Journal of Chromatography
Separation Techniques

Hafez et al., J Chromatograph Separat Techniq 2014, 5:3
DOI: 10.4172/2157-7064.1000226

Research Article Open Access

Quantitative Determination of Amlodipine Besylate, Losartan Potassium, Valsartan and Atorvastatin Calcium by HPLC in their Pharmaceutical Formulations

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Abstract

Amlodipine besylate is a calcium channel blocker which is used in treatment of hypertension alone or in combination with other antihypertensive drugs like angiotensin-II-receptor antagonists (ARA II) group (Losartan potassium and Valsartan) or in combination with anti hyperlipidemic agent like Atorvastatin calcium. RP- HPLC method was developed for the assay of these drugs. The method was performed by reversed phase high performance liquid chromatography using a mobile phase 0.01 M ammonium acetate buffer (pH 5.5): acetonitrile with detection at 240 nm on a spherical monomeric C18 column (250 mm × 4.6 mm, 5 µm) at flow rate of 1.5 ml/min. The proposed method was validated in terms of linearity ranged between [(2-12, 10-60, 16-96, 4-24 µg/ml) corresponding levels of 20-120% w/w of the nominal analytical concentration] with linear regression equations were [{y=64.627x – 3.6383 (r= 0.9998), y=75.385x – 8.3856 (r= 0.9997), y=64.492x – 25.981 (r= 0.9998), y=70.964x – 28.505 (r= 0.9998}], accuracy [100.18 ± 1.38, 100.79 ± 0.59, 100.45 ± 0.58 and 100.8 ± 1.69%], precision [99.29, 99.33, 99.30 and 99.30%], limits of detection [0.03, 0.18, 0.15, 0.007 µg/ml] and limits of quantitation [0.1, 0.54, 0.45, 0.024 µg/ml] for Amlodipine besylate, Losartan potassium, Valsartan and Atorvastatin calcium respectively. Method validation was developed following the recommendations for analytical method validation of International Conference on Harmonization (ICH) and Food and Drug Administration (FDA) organizations.

Keywords: HPLC; Amlodipine Besylate; Losartan potassium; Valsartan; Atorvastatin calcium

Introduction

Hypertension is the "silent killer" of humans because this disease is usually asymptomatic until the damaging effects of hypertension such as coronary heart disease and stroke. *Amlodipine besylate* is chemically described as (3-Ethyl 5-methyl (4RS)-2-[(2-aminoethoxy) methyl]-4-(2-chlorophenyl)-6-methyl-1, 4-dihydropyridine-3, 5-dicarboxylate benzene sulphonate). *Amlodipine besylate* is a calcium-channel blocking agent; a dihydropyridine derivative with an intrinsically long duration of action. *Amlodipine besylate* is an anti-hypertensive and an antianginal agent in the form of the besylate salt [1-3]. *Losartan Potassium* is chemically described as (1H-Imidazole-5-methanol, 2-butyl-4-chloro-1-[[2'-(lH-tetrazol-5-yl)[1,1'-biphenyl]-4-yl]methyl]monopotassiumsalt). *Losartan Potassium* is an angiotensin II receptor (type AT1) antagonist. It is indicated for the treatment of hypertension. It is indicated to reduce the risk of stroke in patients with hypertension and left ventricular hypertrophy [1-3]. Valsartan is chemically described as N-(1-oxopentyl)-N-[[2'-(1H-tetrazol-5-yl) [1,1'-biphenyl]-4-yl] methyl]-L-valine. Valsartan is an angiotensin II receptor antagonist with actions similar to those of Losartan. It is used in the management of hypertension to reduce cardiovascular mortality in patients with left ventricular dysfunction after myocardial infarction and in the management of heart failure [1-3]. *Atorvastatin calcium* is [R-(βR,δR)]-2-(4-fluorophenyl)-β, δ-dihydroxy-5-(1-methylethyl)-3-phenyl-4-[(phenylamino)carbonyl]-1Hpyrrole-1-heptanoic acid, calcium salt (2:1) trihydrate. *Atorvastatin calcium* is a synthetic lipid-lowering agent. Atorvastatin calcium is an inhibitor of 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase. This enzyme catalyzes the conversion of HMG-CoA to mevalonate, an early and rate-limiting step in cholesterol biosynthesis [1-3] (Figure 1).

