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A NEW IMPROVED RP-HPLC METHOD FOR SIMULTANEOUS ESTIMATION OF HYDROCHLOROTHIAZIDE, AMLODIPINE BESYLATE AND VALSARTAN IN BULK AND DOSAGE FORM

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Symmetry C_{18} column, Symmetry $C_{18,}$ Reverse phase, Phosphate buffer

ABSTRACT

Objective: To develop a simple, novel, sensitive, precise and specific RP-HPLC method for the determination of Hydrochlorothiazide, Amlodipine besylate and Valsartan in pure drug and pharmaceutical dosage forms.

Methods: The chromatographic separation was achieved with Symmetry C_{18} (4.6 x 100 mm, 3.5µm) column as stationary phase using a mixture of phosphate buffer and acetonitrile (42:58 v/v) as mobile phase. The flow rate was 0.5 ml/ min and the column was operated at ambient temperature (~25°C). The volume of sample injected was 20 µL and UV detection was made at 240nm wavelength. Prior to injection of the solutions, column was equilibrated for at least 30min with mobile phase flowing through the system. **Results:** The calibration curves for Hydrochlorothiazide, Amlodipine besylate and Valsartan was found to be linear at 12.5 – 62.5µg/ml, 5 - 25 µg/ml and 80 – 400 µg/ml respectively. The correlation coefficient (r^2) value was found to be 0.9994. Precision study showed % RSD values are less than 2% in all selected concentrations. The % recoveries of Hydrochlorothiazide, Amlodipine besylate and Valsartan were in the range of 99.8 -100.90% 99.8 – 100.8% and 99.8 – 101.5% respectively. System suitability parameters remained unchanged when there is a slight change in flow rate and mobile phase composition. **Conclusion:** The developed method had good sensitivity, reproducibility and specificity for the simultaneous determination of Hydrochlorothiazide, Amlodipine besylate and Valsartan in bulk and its tablet dosage forms. This method was simple, fast, accurate, and precise. Hence this method was validated and found to be suitable for determining the purity of Hydrochlorothiazide, Amlodipine besylate and Valsartan in bulk drugs and pharmaceutical formulations. The proposed validated method was successfully used for the quantitative analysis of commercially available dosage form.

INTRODUCTION:

Hydrochlorothiazide is frequently used for the treatment of hypertension, congestive heart failure. Hydrochlorothiazide belongs to thiazide class of diuretics. It reduces blood volume by acting on the kidneys to reduce sodium (Na) reabsorption in the distal convoluted tubule [1]. Amlodipine is used in the management of hypertension and coronary artery disease. Amlodipine is a long acting 1, 4-dihydropyridine calcium channel blocker. Amlodipine is a peripheral arterial vasodilator that acts directly on vascular smooth muscle [2].

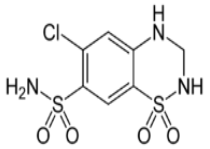


Fig no: 1. Structure of Hydrochlorothiazide

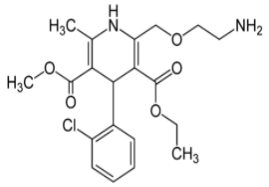


Fig no: 2. Structure of Amlodipine Besylate

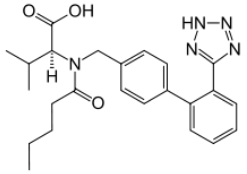


Fig no: 3. Structure of Valsartan.

Valsartan is used to treat uncomplicated hypertension, isolated systolic hypertension and left ventricular hypertrophy. Valsartan is an ARB that selectively inhibits the binding of angiotensin II to AT1 which is found in many tissues such as vascular smooth muscle and the adrenal glands [3]. A survey of literature reveals that good analytical methods are not available for the drugs like Hydrochlorothiazide, Amlodipine besylate and Valsartan.

Even though very few methods of estimation of above drugs are available, many of them suffer from one disadvantage or the other, such as low sensitivity, lack of selectivity and simplicity etc. Our method had better peak response and more number of theoretical plates for Amlodipine and hydrochlorothiazide with provided data which lacks in the method developed by Bodduluri Anil Kumar et al.[4] and better retention time for Amlodipine which is a bit high in the method developed by Younus, Mohammad et al. Hence it was proposed to improve the existing methods and to develop new methods for the assay of Hydrochlorothiazide, Amlodipine besylate and Valsartan in pharmaceutical dosage forms adapting different available analytical techniques like HPLC [5].

