Comparative Study Of Isocratic And Gradient Elution Chromatography In Stability Indicating Assay Of An Antihypertensive Drug Combination.

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ABSTRACT: Inspite that chromatographers are cautioned to avoid gradient elution when isocratic elution will do. In this work, the analytical properties of gradient and isocratic elution applied to separation of a complex sample of (fosinopril, hydrochlorothiazide and their degradation products) which can be done under isocratic condition are compared. Procedures were developed for determining fosinopril and hydrochlorothiazide in presence of each other and their degradation products by HPLC in the gradient elution mode using methanol- 20 mM KH_2PO_4 (PH 2.4) containing 0.1% triethyl amine. In the isocratic mode, the same mobile phase composition was applied in a constant ratio of 60: 40 (Buffer: methanol). Separation was achieved on a cyanopropyl column (4 x 250 mm, 5 μm) known for its high selectivity for polar and hydrophilic compounds and the least retentive of hydrophobic compounds which do not normally elute on standard C18 or C8 columns. The present work shows that gradient elution gave a shorter overall analysis time with similar resolution of the critical pair without sacrificing repeatability in parameters, so many of the reasons given to avoid gradient elution deserve serious reconsideration especially for those samples that can be separated isocratically.

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I. INTRODUCTION

Fosinopril (FOS) and hydrochlorothiazide (HCTZ) are administered as a drug combination used in treatment of hypertension marketed under the name Monozide®. Stability studies confirm that both FOS and HCTZ are liable to hydrolysis yielding fosinoprilat (FOS-at)[1-3] and 6-chloro-2,4-disulfamoyl aniline (DSA)[4-5] respectively. FOS was successfully determined in presence of its degradate in synthetic mixture and biological fluids by chromatographic[1-3,6-13] and CE[14] methods. On the other hand, spectrophotometric[15,16] and HPLC[17,18] methods were applied for determination of HCTZ in presence of its degradate or in its pharmaceutical drug combinations.

Several methods have been also presented for the analysis of pharmaceutical preparation containing mixtures of FOS and HCTZ but the analysis of a quaternary mixture of the two active ingredients simultaneously in presence of their process degradates is considered a challenge.

The complexity of this mixture arises from the great difference in hydrophobicity and physiochemical properties of the analyzed compounds, FOS (being of highest lipophilicity and excessive retention time) and HCTZ (least retentive) in addition to their degradates whose presence lead to the problem of critical pairs of almost similar chromatographic behavior which makes the separation process even more complicated.

Two approaches have been presented in this work for the analysis of this mixture, the first one is based on isocratic elution utilizing the cyanochemistry of cyanocolumns which provides a different selectivity from both phenyl and standard aliphatic (C_4, C_8, C_{18}) reversed phases, having a dual nature with both polar and hydrophobic properties. They therefore can be used as the least polar alternative of NP in normal phase applications and the least hydrophobic alternative to RP-8 in reversed

phase applications. Separations using reversed and normal phase can be carried out using this material, where combination of weak hydrophobic interactions and polar interactions enable successful separation of complex samples of compounds of different physicochemical properties[19].

In the second approach, another modulation is introduced by the use of gradient elution, which involves gradual change in the mobile phase composition by increasing the concentration of the organic modifier during the course of separation. This maintains separation of the critical pair and in the sometime decreases the retention time of the late eluting peaks by reducing the retarding strength of the aqueous component.

Combining the benefits of cyanopropyl column with gradient elution provides a complete separation of the present mixture in a much shorter runtime.

II.EXPERIMENTAL

II.1. Instrumentation

An HPLC unit equipped with an autosampler, thermostated column department, diode array detector IG1315A), quaternary pump (G13154A) and a vacuum degasser from Agilent technologies, USA. The chromatographic column from Merck KGaA, Dermstadt, Germany was lichrospher® 100 CN (4x250 mm i.d., 5µm particle size). Data acquisition was performed on Agilent LC chemstation software. All determinations were performed at 45^{o}C.

