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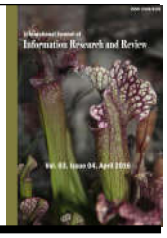

Research Article

DEVELOPMENT AND VALIDATION OF HPLC METHOD FOR SIMULTANEOUS ESTIMATON OF AMLODIPINE BESYLATE AND VALSARTAN

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ABSTRACT

A simple, sensitive, rapid and selective isocratic reversed phase High Performance Liquid Chromatographic method has been developed for simultaneous estimation of Amlodipine Besylate & Valsartan from pharmaceutical dosage form using a mobile phase consisting mixture of triethylamine Buffer: acetonitrile:Methanol (25:37.5:37.5), (pH adjusted to 3.0 using ortho phosphoric acid) at the flow rate of 1.0 mL/min. A Kromasil C-8 (Intersile250 x 4.6 mm,5 µm.) column was used as stationary phase. The retention time of Amlo and Hydro was 3.97 min. and 2.79 min. respectively. Linearity was observed in the concentration range of 10-80 µg/ml, The recovery studies ascertained the accuracy of the proposed method and the results were validated as per ICH guidelines. The eluent were detected at 230 nm. The Results were found to satisfactory and reproducible. The proposed method is precise, accurate, selective and rapid for the simultaneous determination of Amlodipine Besylate & Valsartan. The method can be used for routine analysis of these drugs in bulk and in formulation.

INTRODUCTION

Solubility: Solubility of Amlodipine besylate and Valsartan were performed in different solvents (*Table 1*). Table no. 1

Solubility of drugs

Solvent selection

Depending upon Solubility and stability two solvents were selected and made trials

Column selection

Mobile phase selection

Simultaneously number of mobile phases in different ratio was tried. Some of them are shown in (Table no.2)

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The mobile phase most suitable for analysis was Acetonitrile: Water 65: 35 and pH 3.0 adjusted of water with 0.1% Ortho Phosphoric acid as isocratic system flow rate employed for analysis was 1.0 ml/min.

Selection of separation variable Considering the theoretical information and after several trials separation variables were selected which were constant during whole experiment (Table 3).

System suitability parameters

Separation variables (Table 3) were set and mobile phase Acetonitrile: Water(65:35v/v) was allowed to saturate the column at flow rate 1.0 ml/min and a pH of water 3.0 adjusted with 0.1% Ortho Phosphoric acid to got sharp base line.

Six replicates of reference standard of Amlodipine besylate and Valsartan were injected. Peak report and column performance report were recorded for all chromatogram (Table 4. and Table 5).

Sr.No	Solvent	Solubility	
		Amlodipine besylate	Valsartan
1	Water	-Ve	-Ve
2	0.1 N HCl	-Ve	-Ve
3	Methanol	+Ve	+Ve
4	Acetonitrile	+Ve	+Ve
5	Ethyl alcohol	-Ve	+Ve
6	Ethyl acetate	-Ve	+Ve

Experimental	Observation	Inference
Methanol	Increased retention time	Not suitable
Acetonitrile	Less retention time	Suitable

Experimental	Observation	Inference
C18	Good resolution and sharp peak observed.	Suitable

Preparation of standard solution

For Amlodipine: 10 mg of Amlodipine was accurately weighed and transferred to a 10 ml volumetric flask and dissolved in diluent Acetonitrile (1000 µg/mL),taken 2.5mL further diluted to 25mL (Stock A; 100 µg/mL).

For Valsartan: 25 mg of Valsartan was accurately weighed and transferred to a 25 ml volumetric flask and dissolved in diluent Acetonitrile, (Stock B; 1000µg/mL).

For mix standard: From the stock solutions A, B, aliquots diluted up to 10 ml with acetonitrile to obtain the concentrations (Table no 6.).

Linearity and calibration graph

To establish the linearity, a series of dilution ranging from 2-10 µg/mL for Amlodipine and 64-320µg/mL for Valsartan in the same manner described in Table no.6. All the solutions were filtered through 0.22µm membrane filter and injected, chromatograms were recorded, and it was repeated for six times as in Table 7 and 8.

