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Research Article

ANALYSIS OF SOME BIOMEDICAL COMPOUNDS USING TRIETHANOLAMINE-GLYCEROL-MALEATE COPOLYMER AS HPLC STATIONARY PHASES

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ABSTRACT

A cross-linked polymer was synthesized by condensation reaction between mixture of glycerol and triethanol amine with maleic anhydride. The resulted polymer have high rigidity and easily grinded, with high stability and used as stationary phase for HPLC column. Vitamin E was analyzed with triethanolamine-glycerol-malate column with isocratic eluention 100% acetonitrile as a mobile phase with flow rate of 1ml/min and UV detection of 229nm, some amino acids (tyrosine, tryptophane and phenylalanine) were also examined with this column with isocratic eluention 15% phosphate buffer adjusted pH at 6 in 85% ethanol as a mobile phase with flow rate of 1ml/min and UV detection of 245nm. Furthermore some of drugs, such as amiloride, furosemide, and atenolol were analyzed with this stationary phase. The eluent 20% phosphate buffer at pH 8 in 80% methanol with UV detection at 233 nm has given a good analysis for the drugs using isocratic elution. The retention times for furosemide, atenolol and amiloride were 4.6 min, 7.1 min and 9.2 min, respectively. The separation was improved by with elution gradient (0-20) % phosphate buffer in methanol. Good recoveries were obtained for the drugs ranged from 95-98% by using triethanolamine-glycerol-maleate column. The results obtained were compared with commercial column ODS-C18. The method of analysis was applied for determination of the drugs in pharmaceutical tablets as well as in serum samples.

INTRODUCTION

Cross-linked organic polymer was introduced as packing in column liquid chromatography since 1960[1]. Kwang et al.[2] have prepared cyclodextrin (CD) polymers from the reaction of CD with a hexamethylene diisocyante in dried DMF solution. HPLC column was prepared using CD polymer as a stationary phase for separation of phenol isomers. Kanazawa et al.[6] have proposed a thermo-responsive polymer carrying an amino acid residue as HPLC stationary phase, they have investigated a new concept of chromatography.

They have, also used HPLC adsorbents stationary phase by modifying the surface of micro particulate silica gel using functional polymer[3]. The thermo responsive co-polymer, poly (N-isopropylacrylamide co-n-butyl metaacrylate) was used to modify the silica stationary phase surface. Kobayashi et al.[4] have used a cross-linked poly N-isopropylacrylamide co-acrylic acid grafted silica bead surface and applied as a new column matrix materials that exploit temperature responsive anionic chromatography to separate basic bioactive compounds. Meyer et al.[5] have used three poly ethylene-co-acrylic acid co-polymers with different chain lengths and mass fractions of acrylic acid and covalently immobilized as stationary phase on silica via two variants of molecular spacer namely; 3-aminopropyltriethoxysilane and 3-glycidoxypropyltrimethoxysilane. Sekikawa et al[7] have been determined furosemide in blood by using Shim-pack CLC-ODS column, mobile phase gradient consist of acetonitrile, water, and acetic acid and using fluorescent detection, retention time was 15 min and detection limit was as low as 5 ng/ml.

In this work triethanolamine-cycler-maleate co-polymer was prepared and used as a new stationary phase for HPLC column. It is used for analysis of several arterial hypertension drugs. Isocratic and gradient elution programs were applied for separation as well as the percent composition of the mobile was studied. The results obtained from this column were compared with commercial column ODS-C18.

Experiment

Equipments

1. High performance liquid chromatograph type Shimadzu (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 was used. The HPLC system has been interfaced with computer via a Shimadzu class-VP5 chromatography data system program supplied by the manufacturer.
2. Epson LQ-300 printer model P852A (Japan).
3. Shimadzu Fourier transforms infrared model FTIR 8300 (Japan) was used to measure the IR spectra for the prepared polymers.
4. X-Ray diffract meter, Siemens SRS D500 (Germany).
5. Combination glass electrode was used to measure the pH of polymer solutions (Germany).

Preparation of triethanolamine- glycerol-maleate polymer

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. The temperature was rise gradually to 160^{o}C. The reaction was performed under vacuum. Continued heating at this temperature, for 3 hours, caused 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.