II.2. Samples and reagents

II.2.1. Samples

II.2.1.1. Authentic samples

FOS and HCTZ certified to contain 100.50% and 99.50% respectively by the manufacturer method were kindly supplied by Bristol Myers Squib. 4-amino-6-chlorobenzene-1,3 disulphonamide (DSA) certified to contain 99.25% by the manufacturer method as supplied by Sigma-Aldrich chemie, GmbH, Germany.

II.2.1.2. Market samples

A commercial pharmaceutical preparation (Monozide® tablets manufactured by (Bristol Myers Squib, Egypt, Batch no. L 93731) labeled to contain 20 mg/10 mg FOS/ HCTZ was obtained from the local market.

II.2.2. Reagent

- Methanol used was HPLC grade (Sigma – Aldrich Co., USA)
- Potassium dihydrogen phosphate (BDH, Poole, UK), triethylamine (BDH) and orthophosphoric acid used were analytical grade.
- Distilled water was used throughout the whole work.

II.2.3. Standard solutions

II.2.3.1. Standard stock solutions

II.2.3.1.1. Standard stock solutions of FOS, HCTZ and DSA

Portions equivalent to 500.00 mg of FOS and 100.00 mg of each of HCTZ and DSA were accurately weighed and transferred into separate 100 ml volumetric flasks and dissolved in the minimum amount of methanol, sonicated for 10 minutes and the volume was made up to the mark with methanol to give stock solutions of concentrations 5.0 mg.ml^{-1} and 1 mg.ml^{-1} respectively.

II.2.3.1.2. Standard stock solution of FOS -at

It was prepared by mixing a portion equivalent to 500.0 mg of FOS with 1.0 M NaOH, heated under reflux for 1 hour at 100^{o}C the solution was then cooled and neutralized with a calculated volume of 1.0 M HCl. The obtained precipitate was filtered, washed, transferred quantitatively into 100 ml volumetric flask and the volume was made up to the mark with distilled water to give a standard stock solution of concentration 5.0 mg.ml^{-1}.

II.2.3.2. Standard working solutions of HCTZ and DSA

A portion equivalent to 10.0 mg of HCTZ and DSA was accurately transferred from their standard stock solution into two 100 ml volumetric flasks. The volume was made up to the mark with methanol to give standard working solutions of concentration 100 µg.ml^{-1}.

III. Procedure

III.1. Chromatographic conditions

** For isocratic elution:*

Isocratic elution technique was utilized with the column maintained at 45°C. The mobile phase used was a mixture of 20 mM potassium dihydrogen phosphate containing 0.1% triethylamine adjusted at PH (2.4) using orhtophosphoric acid and methanol in a ratio of (60:40). The mobile phase was filtered thorough a 0.45 μm membrane filtration system (Millipore Corp., Milford, MA, USA) to remove any particulate matter then degassed by sonication for 20 minutes. The flow rate was 1.5 ml/min. samples of 20 μl, were injected onto the column and scanned at 210 nm and 220 nm and the mixtures were scanned over the range of 200-400 nm. All the chromatographic determinations were performed 3 times at 45°C.

** For gradient elution:*

Simultaneous separation and quantification of FOS, FOS-at, HCTZ and DSA was performed by the use of gradient elution with the column maintained at 45°C. The gradient was prepared from 40:60 (v/v) methanol-20 mM aqueous potassium dihydrogen phosphate containing 0.1% triethylamine adjusted at PH 2.4 (component A) and 90:10 (v/v) methanol-20 mM aqueous potassium dihydrogen phosphate containing 0.1% triethylamine adjusted at PH 2.4 (component B). The gradient elution program is presented in the following table. All the changes in gradient were linear and the re-equilibration time was 15 minutes. The mobile phase flow rate was 1.5 ml/minute. Diode array detection was performed for optimum selection of wavelengths used in quantification of the selected compounds.

Time	Component A (%)	Component B (%)
0	100	0
4	100	0
10	0	100

III.2. Method validation

III.2.1. Linearity

Aliquots equivalent to (2.0- 30.0 μg) of HCTZ and (40.0- 1000.0μg) and (6.0-600.0 μg) of FOS for isocratic and gradient elution respectively were accurately transferred from their stock and working solutions respectively into 10 ml volumetric and diluted to the volume with mobile phase. Each of those dilutions were then chromatographed by injecting an aliquot of 20 μl of each into the chromatographic system five times and processed according to the method described in this work. The mean peak areas of five determinations of each concentration were plotted against the same concentration and the regression equations were then computed.

