flow rate 1.2ml/min. Used (0-15) % phosphate buffer in ethanol and detection wavelength 245nm.

The slopes for the linear calibration curves of analytes drugs were ranged from 23268.6-26638.6 depended upon type of drugs. The correlation coefficients ranged from 0.9996-0.9998 and detection limits ranged from 0.05-0.1ppm, using an isocratic eluention, these values are summarized in Table (3-26).

Table (3-26) Linear equation, correlation coefficients R, and detection limit of drugs using acrylamide-bisacrylamide column (25×0.4 cm (id)). Eluent isocratic 10% phosphate buffer-90% EtOH, flow rate 1.2ml/min and detection wavelength 233nm.

Compounds	*Linear Equation y*=mx*+b*	*Conc. range*	*R*	*Detection Limit (ppm)*
Furosmide	Y=23268.6x+12673.4	0.05-10	0.9997	0.05
Atenolol	Y=25872.5x+12905.2	0.05-10	0.9998	0.05
Amiloride	Y=26638.6x+13700.2	0.1-10	0.9996	0.10

***y represent peak area, * x represent concentration**

Prepared standard solutions of the above drugs were injected for at least three times under the same conditions and their concentrations were calculated using linear equation. The recovery ranged from of 97.33-99.00% with an average value of 98.18% and relative errors ranged from 1.00-2.67% with an average value 1.78% as listed in Table (3-27).

Table (3-27) Recovery and percentage relative error of drugs using acrylamide-bisacrylamide column (25×0.4 cm (id)). Eluent isocratic 10% phosphate buffer-90% EtOH, flow rate 1.2ml/min and detection wavelength 233nm.

Drugs	*Conc. Injected (ppm)*	*Conc. Found(calculated*) (ppm)*	*Recovery %*	*Relative error%*
Furosmide	3.00	2.95	98.33	1.67
Atenolol	2.00	1.98	99.00	1.00
Amiloride	1.50	1.46	97.33	2.67

*** using the linear equation for each drugs**

The slopes for the linear calibration curves for amino acid ranged from 60561.6-61002.1 depended upon kind of amino acids. The correlation coefficients ranged from 0.9996-0.9998 and detection limits ranged from 0.05-0.1ppm, using gradient eluention, these values are summarized in Table (3-28).

Table(3-28) Linear equation, correlation coefficients R, and detection limit of amino acids using acrylamide-bisacrylamide column (25×0.4 cm (id)). Flow rate flow rate 1.2ml/min. Used (0-15) % phosphate buffer in ethanol and detection wavelength 245nm.

Compounds	*Linear Equation Y*=mx*+b*	*Conc. range*	*R*	*Detection Limit (ppm)*
Phenylalanine	Y=60561.6x-48153.4	0.1-10	0.9996	0.10
Tryptophane	Y=61002.1x-49544.6	0.05-10	0.9997	0.05
Tyrosine	Y=61316.9x-60122.2	0.1-10	0.9998	0.10

***y represent peak area, * x represent concentration**

Prepared standard solutions of amino acid were injected for at least three times under the same condition and their concentration was calculated using the linear equation. The recoveries ranged from 95.00-97.14% with an average value 96.13% and relative errors ranged from 2.00-5.00% with an average value 5.00% as listed in Table (3-29).

Table (3-29) Recovery and percentage relative error of amino acids using acrylamide-bisacrylamide column (25×0.4 cm (id)). Flow rate flow rate 1.2ml/min. Used (0-15) % phosphate buffer in ethanol and detection wavelength 245nm

Amino acids	*Conc. Injected (ppm)*	*Conc. Found(calculated*) (ppm)*	*Recovery %*	*Relative error%*
Phenylalanine	2.00	1.94	97.03	3.00
Tryptophane	2.00	1.96	97.14	2.00
Tyrosine	1.00	0.95	95.00	5.00

*** using the linear equation for each amino acids**

Calibration curves for the analyzed drugs, amino acids and vitamin E compounds on Octadecaylsilanolol ODS (C-18) column are shown in figure (3-41), figure (3-42) and figure (3-43) respectively. A linear dependence of peak area on the amount injected was evident for all compounds down to the detection limit.