Preparation of standard

A stock solution of 50ppm of standard drugs were prepared by dissolving 5mg furosemide in 0.1M of NaOH and diluted to 100ml with distilled water or dissolving 5mg furosemide in methanol and then diluted to 100ml with distilled water. The same procedure was followed in the preparation of amiloride and atenolol 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 mobile phase employed for their separation. A stock solution of 50ppm of standard amino acids and vitamin E were prepared by similarly.

RESULTS AND DISCUSSION

Synthesis of co-polymers used as a stationary phase in this work was done via condensation reaction of triethanolamine-glycerol with malefic anhydride in which produces a hard and rigid. The high degree of cross linking of the co-polymer prevented the solvation process and for this reason it was difficult to determine the molecular weight and the degree of polymerization. The co-polymer was identified by FTIR in which the appearance of absorption band at 1732 cm^{-1} due to the stretching vibration of the C = O of the formed ester and a band at 1296 cm^{-1} for C-N. Also an X-ray diffraction was used to identify the nature of the polymer whether it is a crystalline or not. The results showed that the polymer have different crystalline forms. Swelling test for prepared polymeric stationary phase was performed according to the ASTM procedure[8]. The degree of cross-linking has been measured using polar, moderately polar and non polar that are usually used in HPLC such as (water, acetone, acetonitrile and hexane). The results of the swelling ranged from 1% to 4% which are theoretically expected, except for unexpected value for water 4% which could be attributed to the presence of hydrogen bonding forming moiety on the polymer surface. 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 polymer insoluble and undecompose in all the above solvents and it is very stable. These results were attributed to high cross-linking of the polymer. However, at pH higher than 10 the polymer being decompose due to hydrolysis of the ester band of the polymer. Column packing was done by using the slurry formed by mixing the triethanolamine-glycerol-maleate powder with 100 ml acetonitrile and homogenized in an ultrasonic bath and placed in the slurry reservoir and the column was packed using down-flow packing system. The study was carried out for the analysis of Vitamin E by using the co-polymer triethanolamine-glycerol-maleate column (25 x 0.4 cm). 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, the resulting chromatogram is showed in Figure1. Also the study was carried out for analysis of amino acids, phenylalanine, tryptophane and tyrosine using this column. The results of pH showed that at pH <6 and at higher pH > 9 the amino acids cannot be detected. The capacity factor **Ќ** using methanol and phosphate buffer as eluent at different pH were calculated and shown in figure 2. The values of **Ќ** were 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. The separation factor (α) values of selectivity α for phenylalanine, tryptophan, and tyrosine were ranged from 1.13-1.07, 1.24-1.33, 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 may indicate that the pH 6.0 is the best pH buffer that can use for separating. The variation of the capacity factor and separation factor for amino acids with different percentage of phosphate buffer (percentage in acetonitrile) ranged from 5% to 25% are listed in table 1. The results indicate that a good competitive interaction of these drugs with the stationary phase and the best mobile phase of acetonitrile at15% of phosphate buffer.

Table 1: Capacity Ќ and separation α factors 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		--	--
Tryptophan	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

Figure 3 shows a chromatogram of the separation of three amino acids; 0.2 ppm furosemide, 0.1 ppm atenolol and 0.5 ppm amiloride, using isocratic elution of 15% phosphate buffer and 85% methanol at pH=6, flow rate of 1 ml/min and wavelength at 245 nm. The retention time and other parameters for separation of the drugs are listed in table 2.

Table 2: Retention time tr, capacity factor Ќ , 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 Ќ	Separation factor α	Resolution	Peak Asymmetry
Phenylalanine	3.12	1.73			1.08
Tryptophan	4.85	1.94	1.12	1.56	1.04
Tyrosine	8.23	2.13	1.10	1.89	1.32

The order of interaction of the drugs with the stationary phase due to the retention time is: Phenylalanine > Tryptophan > Tyrosine. The interaction of these drugs depends on the hydrogen bonding between N-H group of the drug and O-H groups of triethanolamine-glycerol-maleate co-polymer. The separation of these drugs in mixture was improved by using gradient elution programming as shown in figure 4.

The drugs, furosemide, atenolol and amiloride were analyzed by using the co-polymer triethanolamine-glycerol-maleate column (25 x 0.4 cm). The effect of the pH and phosphate buffer concentration of these drugs was studied. The results of pH showed that at pH <6 and at higher pH > 9 the drug cannot be detected. The capacity factor Ќ using methanol and phosphate buffer as eluent at different pH were calculated and shown in figure 5 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. The separation factor (α) values for furosemide, 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 use for separating. 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. The results indicate that a good competitive interaction of these drugs with

the stationary phase and the best mobile phase of methanol at 20% phosphate buffer.