A calibration graph was plotted between the Area Under Curve (AUC) Vs respective concentration and regression equation was derived (fig.1.1, 1.2) Chromatogram of Amlodipine besylate and Valsartan observed at 1.995 min. at 4.945 min.

Table 2. Mobile phase selection

Mobile phase	Ratio V/V	Flow rate	Retention Time in min.		Conclusion
			Amlo	Val	
MeOH:H_2O	50:50	1ml/min	2.7	23	spliting of peak and elution time more
MeOH:H_2O	70:30	0.8ml/min	2.3	21	Elution time is slightly decrease and splitting increased
ACN: H_2O	50:50	1.0 ml/min	2.1	5.1	Fronting and not sharp peak of amlodipine.
ACN: H_2O	65:35	1.0 ml/min	2.09	4.98	Peak of amlodipine not sharp
ACN:H_2O pH2.98 using Ortho Phosphoric acid	65:35	1.0 ml/min	2.04	4.9	Peak sharp and suitable

Table 3. Selection of separation variable

Variable	Condition
Column	
Dimension.	250mm x 4.60mm
Particle Size	5µ
Bonded Phase	Octadecylsilane (C_{18})
Mobile Phase:-	
Acetonitrile (HPLC)	65%
Water(HPLC)	35%
Flow rate	1.0 ml/min
Temperature	Ambient
Sample Size	20µl
Detection wavelength	238nm
Retention time Amlodipine Besylate Valsartan	 2.04 ±0.01 min. 4.9 min.

Table 4. Result of system suitability parameters for Amlodipine besylate

System suitability Parameter →	RT	AUC	No. of theoretical plates	Tailing factor
Sample-1	2.08	194232	2104	1.14
Sample-2	2.09	201375	2121	1.15
Sample-3	2.06	193412	2130	1.12
Sample-4	2.05	192478	2150	1.14
Sample-5	2.04	198248	2098	1.13
Sample-6	2.06	200147	2132	1.11
Mean	2.06	196648.7	2122.5	1.13
S.D.	0.018	3764.178	19.22	0.014
% R.S.D.	0.87	1.91	0.9	1.23

Table 5. Result of system suitability parameters for Valsartan

System suitability Parameter →	RT	AUC	No. Of theoretical plates	Tailing factor
Rep-1	4.92	2498078	4221	1.36
Rep-2	4.91	2545406	4325	1.32
Rep-3	4.98	2590352	4247	1.35
Rep-4	4.95	2524517	4080	1.39
Rep-5	4.94	2548574	4274	1.36
Rep-6	4.95	2598541	4285	1.34
Mean	4.94	2550911	4238.667	1.35
S.D.	0.022	34995.11	85.32917	0.023
% R.S.D.	0.48	1.5	2.01	1.48

Table 6. Preparation of mix standard

Sr.No.	Concentration of amlodipine besylate (µg/mL)	Concentration of Valsartan (µg/mL)
1	2	64
2	4	128
3	6	192
4	8	256
5	10	320

Table 7. Linearity and calibration data of Amlodipine besylate

Conc. (µg/mL)	Area under Curve (AUC) (Replicates)						Mean
	Rep-1	Rep-2	Rep-3	Rep-4	Rep-5	Rep-6	
2	194232	193412	192478	202375	209031	190540	197011
4	387054	362102	386012	387058	394001	380351	382763
6	527712	526120	525897	521219	542000	536105	529842
8	698241	689874	665031	677952	691020	680851	683828
10	869472	856958	829601	824004	832052	863041	845855
r^2	0.9983	0.9998	0.9964	0.9977	0.9973	0.9979	0.9979
Slope	83083.35	82743.2	77663.25	76707.6	77153.05	82275.1	79937.6
Intercept	36842.1	29234	53824.3	62276	70702.5	36527	48234.3