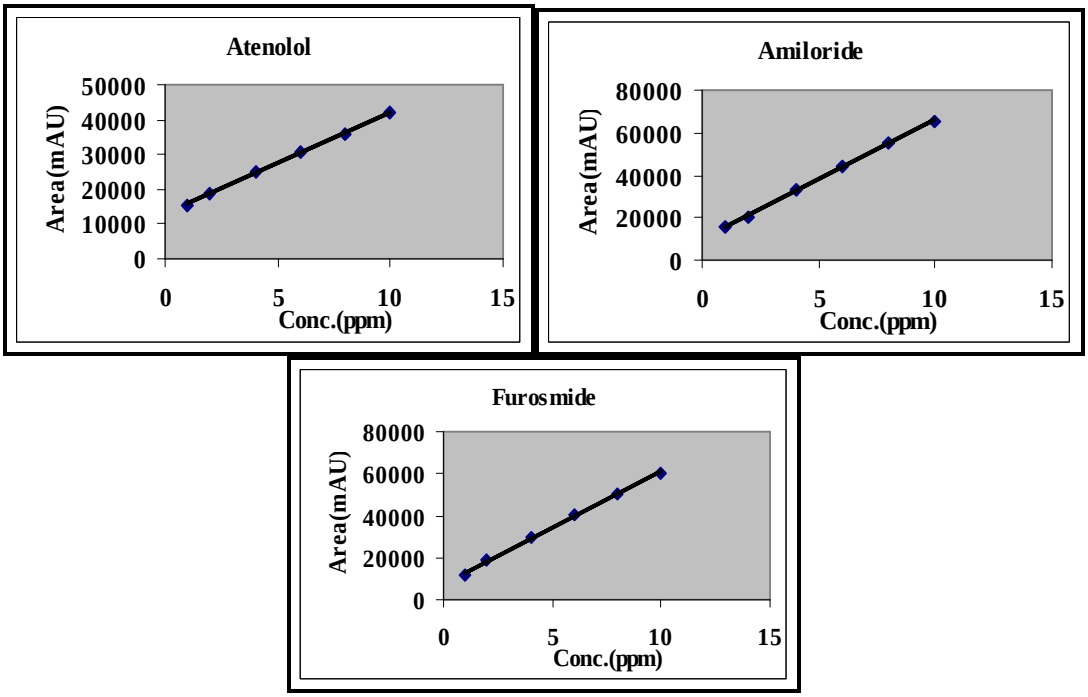


Figure (3-41) Calibration curve of drugs using C-18 column (25×0.4 cm (id)). Different eluent, flow rate 0.8ml/min and detection wavelength 233nm.

The slopes for the linear calibration curve of the analyzed drugs ranged from 23935.6-28636.9. The correlation coefficients ranged from 0.9997-0.9998 and detection limit was 0.05ppm as summarized in Table (3-30).

Table (3-30) Linear equation, correlation coefficients R, and detection limit of drugs using C-18 column (25×0.4 cm (id)). Flow rate flow rate 1.0ml/min. Used deferent eluent and detection wavelength 233nm.

Compounds	*Linear Equation*	*Conc. range*	*R*	*Detection Limit (ppm)*
Amiloride	Y=23935.6x-18138.4	0.05-10	0.9997	0.05
Atenolol	Y=26758.1x-20244.6	0.05-10	0.9997	0.05
Furosemide	Y=28636.9x-22922.2	0.05-10	0.9998	0.05

***y represent peak area, * x represent concentration**

Standard solutions of the analyzed drugs were injected for at least three times under the same condition of their concentration using the linear equation. The recoveries ranged from 96.15-98.25% with an average value 97.21% and relative errors ranged from 1.80-4.60% with an average value 3.07% as listed in Table (3-31).

Table (3-31) Recovery and percentage relative error of drugs using C-18 column (25×0.4 cm (id)). Flow rate flow rate 1.0ml/min. Used deferent eluent and detection wavelength 233nm.

Drugs	*Conc. Injected (ppm)*	*Conc. Found(calculated*) (ppm)*	*Recovery %*	*Relative error%*
Furosmide	5.00	4.91	٩٨,٢٥	1.80
Atenolol	5.00	4.86	97.21	2.80
Amiloride	5.00	4.77	96.15	4.60

*** using the linear equation for each drugs**

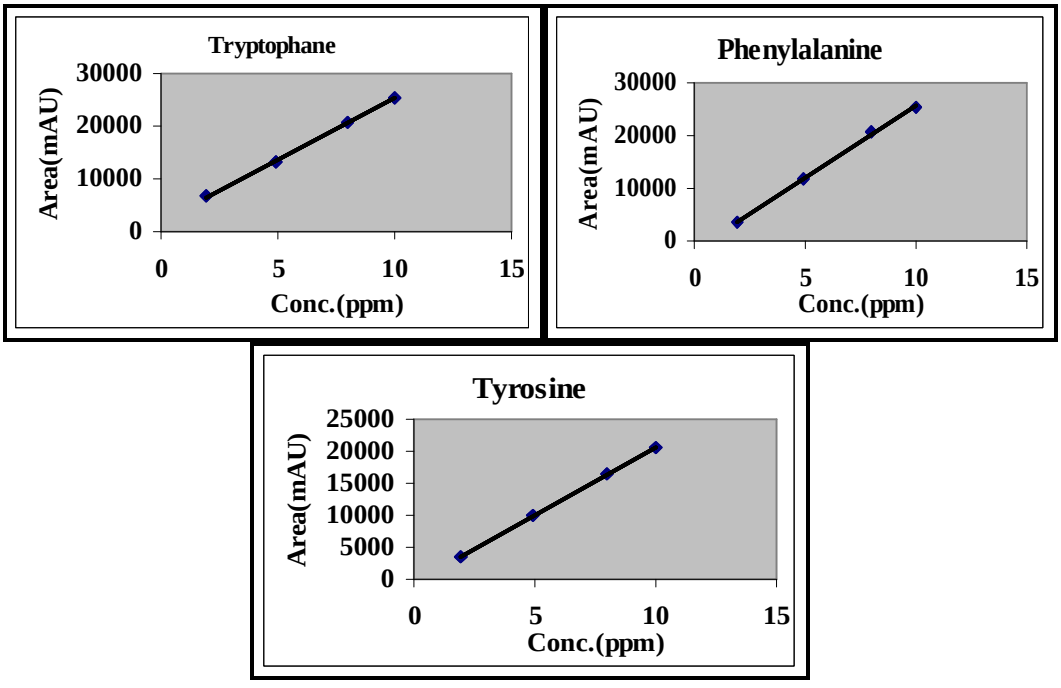


Figure (3-42) Calibration curve of amino acids using C-18 column (25×0.4 cm (id)). Eluent, flow rate 1.0ml/min and detection wavelength 245nm.