Table 3: Capacity Ќ and separation α factors variation with changing of composition of mobile phase for drugs using triethanolamine-glycerol column

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
Furosemide	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	--	--

Figure 6 shows a chromatogram of the separation of three drugs; 0.2 ppm furosemide, 0.1 ppm atenolol and 0.5 ppm amiloride, using isocratic elution of 20% phosphate buffer and 80% methanol at pH=8, flow rate of 1 ml/min and wavelength at 233 nm. The retention time and other parameters for separation of the drugs are listed in table 4.

Table 4: Retention time (tr), capacity (Ќ)and separation (α)factors, resolution and peak asymmetry for drugs using triethanolamine-glycerol-maleate column, eluent gradient 20% phosphate buffer-80% MeOH, flow rate 1ml/min and detection wavelength 233nm

Compounds	Retention time(tr)	Capacity factor(Ќ)	Separation factor (α)	Resolution	Peak Asymmetry
Furosemide	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 order of interaction of the drugs with the stationary phase due to the retention time is: amiloride > atenolol > furosemide.

The interaction of these drugs depends on the hydrogen bonding between N-H group of the drug and O-H groups of triethanolamine-glycerol-maleate co-polymer.

The separation of these drugs in mixture was improved by using gradient elution programming as shown in figure 7. The column triethanolamine-glycerol-maleate column was compared with commercial column ODS-C18. Amiloride, atenolol and furosemide samples were chromatogram on C-18 with flow rate of 0.8 ml/min. The mobile phase for elution the drugs as follows: amiloride was a mixture of 60% phosphate buffer and 40% methanol at pH 2.6 gives retention time of 3.35 min, for furosemide was 46% methanol and 54% phosphate buffer at pH 2.0 gave a retention time of 23.35 min. While for atenolol the mobile was 65% methanol-5% THF- 30% buffer phosphate at pH 2.6, the retention time was 7.55 min. The separation of a mixture cannot be performed because of the different in the composition of the mobile phases. Therefore, ODS-C18 was very good column for determination of each drug individually.

Quantitative analysis was studied from the construction of calibration curves for the vitamin E, amino acids and drugs. The linear calibration curves for these compounds are shown in figure 8. The linear equations and concentration range with the detection limit using triethanolamine-glycerol-maleate and ODS-C18 columns, eluent 100% acetonitrile for vitamin E, eluent gradient (15-85) % phosphate buffer in acetonitrile, flow rate 1ml/min and detection wavelength 245nm for amino acids, and (0-20)% phosphate buffer in methanol, flow rate of 1 ml/min at wavelength 233 nm for drugs are listed in table 5,6 and 7.

Table 5: Linear equation, correlation coefficient and detection limits for the vitamin E using two types of columns

Triethanolamine-glycerol-maleate column				
Compound	**Linear Equation Y*=mx*+b**	**Conc. Range**	**R**	**Detection Limit (ppm)**
Vitamin E	Y=11229.1x-42253.4	0.1-10	0.9998	0.10
ODS-C18 column				
Compounds	**Linear Equation**	**Conc. range**	**R**	**Detection Limit (ppm)**
Vitamin E	Y=16256.6x-23493.4	0.1-10	0.9998	0.10

Table 6: Linear equation, correlation coefficient and detection limits for the amino acids using two types of columns

Triethanolamine-glycerol-maleate column				
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
Tyrosine	Y=57002.1x-45944.6	0.05-10	0.9997	0.05
Tryptophan	Y=59316.9x-46122.2	0.1-10	0.9998	0.10
ODS-C18 column				
Compounds	**Linear Equation**	**Conc. range**	**R**	**Detection Limit (ppm)**
Phenylalanine	Y=28025.6x-23193.4	0.1-10	0.9998	0.10
Tyrosine	Y=30158.8x-24924.1	0.1-10	0.9997	0.10
Tryptophan	Y=32031.9x-25249.9	0.05-10	0.9998	0.05

The slopes for the linear calibration curves using triethanolamine-glycerol-maleate column ranged from 55561.6 – 59316.9 depends upon the kind of amino acids. The correlation coefficients ranged from 0.9996 – 0.9998 with detection limit ranged from 0.05 to 0.10 ppm. While for ODS-C18, the slopes for the linear calibration curves of the analyzed drugs ranged from 28025.6 – 32031.9. The detection limit for the three drugs was 0.05 ppm.