2. MATERIALS AND METHODS:

2.1. Chemicals and reagents:

The working standards of Hydrochlorothiazide, Amlodipine besylate and Valsartan were from Abbott and Novonordisk. Exforge HCT was the dosage form available in local medical shop at Hyderabad was used as test for analysis. Potassium di- Hydrogen Ortho Phosphate, Ortho-Phosphoric Acid, Ammonium Acetate, Ammonium Acetate etc... were from Merck and AR grade. Methanol, Acetonitrile and Water were HPLC grade from Merck and Lobachemi. Filter Paper 0.45 microns was purchased from Millipore Company.

2.2. Instrumentation

UV-3000$^+$ LABINDIA Double beam with UV win 5 software UV-Visible spectrophotometer with 1cm matched quartz cells [1]. WATERS HPLC, Model: Aliance 2695, UV- Visible Dual absorbance Detector 2487, with an automated sample injector. The output signal was monitored and integrated using Empower 2 software. A Symmetry XTerra C18 (4.6 x 100mm, 5 µm, Make: Waters) and XBridge C18 (4.6 x 100mm, 3.5 µm, Make: Waters) column was used for separations.

2.3. Chromatographic Conditions

The elution was isocratic and the mobile phase consisted of a mixture of buffer and acetonitrile (42:58 v/v). The buffer was prepared by dissolving 17.418g of potassium dihydrogen phosphate in 1000 ml water adjusted with ortho phosphoric acid to pH 4.0 $\pm$ 0.1. The buffer was filtered through a 0.45µ (MILLIPORE, Germany) membrane filter. The mobile phase was also filtered through a 0.45-µ (MILLIPORE, Germany) membrane filter prior to use. A Symmetry C_{18} (4.6 x 100 mm, 3.5µm) column was used for determination. The flow rate was 0.5 ml/ min and the column was operated at ambient temperature (~25^{o}C). The volume of sample injected was 20 µL. Prior to injection of the solutions, column was equilibrated for at least 30min with mobile phase flowing through the system. The UV detector was set at wavelength of 240 nm. The Run Time was 15 minutes.

2.4. Preparation of standard stock solution:

Stock solution of Hydrochlorothiazide, Amlodipine besylate and Valsartan was prepared by dissolving 25mg of Hydrochlorothiazide, 10mg of Amlodipine besylate and 160mg of Valsartan in 50 ml volumetric flask with few ml of methanol. Sonicated it for about 30minutes and made upto final volume with methanol. From this, pipette out 7.5ml of stock solution in to 100ml volumetric flask and made upto final volume with mobile phase to attain the concentration of 37.5µg/ml Hydrochlorothiazide, 15µg/ml Amlodipine Besylate, 240µg/ml Valsartan. Inject 20µL of this standard preparation in to HPLC system as per proposed optimized conditions and chromatogram was recorded.

2.5. Sample Preparation (Assay):

Twenty tablets were taken and their average weight was calculated. Tablets were crushed to a fine powder and dose equivalent to 25mg of Hydrochlorothiazide, 10mg of Amlodipine besylate, 160mg of Valsartan was transferred to a 50 ml volumetric flask, dissolved and made up to final volume with methanol. From the above solution, 7.5ml was pipetted out in to 100ml volumetric flask and made upto final volume with mobile phase. This solution was filtered through 0.45 µ membrane filters to get concentration of 37.5µg/ml of Hydrochlorothiazide, 15µg/ml of Amlodipine besylate, 240µg/ml of Valsartan. Inject 20µL of this sample preparation in to HPLC system as per proposed optimized conditions and chromatogram was recorded. Injected 20µL of this sample prepared in to HPLC system as per proposed optimized conditions and chromatogram was recorded.

3. METHOD VALIDATION:

3.1. Specificity:

Specificity is the ability of a method to discriminate between the analyte(s) of interest and other components that are present in the sample. Studies are designed to evaluate the degree of interference, if any, which can be attributed to other analytes, impurities, degradation products, reagent "blanks" and excipients.. This provides the analyst with a degree of certainty that the response observed is due to the single analyte of interest. The degree of specificity testing varies depending on the method type and the stage of validation[6]. Specificity should be evaluated continually through the drug development process.

Acceptance criteria: Chromatogram should not show any peak at the retention times of amlodipine, hydrochlorothiazide and vasartan.