The slopes of the linear calibration curve for amino acid ranged from 28025.6-32031.9. The correlation coefficients ranged from 0.9997-0.9998 and detection limit ranged from 0.05-0.10ppm as summarized in Table (3-32).

Table(3-32) Linear equation, correlation coefficients R, and detection limit of amino acids using C-18 column (25×0.4 cm (id)). Eluent, flow rate 1.0ml/min and detection wavelength 245nm.

Compounds	*Linear Equation*	*R*	*Detection Limit (ppm)*
Phenylalanine	Y=28025.6x-23193.4	0.9998	0.10
Tyrosine	Y=30158.8x-24924.1	0.9997	0.10
Tryptophane	Y=32031.9x-25249.9	0.9998	0.05

Prepared standard solutions of amino acid were injected for at least three times under the same condition and their concentration was calculated using the linear equation. The recovery ranged from of 80.00%-93.50% with an average value of 85.26% and relative errors ranged from 6.50-19.00% with an average value 12.0% as listed in Table (3-33).

Table (3-33) Recovery and percentage relative error of amino acids using acids using C-18 column (25×0.4 cm (id)). Eluent, flow rate 1.0ml/min and detection wavelength 245nm

Amino acids	*Conc. Injected (ppm)*	*Conc. Found(calculated*) (ppm)*	*Recovery %*	*Relative error%*
Phenylalanine	2.00	1.87	93.50	6.50
Tryptophane	1.00	0.81	80.00	19.00

Tyrosine	2.00	1.79	89.50	10.50

*** using the linear equation for each amino acids**

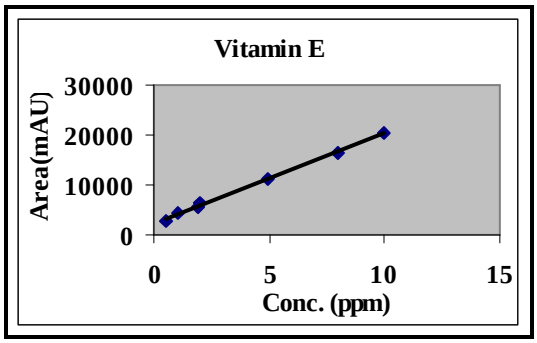


Figure (3-43) Calibration curve of vitamin E using C-18 column (25×0.4 cm (id)). Flow rate flow rate 1.2ml/min. Eluent 5%distilled water-95%ethanol and detection wavelength 229nm.

A slope for the linear calibration curve 30256.6 depended upon kind of vitamin E and type of mobile phase, the correlation coefficient was 0.9998 and detection limit was 0.10ppm. Standard solutions were injected for at least three times under the same condition mentioned earlier by using the linear equation. The recovery was 87.97% and a relative error was 1.80%.

Table (3-34) Correlation coefficients R, detection limit, recovery and percentage relative error of vitamin E, amino acids and drugs using (I)triethanolamine-glycerol-maleate, (II)acrylamide-bisacrylamide, and (III) C-18 column (25×0.4 cm (id)).

Compounds	*Column*	*R*	*Detection limit*	*Recovery*	*Relative error*
Vitamin E	*I*	0.9998	0.10	90.95	0.60
	II	*	*	*	*
	III	0.9998	0.10	89.97	1.80
Phenylalanine	*I*	0.9996	0.10	96.00	4.00
	II	0.9996	0.10	97.03	3.00
	III	0.9998	0.10	93.50	6.5
Tryptophane	*I*	0.9998	0.10	97.00	3.00
	II	0.9997	0.05	97.14	2.00
	III	0.9998	0.05	80.00	19.0
Tyrosine	*I*	0.9998	0.10	90.00	2.00
	II	0.9998	0.10	95.00	5.11
	III	0.9997	0.10	89.50	10.0
Furosmide	*I*	0.9998	0.10	98.00	2.00
	II	0.9997	0.05	98.33	1.67
	III	0.9998	0.05	٩٨,٢٥	4.60
Atenolol	*I*	0.9997	0.05	97.37	2.67
	II	0.9998	0.05	99.00	1.00
	III	0.9997	0.05	97.21	2.80

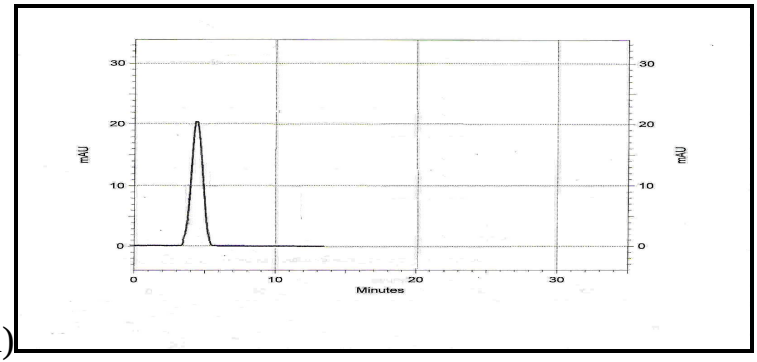
Amiloride	***I***	0.9998	0.10	95.00	5.00
	II	0.9996	0.10	97.33	2.66
	III	0.9997	0.05	96.15	1.80