Table 7: Linear equation, correlation coefficient and detection limits for the drugs using two types of columns

Triethanolamine-glycerol-maleate column				
Compounds	**Linear Equation Y*=mx*+b**	**Conc. Range**	**R**	**Detection Limit (ppm)**
Furosemide	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
ODS-C18 column				
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

The slopes for the linear calibration curves using triethanolamine-glycerol-maleate column ranged from 56221.6 – 59495.9 depends upon the kind of drugs. The correlation coefficients ranged from 0.9996 – 0.9998 with detection limit ranged from 0.05 to 0.10 ppm. While for ODS-C18, the slopes for the linear calibration curves of the analyzed drugs ranged from 23935.6 – 28636.9. The detection limit for the three amino acids was 0.05 ppm. Standard solutions were injected for at least three times under the same condition for both columns triethanolamine-glycerol-maleate and ODS-C18. The recovery ranged from 80% to 97% and relative errors ranged from 3.00% to 19.00%. The results are listed in table 8.

Table 8: Recovery and percentage relative error of amino acids eluted on two columns for the amino acids using gradient eluent (0-15) % phosphate buffer in acetonitrile

Triethanolamine-glycerol-maleate column				
Amino acids	**Conc. Injected (ppm)**	**Conc. Found(calculated*) (ppm)**	**Recovery %**	**Relative error%**
Phenylalanine	1.00	0.96	96.00	4.00
Tryptophan	1.00	0.97	97.00	3.00
Tyrosine	0.50	0.45	90.00	4.00
ODS-C18 column				
Amino acids	**Conc. Injected (ppm)**	**Conc. Found(calculated*) (ppm)**	**Recovery %**	**Relative error%**
Phenylalanine	2.00	1.87	93.50	6.50
Tryptophan	1.00	0.81	80.00	19.00
Tyrosine	2.00	1.79	89.50	10.50

And also the detection limit for the three drugs was 0.05 ppm. Standard solutions were injected for at least three times under the same condition for both columns triethanolamine-glycerol-maleate and ODS-C18. The recovery ranged from 95% to 98% and relative errors ranged from 2.00% to 5.00%. The results are listed in table 9.

Table 9: Recovery and percentage relative error of drugs eluted on two columns for the drugs using gradient eluent (0-20) % phosphate buffer in methanol

Triethanolamine-glycerol-maleate column				
Drugs	**Conc. Injected (ppm)**	**Conc. Found(calculated*) (ppm)**	**Recovery %**	**Relative error%**
Furosemide	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
ODS-C18 column				
Drugs	**Conc. Injected (ppm)**	**Conc. Found(calculated*) (ppm)**	**Recovery %**	**Relative error%**
Furosemide	5.00	4.91	98.25	1.80
Atenolol	5.00	4.86	97.21	2.80
Amiloride	5.00	4.77	96.15	4.60

Solutions of furosemide, atenolol and amiloride obtained from three different tablets were analyzed using triethanolamine-glycerol-maleate column, mobile phase was gradient (0-20) % phosphate buffer in methanol, flow rate 1 ml/min at wavelength 233nm. The %RSD are; 0.845%, 0.841% and 0.809% for furosemide, atenolol and amiloride, respectively are listed in table 10. Also the drugs were determined in serum under the same column. 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 after two hours.

Table 10: Analysis of amiloride (5mg), furosemide (20mg) and atenolol (50mg) in tablets using triethanolamine-glycerol-maleate column

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

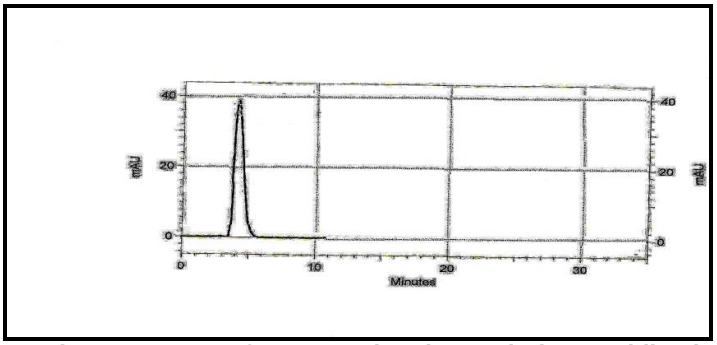

Fig. 1: Chromatogram of 5.0 ppm vitamin E solution, mobile phase 100% acetonitrile, flow rate 1ml/min and detection wavelength 229nm