The preparation of new combinations of drugs in pharmaceuticals for pharmacological activity development, as well as the requirements of modern industrial-scale pharmaceutical analysis, encourages researchers to develop new and efficient methods for multi-quantification with separation procedures. High performance liquid chromatography is a dominant separation technique, especially in pharmaceutical analysis [4]. So, it is necessary to develop a validated analytical method for assay of these drugs in combination with each other in its pharmaceutical preparations. Literature review revealed that USP described RP-HPLC methods for assay of Atorvastatin calcium, Losartan potassium and Valsartan individually and ion pair HPLC for *Amlodipine besylate* [2]. BP described a RP-HPLC method for assay of *Amlodipine besylate* and potentiometric titrations for assay of Atorvastatin calcium, Losartan potassium and Valsartan [3]. Several methods have been published for simultaneous determination of studied drugs in their combinations with each other, these methods depends on different analytical technique like Spectrophotometry [4-11], Spectrofluorimetry [12-14], Capillary Electrophoresis [15-18], HPTLC and TLC [19-21] and HPLC coupled with UV detector [21-29], fluorescence detector [30] and mass spectrometer detector [31-34]. Our scope is development of a validated analytical method for assay

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Received February 19, 2014; **Accepted** May 29, 2014; **Published** May 31, 2014

Citation: Hafez HM, Abdullah AE, Abdelaziz LM, Kamal MM (2014) Quantitative Determination of Amlodipine Besylate, Losartan Potassium, Valsartan and Atorvastatin Calcium by HPLC in their Pharmaceutical Formulations. J Chromatograph Separat Techniq 5: 226. doi:10.4172/2157-7064.1000226

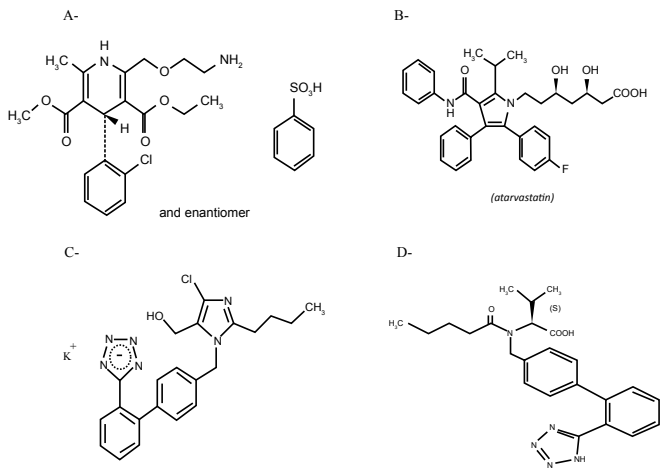

Figure 1: Chemical Structures of a- Amlodipine besylate b- Atorvastatin calcium c- Losartan potassium d- Valsartan

Amlodipine besylate is 3-Ethyl 5-methyl (4RS)-2-[(2-aminoethoxy) methyl]-4-(2-chlorophenyl)-6-methyl-1,4-dihydropyridine-3,5-dicarboxylate benzene sulphonate.

Atorvastatin calcium is R-(βR,δR)]-2-(4-fluorophenyl)-β,δ-dihydroxy-5-(1-methylethyl)-3-phenyl-4-[(phenylamino) carbonyl]-1Hpyrrole-1-heptanoic acid, calcium salt (2:1) trihydrate

Losartan Potassium is 1H-Imidazole-5-methanol,2-butyl-4-chloro-1-[[2'-(lH -tetrazol-5-yl) [1,1'-biphenyl]-4-yl] methyl] monopotassium salt.

Valsartan is N-(1-oxopentyl)-N-[[2′-(1H-tetrazol-5-yl)[1,1′-biphenyl]-4-yl] methyl]-L-valine.

of these drugs in combination with each other in its pharmaceutical preparations and it should be characterized by a simplicity, accuracy, preciseness and sensitivity.