3.10. Analysis of real samples

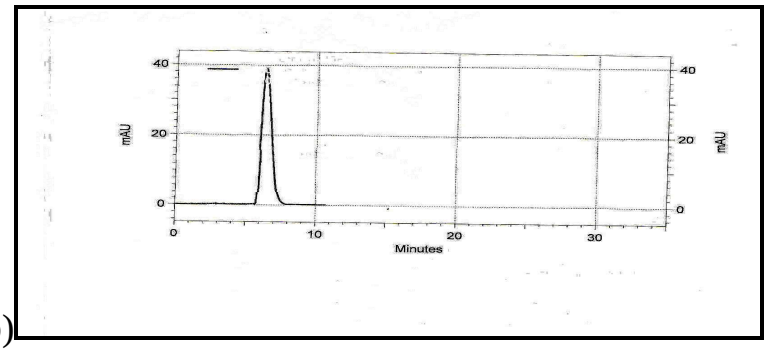
Solutions of furosmide, atenolol, and amiloride obtain from three different tablets manufactures were analyzed using triethanolamine-glycerol-malate column, mobile phase was gradient (0-20)% phosphate buffer in MeOH, flow rate 1ml/min, and detection wavelength 233nm as shown in figure (3-44) the RSD% concentration calculation were averaged 0.845%, 0.841% and 0.809% for furosmide, atenolol and amiloride, respectively as listed in Table (3-35).

Table (3-35) Analysis Amiloride (5mg) in tablet, Furosmide (20mg) in tablet, and Atenolol (50mg) in tablet, using triethanolamine-glycerol-maleate column (25×0.4 cm (id)). Eluent gradient (0-20) % phosphate buffer in MeOH, flow rate 1ml/min and detection wavelength 233nm.

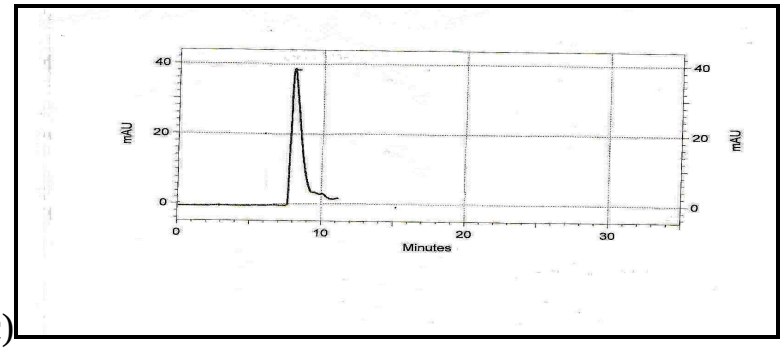
Companies		***Amiloride***	***Furosemide***	***Atenolol***
Samara	***Wt.(mg) injected***	5.00	20.00	50.00
	Wt.(mg) calculated	4.98	19.95	49.97
	Recovery %	99.25	99.77	99.42
India	***Wt.(mg) injected***	5.00	20.00	50.00
	Wt.(mg) calculated	4.84	17.98	48.12
	Recovery %	96.24	88.28	95.64
Egypt	***Wt.(mg) injected***	5.00	20.00	50.00
	Wt.(mg) calculated*	4.91	18.84	48.53
	Recovery %	98.16	94.23	97.44



(a)



(b)



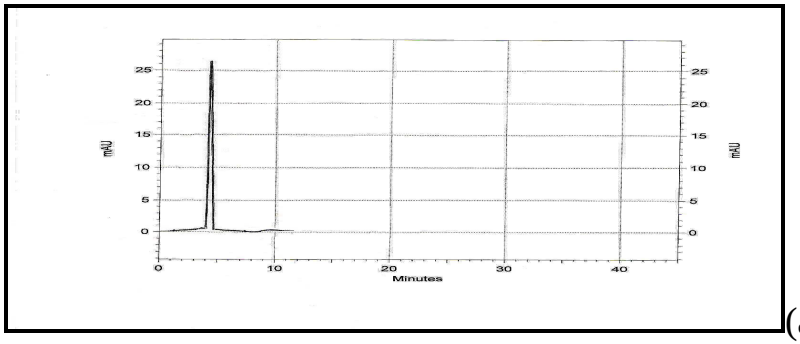
(c)

Figure(3-44) (a)Amiloride 5mg, (b) Furosemide 20mg and (c)Atenolol 50mg , using triethanolamine-glycerol-maleate column (25×0.4 cm (id)). Eluent gradient (0-20) % phosphate buffer in MeOH, flow rate 1ml/min and detection wavelength 233nm.