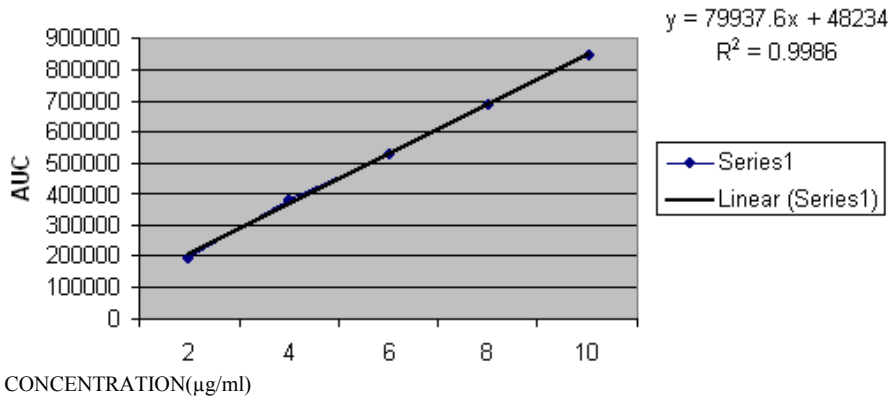

Fig.1..1 Calibration Graph of Amlodipine besylate

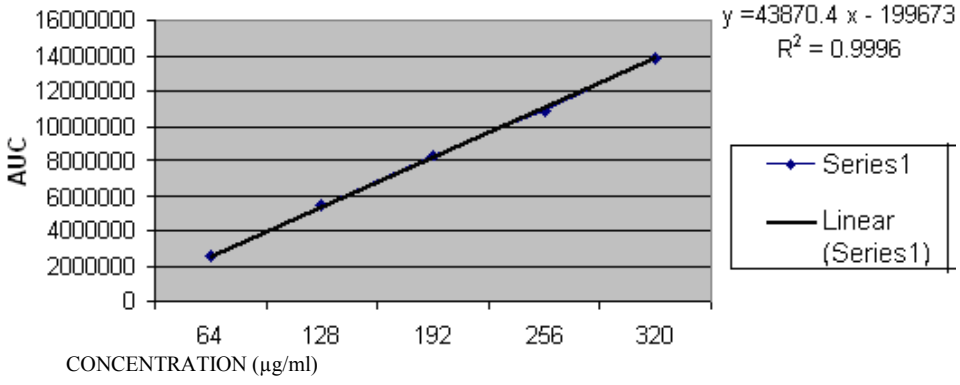

Fig.1.2. Calibration Graph of Valsartan.

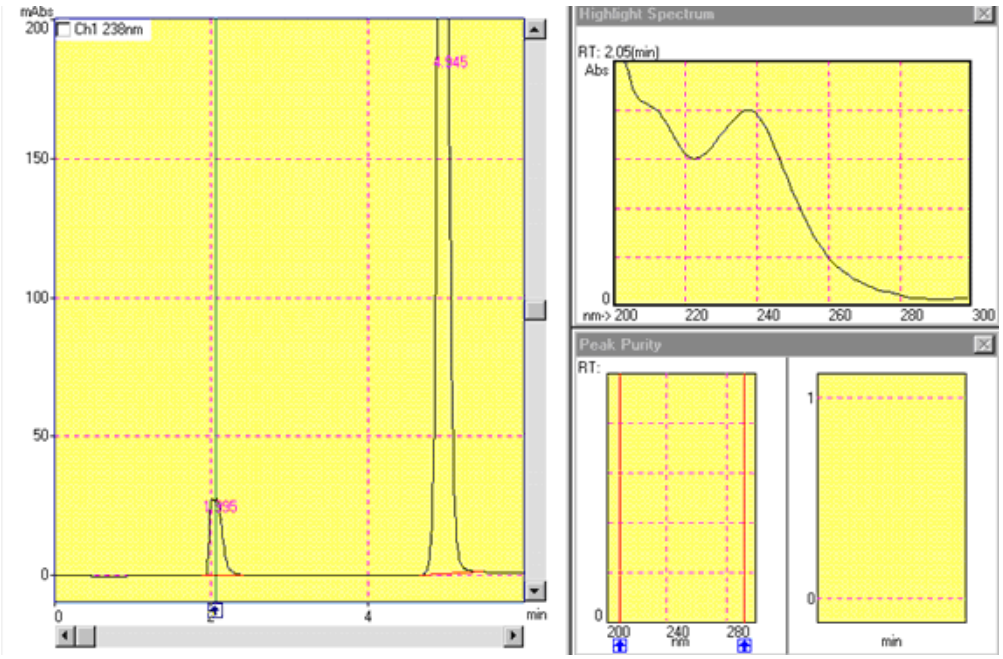

Fig.1.3 Chromatogram of Amlodipine Besylate and Valsartan, highlight spectrum of amlodipine