III.2.2. Laboratory prepared mixture

A laboratory prepared mixture containing 500 $\mu g.ml^{-1}$ of FOS and FOS-at and 10 $\mu g.ml^{-1}$ of HCTZ and DSA was prepared and chromatographed by adopting the procedures mentioned under linearity. The concentrations were determined by referring to the regression equations. The percentage recoveries and the standard deviations were then calculated.

III.2.3. Accuracy

The previously mentioned procedures under linearity were applied for different concentrations of FOS, HCTZ, DSA and FOS-at. The concentrations of the studied compounds were calculated from their corresponding regression equations. The mean percentage recoveries and standard deviations were then calculated.

III.2.4. Precision

III.2.4.1. Intraday precision (Repeatability)

The previously mentioned procedures under linearity were used for the analysis of freshly prepared solutions of concentrations, (2.0, 18.0, 30.0 $\mu g.ml^{-1}$) of HCTZ, (40, 400, 1000 $\mu g.ml^{-1}$) and (6.0, 100.0, 600.0 $\mu g.ml^{-1}$) of FOS by isocratic and gradient elution respectively for the determination of intraday precision (n=5) and the relative standard deviations (R.S.D %) were calculated.

III.2.4.2. Interday precision (Intermediate precision)

The previously mentioned procedures under linearity were used for the analysis of freshly prepared solutions of concentrations, (2.0, 18.0, 30.0 $\mu g.ml^{-1}$) of HCTZ, (40, 400, 1000 $\mu g.ml^{-1}$) and (6.0, 100.0, 600.0 $\mu g.ml^{-1}$) of FOS by isocratic and gradient elution respectively for the determination of interday precision (n=5) and the relative standard deviations (R.S.D %) were calculated.

III.2.5. Application to pharmaceutical preparations

Twenty tablets of Monozide tablets were accurately weighed, and finely ground in a mortar. A portion of the powder equivalent to 100 mg of each of

FOS and HCTZ was separately extracted with the minimum amount of methanol, sonicated for 30 minutes, centrifuged for 10 minutes. The precipitate was then filtered through a micropore filter, washed into two volumetric flasks and the volume was made up to the mark with the same solvent.

An appropriate dilution was made to prepare working solutions of the studied drugs, the aliquot was then chromatographed by adopting the procedures mentioned under linearity. The concentrations of FOS and HCTZ were then calculated from their corresponding regression equations and the mean percentage recoveries were then calculated.

III.2.6. Validation by standard addition technique

This study was performed by adding known amounts of the studied compounds from their working standard solutions to a known concentration of the commercial preparations. The resulting mixtures were chromatographed by adopting the procedures mentioned under linearity.

IV. RESULT AND DISCUSSION:

When working with mixture of analytes in different compound classes, with different structures, physicochemical properties and polarities, selectivity can be a factor of many variables as type of stationary phase used, percentage of organic modifier, temperature of analysis, PH of mobile phase and flow rate.

Developments in column technology have been mainly responsible for the advances in these directions[19,20]. A chemical modification that was made to the surface functional groups by introduction of cyanobonded phases led to development of cyanocolumns which are much less hydrophobic, less sterically restricted and have lower hydrogen bond acidity and considered the least retentive of all reversed phases as discussed earlier[20-24].

Fosinopril is a phosphonate containing ACE inhibitor which is the most hydrophobic (lipophilic) and retentive among all ACE inhibitors due to the hydrophobic side chain added to fosinoprilat to modulate its ionization characteristics and promote its poor oral bioavialbility[25-29] therefore a major difficulty was faced during analysis of a mixture of not only FOS, FOS-at but also HCTZ and its degradate (DSA) due to the diverse nature in the polarity of the investigated compounds.