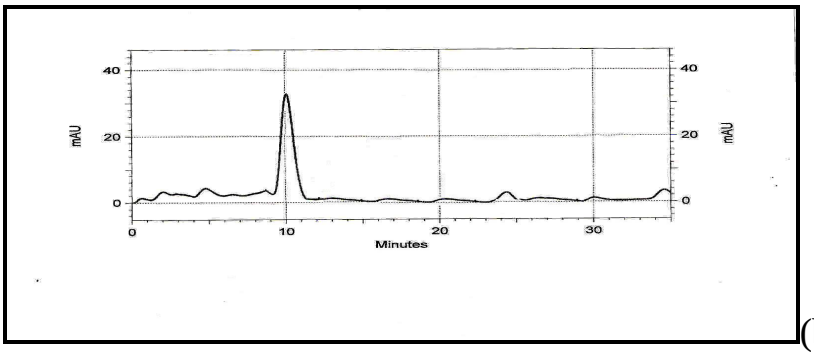
The same method was applied using acrylamide-bisacrylamide column, mobile phase gradient (0-10)% phosphate buffer in EtOH, flow rate 1.2ml/min, and detection wavelength 233nm as shown in figure (3-45). The RSD% for their analysis to determine the concentration ranged from 0.611%, 0.726% and 0.794% for furosmide, atenolol and amiloride as listed in Table (3-36) and.

Table (3-36) Analysis Amiloride (5mg) in tablet, Furosmide (20mg) in tablet, and Atenolol (50mg) in tablet, using acrylamide-bisacrylamide column (25×0.4 cm (id)). Eluent gradient (0-10) % phosphate buffer in EtOH, flow rate 1.2ml/min and detection wavelength 233nm.

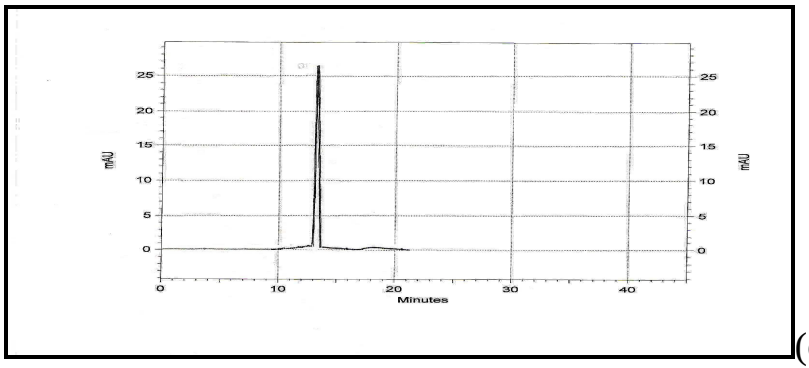
Companies		*Amiloride*	*Furosemide*	*Atenolol*
Samara	***Wt.(mg) injected***	5.00	20.00	50.00
	Wt.(mg) calculated	4.97	19.98	49.99
	Recovery %	99.44	99.90	99.98
India	***Wt.(mg) injected***	5.00	20.00	50.00
	Wt.(mg) calculated	4.80	17.78	48.52
	Recovery %	96.00	88.9	97.04
Egypt	***Wt.(mg) injected***	5.00	20.00	50.00
	Wt.(mg) calculated	4.93	18.99	49.29
	Recovery %	98.60	94.95	98.58



(a)



(b)

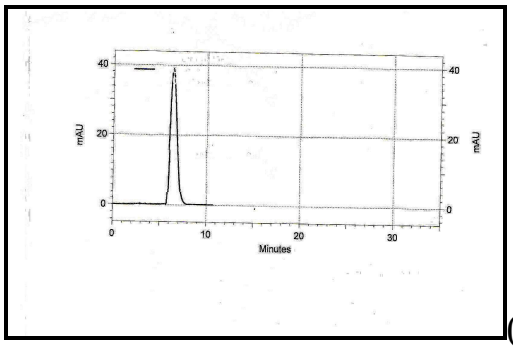


(c)

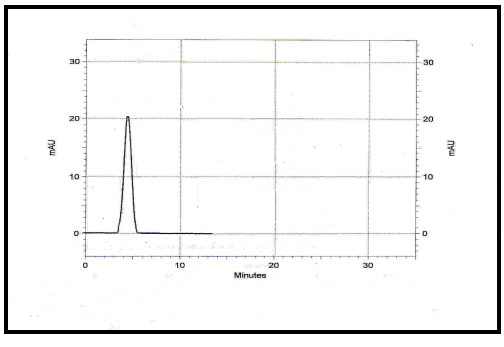
Figure(3-45) (a)Amiloride 5mg, (b) Furosemide 20mg and (c)Atenolol 50mg , using acrylamide-bisacrylamide column (25×0.4 cm (id)). Eluent gradient (0-10) % phosphate buffer in EtOH, flow rate 1.2ml/min and detection wavelength 233nm

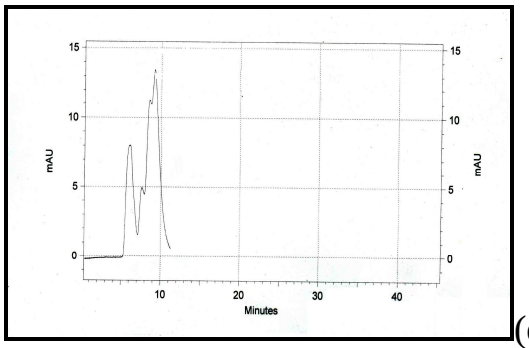
Furosmide, atenolol and amiloride in serum, were analyzed using triethanolamine-glycerol-malate column; mobile phase was gradient (0-20) % phosphate buffer in MeOH, flow rate 1ml/min, and detection wavelength 233nm. The value for lazix ranged from 16.77-17.86 after two hours, for tenormen ranged from 45.44-46.87 after two hours and for amiloride ranged from 4.23-4.41 also after two hours as showed in figure (3-46).