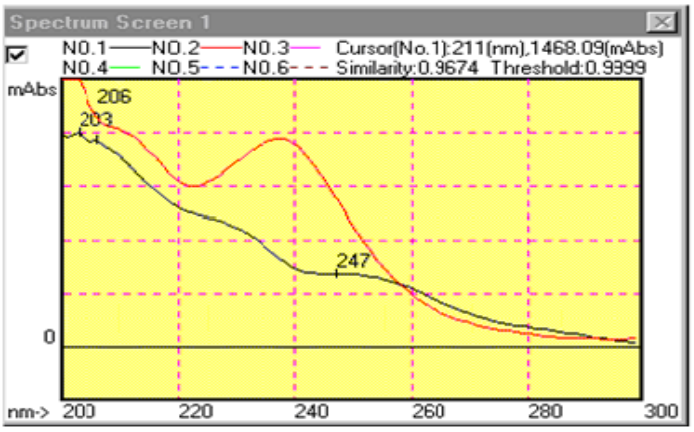

Fig.1.4 Overlay spectra of Amlodipine besylate and Valsartan

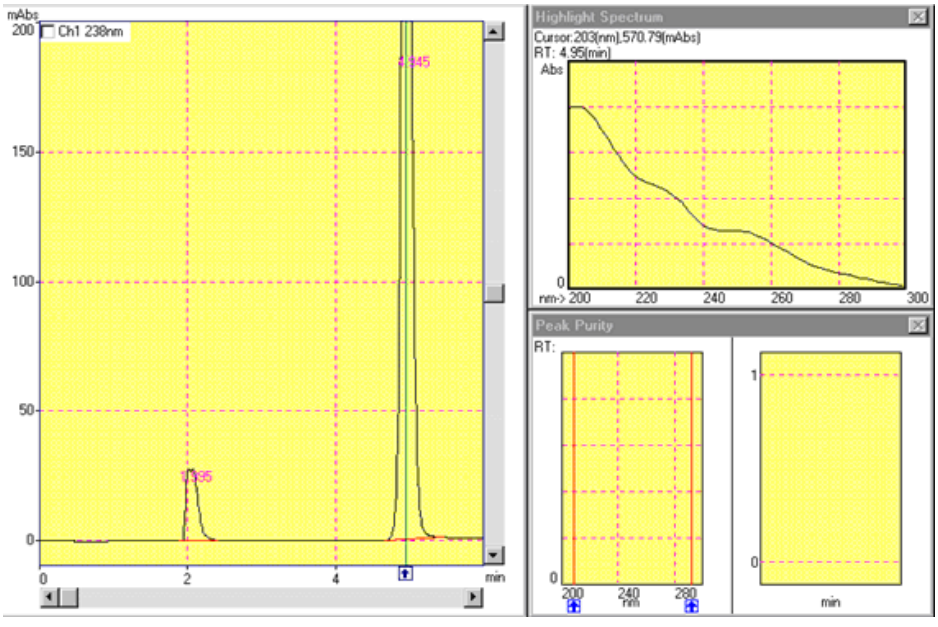

Fig.1.5 Chromatogram of Amlodipine besylate and Valsartan, highlight spectra of valsartan

Table no.8 Linearity and calibration data of Valsartan

Conc. (µg/mL)	Area under Curve (AUC) (Replicates)						Mean
	Rep-1	Rep-2	Rep-3	Rep-4	Rep-5	Rep-6	
64	2485776	2594423	2545406	2590352	2524517	2498544	2539836
128	5505944	5497147	5583385	5298547	5489658	5489571	5477375
192	8279891	8353296	8460969	8197584	8395836	8254923	8323750
256	10920858	10901918	10928452	10915729	10904839	10938725	10918420
320	13811779	13846814	13846841	13891169	13758915	13991542	13857843
r^2	0.9995	0.9995	0.9989	0.9998	0.9992	0.9995	0.9994
Slope	43854.5625	43608.677	43668.652	44091.9	43568.714	44429.922	43870.4
Intercept	-219226.4	-134146.3	-111370.5	-286968.6	-150440.1	-295884	-199673

