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Reversed phase HPLC method development and validation for the analysis of amlodipine besylate in tablets dosage form and human plasma.

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ABSTRACT

Introduction: Several analytical methods for quantitative determination of amlodipine either in pharmaceuticals or biological fluids have suffered different setbacks. Therefore, we aimed at developing a cost effective, simple, robust, and sensitive reversed phase high performance liquid chromatography (RP-HPLC- UV) method for quantification of amlodipine in dosage form and in human plasma.

Method: The separation was performed on an analytical column 150 mm x 4.6 mm i.d., Agilent Eclipse XDB C18 Column packed with 5 µm particle size and protected by a guard column (30 mm x 4.6 mm i.d.). The mobile phase composition contained acetonitrile: phosphate buffer (25 mM KH_2PO_4) adjusted to pH 3.1 with orthophosphoric acid in the ratio of 45:55 v/v. The flow rate was 1.2 mL/min and the UV detector was set at a wavelength of 240 nm. The column was maintained at ambient temperature.

Results: The calibration curve gave good linearity in the concentration range studied. The column efficiency was highly evident by high theoretical plates and small height equivalent to theoretical plate (HETP). The percent relative standard deviation (% RSD) was less than 3% in the inter- and intra-day assay. Amlodipine, 50 ng/mL in plasma stored frozen at -80 °C was stable There was no statistical difference in the precision of amlodipine assayed after 12 months when compared to the initial assay ($P>0.05$). The % recovery of amlodipine in human plasma was 97.79±0.17% to 99.52±0.63%.

Conclusion: The new RP-HPLC method is sensitive, reproducible, precise, accurate and robust. We conclude that the method is suitable for estimation of amlodipine besylate in human plasma for pharmacokinetic studies and assay of pharmaceutical dosage form.

1. Introduction

Amlodipine is a third generation long-acting 1, 4-dihydropyridine calcium channel blocker widely used in the treatment of hypertension and as anti-angina agent with a slow onset of vasodilatory action[1, 2] (Figure 1). Several analytical methods developed for quantitative determination of amlodipine either in tablets or in biological samples have been reported. Among the analytical methods reported are, high performance liquid chromatography with electro chemical detector and photodiode array detector[3, 4, 5], high performance thin layer chromatographic method[6, 7] and liquid chromatography with mass tandem system (LC–MS)[8, 9] and LC-MS-MS[10]. A method for the determination of amlodipine after derivatization with chromogenic agent, 4-chloro-7-nitrobenzofurazan using HPLC with fluorescence detection had also been reported[11] the employment of a chromogenic reagent, 1,2- naphthoquine-4-sulphonate using HPLC with photodiode array detector has also be recorded[2]. In another study, Kadioglu *et al.,* [12] reported spectrofluorimetric

determination of amlodipine in human plasma without derivatization.

However, these methods suffered different setbacks including, poor recovery, delayed elution (long retention time) of the drug, high thermal decomposition of the drug, high cost of analysis, use of sophisticated equipment, appreciably time consuming and high ratio of organic solvents in the mobile phase, which is environmentally inappropriate according to the green chemistry approach[5]. An important principle of green chemistry is to reduce toxic organic solvents and to consume safer chemicals[13, 14]. Alsarra[15] developed an HPLC-UV visible method for quantifying amlodipine following plasma extraction of the drug. This method is time consuming and expensive as it involves the use of lots of reagents. In another work, Kumar and Ram[16] developed an analytical method for the determination of amlodipine in tablet dosage form but failed to apply the method to the assay of the drug in human plasma. Analytical methods may fail when translated to study in biological fluid.

This present study therefore developed and validated a simple, accurate, sensitive and reproducible HPLC–UV visible method to allow rapid determination of amlodipine in tablet and human plasma that does not involve extraction of amlodipine from the sample or biological sample. The method will be applied to detect amlodipine in plasma samples obtained from a pharmacokinetic study.