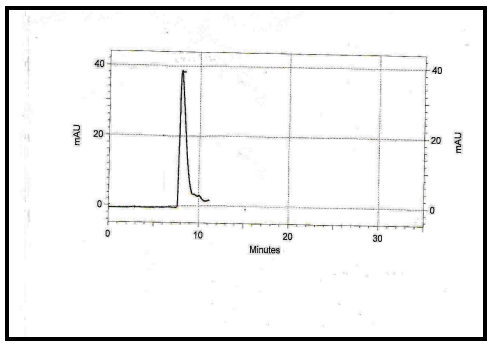
(a)



(b)

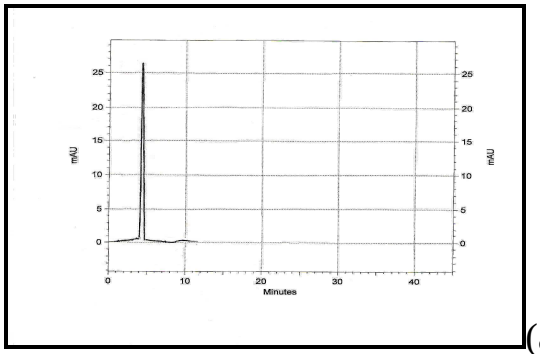


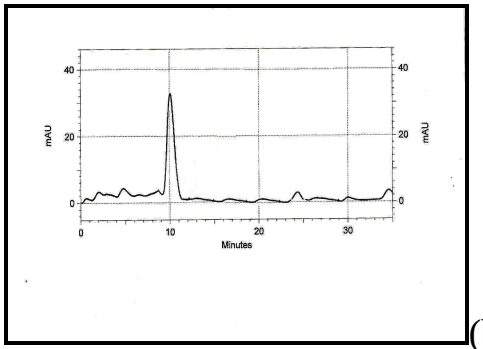
(c) 

(d) 

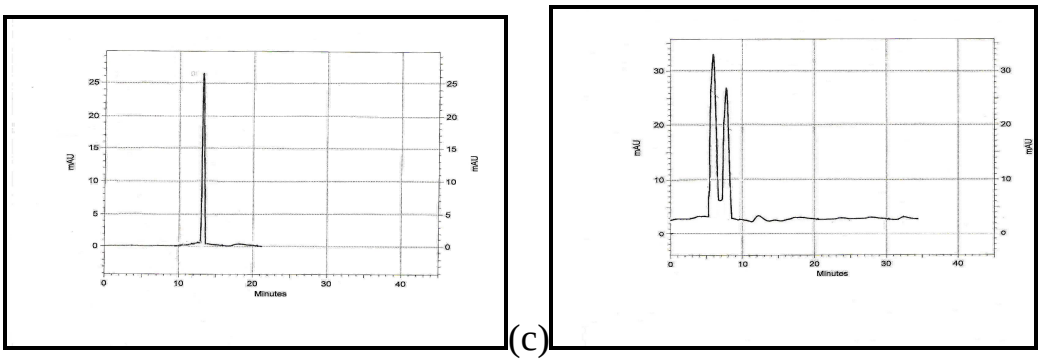
Figure(3-46) (a) Lazix 20mg, (b) tenordin50mg, mixture of Lazix and tenormen and (d) Amiloride 5mg, using triethanolamine-glycerol-maleate column (25×0.4 cm (id)). Eluent gradient (0-20) % phosphate buffer in MeOH, flow rate 1ml/min and detection wavelength 233nm

In analyzed furosmide, atenolol and amiloride in serum, the samples were analyzed using acrylamide-bisacrylamide column. Eluent gradient (0-10) % phosphate buffer in EtOH, flow rate 1.2ml/min, and detection wavelength 233nm three times. The value for lazix ranged from 17.63-18.36 after two hours, for tenormen ranged from 46.58-47.21 after two hours and for amiloride ranged from 4.65-4.83 also after two hours as showed in figure (3-47).



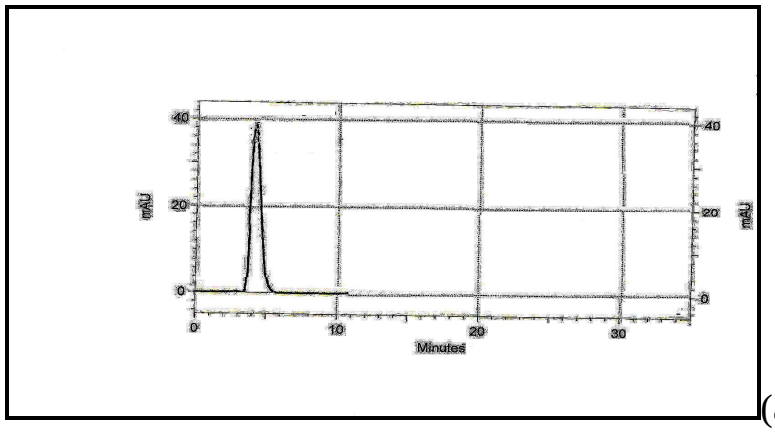
(a) 