3.2. Linearity: The linearity of the calibration curve for Hydrochlorothiazide and Amlodipine besylate, Valsartan were calculated and constructed by plotting the mean peak area *versus* concentration. The correlation coefficients of regression r^2 = 0.9999, 0.9998 and 0.9992 respectively.

3.3. Precision:

Precision is the degree of agreement among individual test results when the method is applied repeatedly to multiple samplings of a homogenous sample [7]. Precision of an analytical method is usually expressed as the standard deviation or relative standard deviation (coefficient of variation) of a series of measurements. Precision of the assay was determined by repeatability (intraday) and intermediate precision (inter-day) for 3 consecutive days. Six sample solutions of HCTZ, AMLO and VAL were prepared and injected into the HPLC system. We determined system and method precisions.

Acceptance Criteria: The % RSD for the area of five sample injections results should not be more than 2.

3.4. Accuracy:

Accuracy was performed in triplicate for various concentrations of Hydrochlorothiazide, Amlodipine besylate, Valsartan equivalent to 50%, 100% and 150% of the standard amounts were injected into the HPLC system. The average %

recovery of Amlodipine besylate, Valsartan, Hydrochlorothiazide was calculated.

3.5. Robustness:

Robustness was done by small deliberate changes in the chromatographic conditions [8] and retention times of Amlodipine besylate, Valsartan, Hydrochlorothiazide were noted. The factors selected were flow rate and variation in the mobile phase composition. The results remained unaffected by small variations in these parameters.

3.6. Solution Stability:

The stability of the diluents used had to be assessed to make sure if any degradants or impurities are produced during the development process [9]. If so they may alter retention times and recoveries of the ingredients. So the stability of solution is assessed after 24 hrs. The evaluation determines the period of time a solution can be held before analysis without compromising with accuracy.

3.7. Intermediate Precision (Ruggedness):

The extent to which intermediate precision should be established depends on the circumstances under which the procedure is intended to be used. Intermediate precision expresses within laboratory variations: different days, different analysts, different equipment, different columns, etc.[10]The procedure followed for assay method in method precision was repeated on two different days, by two analysts, using two different columns and using different HPLC systems. The results for the Intermediate precision are recorded.

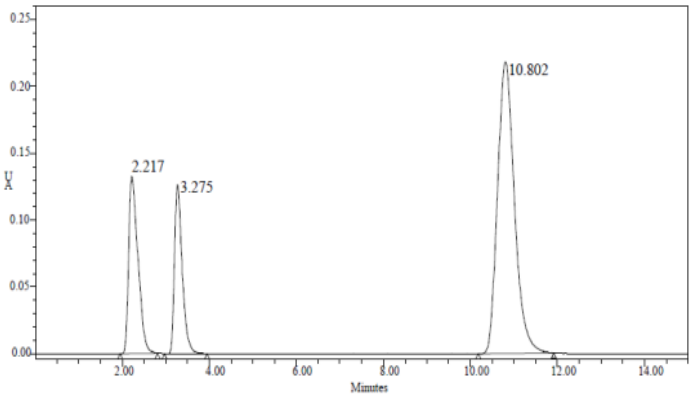
Acceptance Criteria: The relative standard deviation for the assay preparations was not more than 2.0%.

4. RESULTS AND DISCUSSION:

4.1. Method Development: The objective of this experiment was to optimize the method for simultaneous estimation of Hydrochlorothiazide, Amlodipine besylate and Valsartan based on the literature survey made. Following is the best of all trails done.

4.2. Optimized Chromatographic conditions:

The elution was isocratic and the mobile phase consisted of a mixture of buffer and acetonitrile (42:58 v/v). The buffer was prepared by dissolving 17.418g of potassium dihydrogen phosphate in 1000 ml water adjusted with ortho phosphoric acid to pH 4.0 $\pm$ 0.1. The buffer was filtered through a 0.45µ (MILLIPORE, Germany) membrane filter. The mobile phase was also filtered through a 0.45-µ (MILLIPORE, Germany) membrane filter prior to use. A Symmetry C_{18} (4.6 x 100 mm, 3.5µm) column was used for determination. The flow rate was 0.5 ml/ min and the column was operated at ambient temperature (~25ºC). The volume of sample injected was 20 µL. Prior to injection of the solutions, column was equilibrated for at least 30min with mobile phase flowing through the system. The UV detector was set at wavelength of 240 nm.



