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Liquid chromatographic analysis of phenobarbitone, ethosuximide, phenytoin and carbamazepine on a polystyrene-divinyl benzene column

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A liquid chromatographic method for the simultaneous assay of four anticonvulsant drugs, phenobarbitone, ethosuximide, phenytoin and carbamazepine on a polystyrene-divinyl benzene column is described. The method was developed by the systematic study of different types of co-polymer materials, type and concentration of organic modifiers, buffer pH and concentration and column temperature. A PLRP-S 100 Å 8 µm column maintained at 60 °C and a mobile phase consisting of acetonitrile-*tert*-butanol-phosphate buffer (pH 7.6, 0.2 M)-water (25:5:10:60, v/v) were used. The flow rate was 1 ml/min with ultraviolet detection at 220 nm. The method has been validated and used for the analysis of raw materials, finished products and dissolution studies of the drugs.

Key words: Liquid chromatography, co-polymer column, phenobarbitone, ethosuximide, phenytoin, carbamazepine.

INTRODUCTION

Epilepsy is a common cause of morbidity and social stigmatization in Kenya [1-2]. Phenobarbitone (PB), ethosuximide (ESM), phenytoin (PHT) and carbamazepine (CBZ) (Figure1) are the most commonly used drugs for epilepsy management in Kenya. Multiple seizure types and/or refractory disease may require various combinations of these drugs. The use of newer drugs on the market, useful in difficult seizure control, like lamotrigine, oxcarbazine, vigabatrin and gabapentine is as in other resource poor countries, limited due to their cost.

Liquid chromatography (LC) is recognized as the most rapid, specific, precise, sensitive and cost effective method of analysis in multiple drugs. There is no compendial method for the simultaneous analysis of the four drugs [3-4]. However, several silica based reversed-phase (RP) LC methods have been reported.

Kabra *et al.* used µ-Bondapak C-18 column with acetonitrile-potassium phosphate buffer (pH 4.4, 0.1 M), (19:18, v/v) mobile phase to separate the four drugs and primidone in serum with UV detection at 195 nm [5]. Szabo and Browne obtained resolution on a similar column with acetonitrile-methanol-phosphate buffer (pH 5.5) (17:28:55, v/v) as mobile phase. Detection was at 195 nm with a 20 min analysis time [6]. Similarly, Torra *et al.*, obtained separation of lamotrigine and other co-administered anticonvulsants on a 7 µm Chrom Systems C-18 column using acetonitrile-phosphate buffer (pH 3.8) (45:55, v/v) with UV detection at 210 nm [7].

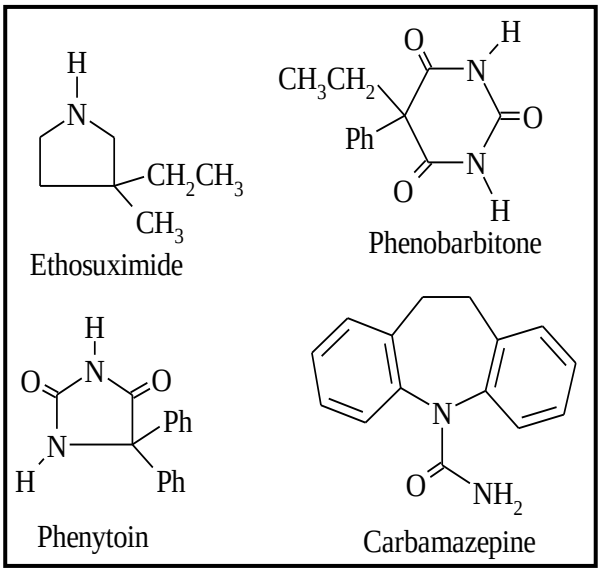

Figure 1. Chemical structures of ethosuximide, phenobarbitone, phenytoin and carbamazepine

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Most of these methods give good results, but have the major disadvantage of column instability in extreme pH and temperature as well as detection instability at the low wavelengths. Although such reversed phase stationary phases still dominate the LC field, polymeric columns are increasing in popularity as their retention characteristics and applications become better understood. One such material, polystyrene-divinylbenzene (PSDVB), is stable in a wide pH range (pH 1- 13) [8].