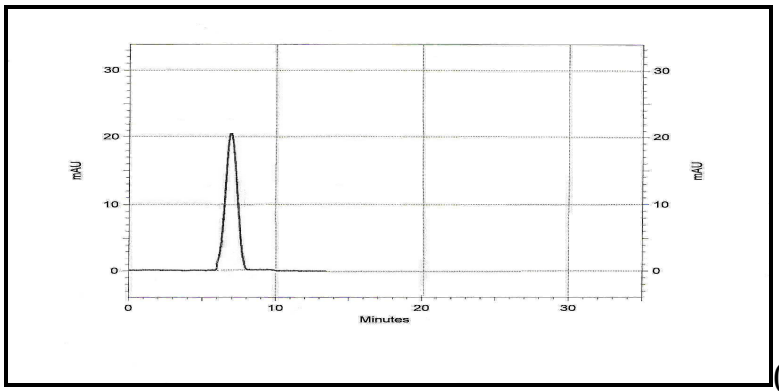
(b)



Figure(3-47) (a) Lazix 20mg, (b) Tenormin50mg, (c) Amiloride5mg and (d) mixture of Lazix and tenor men, using acrylamide-bisacrylamide column (25×0.4 cm (id)). Eluent gradient (0-10) % phosphate buffer in EtOH, flow rate 1.2ml/min and detection wavelength 233nm.

In the analyzed vitamin E in serum the samples prepared diluted to 10ml and filtrated by minipore were analyzed using triethanolamine-glycerol-maleate column. Eluent gradient 100% acetonitrile, flow rate 1.2ml/min, and detection wavelength 229nm three times. The value ranged from 8.63-9.87 for female and ranged from 5.23-6.57 for male as showed in figure (3-48).





(b)

Figure (3-48) (a) female, and (b) male, using triethanolamine-glycerol-maleate column (25×0.4 cm (id)). Eluent gradient 100% acetonitrile, flow rate 1.0ml/min and detection wavelength 229nm

Conclusion

New stationary phases were prepared. The first polymer was synthesized by condensation reaction between glycerol, triethanolamine with maleic anhydride. The second polymer was synthesized by addition polymerization of free radical for acrylamide, bisacrylamide with ammonium persulphate and N, N,N,N-tetramethyldiamine (TEMED). The products were characterized by FTIR & X-ray. The resulted polymer have high rigidity and easily grinded, with high stability. The polymers were used as a stationary phase for HPLC. This stationary phase has been packed into stainless steel column. The chromatographic performances of the newly packed columns were characterized.

The new triethanolamine-glycerol-maleate stationary phases packed in to HPLC column was used for the analysis of vitamin E. The average value of capacity factors was 1.39, peaks asymmetry value was 1.11 and retention times was 4.68min, using 5%water and 95% ethanol as mobile phase. In the analysis of amino acids with the same column, the average value of plate numbers N was 1233.5. The plate heights was 0.06cm, capacity factors were ranged from 1.73-2.13, separation factors were ranged from 1.10-1.12, peaks asymmetry values were ranged from 1.04-1.32 and retention times were ranged from 3.12-8.23for phenylalanine, tryptophane and tyrosine, using gradient elution of (0-15)%phosphate buffer adjusted at pH 6.0 in acetonitrile as mobile phase.

However, in the analysis of drugs with the same column, the average value of plate numbers N was 779.3. The plate heights was 0.02cm, capacity factors were ranged from 1.52-2.13, separation factors were ranged from 1.22-1.27, peaks asymmetry values were ranged from 0.99-1.14 and retention times were ranged from 4.65-9.16 for furosmide,

atenolol and amiloride, using gradient elution of (0-20)%phosphate buffer adjusted at pH 8.0 in methanol as mobile phase.

In the analysis of drugs using acrylamide-bisacrylamide column, the average value of plate numbers N was found to be 1089.5. The plate height was 0.03cm, capacity factors were ranged from 2.30-3.02, separation factors were ranged from 1.13-1.15, peaks asymmetry values were ranged from 1.01-1.14 and retention times were ranged from 4.91-13.24 for furosmide, atenolol and amiloride, using isocratic elution of 10%phosphate buffer adjusted at pH 6.0 and 90% ethanol as mobile phase.

However, in the analysis of amino acids with the same column, the average value of plate numbers N was 1154.5. The plate heights was 0.05cm, capacity factors were ranged from 1.71-2.11, separation factors were ranged from 1.06-1.15, peaks asymmetry values were ranged from 0.99-1.23 and retention times were ranged from 2.72-7058 for phenylalanine, tryptophane and tyrosine, using gradient elution of (0-15)%phosphate buffer adjusted at pH 6.0 in ethanol as mobile phase.

Calibration curves for all analyzed compounds were linear from their detection limit to at least 10.0ppm with correlation coefficient ranged from 0.9996-0.9998, the detection limit ranged from 0.05-0.10 at signal to noise ratio of three or more, recovery percentage ranged from 82.00-91.23 and relative error ranged from 0.60-5.00.

Suggestions for future studies

1. A analysis of organic compounds using new prepared columns.
2. Using small particles size of triethanolamine-glycerol-maleate column and acrylamide-bisacrylamide column for preparation of new stationary phases.
3. The use of other chromatographic detector refractive index (RI) for detection of non UV-Visible absorbing species after separated with new columns.

CHAPTER ONE

INTRODUCTION

CHAPTER TWO

EXPERIMENTAL

CHAPTER THREE

RESULTS AND DISCUSSIONS

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Republic of Iraq
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Synthesis and characterization of new cross linked polymers as stationary phases for HPLC separation

A thesis
Submitted to the College of Science
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In partial fulfillment of Requirements
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Chemistry

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Supervisor certification

We certify that this thesis was prepared under our Supervision in the Department of Chemistry, College of Science, and Al-Nahrain University as partial requirements for the degree of doctor of philosophy in chemistry.