Method development started with the use of different columns including C8, C18 and CN columns with different lengths (15 cm and 25 cm) and CN column (25 cm) was the only one capable of eluting FOS under the chromatographic conditions which resolve the rest of the mixture isocratically but the problem faced was the very long retention time of FOS which makes the runtime very long and it being eluted as a broad peak with extensive tailing. This means that the selectivity of the stationary phase was not enough for solving this analytical problem, there arises the need for manipulating another parameters which is the column temperature.

Operation at elevated temperatures decreases the mobile phase viscosity, the column back pressure allowing the use of not only higher flow rates but also longer columns thus offers better separation for complex mixtures[19,20].

In the present work, a long cyanocolumn, 25 cm long was under being operated at 45^{o}C, where significant reduction in the column back pressure allowed the use of a flow rate of 1.5 ml/min which was considered a limitation when being used the same column at room temperature and in the same time led to faster elution of FOS as a sharp peak instead of the broad peak obtained by operating at room temperature.

Several mobile phase compositions of methanol-water were investigated but no peaks were obtained, water was then replaced by 20 mM potassium dihydrogen phosphate with subsequent adjustment of PH at different values. It was found that very minor changes in PH can have a major effect on the peak shape and the chromatographic behavior of FOS and FOS-at at where at PH 3.0, distorted or irregular shaped peaks were obtained while at PH 2.4, sharp and symmetrical peaks were obtained instead.

The composition ratios of buffer and methanol were varied to study the effect of the % of organic modifier. It was found that minor changes lead to either:

1. Complete retention of FOS, where there exists a critical concentration of methanol below which FOS is retained on the column.

2. Overlapping of the critical pair (HCTZ and DSA).

Therefore fine tuning of the mobile phases composition is considered a must in order to establish good resolution of the critical pair and elution of FOS from the column with a reasonable retention time. Triethylamine (0.1%) was added to minimize peak tailing and enhance peak symmetry. Thus the mobile phase used was a mixture of 20 mM potassium dihydrogen phosphate containing 0.1% TEA and methanol in a ratio of 40: 60 (v/v) and a representative chromatogram of the mixture is presented in figure (2).

In gradient elution:

A further refinement made to HPLC by changing the mobile phase composition during the coarse of separation termed a solvent gradient elution was generally utilized for separation of complex mixtures with a wide retention range with no loss of resolution and applied specially in the case presented here to decrease the overall runtime and in the same time the cost of analysis.

Successful separation of the investigated compounds (FOS, FOS-at, HCTZ and DSA) was carried out but with an important limitation which is the prolonged analysis time where each run lasts about 30 minutes, therefore a modification was introduced to the method which involves gradual increase in the mobile phase strength as the separation proceeds in such away that the elution of the late elute compounds (FOS and FOS-at) was continuously reduced.

The model of gradient solvent profile applied was linear, gradient shown in figure (1) where the rate of the change in methanol is linear overtime because it permits greater separation selectivity between the earlier peaks (HCTZ and DSA) as well as later peaks (FOS and FOS-at).

It starts with an isocratic mode of 60% buffer: 40% methanol for the first 4 minutes of the run to ensure complete separation of the critical pains (HCTZ and DSA) followed by linear gradient over the next 6 minutes where methanol reaches 90% by the end of the run and this produces early elution of FOS-at and FOS with no loss of their resolution.

The column was allowed to re-equilibrate for 10 minutes with less than two column volumes of the initial eluent before the next run. The detector was operated on the DAD mode, allowing scanning of the absorption of the investigated compounds for confirmation of their identity and for selection of optimum wavelength for measurement.

The main problem faced during the analysis was that the wavelength chosen for quantitative determination of HCTZ and FOS was 210 nm which is close to the cut-off λ of the solvent used (methanol) which cause slight drift in the baseline in the positive direction, therefore the linear gradient model was chosen because it involved small change in the eluent strength relative to time which helped minimize the baseline disturbance. The same flow rate (1.5 ml/min) was applied throughout the whole run for the same season. Representative chromatograms of the laboratory prepared mixture of the investigated compounds by the gradient mode are shown in figure (3).