RT- 2.22 - HCTZ

RT- 3.27 - AMLO

RT- 10.82 - VAL

Fig.4. Showing optimized chromatogram

Observation: Good separation and resolution was observed. Tailing was observed 1.6, 1.5 and 1.2 within the limits.

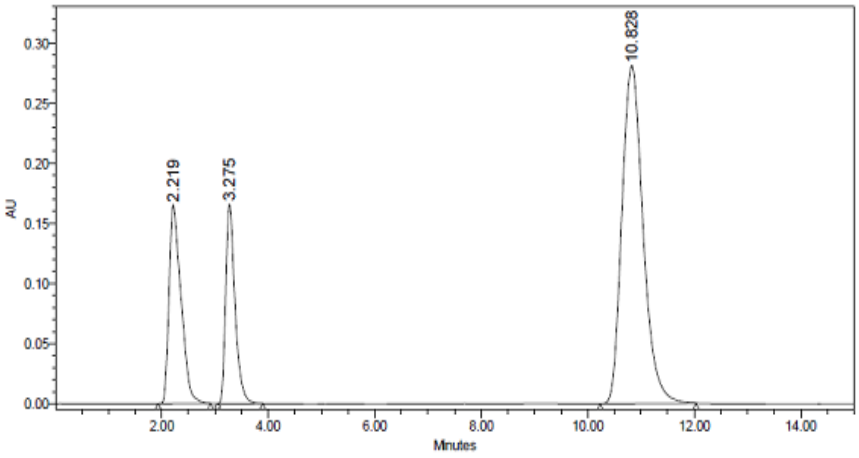


Fig no. 5. Chromatogram of standard HCT, Amlo and Val

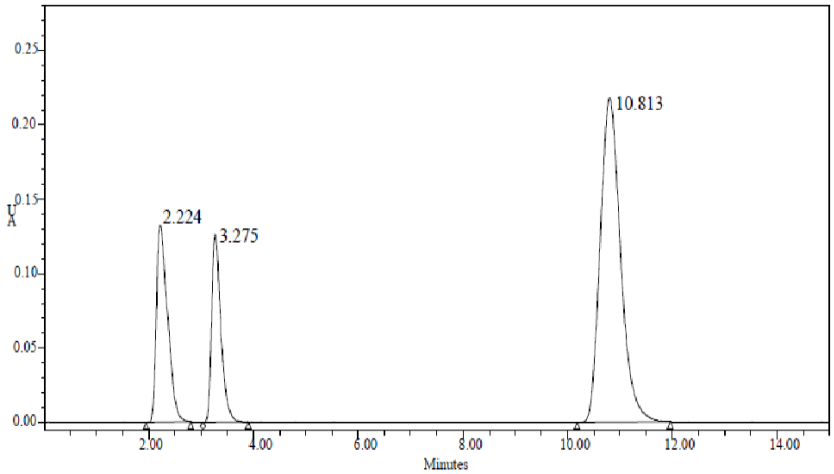


Fig no. 6. Chromatogram of formulation HCT, Amlo and Val

4.3.1. System Suitability:

Table no.1. Showing system suitability of Hydrochlorothiazide, Amlodipine besylate, Valsartan.

Injection	Hydrochlorothiazide area	Amlodipine area	Valsartan area
Injection1	1961421	1536781	5887751
Injection2	1965272	1538201	5880942
Injection3	1960978	1530754	5882176
Injection4	1959521	1528124	5878671
Injection5	1969720	1521658	5889024
Average	1963382	1533104	5883713
Standard Deviation	4131.416	4240.093	4471.43
% RSD	0.210	0.276	0.075
Theoretical plates	4449	4314	5740
Tailing factor	1.6	1.5	1.2

Observation: The chromatographic parameters such as number of theoretical plates and tailing factors were calculated and are in limits.