Table 9. Response ratio data of Amlodipine besylate

Replicates	Concentration (µg/mL)	Mean AUC	Response Ratio
Rep-1	2	193412	96706
Rep-2	4	362102	90525.5
Rep-3	6	526120	87686.6
Rep-4	8	689874	86234.25
Rep-5	10	856958	85695.8
Mean of Response ratio			89369.63
S.D. of Response ratio			4508.841
%R.S.D. of Response ratio			5.04

Table no.10. Response ratio data of Valsartan

Replicates	Concentration (µg/mL)	Mean AUC	Response Ratio
Rep-1	64	2545406	39771.9
Rep-2	128	5583385	43620.1
Rep-3	192	8460969	44067.5
Rep-4	256	10928452	42689.2
Rep-5	320	13846841	43271.3
Mean of Response ratio			42684
S.D. of Response ratio			1704.086
%R.S.D. of Response ratio			3.9

Table no.11. Recovery data study for accuracy of Amlodipine besylate

Conc. Of drug in sample (µg/mL)	Std. drug sol. added (µg/mL)	Recovered amount (µg/mL) in replicates						% Mean recovered
		Rep-1	Rep-2	Rep-3	Rep-4	Rep-5	Rep-6	
1.81	2	1.99	1.98	1.89	1.88	1.89	1.95	96.5
4.93	5	4.95	4.88	4.92	4.82	4.89	4.84	97.84
25.89	6	5.98	5.85	5.67	5.49	5.79	5.94	96.44
						Mean		96.93
						S.D.		0.79
						%R.S.D.		0.82

Table no.12. Recovery data study for accuracy of Valsartan

Conc. Of drug in sample (µg/mL)	Std. drug sol. added (µg/mL)	Recovered amount (µg/mL) in replicates						% Mean recovered
		Rep-1	Rep-2	Rep-3	Rep-4	Rep-5	Rep-6	
63.81	64	63.45	63.91	63.98	63.78	62.99	63.87	99.48
127.43	128	127.8	127.76	127.88	127.98	127.79	127.99	99.9
159.27	160	158.59	158.89	159.39	159.79	158.99	159.49	99.5
						Mean		99.63
						S.D.		0.24
						%R.S.D.		0.24

Table no.13. Repeatability data for precision

Amlodipine				
Std. Conc	Concentration found (□g/mL) in replicates			
(□g/mL)	Rep-1	Rep-2	Rep-3	% Mean
2	1.73	2.17	2.07	99.5
4	4.15	3.89	3.78	98.5
6	5.97	6.21	5.89	100.3
8	7.79	7.99	8.41	100.7
10	9.94	10.12	10.18	100.8
			Mean	99.96
			S.D.	0.96
			%R.S.D.	0.96

The complete elution of both drugs was achieved in 7 min.at 238nm, resolution was 6.0.-8.0 and tailing is below than 1.5. Chromatogram, Overlay spectrum and 3D Cromatogram of Amlodipine besylate and Valsartan shown in Fig.1.3,1.4,1.6