This paper reports the development of a rapid, specific, selective and cost effective LC method on a polymer column for the analysis of four commonly used anticonvulsant drugs.

EXPERIMENTAL

Reagents and reference materials

HPLC grade acetonitrile and methanol were obtained from Fischer Scientific, UK Ltd., (Leicestershire, UK) while tertiary butanol, analytical grade K_2HPO_4 and KH_2PO_4 were sourced from Acros Organics /Chemica (New Jersey, USA). Water was distilled in glass apparatus. Working standards, used as reference material, were carbamazepine from Norvatis (Basle, Switzerland), phenytoin and phenobarbitone, obtained courtesy of Laboratory and Allied Ltd. (Nairobi, Kenya), and ethosuximide (extracted from Zarontin capsules (Parke Davis, Hampshire, UK). All other reagents were of analytical grade.

Chromatographic system

The LC system consisted of a Merck Hitachi model L 6000-a pump (Hitachi Ltd., Tokyo, Japan) set to deliver 1 ml/min, a sample injection valve, model CV-6-UHPa-N60, from Valco company (Texas, USA) equipped with a 25 µl loop, a Greenford III spectromonitor, model 40014, variable UV detector set at 220 nm and a Hewlett Packard model 3394A integrating recorder (Avondale, PA, USA). The stationary phases studied, PLRP-S 8 µm 100 Å, 300 Å and 1000 Å (Polymer Laboratories, Church Stretton, Shropshire, UK), PRP-1 10µm (Hamilton, Reno, N.Y., USA) and Rogel 8 µm (RSL-BioRad, Eke, Belgium) were laboratory packed each in a 250 x 4.6 mm I.D. column and the temperature was maintained at 60 ^{0}C by means of a water bath.

Sample preparation

The working standards were dissolved in the mobile phase to give stock solutions of 62.5, 500, 125 and 62.5 µg/ml of PB, ESM, PHT and CBZ, respectively. These solutions were serially diluted (n=7) and injected in replicate for linearity range determinations. Serial dilutions of PB 1.25, ESM 10, PHT 1.25 and CBZ 0.625 µg/ml solutions were injected in replicate for the limit of detection (LOD) and limit of quantitation (LOQ) determinations at the signal to noise ratio of 3 and 10, respectively.

RESULTS AND DISCUSSION

Method development

An overlay of phenobarbitone, ethosuximide, phenytoin and carbamazepine individual UV absorption scans, gave the 220 nm detection wavelength, being the lowest practical to give the best sensitivity and selectivity. In the preliminary studies, five co-polymer stationary phase materials' performance was compared using an acetonitrile-phosphate buffer-water (25:10:65, v/v) mobile phase at 60 ^{o}C. The chromatographic parameters were calculated as per the European Pharmacopoeia guidelines [9]. The PLRP-S materials gave better chromatographic performance with its selectivity dependent on the particle pore size. The PLRP-S 100 Å gave the highest column efficiency, best selectivity and symmetry (table 1). This column was then used in further method optimization.

The column performance was investigated in terms of the influence of the organic modifier type and content in the mobile phase, mobile phase pH, buffer concentration and column temperature.

Influence of organic modifier: Acetonitrile (ACN) was chosen as the main organic modifier because of its strong eluting power, better UV transparency and low backpressure. Satisfactory retention times and peak symmetry were obtained in comparison to methanol. The effect of acetonitrile concentrations on capacity factors is shown in figure 2.

Table 1. Chromatographic parameters in the separation of phenobarbitone, ethosuximide, phenytoin and carbamazepine on different polymer columns

Column	Drug	k'	A_s	N (CBZ peak)	R_s (PB/ESM)
PLRP-S 1000 Å 8 µm	PB	0.2	a		
	ESM	0.3	a	70	b
	PHT	1.0	1.9		
	CBZ	2.3	2.3		
PLRP-S 300 Å 8 µm	PB	0.5	0.7		
	ESM	0.6	0.8	2050	b
	PHT	2.4	1.8		
	CBZ	3.1	2.1		
PLRP-S 100 Å 8 µm	PB	0.5	0.8		
	ESM	0.9	0.8	2180	3.4
	PHT	3.5	1.6		
	CBZ	5.2	1.9		
PRP-1 8 µm	PB	0.8	a		
	ESM	0.9	a	730	b
	PHT	4.2	1.1		
	CBZ	5.2	0.9		
RoGel RP	PB	1.4	a		
	ESM	1.6	a	120	b
	PHT	7.4	a		
	CBZ	9.8	a		

Column temperature: 60 ^{o}C. Mobile phase: Acetonitrile- phosphate buffer (pH 7.5, 0.2 M)-water (25: 10: 65, v/v). Flow rate: 1 ml/min. Detection: UV at 220 nm. a: no separation at 0.05 of the peak height. b: no separation at 0.5 of the peak height

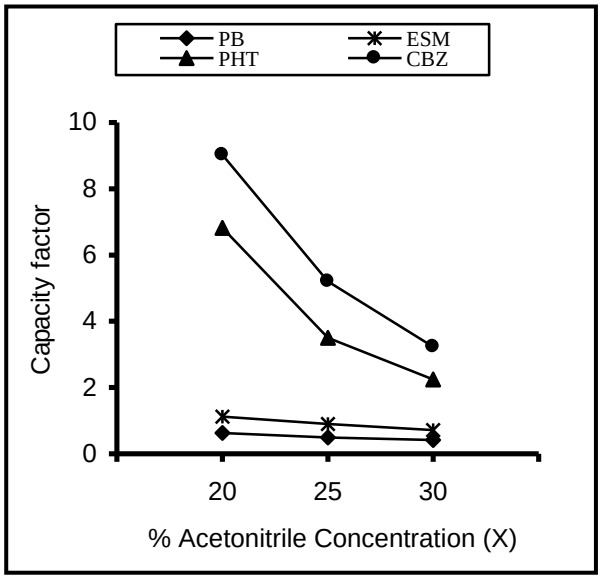

Figure 2. Effect of acetonitrile concentration in mobile phase on capacity factors of phenobarbitone (PB), ethosuximide(ESM), phenytoin(PHT) and carbamazepine(CBZ). Column: PLRP-S 8 µm 100 Å. Column temperature: 60 ^{0}C. Mobile phase: Acetonitrile-phosphate buffer (pH 7.5, 0.2 M)-water (X: 10: 90-X, v/v). Flow rate: 1 ml/min. Detection: UV at 220 nm.

As expected, a decrease in capacity factors, k', was observed with increase in acetonitrile concentration. The optimal selectivity combined with symmetry and analysis time was obtained at 25 % ACN and this was selected for further work. Subsequent modifications were aimed at improving chromatographic separation. Introduction of *tert*-butanol (t-BuOH), as an additional organic modifier in the mobile phase, improved peak symmetry and k' values markedly (table 2). The best symmetry and analysis time were obtained with 5 % of t-BuOH. No further investigation on organic modifiers was carried out after attainment of these desirable attributes.

Influence of mobile phase pH: The effect of the mobile phase pH (6.5-8.5), at half unit intervals, on the k' values is shown in figure 3. As the pH increased, the k' values decreased. Good separation was observed between pH 7.5 and 8.0. Further investigations between pH 7.5 and 8.0 for the early eluting drugs, ESM and PB, achieved best separation and k' values at pH 7.6.

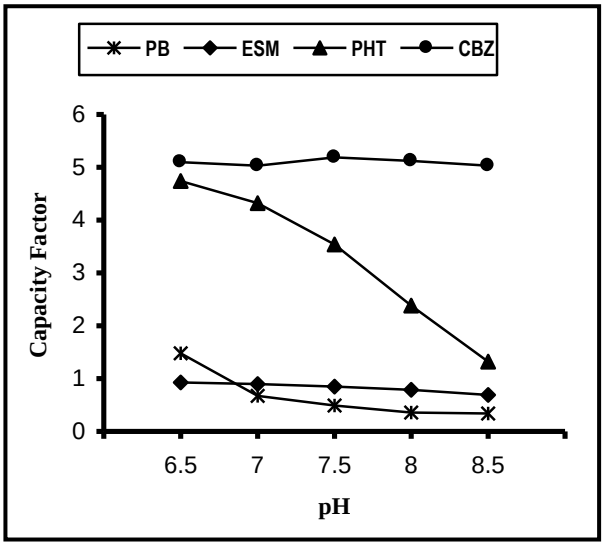

Figure 3. Effect of mobile phase pH on capacity factors of phenobarbitone (PB), etho-suximide (ESM), phenytoin (PHT) and carbamazepine (CBZ). Mobile phase: Acetonitrile-*tert*-butanol- phosphate buffer (pH 7.5, 0.2 M) - water (25:X: 10: 65-X, v/v). Flow rate: 1 ml/min. Column: PLRP-S 8 µm 100 Å. Column temperature: 60 ^{0}C. Detection: UV at 220 nm.

The compounds under review are weakly acidic and exhibit keto-enol tautomerism with pK_a values of 7.4, 9.5, 8.3 and 7.0 for PB, ESM, PHT and CBZ respectively [10]. On this chromatographic system there is preference for the elution of the polar ionized form of a compound due to its repulsion on the hydrophobic stationary phase. An increase in the ionic species leads to a drastic drop in k' value due to this repulsion. This increase in ionic species occurs from one pH unit less than the corresponding pK_a of the compound. The point for this effect for ESM occurs outside the pH range in the study. It is worth noting that there are instances when a much-pronounced size and bulkiness effect of the migrating species could hinder movement than the ionic species repulsion [11]. This could explain the apparent minimal effect of pH on the CBZ retention.

Influence of phosphate buffer concentration: The concentration of the buffer in the mobile phase had little or no effect on k' values within the 5 - 20 % range studied (results not shown). This is because buffer concentration does not affect the dissociation of the analyte species, but may affect the selectivity by influencing the extent of salting out. The 10 % concentration was chosen for further work.

Influence of column temperature: An increase in temperature resulted in improved k' and peak symmetry (figure 4). The temperature of 60 ^{0}C was selected for subsequent work. Increasing the temperature to 70 ^{0}C had no added advantages but instead gave baseline instability with the use of the low boiling point solvents. A typical chromatogram of the developed chromatographic conditions is shown in figure 5. The optimized method employs a PLRP-S 100 Å 8 µm stationary phase material column maintained at 60 ^{0}C, with a mobile phase of acetonitrile-tertiary butanol-phosphate buffer (0.2 M, pH 7.6) -water (25:5:10:60, v/v). A flow rate of 1.0 ml/min and UV detection at 220 nm is used.

Method validation

Linearity and Precision: The results for both linearity of detector response and precision are shown in table 3. The linearity of detector response over the concentration range studied showed good correlation from linear regression analysis for the assay of the four drugs. Where, y is the peak area ($x10^6$) and x, the injected concentration in µg. Similarly, acceptable precision was observed for all the compounds.

Sensitivity: The limits of detection (LOD) values and limit of quantitation (LOQ) values were determined at the signal to noise ratio of 3 and 10 respectively. The LOD values were 2, 15.6, 4 and 2 ng, while the LOQ values were 7.5, 62.5, 32.5 and 15.6 ng for PB, ESM, PHT and CBZ, respectively. The LOQ peak area coefficient of variation (CV) values of 11.0, 3.3 14.0 and 8.0 % obtained for PB, ESM, PHT and CBZ respectively, are indicative of a method that can be used in quantification of low concentrations such as those encountered in biological materials. The therapeutic ranges of plasma concentrations in adults, for the drugs in study, are 10-40, 40-100, 10-25 and 4-12 µg/ml for PB, ESM, PHT and CBZ, respectively [12].

Method robustness: The effect of small changes in ACN concentration, pH and column temperature was studied. The degree of variation of peak areas over each set of conditions is shown in table 4.

Table 2: Effect of *tert*-butanol concentration in the mobile phase on peak symmetry and retention times of phenobarbitone(PB), ethosuximide(ESM), phenytoin(PHT) and carbamazepine(CBZ)

Tert-butanol (%)	Asymmetry factor				Capacity factor			
concentration (X)	PB	ESM	PHT	CBZ	PB	ESM	PHT	CBZ
0	0.8	0.8	1.6	1.9	0.5	1.1	5.6	7.8
2	0.8	1.0	1.6	1.6	0.6	1.1	5.5	7.6
4	0.8	0.9	1.4	1.0	0.6	1.0	4.4	5.7
5	0.8	0.8	1.1	1.0	0.5	0.9	3.8	5.2
6	0.7	0.8	1.4	1.5	0.5	0.9	3.5	4.4
8	0.7	0.7	1.6	1.6	0.5	0.8	2.9	3.5

Mobile phase: Acetonitrile-*tert*-butanol- phosphate buffer (pH 7.5, 0.2 M) - water (25:X: 10: 65-X, v/v). Flow rate: 1 ml/min. Column: PLRP-S 8 µm 100 Å. Column temperature: 60 ^{0}C. Detection: UV at 220 nm.

Table 3. Linearity of detector response range and precision parameters for phenobarbitone (PB), ethosuximide (ESM), phenytoin (PHT) and carbamazepine (CBZ).

Drug	Concentration range (µg)	R	Slope	y-intercept	$S_{y,x}$	Within-day CV (%)	Between-day CV (%)
PB	0.005-3.1	0.9999	2.3559	0.0538	1.3	1.7	1.8
ESM	0.05-25.0	0.9993	0.0650	-1.6275	8.4	2.0	6.9
PHT	0.005-3.1	0.9999	2.4604	-0.01432	1.1	1.3	1.4
CBZ	0.003-1.6	0.9999	7.4894	-0.9466	2.1	1.1	1.8

Mobile phase: Acetonitrile- *tert*-butanol-phosphate buffer (pH 7.6, 0.2 M)-water (25:5:10:60, v/v). Flow rate: 1 ml/min. Column: PLRP-S 8 µm 100 Å. Column temperature: 60 ^{o} C. Detection: UV at 220 nm.

Table 4. Effect of minor variation of temperature, pH, and acetonitrile concentration on the peak areas of phenobarbitone, ethosuximide, phenytoin and carbamazepine

Condition varied		Peak area CV (%)
Temperature (58,60 and 62º C)	PB	0.7
	ESM	3.4
	PHT	0.3
	CBZ	0.9
pH (7.5, 7.6 and 7.7)	PB	1.5
	ESM	6.0
	PHT	1.3
	CBZ	1.2
% ACN in mobile phase (23, 25 and 27)	PB	1.2
	ESM	3.4
	PHT	0.5
	CBZ	0.4

Flow rate: 1ml/min. Column: PLRP-S 8 µm 100 Å. Detection: UV at 220 nm.

Changes in the ACN concentration gave higher retention time variation (> 10 %) while pH variation showed the least effect. However, the pH variation effect on peak area was marked, but did not affect quantitation. This is indicative of a rugged enough method.

Column stability: The satisfactory peak symmetry, selectivity and column stability was maintained over the approximately 7 months study period at 60 ^{0}C, without any aggressive column solvent clean up.

Stability of sample solutions: Triplicate daily injections of solutions kept at 4 ^{o}C, room temperature (21 ^{o}C) and 40 ^{o}C in closed containers over time were investigated. The peak areas change (more than 2 %) and/or extra peaks emergence were considered as indications of altered stability. All solutions kept at 4 ^{o}C, 21 ^{o}C and 40 ^{o}C showed significant changes only after 28, 8 and 5 days respectively. Thus, solutions for the study can be kept at 4 ^{o}C for not more than 28 days.

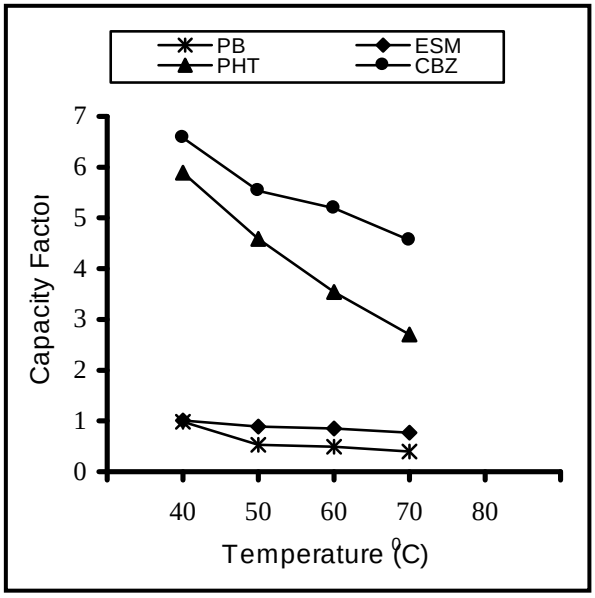

Figure 4. Effect of column temperature on capacity factors of phenobarbitone (PB), ethosuximide (ESM), phenytoin (PHT) and carbamazepine (CBZ). Mobile phase: Acetonitrile-*tert*-butanol-phosphate buffer (pH 7.6, 0.2 M) - water (25:5:10:60, v/v) Flow rate: 1 ml/min. Detection: UV at 220 nm. Mobile phase: Acetonitrile-tert-butanol-phosphate buffer (pH 7.6, 0.2 M)-water (25:5:10:60, v/v). Flow rate: 1 ml/min. Column: PLRP-S 8 µm 100 Å. Column temperature: 60 ^{o} C. Detection: UV at 220 nm.

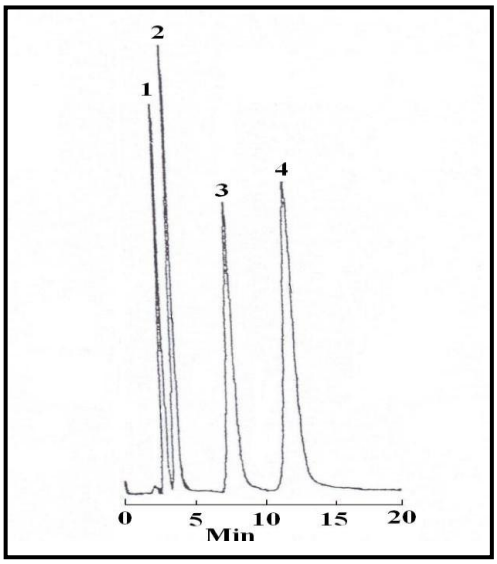

Figure 5. A typical chromatogram of phenol-barbitone (1) 0.6 µg, ethosuximide (2) 5.0 µg, phenytoin (3) 0.6 µg and carbamazepine (4) 0.3 µg on PLRP-S 100Å 8 µm column. Column temperature: 60 ^{o}C Mobile phase: Acetonitrile-*tert*-butanol-phosphate buffer (pH 7.6, 0.2 M) - water (25:5:10:60, v/v) Flow rate: 1 ml/min. Detection: UV at 220 nm.

CONCLUSION

The analytical method described here using a polymeric column enables the simultaneous separation of phenobarbitone, ethosuximide, phenytoin and carbamazepine within 15 min. This selective method that has good repeatability, linearity, sensitivity and robustness was used in analyzing the content and dissolution properties of three of the drugs, phenobarbitone, phenytoin and carbamazepine in several locally manufactured commercial samples on the Kenyan market [13]. No ethosuximide commercial samples are manufactured locally. The method may similarly find use in raw material analysis as well as in the analysis of any future fixed dose combinations on the Kenyan market. Such combinations do appear on other markets [14]. In addition, it may be useful in trace analysis or pharmacokinetic studies of the drugs used in combination.

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