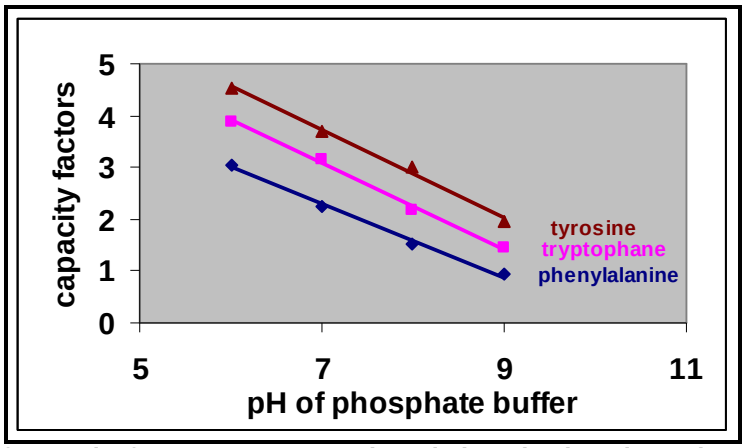

Fig. 2: Plot of Capacity factor versus pH, using triethanolamine-glycerol-maleate column (25×0.4 cm (id)), flow rate 1ml/min, detection wavelength 233nm, 10ppm of phenylalanine, tryptophane and tyrosine as a samples, respectively

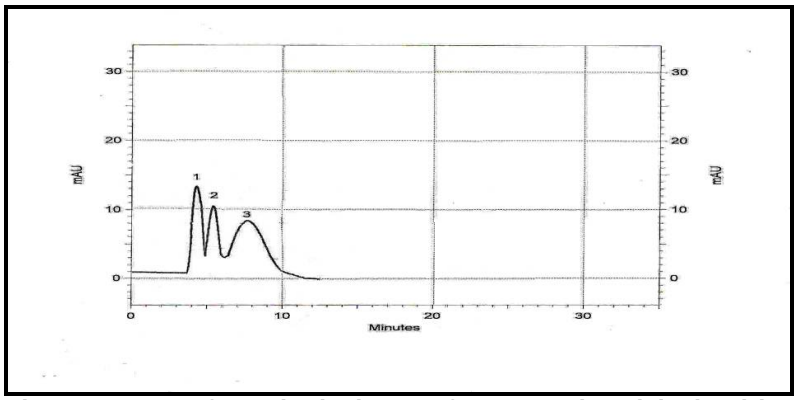

Fig. 3: Chromatogram of standard mixture of 0.2ppm phenylalanine (1), 0.1ppm of tryptophan (2)and 0.5 ppm of tyrosine (3), using triethanolamine-glycerol-maleate column (25×0.4 cm (id)). Eluent 15% phosphate buffer-85% acetonitrile, flow rate 1ml/min and detection wavelength 245nm

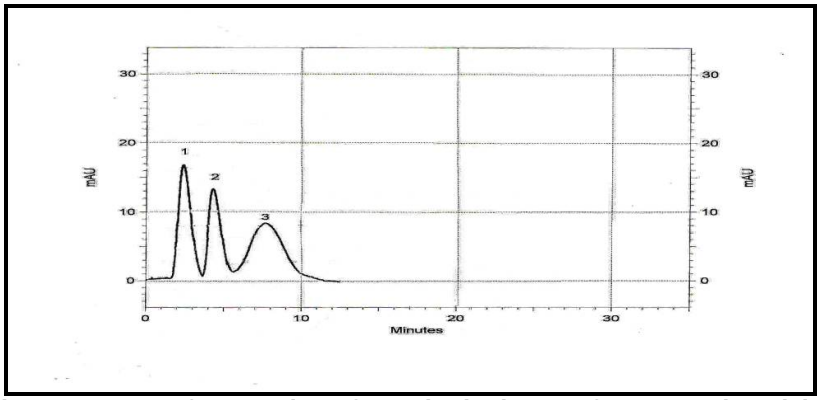

Fig. 4: Chromatogram of separation of standard mixture of 0.2ppm phenylalanine(1), 0.1ppm of tryptophane(2) and 0.5 ppm of tyrosine (3), using triethanolamine-glycerol-maleate column (25×0.4 cm (id)). Eluent gradient (0-15) % phosphate buffer in acetonitrile, flow rate 1ml/min and detection wavelength 245nm