4.3.2. Specificity:

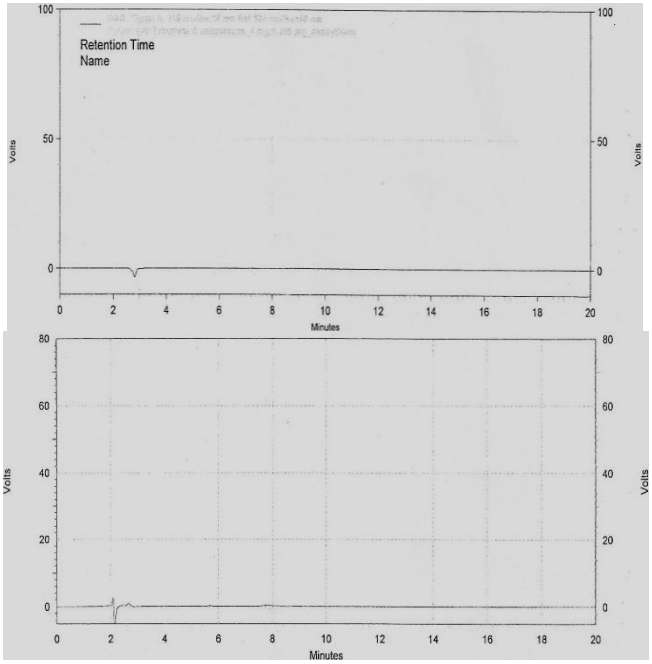


Fig.7. Blank Chromatogram & Fig .8. Chromatogram showing no interferences.

Linearity of Hydrochlorothiazide:

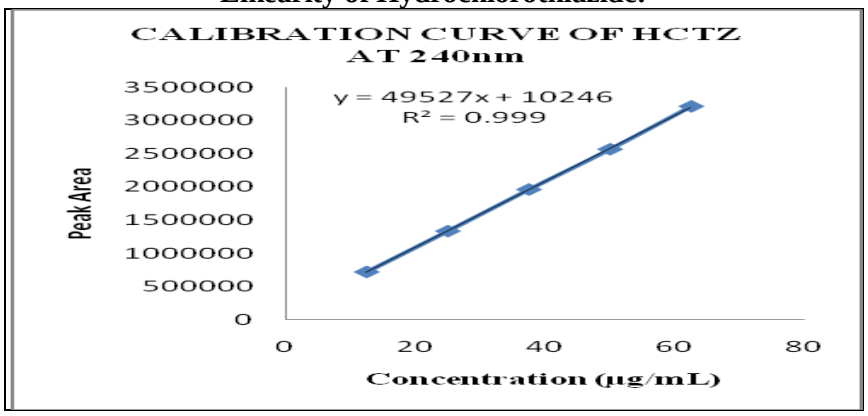


Fig.9. Linearity plot of Hydrochlorothiazide.

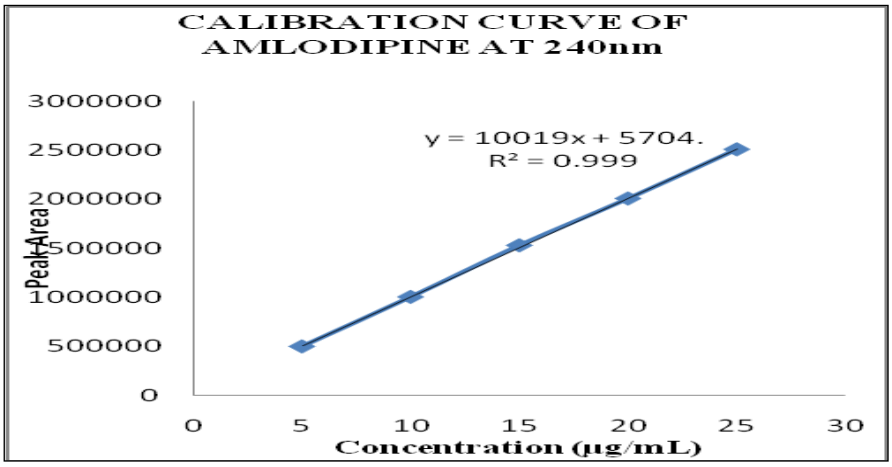


Fig.10. Linearity plot of Amlodipine.

S.No	Linearity Level	Concentration	Area
1	I	12.5ppm	726059
2	II	25ppm	1337825
3	III	37.5 ppm	1961654
4	IV	50 ppm	2565253
5	V	62.5 ppm	3207761
Correlation Coefficient			0.999

Table.2. Showing linearity data of HCT.

S.no	Linearity Level	Concentration	Area
1	I	5 ppm	500508
2	II	10 ppm	1003914
3	III	15 ppm	1528727
4	IV	20 ppm	2004809
5	V	25 ppm	2504809
Correlation Coefficient			0.999

Table.3. Showing linearity data of Amlodipine.

S.no	Linearity Level	Concentration	Area
1	I	80ppm	2349577
2	II	160ppm	4007422
3	III	240ppm	5904625
4	IV	320ppm	7747436
5	V	400ppm	9725525
Correlation Coefficient			0.999

Table.4. Showing linearity data of valsartan.

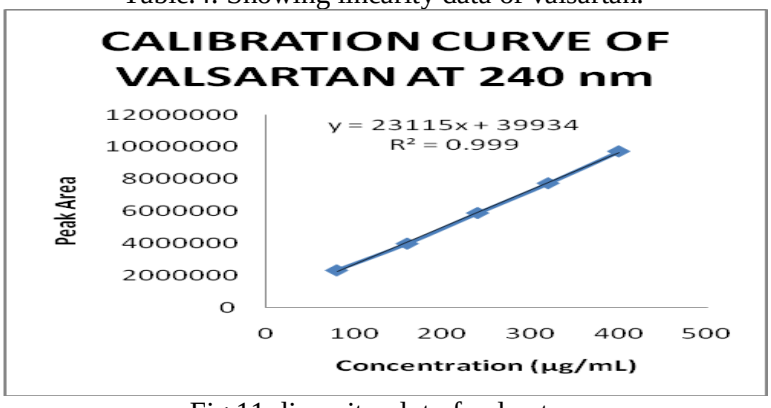


Fig.11. linearity plot of valsartan

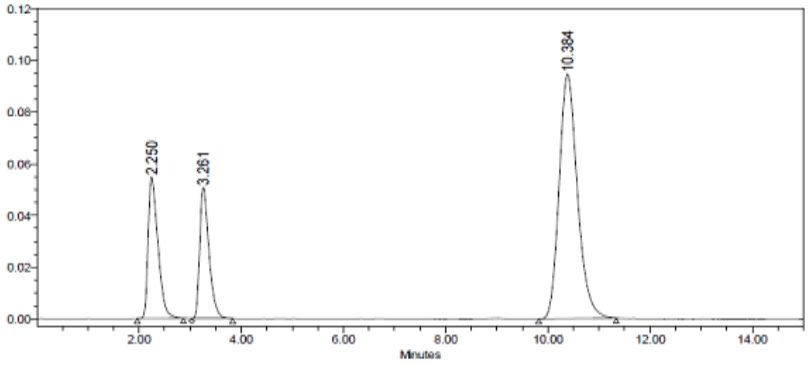


Fig no.12. Chromatogram showing solution stability.

4.3.4. Precision:

4.3.4.1. System Precision: Table.5. Showing system precision data:

Injection	Hydrochlorothiazide area	Amlodipine area	Valsartan area
Injection1	1961421	1536781	5887751
Injection2	1965272	1538201	5880942
Injection3	1960978	1530754	5882176
Injection4	1959521	1528124	5878671
Injection5	1969720	1521658	5889024
Injection6	1960971	1530750	5882177
Average	1962981	1531045	5883457
Std Deviation	3824.14	6015.48	4048.22
% RSD	0.19	0.39	0.07

Observation: The % RSD for the area of five standard injections results are found to be 0.210, 0.276, 0.075 and they were in limits.

4.3.4.2. Method Precision: Table 6. Showing method precision data:

INJECTION	**Hydrochlorothiazide area**	**Amlodipine area**	**Valsartan area**
Injection1	1971625	1539062	5894932
Injection2	1972013	1538913	5893863
Injection3	1971815	1538623	5892462
Injection4	1971923	1538513	5894813
Injection5	1970516	1538408	5894560
Injection6	1971578	1538704	5894126
Average	1971578	1538704	5894126
Std Deviation	546.7	246.0	910.8
% RSD	0.03	0.02	0.02

Observation: The % RSD for the area of five standard injections results were found to be 0.0310, 017, 0.017 and they were in limits.