Experimental

Instrumentation and chromatographic condition

Analysis was performed on a chromatographic system of AGILENT 1200 separation module connected to AGILENT 1200 Diode Array detector (DAD) detector. The system equipped by Agilent chemistation PC program. The chromatographic separation was achieved by injection of 50 µl of drugs sample solutions on Spherical monomeric C18 column (250×4.6 mm, 5µ) and the mobile phase is consisting of Ammonium acetate (pH 5.5, 0.01M) - acetonitrile (45:55, V/V) which pumped at a flow rate equals to 1.5 ml/min at 40°C and monitored at lambda=240 nm.

Ammonium acetate (0.01 M) was prepared by dissolving 0.77 g Ammonium acetate in approximately 950 ml distilled water. The pH was adjusted to 5.5 with glacial acetic acid. Water was added to 1000 ml. Mobile phase was filtered through a 0.45 µl Nylon membrane filter (Millipore, Milford, MA, USA) under vacuum and degassed by ultrasonication (Cole Palmer, Vernon Hills, USA) before usage.

Chemicals and reagents

All reagents used were of analytical grade or HPLC grade. Ammonium acetate, Glacial acetic acid and Sodium hydroxide (NaOH) were supplied by (Merck, Darmstadt, Germany), Acetonitrile and Methanol HPLC grade were supplied by (Fischer scientific, U.K.) and Distilled water. (Note: The water used in all the experiments was obtained from Milli-RO and Milli-Q systems (Millipore, Bedford, MA). *Amlodipine besylate* (99.8% as anhydrous), Losartan potassium (99.8% as is), Valsartan (99.3% as anhydrous) and Atorvastatin calcium (99.5% as anhydrous) working standard powders and all used excipients were kindly supplied by Egyptian international pharmaceutical industries company (EIPICO) (10th Ramadan, Egypt), and were used without further purification.

Pharmaceutical preparation

Exforge 10/160 tablets Novartis (Egypt) contain (10 mg Amlodipine as *Amlodipine besylate* and 160 mg Valsartan) per tablet B.NO: 10028/S0098. Caduet 5/10 tablets Pfizer Company (Egypt) contains (5 mg Amlodipine as *Amlodipine besylate* and 10 mg Atorvastatin as Atorvastatin calcium) per tablet B.NO: 0996099. Ator 10 tablets EIPICO (Egypt) contain (10 mg Atorvastatin as Atorvastatin calcium) per tablet B.NO: 1102999. Losarmepha tablets Mepha (Switzerland) contain 50 mg Losartan potassium per tablet B.NO:02862.

Preparation of stock standard solutions

Stock standard solutions containing (0.1, 0.5, 0.8, 0.2 mg/ml) of Amlodipine besylate, Losartan potassium, Valsartan and Atorvastatin calcium respectively were prepared by dissolving (5, 25, 40, 10 mg) of each in methanol in 50 ml volumetric flask respectively. It was then sonicated for 15 minutes and the final volume of solutions was made up to 50 ml with methanol to get stock standard solutions.

Preparation of calibration plot (working standard solutions)

To construct calibration plots, The stock standard solutions were diluted with the mobile phase to prepare working solutions in the concentration ranges (2-12, 10-60, 16-96, 4-24 µg/ml) for Amlodipine besylate, Losartan potassium, Valsartan and Atorvastatin calcium respectively. Each solution (n=5) was injected in triplicate and chromatographed under the mentioned conditions above. Linear relationships were obtained when average drug standard peak area were plotted against the corresponding concentrations for each drug. Regression equation was computed.

Sample preparation

Ten units of Exforge tablets, Caduet tablets, LosarMepha tablets and Ator tablets were prepared by grinding them to a fine, uniform size powder, triturated using mortar and pestle. After calculating the average tablet weight, amounts of powder equivalent to (10, 80, 20 and 50 mg) for *Amlodipine besylate* , Valsartan, Atorvastatin calcium and Losartan potassium of tablets were accurately weighed and transferred separately to 100 ml volumetric flasks respectively. Complete with methanol up to 100 ml. Solutions were sonicated for 15 min and the solutions were then filtered through 0.45 lm Nylon membrane filters (Millipore, Milford, MA, USA). Aliquots of appropriate volume (10 ml) were transferred to 100 ml calibrated flasks and diluted to volume with mobile phase to furnish the mentioned concentration above. The diluted solutions were analyzed under optimized chromatographic conditions and chromatogram is depicted in (Figure 2).

Results

Method validation

Selectivity: Specificity of the method was evaluated by assessing whether excipients present in the pharmaceutical formulations interfered with the analysis or not [35]. Inactive ingredients of studied tablets are Calcium Carbonate, Croscarmellose Sodium,

Microcrystalline Cellulose, Pregelatinized Starch, Polysorbate 80, Hydroxypropyl Cellulose, Colloidal Silicon Dioxide (anhydrous), Magnesium Stearate, titanium dioxide and talc. A placebo was prepared by mixing the respective excipients and solutions were prepared by

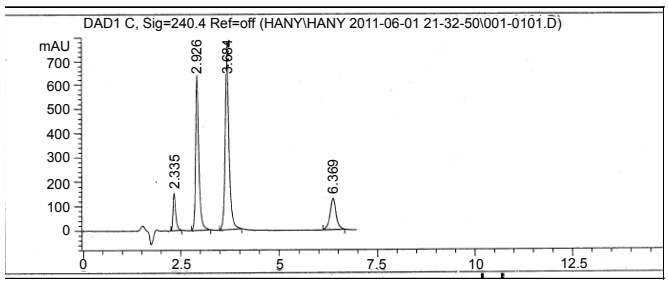

Figure 2: HPLC chromatogram of Amlodipine besylate (2.33 min), Losartan potassium(2.92 min), Valsartan (3.68 min) and Atorvastatin calcium (6.36 min) respectively on spherical monomeric C18 Column and mobile phase consisted of (A) acetonitrile and (B) acetate buffer pH=3.5 in ratio 55:45% at flow rate =1.5 ml/min by an isocratic technique.

Drug name	Average µg/ml	Average %	RSD
Amlodipine besylate	5.04	100.8	0.30%
Losartan potassium	49.60	99.20	0.70%
Valsartan	78.9	98.63	0.79%
Atorvastatin calcium	9.91	99.18	0.33%

Table 1: Repeatability of Amlodipine besylate, Losartan potassium, Valsartan and Atorvastatin calcium respectively.

Drug name	1st day µg/ml	2nd day µg/ml	3rd day µg/ml	pooled average	pooled average %	RSD
Amlodipine besylate	5.03	4.91	5.00	4.98	99.29	1.33%
Losartan potassium	49.60	49.21	50.18	49.66	99.33	0.98%
Valsartan	78.90	79.10	80.4	79.48	99.30	1.03%
Atorvastatin calcium	9.91	101.0	9.77	9.93	99.30	1.68%

Table 2: Intermediate precision Amlodipine besylate, Losartan potassium, Valsartan and Atorvastatin calcium respectively.

Drug name	Recovery at 80% conc. (%)	Recovery at 100% conc. (%)	Recovery at 120% conc. (%)	Average Recovery (%)	RSD
Amlodipine besylate	101.57	98.80	100.17	100.18	1.38%
Losartan potassium	99.91	99.68	99.87	100.79	0.59%
Valsartan	100.25	100.00	101.10	100.45	0.58%
Atorvastatin calcium	98.37	101.61	99.13	100.8	1.69%

Table 3: Recovery results for standard solution plus excipients for Amlodipine besylate, Losartan potassium, Valsartan and Atorvastatin calcium respectively.

following the procedure described in the section of sample preparation. The commonly used tablet excipients did not interfere with the method. The diluent chromatogram shows that the tablet diluent has negligible contribution after the void volume at the method detection wavelength of 240 nm.

Linearity and range: The linearity of the method was evaluated by analyzing different concentration of the drugs. According to ICH recommendations [35] at least five concentrations must be used. In this study five Concentrations were chosen, in the ranges (2-12, 10-60, 16-96, 4-24 µg/ml) corresponding levels of 20-120% w/w of the nominal analytical concentration for Amlodipine besylate, Losartan potassium, Valsartan and Atorvastatin calcium respectively. The linearity of peak area responses versus concentrations was demonstrated by linear least square regression analysis. The linear regression equations were {y=64.627x – 3.6383 (r=0.9998), y=75.385x – 8.3856 (r=0.9997), y=64.492x – 25.981 (r=0.9998), y=70.964x – 28.505(r=0.9998} for Amlodipine besylate, Losartan potassium, Valsartan and Atorvastatin calcium respectively. Where Y is the peak area of standard solution and X is the drug concentration.

Precision: According to ICH recommendations [35], The precision of the assay was investigated by measurement of both repeatability and Intermediate precision.

Repeatability: Repeatability was investigated by injecting 6 determinations at 100% of the test concentration percentage RSD were calculated in Table 1.

Intermediate precision: In the inter-day studies, standard and sample solutions prepared as described above, were analyzed in triplicate on three consecutive days at 100% of the test concentration and percentage RSD were calculated (Table 2).

Accuracy: According to ICH recommendations [35], Accuracy was assessed using 9 determinations over 3 concentration levels covering the specified range (80,100 and 120%). Accuracy was reported as percent recovery by the assay of known added amount of analyte in the sample (as standard addition method) (Table 3).

Limits of detection and Limits of quantitation: According to the ICH recommendations [35], Determination of limits of detection and quantitation was based on the standard deviation of the y-intercepts of regression lines and the slope of the calibration plots (Table 4).

Robustness: Robustness of an analytical procedure is a measure of its capacity to remain unaffected by small variations in method parameters and provides an indication of its reliability during normal usage [35]. Robustness was tested by studying the effect of changing mobile phase pH by ± 0.1, the amount of acetonitrile in the mobile phase by ± 2%, temperature ± 2°C, different column and flow rate ± 0.05 ml/min had no significant effect on the chromatographic resolution of the method.

Stability of analytical solution: Also as part of evaluation of robustness, solution stability was evaluated by monitoring the peak area response. Standard stock solutions in methanol were analyzed right after its preparation 1, 2 and 3 days after at 5°C and for a day at room temperature. The change in standard solution peak area response over 3 days was (1.67, 1.03, 1.10 and 1.56%) for Amlodipine Besylate, Losartan potassium, Valsartan and Atorvastatin calcium respectively. Their solutions were found to be stable for 3 days at 5°C and for a day at room temperature at least.

Item	Amlodipine besylate	Losartan potassium	Valsartan	Atorvastatin calcium
Linear range (µg/ml)	2-12	10-60	16-96	4-24
Detection limit (µg/ml)	0.03	0.18	0.15	0.007
Quantitation limit (µg/ml)	0.1	0.54	0.45	0.024
Regression data				
N	5	5	5	5
Slope (b)	64.62	75.39	64.49	70.96
Standard deviation of the slope	0.71	0.1	0.065	0.69
Intercept (a)	6.0	8.39	30.19	33.99
Standard deviation of the intercept	0.62	4.09	2.95	0.17
Correlation coefficient®	0.9998	0.9997	0.9998	0.9998
Standard error of regression	0.07	0.40	0.47	0.17

(Y = a + bC, where C is the concentration of the compound (µg/ml) and Y is the drug peak area

Table 4: Calibration data was resulted from method validation of Amlodipine besylate, Losartan potassium, Valsartan and Atorvastatin calcium respectively.

Drug name	Resolution	HETP	Capacity Factor	Tailing Factor
Amlodipine besylate	-	6070	1.12	0.65
Losartan potassium	4.61	7184	1.66	0.75
Valsartan	4.92	7534	2.35	0.8
Atorvastatin calcium	12.45	9564	4.79	0.95

Table 5: Chromatographic parameters for Amlodipine besylate, Losartan potassium, Vasartan and Atorvastatin calcium respectively.

Drug name		Recovery ± SD		Calculated t- values	Calculated F- values
		Proposed methods	Reference method		
Exforge®	Aml. (%)	95.81 ± 0.83	95.79 ± 0.65 [26]	0.06	1.64
	Val. (%)	97.46 ± 0.49	97.09 ± 0.51[26]	1.04	0.92
LosarMepha® Ator®	Los. (%)	101.15 ± 0.95	101.10 ± 0.45 [21]	0.11	4.54
	Ator. (%)	99.98 ± 1.35	100.65 ± 1.34 [21]	0.91	1.02
Caduet®	Aml. (%)	96.70 ± 0.61	96.68 ± 0.63 [22]	0.05	0.92
	Ator. (%)	96.41 ± 0.26	96.93 ± 0.36 [22]	2.26	0.54

(Where the Tabulated t-values and F-ratios at p = 0.05 are 2.57 and 5.05)
[21,22,26] are the reference numbers of reported method used in the comparison

Table 6: Statistical comparison of the proposed and published methods for determination of Amlodipine besylate, Losartan potassium, Valsartan and Atorvastatin calcium respectively in their dosage forms by reported method (T-student test) and (F–test for variance).