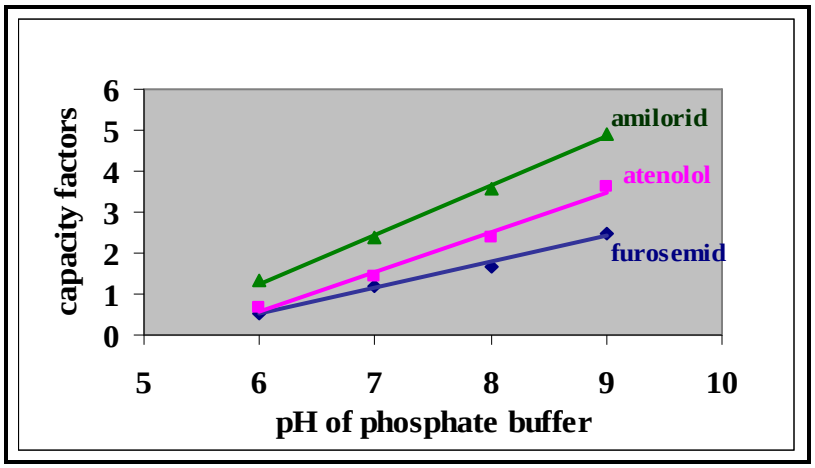

Fig. 5: Plot of Capacity factor, versus pH, using triethanolamine-glycerol-maleate column, flow rate 1ml/min, detection wavelength 233nm and 10ppm furosemide, atenolol and amiloride sample

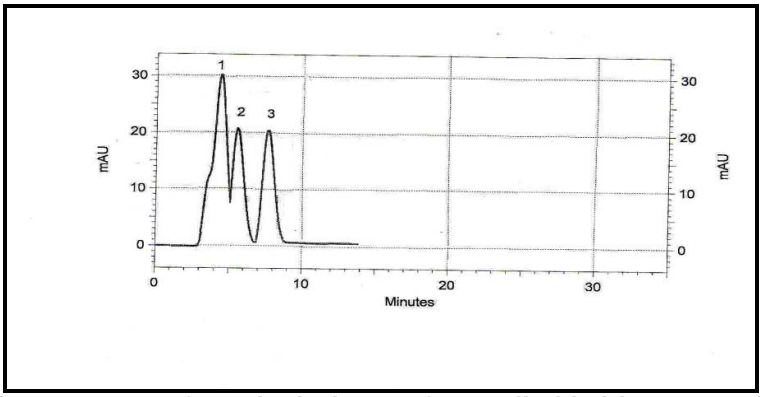

Fig. 6: Chromatogram of standard mixture of 0.2 amiloride (1), 0.1ppm of column. Furosemide (2) and 0.5 ppm of atenolol (3), using triethanolamine-glycerol-maleate. Eluent 20% phosphate buffer-80% MeOH, flow rate 1ml/min, and detection wavelength 233nm

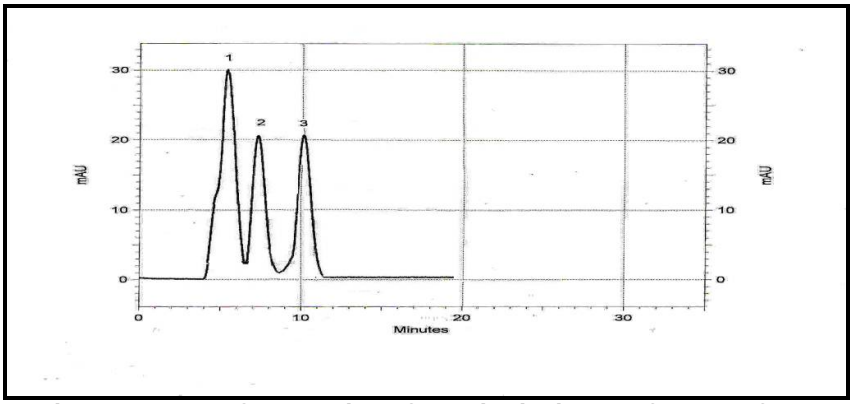

Fig. 7: Chromatogram of Separation of standard mixture of 0.2ppm furosemide (1), 0.1ppm of atenolol (2) and 0.5 ppm of amiloride (3), using triethanolamine-glycerol-maleate column. Eluent gradient (0-20) % phosphate buffer in MeOH, flow rate 1ml/min at wavelength 233nm