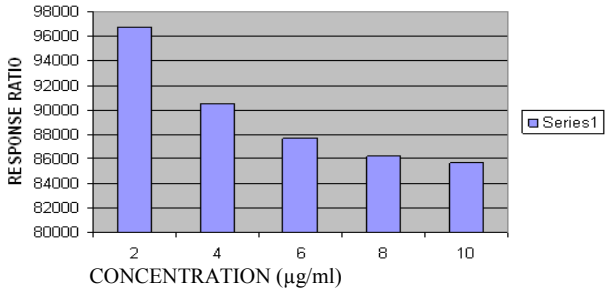

Fig.1.7. Graph of response ratio for Amlodipine besylate

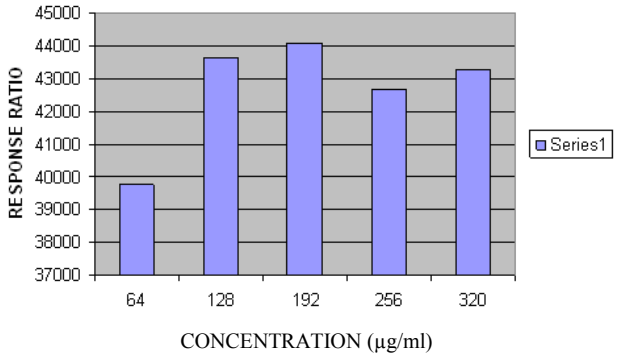

Fig.1.8. Graph of response ratio for Valsartan

Table no.14 Repeatability data for precision

Valsartan				
Std. Conc.	Concentration found (□g/mL) in replicates			
(□g/mL)	Rep-1	Rep-2	Rep-3	% Mean
64	64.5	63.5	64.01	100
128	128.2	127.4	128.3	99.97
192	192.5	193.4	191.8	100.3
256	257.5	256.1	255.8	100.2
320	320.2	319.4	319.4.	99.4
			Mean	99.974
			S.D.	0.35
			%R.S.D.	0.35

VALIDATION OF DEVELOPED METHOD

Linearity

From the mean of AUC observed and respective concentration value Table no.9 and 10 the response ratio (response factor) were found by dividing the AUC with respective concentration. The curve was plotted between response ratios vs. concentration (Fig 1.7 and 1.8)

Table no.15. Intermediate precision: day to day

Amlodipine			Valsartan		
Conc. (□g/mL)	Day-1	Day-2	Conc. (□g/mL)	Day-1	Day-2
2	98.21	99.87	64	101.64	99.21
4	96.13	101.18	128	99.83	98.49
6	100.5	100.15	192	97.66	101.61
8	100.9	99.91	256	99.84	100.94
10	100.1	100.95	320	98.8	99.72
Mean	99.17	100.41	Mean	99.74	99.99
Mean		99.79	Mean		99.865
S.D.		0.88	S.D.		0.18
%R.S.D.		0.88	%R.S.D.		0.18

Table no 16 Intermediate precision: analyst-to-analyst

Amlodipine			Valsartan		
Conc. (□g/mL)	A-1	A-2	Conc. (□g/mL)	A-1	A-2
2	98.28	99.81	64	99.87	100.21
4	96.98	100.18	128	98.83	99.49
6	100.7	100.01	192	98.66	100.61
8	100.99	99.5	256	100.84	98.94
10	100.6	100.4	320	98.1	99.92
Mean	99.51	99.98	Mean	99.26	99.83
Mean		99.74	Mean		99.46
S.D.		0.33	S.D.		0.4
%R.S.D.		0.33	%R.S.D.		0.4

Precision

Repeatability

As per mix standard of Amlodipine besylate and Valsartan were prepared and analyzed in triplicates for repeatability in same day and statistical validation is carried out (Table no.13 and 14)

Intermediate precision: As per mix standard dilutions were prepared and its analysis was carried out in different days in different concentration and analyst to analyst intermediate precision done. The results were validated statistically (Table no.15 Table.16.).

Robustness: As per ICH guidelines, small, but deliberate variations in concentration of the mobile phase were made to check the method's capacity to remain unaffected. The ratio of mobile phase was acetonitrile: Water 65:35(V/V) adjusted pH 3.0 with 0.1% ortho phosphoric acid change in ratios of mobile phase from 65:35(V/V) to 66:34(V/V) and 64:36(V/V) for robustness. The changes in mobile phase concentration shown in Table no.17.

Table no.17 Change in ratio of mobile phase

Amlodipine			Valsartan		
Conc. (µg/mL)	Ratio 66:34	Ratio 64:36	Conc. (µg/mL)	Ratio 66:34	Ratio 64:36
6	98.01	98.67	192	101.17	105.65
6	100.65	101.49	192	105.95	105.95
6	100.97	102.25	192	106.12	103.44
6	101.42	101.43	192	106.62	103.91
6	101.44	101.44	192	105.83	103.46
Mean	100.50	101.05	Mean	105.14	104.48
Mean		100.78	Mean		104.81
S.D.		0.39	S.D.		0.47
%R.S.D.		0.39	%R.S.D.		0.45