Discussion

Optimization of chromatographic condition

To establish and validate an accurate method for analysis of these drugs in pharmaceutical formulations, preliminary tests were performed with the objective of selecting optimum conditions. To reach our goal, BDS Hypersil column (25 cm) and Spherical monomeric C18 column (250 mm × 4.6 mm, 5 µm) were tried for simultaneous determination of the drugs. Spherical monomeric C18 column (25 cm) is less hydrophobic stationary phase so, it gave good separation between these drugs but drugs eluted very slowly especially Atorvastatin.

Acetonitrile was better than methanol in separation between all drugs, at higher percent (75% and 65%), interference between drugs and bad resolution obtained. At lower percent of acetonitrile (45% and 35%), atorvastatin eluted very lately. 55% of organic modifier is the most appropriate one. The effect of pH of aqueous mobile phase composition was also studied. Where at lower pH=3.5, all drugs except amlodipine eluted at higher retention time. After pH had been raised to 5.5 [greater than pka of ionizable drugs like Atorvastatin (4.5) and Valsartan (4.7)], they eluted faster due to containing of carboxylic acid group and Losartan (pKa=3.15) eluted faster due to presence of ionizable tetrazole group. Good separation between these drugs was achieved at pH=5.5. The optimum wavelength for detection was 240 nm at which much better detector responses for four drugs were obtained. The best resolution with reasonable retention time was obtained at 45% ammonium acetate and 55% acetonitrile as organic modifier. A major reason for using a concentration of 10 mM was achieving maximum sensitivity of UV detection at low wavelengths. After all previous trial had done, the most appropriated chromatographic condition was consisted of a mobile phase 0.01 M ammonium acetate buffer (pH 5.5): acetonitrile with detection at 240 nm on a spherical monomeric C18 column (250 mm × 4.6 mm, 5 µm) at flow rate of 1.5 ml/min. According to USP [2], system suitability tests are an integral part of an LC method. System suitability tests are used to verify that the Capacity Factor, Selectivity Factor (Resolution), Tailing Factor and Reproducibility of the chromatographic system are adequate for the analysis. System suitability tests were carried out on freshly prepared standard stock solutions of all drugs. The system was found to be suitable as shown in Table 5.

Application on pharmaceutical Preparation

The proposed methods were successfully used to determine Amlodipine besylate, Losartan potassium, Valsartan and Atorvastatin calcium respectively in their dosage forms e.g. Exforge tablets, LosarMepha tablets, Ator tablet and Caduet tablets respectively. Five replicate determinations were performed. Satisfactory results were obtained for each compound in good agreement with label claims. The results obtained were compared statistically with those from reported methods [21,22,26] by using Student's t-test and the variance ratio F-test. The results showed that the t and F values were smaller than the critical values. So, there were no significant differences between the results obtained from this method and published methods (Table 6).

Conclusion

A simple, accurate, precise, robust and reliable LC method has been established for simultaneous determination for Amlodipine besylate, Losartan potassium, Valsartan and Atorvastatin calcium in their formulations. The method has several advantages:

The first is using HPLC-UV which is the most available instrument in pharmaceutical analysis in companies with using of simple reversed phase mobile phase to lengthen column lifetime. High sensitive method has LOD range (0.007-0.18) µg/ml and LOQ range (0.024-0.54) µg/ml. Fast analysis of the four drugs had obtained, run time is less than 7 minutes, in addition to simplicity of sample preparation and extraction.

It is suitable for analysis of antihypertensive agents in their formulations in a single run, in contrast with previous methods. This makes the method suitable for routine analysis in quality-control laboratories.

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