It was found that gradient elution gave a shorter overall analysis time with similar resolution of the critical pairs compared to the isocratic elution without sacrificing repeatability in retention time, peak area, peak height or linearity of the calibration curve and this was confirmed by the values of the correlation coefficients of calibration curves constructed for Fos and HCTZ which are close to 1. The regression equations were

P.A = 18.961 C + 574.070 r =0.9999 for FOS (by isocratic elution)
P.A = 24.028 C + 11.039 r =1
for FOS (by gradient elution)
P.A = 71.534 C + 13.348 r =1
for HCTZ.

Linearity was obtained over the range of (2.0-30.0 $\mu g.ml^{-1}$) for HCTZ and (40.0 -1000.0 $\mu g.ml^{-1}$) and (6.0-600.0 $\mu g.ml^{-1}$) for FOS by isocratic and gradient elution respectively.

The results obtained from the determination of accuracy are listed in table (1). The good recoveries and low standard deviation values indicate that the method is accurate. The mean percentage recoveries ±SD were found to be 100.14±0.530 for HCTZ and 99.96±1.227 and 100.51±0.962 for FOS using isocratic and gradient elution respectively.

The methods were found selective and valid for the determination of the two intact drugs simultaneously in presence of their degradates with mean percentage recoveries 100.14±0.894 for HCTZ, 99.59±1.917 and 100.05±1.012 for FOS by isocratic and gradient elution respectively.

Satisfactory results were obtained for the recovery of the analyzed drugs in its pharmaceutical preparation (Monozide®) and were in agreement with the labeled claims. Results indicate there was no interference from the excipients used in the formulation of tablets.

The results of the assay validation parameters are discussed in table (3). The data revealed that the proposed method was found suitable for the determination of the drugs in pure powder form, pharmaceutical preparation and was proved to be precise, accurate and suitable for quality control laboratories where economy and time are essential provided that the instrument with the right specification is available. The results of the proposed method presented in table (2) were compared with the reported method, student's t- and F tests at 95% confidence level were applied and the results showed that the calculated t- and F-values didn't exceed the theoretical ones indicating no significant difference between the results obtained by the proposed method and these obtained by the reported method.

Table (1): Accuracy of the proposed HPLC method for the analysis of pure samples of fosinopril and hydrochlorothiazide.

Fos						HCTZ		
Isocratic			Gradient					
Taken ($g.ml^{-1}$)	Found ($g.ml^{-1}$)	Recovery* (%)	Taken ($g.ml^{-1}$)	Found ($g.ml^{-1}$)	Recovery (%)	Taken ($g.ml^{-1}$)	Found ($g.ml^{-1}$)	Recovery* (%)
50	49.72	99.44	230	30.45	101.5	4	3.97	99.25
100	99.56	99.56	50	50.32	100.64	8	8.06	100.75
300	297.2	99.07	150	148.2	98.8	12	12.01	100.08
500	501.67	100.33	250	253.99	101.6	16	15.95	99.69
700	717.58	102.51	350	349.62	99.89	20	20.08	100.4
900	889.89	98.88	450	453.32	100.72	24	24.15	100.63
100	999.58	99.96	550	552.24	100.41	28	28.06	100.21
Mean±SD		99.6±1.227			100.51±0.962			100.14±0.530

* Average of five determinations.

Table (2): Quantitative determination of fosinopril and hydrochlorothiazide in pharmaceutical preparation and application of standard addition technique by the proposed HPLC methods

Pharmaceutical formulation			Taken ($g.ml^{-1}$)	Found* ($g.ml^{-1}$)	Standard addition technique		
					Pure added ($g.ml^{-1}$)	Pure found ($g.ml^{-1}$)	Recovery (%)*
Monozide® plus tablets claimed to contain 20 mg/12.5 mg (Fos/HCTZ) tablets (Batch no. L 93731)	Fosinopril	Isocratic	40 $ug.ml^{-1}$	99.34±1.264	200	204	102
					400	395.38	98.85
					600	605.58	100.43
					800	793.42	99.18
							100.12±1.429
		Gradient		99.28±1.212	200	199.41	99.71
					300	299.1	99.7
					400	401.72	100.43
					500	497	99.4

	Hydrochlorothiazide	25 ug.ml^{-1}	100.56±0.602	2 3 4 5	2 2.99 4.02 5.03	100 99.67 100.5 100.6 100.19±0.436

* Average of five determinations.

