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NOVEL RP-HPLC METHOD FOR SIMULTANEOUS DETERMINATION OF OLMESARTAN MEDOXOMIL, AMLODIPINE BESYLATE AND HYDROCHLOROTHIAZIDE IN TABLET DOSAGE FORM

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ABSTRACT

A convenient, rapid, simple, specific, accurate, precise, economical isocratic Reversed Phase-High Performance Liquid Chromatography (RP-HPLC) method was developed and validated for simultaneous estimation of new combination of Olmesartan medoxomil (OLM), Amlodipine besylate (AMD) and Hydrochlorothiazide (HCTZ) in its dosage form. RP-HPLC method was carried out by utilizing Welchrom C₁₈ Column (4.6 X 250 mm, 5µm), Shimadzu LC-20AT Prominence Liquid Chromatograph. The mobile phase comprised of 10 mM Phosphate buffer (pH-3.0): Acetonitrile (50:50 v/v). The flow rate was adjusted to 1.0 mL/minute with the responses measured at 262 nm utilizing Shimadzu SPD-20A Prominence UV-Vis detector. The chromatogram showed a peak of 5.410 minutes for OLM, 4.067 minutes for AMD and 3.353 minutes for HCTZ. Good linearity was obtained in the range of 8-40 µg/ mL, 2-10 µg/ mL and 5-25 µg/mL for OLM, AMD and HCTZ respectively. The linear regression equations for OLM, AMD and HCTZ were $y=27.61x + 0.58$, $y = 55.80x + 0.988$ and $y = 37.75x + 0.345$ respectively. The limit of detection (LOD) for OLM, AMD and HCTZ were found to be 0.150 µg/mL, 0.0795 µg/mL and 0.1272 µg/mL respectively and the Limit of quantitation (LOQ) for OLM, AMD, HCTZ were found to be 0.456 µg/mL, 0.2410 µg/mL, 0.3856 µg/mL respectively. The proposed method has resulted high recovery values (99.74, 99.86 and 99.81 for OLM, AMD and HCTZ respectively). The assay content of OLM, AMD and HCTZ were determined and mean assay values were found to 99.564 %, 99.786 % and 99.567 % for OLM, AMD and HCTZ respectively in marketed tablets. Validation of the developed method was carried out for its accuracy, precision and ruggedness according to ICH Q2 (R1) guidelines. Thus the present study is an excellent method for the simultaneous separation and determination of all the three drugs in combined dosage form without any interference of excipients.

Key Words: Olmesartan medoxomil, Amlodipine besylate, Hydrochlorothiazide, Isocratic RP-HPLC.

INTRODUCTION

Currently multiple therapies are becoming extremely useful and frequent in pharmaceutical dosage forms. As a result, numerous combinations of two or more

drugs are being introduced into the market. Out of these, anti-Hypertensive drugs are one of the most frequently prescribed cardiovascular drugs. OLM is an angiotensin II receptor blocker. OLM blocks the vasoconstrictor effects of angiotensin II by selectively blocking the binding of angiotensin II to type I receptor in vascular smooth muscle. Chemically it is 4-(1-Hydroxy-1-methylethyl) – 2 -propyl-1 - [2'-(1H-tetazol-5-yl) [1, 1'-biphenyl]-4-yl] methyl] -1H-imidazole-5-carboxylic acid (5-Methyl-2-oxo-1, 3-dioxol-

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4-yl) methyl ester. Chemical structure of OLM is shown in Figure 1.

AMD is the besylate salt of Amlodipine and a long acting calcium channel blocker that inhibits the transmembrane influx of calcium ions into vascular smooth muscle and cardiac muscle. AMD inhibits calcium ion influx across cell membrane. Chemically it is [2-(Amino ethoxy) methyl] - 4 - (2-chlorophenyl) - 3 - ethoxycarbonyl- 5- methoxycarbonyl-6-methyl 1,4-dihydropyridine benzene Sulfonate. Chemical structure of AMD is shown in Figure 2.

HCTZ is 6-Chloro-3, 4-dihydro-2H-1, 2, 4-benzothiadiazine-7-sulfonamide 1, 1-dioxide. It reduces the amount of water in the body by increasing the flow of urine, which helps lower the blood pressure. Its molecular weight is 297.7 g/mol. It has a pKa of 8.5. Indirectly, the diuretic action of HCTZ reduces plasma volume with consequent increases in plasma rennin that activity increases in urinary potassium loss and decrease in serum potassium. Chemical structure of HCTZ is shown in Figure 3.

Tribenzor is a fixed-dose triple combination has the potential to simplify dosing regimens, reduce pill burden, and reduce cost. These three medicines allow blood vessels to relax so that blood can flow more easily. The use of drug combinations is intended to increase the efficiency in chronic disease management due to synergic effect.

An extensive literature survey reveals that there are various analytical methods have been reported for the determination of the above selected drugs either separately or in combination with other drugs using spectrophotometric methods (Sharma H *et al.*, 2011; Sharma H *et al.*, 2012; