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A Liquid Chromatographic Method for the Simultaneous Determination of Amlodipine, Valsartan and Hydrochlorothiazide in Tablets

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A simple, rapid, sensitive, specific, accurate, precise and fast high performance liquid chromatographic method for the determination of antihypertensive drugs amlodipine, valsartan and hydrochlorothiazide singly or in combination was developed and validated. Separation of the analytes was achieved on a Hypersil C-18 (250 mm × 4.6 mm, 5 µm) column using a mobile phase consisting of acetonitrile-KH_2PO_4 pH 3.0-water (75:6:19 % v/v/v) delivered at 1 ml/min, UV detection at 229 nm and 40 ^oC column temperature. The precision of the method was demonstrated through repeatability (coefficient of variation = 0.298-0.724) as well as intermediate precision (coefficient of variation = 0.435-1.412). The detector response was linear over the 25-150 % range with $R^2 \geq 0.99$ for each of the three analytes. The limit of detection for hydrochlorothiazide, valsartan and amlodipine were 10.72, 21.20 and 14.45 ng, while the limits of quantification were 35.76, 71.23 and 48.16 ng, respectively. The method showed satisfactory robustness and accuracy with a recovery of 99.7-100.6 %. The method was applied in the assay of 6 commercial products containing drugs under study. The results obtained revealed quality problems among the samples analyzed.

Keywords: HPLC, amlodipine, valsartan, hydrochlorothiazide, antihypertensives

INTRODUCTION

Treatment of hypertension using one drug is desirable due to good compliance, lower cost and fewer adverse effects. However, most patients with hypertension require two or more drugs, preferably acting by different mechanisms to yield the desired therapeutic outcome [1]. Some of the commonly used oral antihypertensive tablets in Kenya include valsartan, amlodipine and hydrochlorothiazide. Valsartan is a free acid with two acidic hydrogens (pK_a 3.9 and 4.7) acting as angiotensin receptor blocker [2], amlodipine is a calcium channel blocker formulated as the long acting besylate salt while hydrochlorothiazide is a thiazide diuretic with a pK_a of 7.0 [3, 4]. The chemical structures of amlodipine, valsartan and hydrochlorothiazide are shown in Figure 1.

There are several published methods for the estimation of amlodipine, valsartan and hydrochlorothiazide either individually or in combination. In one such method, Jothieswari *et al.* achieved separation on a C-18 (150 mm × 4.6 mm, 5 µm) column using a mobile phase composed of acetonitrile-methanol-50mM phosphate buffer pH 3.0 (20:50:30, % v/v/v) and UV detection at 239 nm with a run time of about 10 min [5]. The order of elution of the components was hydrochlorothiazide, amlodipine and valsartan. Similarly, Varghesea *et al.* used a C-18 column of similar dimensions, 10 mM ammonium acetate buffer pH 6.7 and methanol as mobile phase in solvent gradient elution with photodiode array detection at 238 nm whereby the order of elution was hydrochlorothiazide, valsartan and amlodipine with a run time of 11 min [6]. The present study reports the development and validation of a method utilizing a commonly available and affordable C-18 (250 × 4.6 mm, 5 µm) column, to achieve reasonable separation of the study analytes with acceptable chromatographic parameters.

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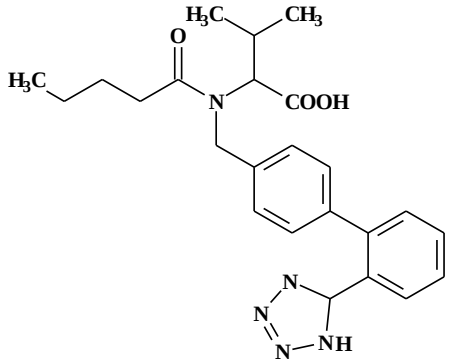
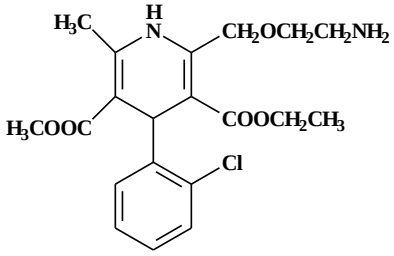
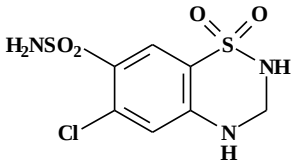


Valsartan



Amlodipine



Hydrochlorothiazide

Figure 1. Chemical structures of valsartan, amlodipine and hydrochlorothiazide.

MATERIALS AND METHODS

Chemicals and reagents

High performance liquid chromatography (HPLC) grade solvents including methanol (Rankem, RFCL Limited, Mumbai, India) and acetonitrile (Fisher Scientific UK Limited, Madison, East Grinstead, UK) together with analytical grade orthophosphoric acid (May and Baker Ltd, Dagenham, England) and potassium dihydrogen phosphate (LobaChemie, PVT Ltd, Mumbai, India) were used for chromatographic work. Freshly double-distilled water was used for all experiments.

Working reference standards

The working standard substances amlodipine and hydrochlorothiazide were from AurobidoPharma (Mumbai, India) while valsartan was from Ranbaxy Pharma (Mumbai, India).

Commercial samples

Samples of tablets on the market were purchased from randomly selected retail pharmacies located within the Thika town, Kenya. A total of 6 commercial brands containing a combination of one, two or three of the study drugs were obtained and coded A-F. Three batches of each product were tested for batch consistency. The samples analyzed had at least six months of their self-life remaining.

Instrumentation

The HPLC system consisted of a Cyberlab LC 100 HPLC pump (Cyberlab Corporation, Milbury, USA), equipped with universal 20 µl loop injector, Rheodyne 7725 (Rheodyne Inc, Cotati, CA, USA) and LC 100 UV detector (Wufeng Instruments Co., Shangai, China) controlled by a desktop computer equipped with WS-100 work station software (Wufeng Instruments Co., Shangai, China). Analytes were separated on a Hypersil® C18 (250 mm × 4.6 mm, 5 µm) column (Thermo Electron Corporation, Waltham, MA, USA). Other columns tested included Nucleosil® 100-5 C18 (125 mm × 4 mm, 5 µm) (SMI-LabHut Ltd, Gloucester, GL2 8AX, UK), Phenomenex® C-8 (250 mm × 4.6 mm, 5 µm) (Phenomenex Inc., Foster City, CA, USA) and Capcell Pak® C18 (250mm × 4.6 mm, 5 µm) (Shiseido, Tokyo, Japan). The column temperature was thermostated using a Lab Tech water bath (Daihan Labtech Co., Mumbai, India).

Fixed operational parameters

The mobile phase flow rate (1.0 ml/min), injection volume (20 µl) and detection wavelength (229 nm) were fixed during method development.

Preparation of mobile phase

The mobile phases consisted of varying mixtures of acetonitrile, distilled water and phosphate

buffer. A stock solution of 0.1M KH_2PO_4 buffer was prepared and adjusted to the required pH with equimolar H_3PO_4 before use. Aliquots of 0.1 M KH_2PO_4 were mixed with water and acetonitrile to yield the desired buffer concentration in the mobile phase. Mobile phases were degassed by ultra-sonication before use.

Working standard solution

A stock solution containing 12.5 mg amlodipine, 100 mg valsartan and 25 mg hydrochlorothiazide in 50 ml acetonitrile was prepared. The working standard solution was prepared by mixing 1.0 ml of each of the stock solutions in a 25.0 ml volumetric flask and making up to volume with acetonitrile-water (50:50 % v/v) to give a final concentration of the 10 µg, 80 µg and 20 µg per ml of amlodipine, valsartan and hydrochlorothiazide, respectively. These concentrations reflect the relative proportions of the three drugs in commercial products.

Method validation

Accuracy

The accuracy of the method was evaluated by spiking commercial products with working standards of the compounds under study before HPLC analysis. The recovery of the analytes was determined and assessed against the International Committee on Harmonization (ICH) acceptance criteria [7].

Precision

Repeatability was determined by carrying out six injections of each of three freshly prepared working standard solutions on the same day. The intermediate precision was determined by making six injections of freshly prepared standard solutions daily over three consecutive days. In each case, the peak areas of the components were normalized and the coefficient of variation (CV) of the normalized areas computed and used as the measure of precision [7].

Linearity and range

The linearity of the method was determined by running freshly prepared working standard solution at the 25, 50, 75, 100, 125 and 150 % levels. The resulting peak areas were subjected to regression analysis against the corresponding concentrations. The coefficient of determination (R^2) was computed to confirm linearity [7].

Sensitivity

A solution containing 10 µg hydrochloro-thiazide, 40 µg valsartan and 5 µg amlodipine per ml was serially diluted and analysed for signal to noise ratio (S/N) against the mobile phase (blank). The LOD and LOQ were established at concentrations that yielded S/N of 3 and 10, respectively [7].

Robustness

The influence of the chromatographic factors was tested at 3 levels, low (-1), central (0) and high (1) as shown in Table 1. Six runs of the working standard solution were made after adjusting the factor levels. The capacity factors, k' and CV of the peak areas were computed and used as a measure of robustness. For this purpose, the k' values were plotted against each factor variation as an indicator of selectivity.

Table 1: Robustness testing levels for the chromatographic factors

Factor level	pH	Temperature (°C)	Methanol concentration (% v/v)
1	3.5	45	80
0	3.0	40	75
-1	2.5	35	70

Analysis of commercial samples

Twenty tablets were crushed and powders equivalent to the required quantity of analyte weighed and dissolved in acetonitrile in 50 ml volumetric flasks as the stock solution. The stock solutions were appropriately diluted in mobile phase to make 10, 80 and 20 µg/ml of amlodipine, valsartan and hydrochlorothiazide sample solutions, respectively. Due to non-availability of specifications for the combination products, the British Pharmacopoeia (BP) and United States Pharmacopoeia (USP) limits for the single component products, 90-110% of label claim, were used as a basis for assessment [8, 9].

RESULTS AND DISCUSSION

Method development

An overlay of the UV spectra of the individual three analytes was used to establish the optimum wavelength of detection as 229 nm. Reversed phase silica columns were chosen on account of their high mechanical strength, high efficiency and superior chromatographic parameters especially with basic analytes. In preliminary experiments, four RP columns maintained at 40 ^{o}C temperature were tested using acetonitrile-0.1 M KH_2PO_4 pH 2.0-water (60:4:36, % v/v/v) as mobile phase. The columns Phenomenex C-8, Nucleosil 100-5 C-18, Capcell Pak C-18 and Hypersil C-18 were evaluated. Due to good peak shapes and better resolution, Hypersil C-18 (250 mm × 4.6 mm, 5 µm) was chosen for further work.

Experiments using acetonitrile-water (60:40 % v/v) as mobile phase yielded two poorly resolved peaks with amlodipine and valsartan co-eluting with run time 4 min. Incorporation of 4 % v/v 0.1 M KH_2PO_4 (pH 4.3) buffer in this mobile phase composition caused co-elution of amlodipine and hydrochlorothiazide with reversal of elution order. Decreasing the buffer pH to 2.0 caused resolution of all the three peaks. At this pH, the asymmetry factor was improved by increasing the acetonitrile content of the mobile phase to 75% v/v (Figure 2).

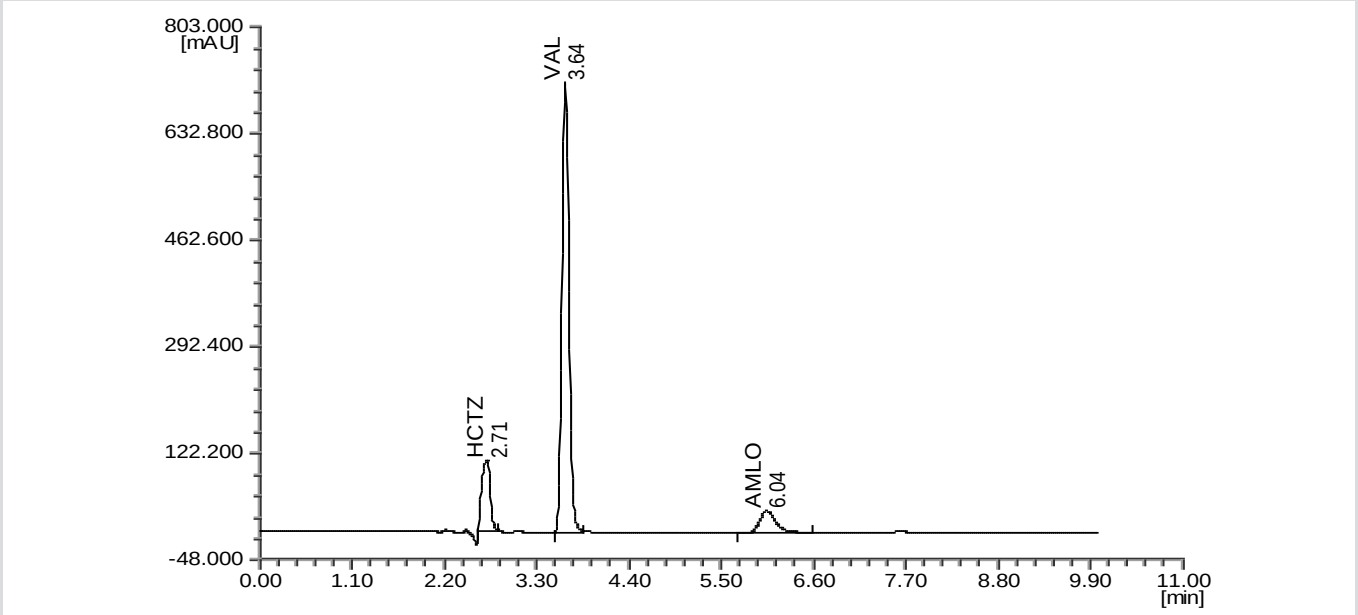


Figure 2. Typical chromatogram of hydrochlorothiazide (HCTZ), valsartan (VAL) and amlodipine (AMLO). Column: Hypersil C-18 (250 mm × 4.6mm, 5 µm); Mobile phase: acetonitrile-0.1 M potassium dihydrogen phosphate pH 2.0-water (75:4:21, % v/v/v); Column temperature: 40 ^{o}C.

Figure 3 shows the effect of buffer pH (2.0, 2.5 and 3.0) on the capacity factors and selectivity for the component peaks. Increasing the pH increased k' due to increased retention of amlodipine, the last eluting peak. Based on the peak symmetry, resolution and run time, pH 3.0 was taken as optimum.

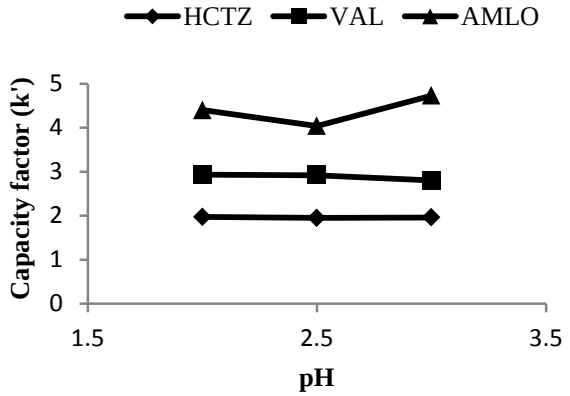


Figure 3. Effect of pH on capacity factor for the separation hydrochlorothiazide (HCTZ), valsartan (VAL) and amlodipine (AMLO). Column: Hypersil C-18 (250 mm × 4.6 mm, 5 µm); Mobile phase: acetonitrile-0.1 M potassium dihydrogen phosphate-water (75:6:19, % v/v/v).

It was further observed that varying buffer concentration affected the retention times of all the peaks with a profound effect on the shape of the hydrochlorothiazide peak. A buffer concentration of 6 % v/v (0.006 M) was established as optimum.

Column temperature was investigated at 25, 30, 35, 40 and 45 ^{o}C. An increase in temperature improved symmetry of the peaks with concomitant reduction of run times due to enhanced mass transfer. The optimum temperature was found to be 40 ^{o}C as compromise of chromatographic parameters and column stability. The use of mobile phase as the sample solution diluents instead of acetonitrile-water (50:50, % v/v) showed improved peak symmetry especially for hydrochlorothiazide.

The optimized chromatographic conditions were: Hypersil C-18 (250 mm × 4.6mm, 5 µm) column with a mobile phase consisting of acetonitrile-0.1M KH_2PO_4 pH 3.0-water (75:6:19, % v/v/v) and 40 ^{o}C column temperature. A flow rate of 1 ml/min, detection at 229 nm and an injection volume of 20 µl of samples diluted in mobile phase were maintained. Figure 4 is a typical chromatogram obtained under these conditions.

Method validation

Accuracy

The accuracy for the developed method was determined by spiking a predetermined concentration of the commercial products with working standards of the compounds under study. The difference between the spiked and unspiked samples was calculated as percentage of the added analyte (Table 2). The percentage recovery obtained showed compliance with ICH acceptance criteria for accuracy (98-103 %) [7].

Table 2: Percentage recovery of hydrochlorothiazide, valsartan and amlodipine

Drug	% recovery	Coefficient of variance (CV) n = 9
Hydrochlorothiazide	100.6	1.82
Valsartan	99.9	0.45
Amlodipine	99.7	1.24

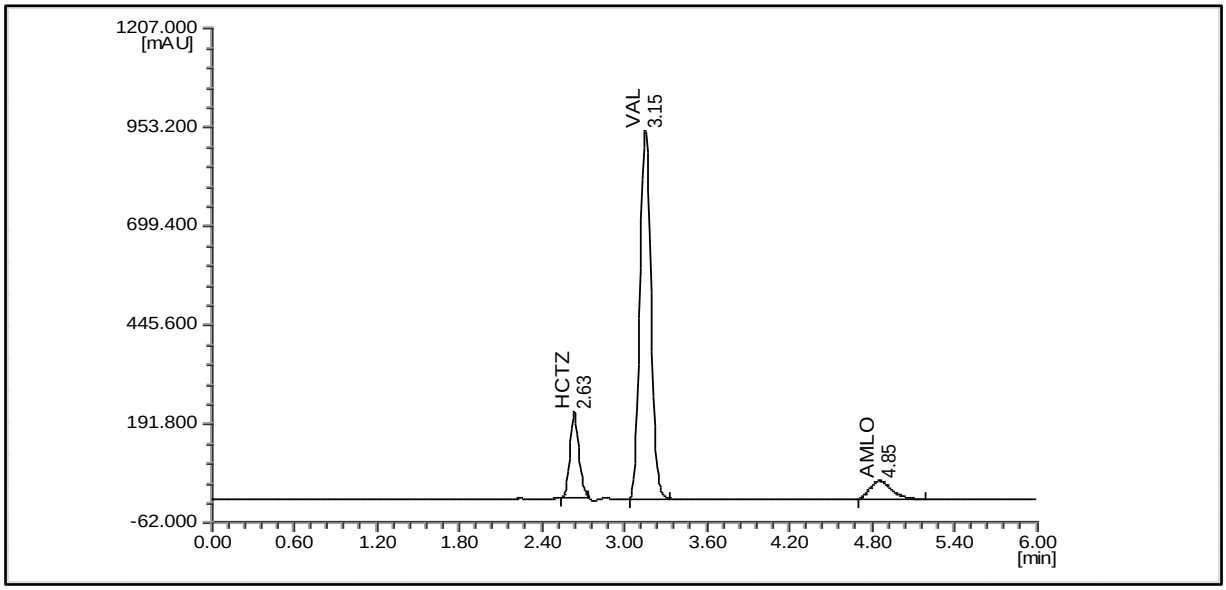


Figure 4. A typical chromatogram for the separation of a mixture of hydrochlorothiazide (HCTZ), valsartan (VAL) and amlodipine (AMLO) at optimized conditions. Column: Hypersil C-18 (250 mm × 4.6 mm, 5 µm); Mobile phase: acetonitrile-0.1 M potassium dihydrogen phosphate pH 3.0-water (75:6:19, % v/v/v); Column temperature: 40 ^{o}C.

Precision

The results obtained for repeatability and intermediate precision are summarized in Table 3. The CV values for all the components were < 2 thus indicative of good precision.

Linearity

The linearity data obtained were further subjected to regression analysis and generated the parameters recorded in Table 4. The R^2 > 0.99 values for each of the three compounds indicate a close correlation between the analyte concentrations and the peak areas in the range 25-150 %. The ICH guidelines recommend linearity establishment for a minimum of five concentrations over the 80 to 120 % range [7].

Table 3: Precision data for hydrochlorothiazide, valsartan and amlodipine

Drug	Repeatability peak areas	Intermediate precision
	Coefficient of variation (n=18)	Coefficient of variation (n=36)
Hydrochlorothiazide	0.629	1.080
Valsartan	0.298	0.435
Amlodipine	0.724	1.412

Table 4: Linear regression analysis for hydrochlorothiazide, valsartan and amlodipine

Drug	Slope	Y intercept	R^2 value
Hydrochlorothiazide	4537	26628	0.991
Valsartan	5344	124106	0.997
Amlodipine	4227	9893	0.995

Sensitivity

The obtained LOD and LOQ values are summarized in Table 5. The method exhibits satisfactory precision (CV < 2.0) for each peak at the limit of quantification for all three analyte compounds.

Robustness

At the pH range tested, the k' and selectivity for hydrochlorothiazide, valsartan and amlodipine remained generally unaffected (Figure 5). An increase in acetonitrile concentration caused a gradual decrease in capacity factor for amlodipine with little effect on hydrochlorothiazide and valsartan while temperature decreased the capacity factor of amlodipine alone. Notably, none of the peaks co-eluted within the ranges investigated thus demonstrating the robustness of the method. Furthermore, the CV for the variation in peak areas was > 2% in all cases. However, the interactive effects of the factors were not elucidated due to lack of the necessary software for experimental design and data analysis.

Table 5: Limit of detection and limit of quantitation values for hydrochlorothiazide, valsartan and amlodipine

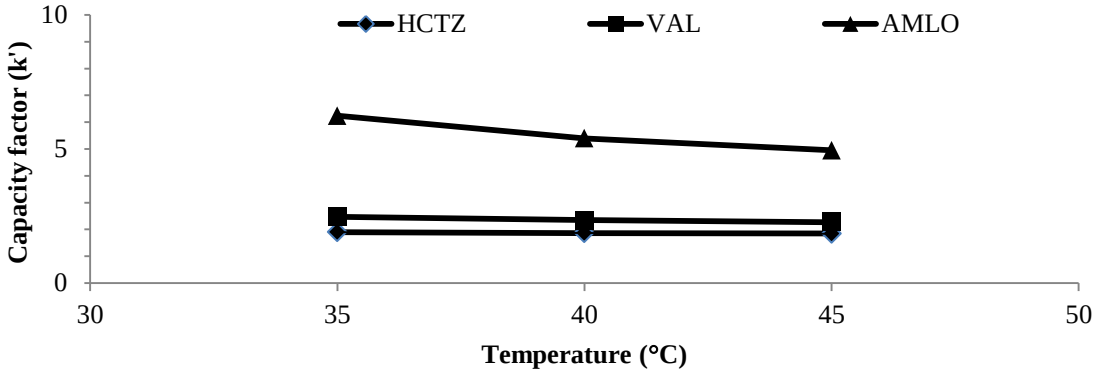
Analyte	LOD (ng)	LOQ (ng)	CV of peak areas at LOQ
Hydrochlorothiazide	10.72	35.76	1.23
Valsartan	21.20	71.23	1.55
Amlodipine	14.45	48.16	1.76

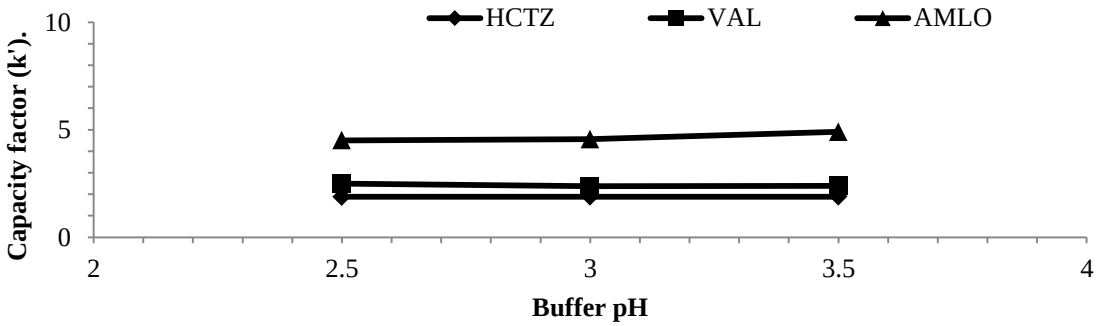
LOD - limit of detection; LOQ - limit of quantitation.

Analysis of commercial samples

The assay results obtained for the six products analyzed are listed in Table 6. The BP and USP limits (90-110 %) for single component products were used as a basis for determining whether the products met specifications. The amlodipine content of seven out of nine batches (77.8 %) tested was found not to meet the pharmacopoeial specifications. The only exceptions were two batches of product A although they still exhibited significant batch variation (< ±10%).

The hydrochlorothiazide assay in eight out of nine samples (88.9 %) was below the lower limit while the compliance rate for valsartan content of the samples analyzed was 33.3 %. Products C and D varied significantly in valsartan content. The two samples that complied with the assay of the component APIs, were one containing valsartan only (product D) and the other with the three drugs in combination (product A). Only one batch out of the three analyzed was compliant in both cases.





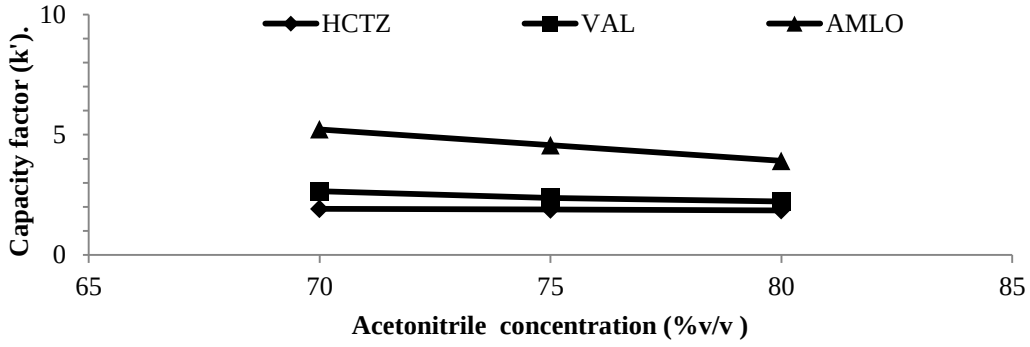


Figure 5. Effects of changes in temperature, mobile phase pH, and acetonitrile concentration on capacity factor (k') for the test compounds. AMLO = amlodipine; HCTZ = hydrochlorothiazide; VAL = valsartan.

Table 6: Content analysis of hydrochlorothiazide, valsartan and amlodipine in commercial samples

Product code	Batch number	% Label claim (CV)		
		HCTZ	VAL	AMLO
A	S0157	81.3 (0.82)	84.3 (0.94)	123.7 (1.25)
	S0100B	100.3 (1.65)	100.5 (1.61)	99.3 (0.55)
	S0182	89.1 (1.72)	96.5 (1.94)	95.6 (0.75)
B	B8136	-	88.9 (1.82)	122.4 (1.69)
	S0260	-	88.0 (1.87)	122.7 (1.45)
	B5208	-	92.8 (1.28)	129.2 (1.32)
C	T6017	74.7 (1.85)	79.9 (1.10)	-
	T3287	80.6 (1.55)	79.6 (1.47)	-
	T4530	77.3 (1.59)	89.7 (0.41)	-
D	D8258	-	79.9 (1.11)	-
	B8067	-	100.1 (1.23)	-
	D8124	-	77.4 (0.13)	-
E	ACTP0041	-	-	123.4 (1.58)
	SKK2300	-	-	127.6 (1.40)
	EII276	-	-	129.6 (1.21)
F	54562	73.2 (1.17)	-	-
	20725	71.4 (1.11)	-	-
	30422	79.6 (1.92)	-	-

Figures in parentheses represent the coefficient of variation. AMLO = amlodipine; HCTZ = hydrochlorothiazide; VAL = valsartan.

CONCLUSION

A rapid simple, reliable, precise and robust isocratic reverse phase HPLC method with UV detection was developed and validated for the simultaneous determination of amlodipine, valsartan and hydrochlorothiazide. The method can be applied in the determination of drugs in bulk samples and dosage forms.

However, the high failure rate as revealed by assay test results is of great concern. This shows a failure of quality assurance during the manufacture of the anti-hypertensive medicines sampled. Sustained market surveillance needs to be carried out in Kenya to scout for substandard medicines which should be subjected to appropriate regulatory action.

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