RESULTS AND DISCUSSION

The objective of present work was to develop an analytical method for simultaneous estimation of Amlodipine besylate and Valsartan and its validation. HPLC determination of the drugs was carried out by maintaining optimized chromatographic conditions throughout the method. These conditions are as follows:

- Column- Inertsil (250mm x 4.60mm), partical size 5µ
- Detection wavelength- 238 nm
- Temperature- Ambient
- Injection volume- 20 ml
- Mobile phase- Acetonitrile:Water (65:35) pH 3.0 by Orthophosporic acid of water

- Flow rate (mL/min)- 1.0ml/min
- Diluents -100% Acetonitrile

For estimation of Amlodipine besylate and Valsartan series of mixed standards were prepared in different concentration range 2-64, 5-160and 6-128 µg/mL respectively. The calibration curve was plotted between concentration and AUC observe at the selected wavelength 238 nm and concentration of drugs. The concentration of drugs in the tablet was found by using slope and 'Y' intercept of linearity curve. Validation challenges the performance of analytical procedure as intended; it means that a method shows reproducibility when carried out by different persons, in same or different laboratories, using different reagents,etc. The various parameters, which are necessary to validate method, are

- Linearity
- Range
- Accuracy
- Precision (Repeatability Intermediate precision and Reproducibility)
- Robustness
- Specificity

Results of the developed method using the validation parameters

The developed method was validated and results with respect to the various validation parameters are given below:

Linearity: The results of linearity analysis indicates that the drug components are linear with respect to the concentration range; obtained in the Table no.18

Table no.18. Statistical data for linearity

Data for linearity	Amlodipine besylate	Valsartan
Correlation Coefficient (r^2)	0.997	0.994
Slope (m)	79937.6	43870.4
Y-Intercept	48234.3	-199672.7
Linearity rang (µg/mL)	2-10	64-320

Accuracy

Accuracy for the developed method studied and results shows that the percent recovery was found within the limit, percent relative standard deviation shown in Table no.19

Table 19. Statistical data for accuracy (recovery)

Statistical data	Amlodipine besylate	Valsartan
% Mean	96.93	99.63
SD	0.79	0.24
%R.S.D.	0.82	0.24

Precision

Precision was also determined to assure the repeatability of the method. The R.S.D. was found less than 2.0% which lies within the limit shown in Table no.20

Table 20. Statistical data for precision

Statistical parameter	Amlodipine besylate		Valsartan	
	SD	%RSD	SD	%RSD
Repeatability	0.96	0.96	0.35	0.35
Intermediate Precision				
a. Day to day	0.8	0.8	0.18	0.18
b. Analyst to Analyst	0.33	0.33	0.4	0.4

Robustness

As per ICH guidelines, small, but deliberate variations in concentration of the mobile phase were made to check the method's capacity to remain unaffected. The ratio of mobile phase was Acetonitrile: Water (pH 3.0 adjusted with 0.1% ortho phosphoric acid) 65:35. changes in ratios of mobile phase from 65:35(v/v) to 64:36(v/v) and 66:34 (v/v)for robustness. Robust as RSD is found Less than 2.0% shown in Table 21.

Table no.21 Statistical data for robustness

Parameter	Amlodipine besylate	Valsartan
Change in ratio of mobile phase		
SD	0.39	0.47
%RSD	0.39	0.45

RESULTS OF TABLET ANALYSIS

Simultaneous estimation of the marketed formulation of Amlodipine besylate and Valsartan. The results of tablet analysis are given in the Table no.22.

Table no. 22. Results of tablet analysis

Parameter	Amlodipine besylate	Valsartan
%mean	100.134	99.986
SD	0.4	0.099
%RSD	0.34	0.09

DISCUSSION

The developed method can be used for routine analysis because the linearity found of Amlodipine besylate and Valsartan is nearby to 1 i.e. not a single component lies below 0.99 The system suitability parameters are concern the %RSD of each parameter lies below the limit of 2% as per the ICH guidelines. Good recovery obtained by this developed method and the "% relative standard deviation" for each component is not more than 2%.and method can be used for the routine analysis. The temperature of working area, pH of water used in mobile phase, sudden fluctuation in pressure (kgf) of any one pump of HPLC, back pressure on pump, unadiquate saturation of mobile phase in C18 column (phenomenex) will affect the system suitability.

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