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We the examining committees, certify that we read this thesis and examined the student ***Noor Mustafa Ali***, in its contents and that, according to our opinion, is accepted as a thesis for the degree of Doctor of philosophy, in chemistry.

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جمهورية العراق
وزارة التعليم العالي والبحث العلمي
جامعة النهرين
كلية العلوم
قسم الكيمياء

تحضير وتشخيص بوليمرات جديدة كأطوار ثابتة محتمله للفصل في الكروماتوغرافي السائل عالي الأداء

رسالة
مقدمة الى كلية العلوم- جامعة النهرين
وهي جزء من متطلبات نيل درجة الدكتوراه فلسفة في الكيمياء

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نيسان ٢٠٠٨ **ربيع الأول١٤٢٩**

بِسم الله الرحمن الرحيم

نـرفَـعُ دَرَجَـاتٍ مَـن نـَشـــَاءُ

وَفـَوقَ كُـلِ ذي عِـلمٍ عَـلـيمُ

صَدَقَ الله العلـي آلَعَظِيم

سورة يوسف (٧٦)

الإهداء

إلى مثلي الأعلى ...وقدوتي في الحياة ...إلى نبع المحبة الصافي
إلى من علمني معنى العطاء و التضحية

والدي

إلى من صبرت وسهرت من اجل راحتي ... إلى فيض الحنان الدافق

والدتي

إلى من شرفني الله بوجودهم ... إلى سندي و مشجعي في الشدائد

إلى مهد طفولتي و مرح شبابي ... و أحباب قلبي ...

أخوتي

إلى كل من أحبني ... و أراد مودتي ...

أهدي هذا الجهد المتواضع

نور

الخلاصة

تم تحضيراثنان من البوليمرات متشابكة الأول بواسطة بلمرة التكثيف من تفاعل (Glycerol) و(Triethanol amine) مع(Maleic anhydride) . والبوليمر الثاني بواسطة بلمرة الجذور الحرة من تفاعل (Acrylamide) و (Bisacrylamide) مع (Ammonium persulphate)و(TEMED)

تم تشخيص الناتج بواسطة تحاليل الاشعة تحت الحمراء و ال(X-ray) حيث ا وجد ان البوليمر الناتج من هذا المزيج يملك الصلادة العالية وسهولة الطحن. استخدم البوليمر الناتج كطور ثابت في الفصل الكروماتوغرافي السائل العالية الأداء.

تم تعبئة هذا الطور الثابت في عمود من الفولاذ المقاوم للصدأ، وتم قياس كفاءة العمود المعبأ الجديد بحساب عدد الصفيحات النظرية. الارتفاع المكافيء للصفيحات النظرية، عوامل الاستيعاب، عوامل الانتقائية، وعدم تماثل القمة بواسطة محاليل مختلفة على العمود الجديد باستخدام اطوار متحركة مختلفة النسب وسرعة جريان ثابتة.

تم تحليل المركبات الدوائية باستخدام العامود الاول، حيث تم فصل(Amiloride , Furosmide و Atenolol)

بواسطة استخدام خليط بنسب مختلفة

(buffer phosphate at pH 8: 20% methanol: 80%) كطور متحرك ومعدل جريان (1ml/min) وطول موجي 233nm وكذلك باستخدام العامود الثاني، تم فصل المركبات الدوائية باستخدام خليط بنسب مختلفة

(Buffer phosphate at pH 6: 10% ethanol: 90%) كطور متحرك ومعدل جريان (1.2ml/min) وطول موجي 233nm

وحللت بعض الأحماض الأمينة (Phenylalanine, Tryptophane, Tyrosine)باستخدام (buffer phosphate at pH 6: 15% methanol: 85%) كطور متحرك ومعدل جريان (1ml/min) وطول موجي 245nm باستخدام العامود الأول وكذلك تم تحليل هذه الأحماض الأمينة باستخدام (buffer phosphate at pH 6: 5% ethanol: 95%) كطور متحرك ومعدل جريان (1.2ml/min) وطول موجي 245nm باستخدام العامود الثاني

و كذلكم ت تحليل فيتامين E (Acetonitrile 100%) كطور متحرك ومعدل جريان (1ml/min) وطول موجي 229nm باستخدام العامود الأول

و ان فصل جميع هذه المركبات قورن مع فصل هذه المركبات باستخدام عامود C-18. وكان لكل من المركبات المحللة وقت احتجاز مختلف عن الاخرى بالاضافة الى انتقائية مختلفة مما سمح بفصل المركبات الدوائية ,الأحماض الأمينة و فيتامين E المحللة وقت احتجاز مختلف عن الأخرى بالإضافة إلى انتقائية مختلفة مما سمح بفصل المركبات الدوائية والأحماض الأمينة وكان المنحنيات المعيارية للمركبات المحللة كانت خطية من الحد الأدنى لكشفها إلى ١٠ جزء بالمليون مع عامل ترابط يتراوح بين ٠,٩٩٩٣ ــ ٠,٩٩٩٩ وكانت حدود الكشف تتراوح من ٠,٠٥ ـ ٠,١ جزء بالمليون.