Table (3): Results of assay validation parameters obtained by applying the proposed HPLC method for determination of FOS and HCTZ.

	Fosinopril		**Hydrochlorothiazide**
Parameters	**Isocratic**	**Gradient**	
Linearity	40-1000µg.ml^{-1}	6-600 µg.ml^{-1}	2-30 µg.ml^{-1}
Correlation coefficient (r)	0.999	0.9999	
Slope	18.96123495	24.02847116	13.34805027
Intercept	574.0739297	-11.03926857	71.53375862
Standard error of the slope	0.066929187	0.043138751	0.206237883
Confidence limit of the slope	18.78918799-19.1332819	23.92899303-24.1279493	71.0291127-72.03840453
Confidence limit of the intercept	37.62008927	13.03501438	3.802838654
Confidence limit of the intercept	477.3684116-670.7794478	-41.09806562-19.01952848	4.042839314-22.65326122
Standard error of estimation	59.97862445	28.45772849	5.346296957
Accuracy (mean±SD)	99.96±1.227	100.51±0.962	100.14±0.530
Selectivity			
Precision (RSD%)			
Repeatability*	0.596	1.018	1.380
Intermediate precision*	0.739	1.260	1.734

Table (4): Statistical comparison between the results obtained by the proposed HPLC method and the reference methods for determination of fosinopril and hydrochlorothiazide.

Items	HPLC method			Reported method*	
	Fosinopril		Hydrochloro-thiazide	Fosinopril	Hydrochloro-thiazide
	Isocratic	Gradient			
Mean	99.34	99.28	100.56	100.6	100.75
SD	1.264	1.212	0.602	0.528	0.404
RSD%	1.272	1.221		0.525	0.401
N	5	5	5	5	5
Variance	1.598	1.468	0.363	0.279	0.163
Student's t-test (2.306)*	2.05	2.23	0.586		
F-value (6.388)*	5.727	5.256	2.227		

* The values in parenthesis are the tabulated values.

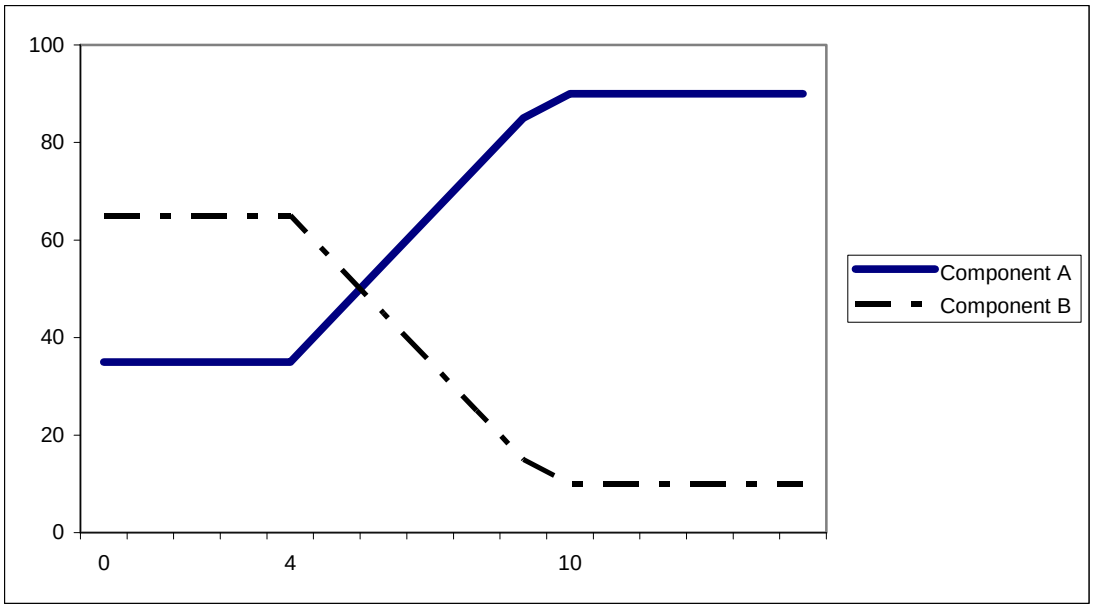

Figure (1): Linear gradient model applied in HPLC of mixture.