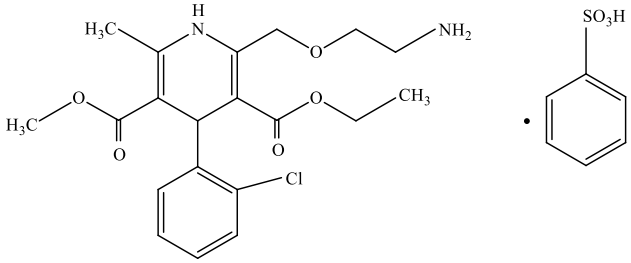

Figure 1 Structure of amlodipine besylate

Materials and methods

2.1 Reagents and chemicals

Acetonitrile, methanol, potassium dihydrogen phosphate were of analytical grade (Sigma-Aldrich, St. Louis, USA). Amlodipine besylate standard powder was a gift from Bond Chemical Industries, atorvastatin standard powder used as internal standard was a gift from Fidson Pharmaceutical Company. Amoldipine besylate tablets were purchased from a pharmaceutical shop in Mushin area of Lagos State, Nigeria.

2.2 Preparation of stock and working solutions

From the stock solution of amlodipine besylate, 500 ng/mL in methanol was prepared varying concentrations of working standard solutions (0, 10, 25, 50, 70, 80 and 100) ng /mL used to construct the calibration curve. The solutions were filtered through a 0.22 µm membrane filter before injection.

2.3 Chromatographic System and Conditions

The Hewlett-Packard high performance liquid chromatographic system, (Agilent 1260 series, Hewlett-Packard, Germany) consisted of an auto sampler (Serial No. DEAAC15585, Product No. G1329B) having Rheodyne 7725i (20 µL capacity loop) injector and a quat pump (Serial No. DEAB706149, Product No. G1311B) which, maintained a constant flow of mobile phase through the column. The analytical column was Agilent Eclipse XDB C18 (ODS) packed with 5 µm particle size (stainless steel column of 150 mm x 4.6 mm i.d.) protected by a guard column, 30 mm x 4.6 mm i.d., placed between the injector and the analytical column; the degasser (Serial No. DEACN16928, Product No. G1316A) was used to remove gasses from the mobile phase. The detector was a UV-Visible spectrophotometer (Serial No. DEABB05196, Product No. G1314F) set at a wavelength of 240 nm.

2.4 Optimization of HPLC chromatographic conditions

The HPLC method was optimized for the analysis of amlodipine besylate in tablet and human plasma to give a satisfactory and good resolution. Mobile phases of different polarity, from non-polar to polar solvent combination (methanol: acetonitrile: phosphate buffer and ratios of acetonitrile: methanol adjusted to different pH with phosphoric acid) and varying flow rates to find the best condition for the quantification of amlodipine besylate with atorvastatin as the internal standard using Agilent Eclipse XDB C18 (ODS) Column packed with 5 µm particle size and protected by a guard column (30 mm x 4.6 mm i.d.).

2.5 Validation Assay Methods

The validation of assay method followed the ICH[17] and USP guidelines[18] for linearity, specificity, accuracy, precision, ruggedness and robustness as well as limit of detection (LOD) and limit of quantification (LOQ).

2.5.1 Calibration plot

All assays were performed under subdued light. The linearity assays were evaluated by linear regression by plotting of the peak area ratio of amlodipine to atorvastatin (I.S) against their corresponding concentration. A linear regression was calculated to obtain the linearity, slope, y-intercept and correlation coefficient.

2.5.2 Specificity, Accuracy and Precision

A mixture of solution containing amlodipine (50 ng/ml) and some excipients: - codeine (50 µg/ml) and nifedipine (50 µg/ml) were filtered with a 0.22 µm membrane filter. An aliquot, 20 µl of the solution was injected into the HPLC. Similarly, excipient solutions were also injected individually. Accuracy, assessed by percent recovery was determined. The intra-day calibration curves were obtained at fixed time interval within the same day at five different concentrations. The inter-week calibration curves were carried out once every week for four weeks at five different concentrations. Working standards were made fresh each week of the assay. The relative standard deviation (RSD) or coefficient of variation of the estimated concentrations were determined and used for the assessment of precision.

2.5.3 Robustness of assay method

The robustness of the method was affirmed by changing the experimental conditions such as the flow rate, mobile phase composition, type of column and working temperature of the column. Results obtained were compared.

2.5.4 Assay stability of amlodipine

Stability of amlodipine in plasma at room temperature and that stored at -80 °C was evaluated by spiking human plasma with the drug and assaying within a four-week period. The drug solution (n = 5, 50 ng/mL) freshly prepared in amber bottle at room temperature was assayed once a week for the period of study. The concentration of amlodipine in the spiked plasma was determined on the day it was prepared and thereafter stored at – 80 °C. On the day of assay, the plasma was thawed, re-assayed and frozen for the period of the study. The percent recovery of amlodipine in the plasma was evaluated by comparing with direct injection of standard stock solution.

2.5.5 Limit of detection (LOD) and Limit of Quantification (LOQ)

The lowest amount of the drug in a sample that can be detected in the sample (LOD) and the lowest amount of drug in a sample that can be quantified with acceptable precision and accuracy (LOQ) were determined according to the formulae: - LOD=3.3×SDa/b, and LOQ=10×SDa/b. Where SDa, is the standard deviation of the intercept and b is the slope of the calibration curve under the ICH guidelines[18].

2.5.6 System suitability

System suitability was determined by monitoring the consistency of the retention time of amlodipine and the internal standard as recorded from their typical chromatograms. This gives information on the resolution between the amlodipine and the internal standard peaks.

2.5.7 Ruggedness

An aliquot, 50 ng/mL, was prepared and analyzed by three different researchers using similar experimental conditions at different times. The peak areas were measured for same concentration in seven replicates. The percent relative standard deviation (%RSD) was estimated.

2.6 Application of the developed HPLC method to the assay of amlodipine in dosage form

Four brands of amlodipine dosage form were assayed using the developed method. Twenty tablets of each brand of amlodipine besylate were weighed and triturated in a porcelain mortar and pestle. Each of the powered samples, equivalent to 10 mg of amlodipine was dissolved in 500 mL methanol. The solution was then filtered through a 0.22 µm filter. Thereafter, 100 ng/mL solutions were prepared from each of the sample solutions and internal standard, 50 ng/mL was added. The percentage of amlodipine in each of the samples was calculated by evaluating the peak area ratio of the sample with that of the reference standard.

2.7 Application of the developed HPLC method in the assay of amlodipine in spiked plasma sample

Drug-free plasma, 1.0 mL was spiked with amlodipine to yield concentrations of 25 to 80 ng/mL. To each spiked plasma, were added atorvastatin (I.S.) and acetonitrile and vortexed. The mixture was centrifuged at 5,000 g for 10 min. The clear supernatant was separated using Pasteur pipette and 20 µL of the supernatant was injected into the HPLC. The percentage recovery of amlodipine from each concentration was evaluated.

2.8 Statistical analysis

Data were expressed as mean ± SD (standard deviation). The statistical difference was accessed by means of an unpaired Student's t- test using Graph pad with the level of

statistical significance (P) set at 0.05.

3. Results

3.1 Wavelength of absorption

Amlodipine was scanned with ultraviolet spectrophotometer to determine the optimum wavelength for detection. The maximum wavelength was exhibited at wavelength of 240 nm. The wavelength of absorption obtained was used as the wavelength of detection in the HPLC study.

3.2 Optimization of HPLC chromatographic conditions

The chromatographic conditions were optimized by changing the composition of mobile phase, pH and flow rate; following the number of theoretical plates and peak shape, the chromatographic condition was selected. The optimized chromatographic condition produced stable and acceptable peak shapes for amlodipine. The ideal mobile phase composition selected contained acetonitrile: phosphate buffer (25 mM KH_2PO_4) adjusted to pH 3.1 with orthophosphoric acid in the ratio of 45:55 v/v pumped at a flow rate of 1.2 mL/min and monitored by ultraviolet detection wavelength set at 240 nm. Representative chromatogram of amlodipine and atorvastatin (internal standard) is presented in Figs. 2a-d.

3.3 Linearity and quantification limits

The results of the linearity and quantification of amlodipine in drug-free plasma and methanol are presented in Table 1. The results were obtained by linear regression analysis using peak area ratio of amlodipine to the atorvastatin at different concentration. The result gave the correlation coefficient, slope and intercept for standard curves constructed in methanol and in plasma. The results showed a linear relationship in the range of the concentrations studied**.** Column efficiency was determined by calculating the number of theoretical plates (N) and height equivalent to theoretical plates (HETP).

Table 1: Calibration parameters for the developed HPLC method

	values	
Parameters	**Amlodipine in methanol**	**Amlodipine in plasma**
Linear range (ng/mL)	10 - 100	10 - 100
Intercept	+0.0003	- 0.0021
Slope	0.0001	0.0004
Correlation Coefficient (r)	0.9998	0.9971
LOD (ng/mL)	1.726	1.814
LOQ (ng/mL)	5.182	5.678
Number of theoretical plates	1626.37	1634.5
HETP	0.0922	0 0917

3.4 Intra and inter-day assay of amlodipine

The intra-day and inter-day precision of the method evaluated by replicate analysis and expressed as relative standard deviation (RSD) was less than 3 % (n=5) in each concentration considered (Table 2). The HPLC method was optimized to give sharp peak of amlodipine with no interference peak from the plasma, internal standard and excipients. Figures 2a-g shows the representative chromatograms of plasma sample, spiked plasma with amlodipine or atorvastatin, with mixture of amlodipine and atorvastatin, amlodipine in methanol, mixture of amlodipine and codeine (excipient), amlodipine and nifedipine (excipient) respectively. Accuracy expressed as percentage bias (percent relative error, %RE) was -1.31% or -0.76% for the intra and inter day assays respectively (25 µg/mL; n = 5). Accuracy, assessed by percent recovery was determined by comparing the peak area ratio of the spiked drug-free plasma with the peak area ratio obtained by direct injection of the standard solutions of the same concentration. The mean percent recovery for intra-day and inter-day assays for the concentrations studied ranged from 97.79 ± 0.17 to 99.52 ± 0.63 %.

Table 2: Intra-day and Inter-day precision and accuracy of amlodipine in methanol and spiked plasma

Nominal conc. (ng/ml)	amlodipine in solvent				amlodipine in spiked plasma			
	Conc. found [a] (ng/mL)	R.S.D. [b] (%)	Accuracy [c] (%)	Recovery [a] %	Conc. found [a] (ng/mL)	R.S.D. [b] (%)	Accuracy [c] (%)	Recovery [a] %
Intra-day (n = 5)								
25	25.07±0.03	0.12	0.29	100.29±0.46	24.97±0.16	0.15	-1.31	98.69±0.43
50	50.03±0.04	0.12	0.07	100.07±0.38	49.03±0.12	0.24	-1.94	98.06±0.28
70	69.16±0.03	0.04	-1.20	98.79±0.25	69.30±0.09	0.13	-1.00	99.00±0.16
Inter-day (n = 5)								
25	24.56±0.03	0.13	-1.76	98.24±1.43	24.81±0.29	1.16	-0.76	99.28±0.21
50	49.74±0.50	1.01	-0.52	99.48±1.63	48.90±0.73	1.49	-2.21	97.79±0.17
70	68.94±0.26	0.26	-1.51	98.49±2.08	69.67±0.64	0.91	-0.47	99.52±0.63

[a] Values are mean ±SD
[b] coefficient of variation
[c]% bias = (conc. found – nominal conc.)/nominal conc. X 100

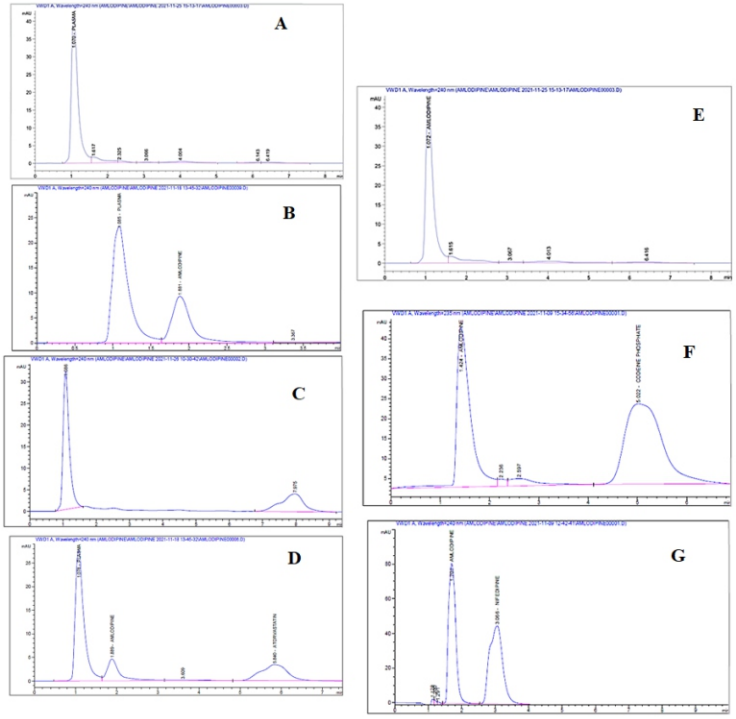

Figure 2A-G Representative chromatogram of (A) plasma sample, (B) plasma spiked with amlodipine, (C) plasma spiked with atorvastatin and (D) plasma spiked with amlodipine and atorvastatin, (E) amlodipine in methanol, (F) amlodipine and codeine (excipient) in methanol and (G) amlodipine and nifedipine (excipient) in methanol.

3.5 Stability of Amlodipine

The results of the stability of amlodipine in plasma at room temperature and that stored at -80 °C are shown in Table 3. There was no statistical significance in the percent recovery of amlodipine stored at -80 °C when compared to amlodipine at room temperature ($p>0.05$). The precision of the stability expressed as relative standard deviations (%RSD) of amlodipine in the stored samples were less than 5% for each week of the experiment.

Table 3: Stability of amlodipine in stored plasma at room temperature and at -80 °C

Periods (weeks)	Initial Concentration (50 ng/mL)					
	Room Temperature			- 80°C		
	Final conc. ± S. D	R.S.D. (%)	Recovery (%)	Final conc. ± S. D	R.S.D. (%)	Recovery (%)
0	50.1±1.43	2.85	100.20±1.84	49.92± 1.32	2.64	99.84±2.73
1	50.0± 2.14	4.28	100.00±3.58	50.1±1.61	3.21	100.20±2.42
2	49.8±1.94	3.89	99.60±2.73	50.0±1.10	2.20	100.00±2.01
3	48.1±1.31	2.70	96.20±3.74	49.7± 1.02	2.05	99.40±1.04
4	49.3±1.83	3.71	98.6±2.71	49.9±1.31	2.63	99.80±1.52

Values are mean ±SD

3.6 Robustness and Ruggedness

The results of the robustness and ruggedness of the developed method are shown in Table 4. The robustness of the method was evaluated by interchanging some parameters such as the ratio of mobile phase, flow rate, column temperature, wavelength and analysts (Table 4). The ruggedness of the method expressed as %R.S.D. of amlodipine was less than 3% when different analysts prepared and injected the samples at different weeks. The results showed no statistical differences when parameters were altered as well as between different analysts.

Table 4: Variation of analytical parameters for system suitability in the HPLC method for assay of amlodipine

Parameters	Optimized	used	Peak area ratio	Retention time (min)		% R.S.D.	Tailing factor
				Amlodipine	Atorvastatin		
Flow rate	**1.2 ml/min**	0.8	0.0141± 0.0002	2.65	7.12	1.42	1.68
		1.0	0.0140 ± 0.0001	2.2	6.7	0.71	1.64
		1.2	0.0139± 0.00021	2.25	6.93	1.5	1.31
Mobile phase composition	**ACN / KH_2PO_4**	45:55	0.0140 ± 0.0001	2.25	6.85	0.71	1.38
		50:50	0.0139 ± 0.0002	2.28	6.94	1.43	1.57
	(±5 %) 45:55	55:45	0.0140 ± 0.0001	2.31	7.01	0.71	1.92
Analyst	**50ng (n=7)**	First	49.03 ± 0.12	2.25	6.85	0.24	1.64
		Second	50.01± 0.13	2.31	6.86	0.26	1.39
		Third	49.3 ± 0.43	2.3	6.82	0.87	1.59

Column Temperature	**25^{0}C**	22^{0}C	0.0140 ± 0.0002	2.29	7.06	1.43	1.68
		25^{0}C	0.0139 ± 0.0002	2.25	6.93	1.43	1.43
		35^{0}C	0.0138± 0.00021	2.25	6.72	1.52	2.10
Wavelength	**240nm**	236	0.0141± 0.0002	2.25	6.85	1.42	1.63
		238	0.0139 ± 0.0003	2.31	6.86	2.16	1.56
		240	0.0140 ± 0.0001	2.3	6.82	0.71	1.58

Values are mean ±SD

3.7 Method repeatability

The repeatability of the developed method was evaluated by analyzing amlodipine in the dosage form after twelve months (Table 5). Precision measured as %RSD was less than 2% and accuracy measured as %bias (% relative error, RE) ranged from -1.66 to -0.08. There was no statistical difference between the 12 months evaluation and the initial evaluation ($p>0.05$).

Table 5: Evaluation of precision and accuracy of the HPLC method developed for the assay of amlodipine after 12 months.

Nominal conc. (ng/mL)	1st month evaluation			12th month evaluation		
	Conc. found [a] (ng/mL)	R.S.D. [b] (%)	Accuracy [c] (%)	Conc. found [a] (ng/mL)	R.S.D. [b] (%)	Accuracy [c] (%)
25	25.07±0.03	0.12	0.29	24.98±0.02	0.08	-0.08
50	50.03± 0.04	0.12	0.07	49.17±0.06	0.13	-1.66
70	69.16±0.03	0.04	-1.20	69.21±0.04	0.06	-1.13

Values are mean ±SD

3.8 Application of the proposed method to analysis of amlodipine in plasma and tablet dosage form

Four different brands of amlodipine tablets sold locally in pharmaceutical shop were randomly sampled and assayed in replicates using the developed HPLC method. The percentage purity was 100.10±0.17; 98.13±0.66; 98.04±0.85 and 99.47±1.10 for the Pfizer, Ever destiny, De sharp and Elbe pharma products respectively (Table 6). The percent recovery of amlodipine obtained from spiked plasma (Table 2) was good ranging from 97.79±0.17 to 99.52±0.63.

Table 6: Application of the developed HPLC method for the assay of amlodipine in dosage form

Drug	Label claim (mg/tablet)	Amount estimated (mg/tablet)	% purity
Pfizer Norvasc® BATCH NO: 00026288 NAFDAC NO: 04-5354	10	10.01 ± 0.06	100.10 ± 0.17
Ever destiny Pharmaceutical ltd (Amlodipine®) BATCH NO : GT19387 NAFDAC NO: B4-9888	5	9.81 ± 0.26	98.13 ± 0.66
De sharp Pharmaceutical ltd (Amlodipine®) BATCH NO: (10) ET-349 NAFDAC NO: C4-0331	10	9.80 ± 0.34	98.04 ± 0.85
Elbe pharma (Amlong ®) BATCH NO: ABGH0055 NAFDAC NO: A4 0445	10	9.94± 0.44	99.47 ± 1.10

Values are mean ±SD

4. Discussion

Several analytical methods have been developed for determination of amlodipine either in tablets or in biological samples with setbacks including, poor recovery, and delayed elution (long retention time), high thermal decomposition of the drug, high cost of analysis, use of sophisticated equipment and appreciably time consuming. Also validation of analytical technique is very crucial as it is aimed at confirming and obtaining evidences that the technique can give accurate, reliable and precise results that can be used for its intended purpose. The validation of our developed method was evaluated according to the International Conference of Harmonization (ICH) and USP guidelines[17,18].

The present study developed sensitive, precise, and reproducible RP HPLC–UV visible method for rapid determination of amlodipine in tablets and human plasma. Unlike the method of Alsarra[15], the present developed method is cost effective, not time consuming and uses little quantity of reagents. Wavelength of maximum absorption was 240 nm. The chromatographic conditions were optimized to give good resolution of peaks while preventing interference from internal standard and endogenous plasma components as well as excipients (Figs. 2a-g). In all, the HPLC method developed was specific for amlodipine; excipient, plasma components and atorvastatin did not interfere with the amlodipine peak as it showed single sharp peaks. Interchanging some analytical parameters did not affect the outcome of the results. In all parameters interchanged the % RSD of amlodipine was less than 3% (Table 4). These results showed no significant differences ($p>0.05$) between different interchanged parameters suggesting system suitability, robustness and method ruggedness.

Calibration curve which presents the relationship between concentrations of drug and the peak area ratio showed good linearity in the range of the concentrations studied with correlation coefficient (r^2) of 0.9998 for amlodipine in solvent and 0.9971 for amlodipine in plasma (Table 1). The result indicates that the method is sensitive. The number of theoretical plates which is an index that indicates column efficiency was good; evident by sharp peaks thus good resolution. The smaller the height equivalent to theoretical plate (HETP), the narrower the solute peak width and the more efficient the separation[19]. In this study, the HETP was less than 1 indicating high column efficiency which is not significantly different from the study of El-Gizawy[20]. The method developed detected the drug in solvent and plasma in nanogram concentration evident by the low LOD and LOQ values, which again is an indication of the sensitivity of the method. Precision and accuracy of the analytical method was determined by intra-day and inter-day

repeatability. Precision and accuracy of the method in solvent and plasma (Table 2) showed acceptable %RSD (less than 3%) and %RE values expressed as percentage bias (less than 2%). Amlodipine in stored plasma frozen at -80 °C was stable during the study period (Table 3) indicating that prolong storage and repeated freezing and thawing of the plasma did not alter the concentration of amlodipine in this study. These facts give evidence that the developed method is suitable for the assay of amlodipine in human plasma and their pharmaceutical dosage form.

Following our simple and rapid method for the determination of amlodipine, the percent recovery in the tablet dosage form and plasma were statistically significant ($p<0.05$) with a mean recovery rate of 97.79 ± 0.17 to 99.52 ± 0.63 % giving credence to the accuracy of the developed method. The lower the % RSD values, the more precise and reproducible the method. The assay performed in the different laboratory by different analysts, gave the % RSD of less than 1, indicating that the method is reproducible. The analytical technique developed was re-evaluated after 12 months (Table 6). The accuracy of the method, assessed as %RSD was less than 3% and comparable to the result obtain in the first month of evaluation. The result obtained confirms the suitability, repeatability of the analytical method developed.

The method developed was applied for the assay of amlodipine in spiked human plasma (Table 2 and Figs. 25a-d) and in tablet dosage form (Table 6). The % recovery in human plasma ranged from 97.79±0.17 to 99.52±0.63. The results obtained agreed with the labeled claim in each tablet and were statistically comparable to the United States Pharmacopeia specification[21]. This result indicates that the developed method can be employed in the determination of amlodipine in human plasma and pharmaceutical dosage form.

CONCLUSION

The RP-HPLC method offers several advantages compared to the previously reported methods using the liquid-liquid extraction technique. The method developed is sensitive, precise, accurate and cost effective. It allows for rapid quantitative assay of amlodipine besylate in human plasma and dosage form. This method may be applied in the evaluation of amlodipine concentration in human plasma samples obtained from a pharmacokinetic study

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COMPETING INTEREST

The authors declare no conflicts of interest.

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