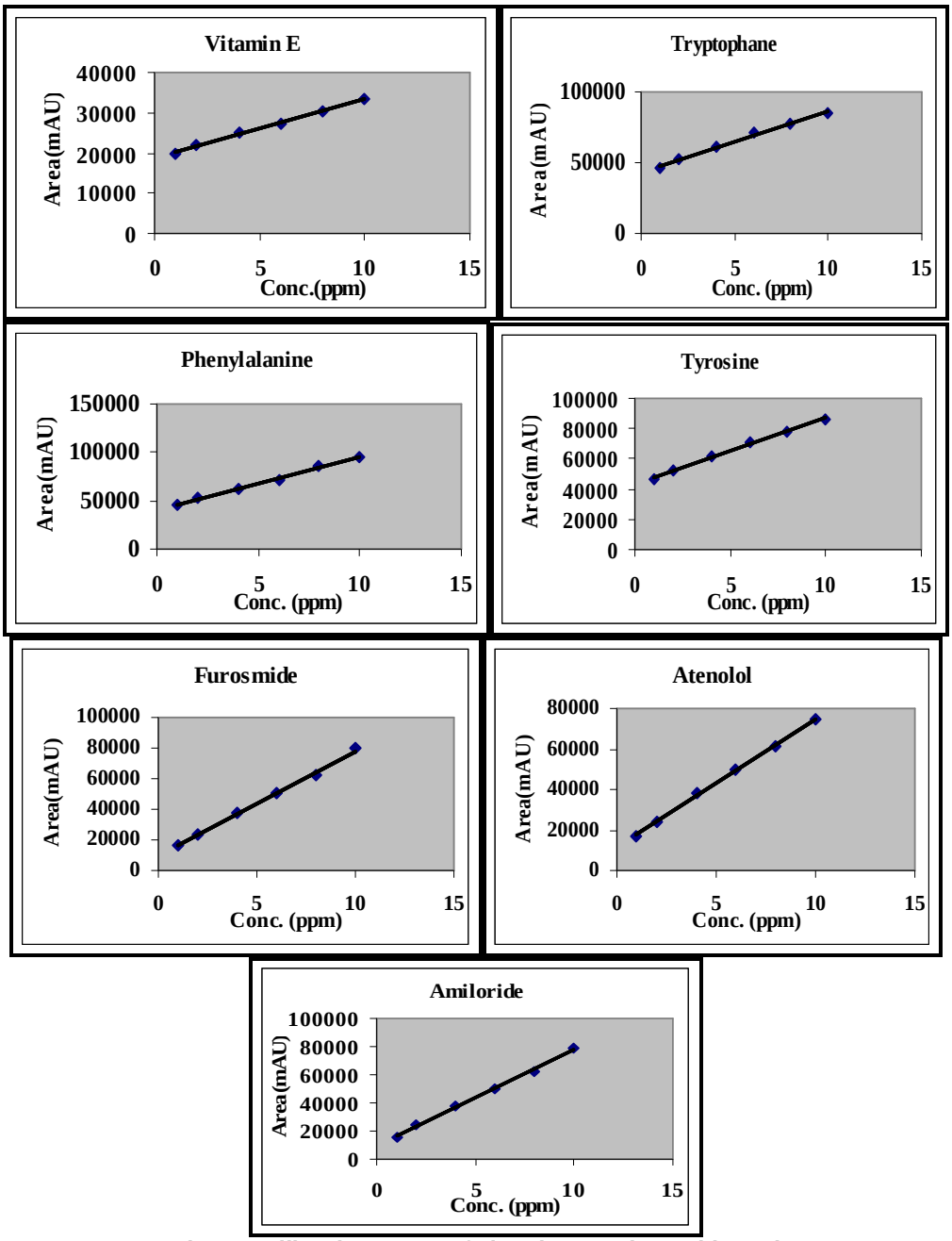

Fig. 8: Calibration curve of vitamin E, amino acids and drugs using triethanolamine-glycerol-maleate column (25×0.4 cm (id))

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