4.3.5. Accuracy: Table 7. Showing Accuracy data of Hydrochlorothiazide, Amlodipine and Valsartan**:**

Sample ID	**Concentration (□g/mL)**		**Mean %Recovery**
	Pure drug	**Formulation**	
Hydrochlorothiazide:			
S1 50%	18.75	37.5	99.9
S4 100%	37.5	37.5	100
S7 150%	56.25	37.5	99.8
Amlodipine:			
S1 50%	7.5	15	99.7
S6 100%	15	15	99.8
S9 150%	22.5	15	100.1
Valsartan:			
S1 50%	120	240	99.9
S6 100%	240	240	100.2
S7 150%	360	240	100.1

4.3.6. Robustness:

Table. 8. Showing robustness of hydroachlorthiazide, Amlodipine and Valsartan in Flow rate:

S.No	Flow Rate (ml/min)	Area	%RSD	System Suitability Results	
				Plate Count	Tailing
Hydrochlorothiazide:					
1	Less flow 0.4	1946869 1947968 1940707	0.2011	4451	1.5
2	Actual flow 0.5	1956734 1967327 1965713	0.29	4449	1.6
3	More flow 0.6	1978371 1984173 1980109	0.1503	4427	1.5
Amlodipine:					
1	Less flow 0.4	1572749 1521691 1532178	1.748	4345	1.4
2	Actual flow 0.5	1573758 1527107 1531461	1.6688	4314	1.5
3	More flow 0.6	1592169 1589301 1537321	1.9627	4298	1.4
Valsartan :					
1	Less flow 0.4	5972118 5997831 5923166	0.635	5776	1.2
2	Actual flow 0.5	5893163 5896871 5892185	0.0419	5740	1.2
3	More flow 0.6	5573072 5813271 5521311	2.7641	5712	1.3

Table: 10 showing the assay of formulation.

Sample No.	**Sample Weight (µg/ml)**			**Sample Area**			**% Assay**		
	HCTZ	**AMLO**	**VAL**	**HCTZ**	**AMLO**	**VAL**	**HCTZ**	**AMLO**	**VAL**
1	37.5	15	240	1961427	1536795	5887758	99.9	100	99.8
2	37.5	15	240	1965272	1538221	5880952	100.4	100.3	99.7
3	37.5	15	240	1960971	1530744	5882166	100.1	100.1	100.1
4	37.5	15	240	1959529	1528134	5878671	99.8	99.9	100.3
5	37.5	15	240	1969720	1521758	5889034	100	99.8	99.9
6	37.5	15	240	1963482	1533102	5883703	100.2	100.3	99.6
AVG							100.07	100.3	99.9
STD							0.216	0.207	0.261
%RSD							0.216	0.206	0.261

Table 9. Showing robustness of hydroachlorthiazide : in mobile phase composition:

S.No	Mobile phase composition	Area	%RSD	System Suitability Results	
				Plate Count	Tailing
Hydrochlorothiazide:					
1	Less Org	1923642 1923424 1932429	0.266	4414	1.5
2	Normal	1932842 1923472 1923427	0.2814	4449	1.6
3	More Org	1974575 1974258 1945739	0.8426	4462	1.5
Amlodipine:					
1	Less Org	1572985 1523793	0.8426	4298	1.4
	Normal	1527382			
2		1523747 1523742	0.0002	4314	1.5
	More Org	1523748			
3		1532798 1523922 1523792	0.3381	4396	1.4
Valsartan :					
1	Less Org	5792836 5239823 5892373	0.231	5689	1.2
2	Normal	5823023 5823782 5837286	0.137	5740	1.2
3	More Org	5332892 5322922 5327222	0.0938	5759	1.3

4.3.8. Intermediate Precision (Ruggedness):

Table 11. Shows the results of ruggedness: Day 1 and Day 2.

		Instrument	Column	Assay(AVG)	%RSD
Analyst I day 1	Amlodipine	WATERS HPLC Aliance 2695	Symmetry c18 (100×4.6mm, 3.5µ)	99.8	0.1002
	Hydrochlorthizide			99.83	0.115
	Valsartan			99.86	0.057
Analyst 2 day 2	Amlodipine	SHIMADZU HPLC LC-2010	Prontosil c18 (100×4.6mm, 3.5µ)	99.58	0.12
	Hydrochlorthizide			100.1	0.32
	Valsartan			99.83	0.11

Theoretical plates were 4449, 4314 and 5740 and limit was more than 2000. And it was the final optimized trail. Retention time for the drugs was found to be 2.22min, 3.27min, 10.82min for Hydrochlorothiazide, Amlodipine besylate, Valsartan respectively.

4.3. Validation: The method was validated with respect to parameters including linearity, Robustness, Ruggedness, Specificity, suitability, precision and accuracy.

4.3.3. Linearity: The linearity of the calibration curve for Hydrochlorothiazide and Amlodipine besylate, Valsartan were calculated and constructed by plotting the mean peak area *versus* concentration. The correlation coefficients of regression r^2 = 0.9999, 0.9998 and 0.9992 respectively over a concentration range (12.5ppm of HCTZ, 5ppm of AMLO, 80ppm of VAL to 62.5ppm of HCTZ, 25ppm of AMLO, 400ppm of VAL).The representative linear regression equations for HCTZ, AMLO and VAL *Y* = 49527x+102461, *Y* = 100190x+5704.3and y = 23115x + 399344 respectively as shown in the below figures, and the corresponding results given in the table.

Observation: Absorbance of resulting solutions was measured and the calibration curve was plotted between peak area and concentration of the drug. Chromatograms were shown above. The response was found to be linear in the range 12.5-62.5 µg/ml for Hydrochlorothiazide, 5-25 µg/ml for Amlodipine besylate, 80-240 µg/ml for Valsartan. The data is given in tables above.

4.3.7. Solution Stability:

The stability of the diluents used has to be assessed to make sure if any degradants or impurities are produced during the development process. If so they may alter retention times and recoveries of the ingredients. So the stability of solution is assessed after 24 hrs. The evaluation determines the period of time a solution can be held before analysis without compromising with accuracy [11].

Determination of the main drug in bulk and tablet dosage form (Assay)

Six solutions of HCTZ, AMLO and VAL were prepared from the bulk drug and tablet dosage form and analyzed with the same experimental conditions and found to be drug content within the specified limits.

5. CONCLUSION:

The proposed method was found to be simple, precise, accurate and rapid for determination of Hydrochlorothiazide, Amlodipine and Valsartan from pure and its dosage forms. The mobile phase is simple to prepare and economical. The sample recovered in the formulation was in good agreement with their respective label claims and they suggested non-interference of formulation excipients in the estimation. This method proved that it has good peak response and better retention time when compared to those existing methods in case of Hydrochlorothiazide and Amlodipine. Hence, this method can be easily and conveniently adopted for routine analysis of Hydrochlorothiazide, Amlodipine and Valsartan in pure form and its dosage form and also can be used for dissolution or similar studies.

Conflict of interests:

No conflict of interests expressed by all the authors of this article

REFERENCES:

1. Messerli Franz, Antihypertensive Efficacy of Hydrochlorothiazide as Evaluated by Ambulatory Blood Pressure Monitoring A Meta-Analysis of Randomized Trials. J. Am.Coll.Cardiol 2011; **57** (5): 590–600. doi:10.1016/j.jacc.2010.07.053.
2. Amlodipine Besylate. Drugs.com. American Society of Hospital Pharmacists. Retrieved 22 July 2016.
3. Goodman & Gilman's: The Pharmacological Basis of Therapeutics 2011; 12th edn: New York: McGraw-Hill. ISBN 978-0-07-162442-8.

4. Bodduluri Anil Kumar1 and Pankaj Kumar Sharma: HPLC method development and validation for simultaneous estimation of amlodipine, hydrochlorothiazide and valsartan in bulk and tablet dosage form. International journal of research in pharmacy and chemistry India 2014; 4(4).
5. Klaus Florey: Analysis Profile of Drugs Substances. Academic Press New York, 2005; 76-84.
6. ICH Draft Guidelines on Validation of Analytical procedures: Definitions and Terminology. Federal Register 60. IFPMA Switzerland 1995.
7. Method validation guidelines International Conference on harmonization GENEVA; 1996.
8. Berry RI, Nash: AR. Pharmaceutical Process Validation, Analytical method validation. Marcel Dekker Inc. New York. 1993; 57:411-28
9. Rasha A.Shaalan: Validated Stability-Indicating HPLC-DAD method of analysis for the Anti hypertensive triples mixture of Amlodipine besylate, Valsartan and Hydrochlorothiazide in their Tablets. Arabian Journal of Chemistry 2013;
10. Validation of Analytical procedures. Text and Methodology. International Conference on Harmonization, Draft Revised Guidance on Q2 (R1), Federal Register, 1995; 60:
11. Jothieswari.D: Absorption correction method for estimation of Amlodipine besylate, Valsartan and Hydrochlorothiazide in bulk and in combined tablet dosage form. International Journal of pharmacy and pharmaceutical sciences 2011; 3(3): 22-23.