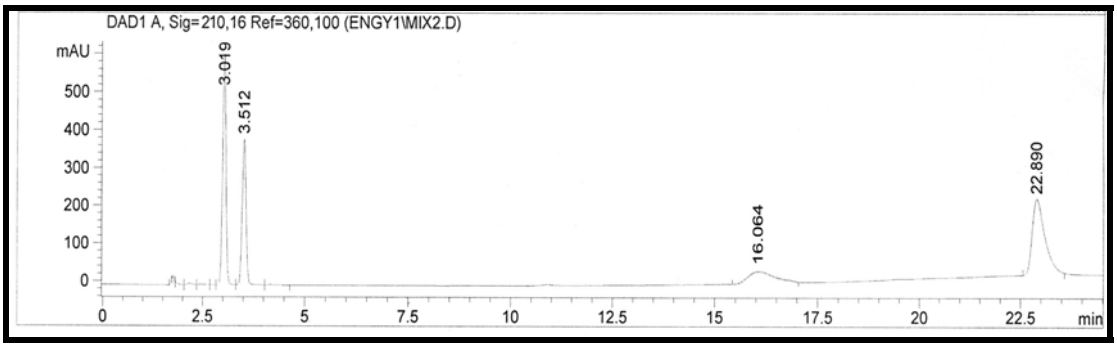

Figure (2): Scanning profile of HPLC chromatogram of fosinopril (500µg.ml^{-1}), hydrochlorothiazide (10 µg.ml^{-1}) and their process degradates fosinoprilat (500µg.ml^{-1}) and 4-amino-6-chlorobenzene-1,3 disulphonamide (10 µg.ml^{-1}).

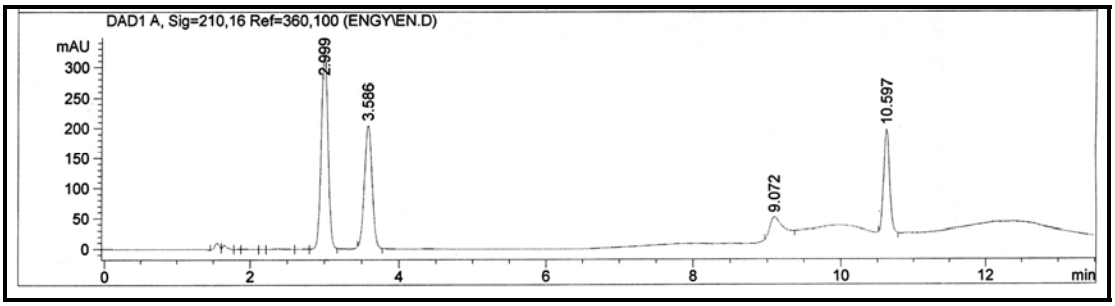

Figure (3): Scanning profile of HPLC-GE chromatogram of fosinopril (400μg.ml^{-1}), hydrochlorothiazide (10μg.ml^{-1}) and their process degradates FOS-at ((400μg.ml^{-1}) and DSA (10 μg.ml^{-1})

V. CONCLUSION

Delivering competent analytical judgment on samples in a timely manner is becoming more difficult as the sample load in quality control laboratories continues to increase. These economic pressures prompted the development of analytical technologies which can deliver high qualitative and quantitative information in a high throughout environment. Cyanopropyl columns combined with elevated temperature was presented as an approach for this purpose as well as gradient elution. Gradient elution technique was found to present tremendous chemical selectivity as well as precise retention time data under well controlled conditions. It was successful in separation of a complex mixture of fosinopril, hydrochlorothiazide and their degradates in less than 1/2 the time consumed in isocratic elution with no loss of accuracy or precision of data.

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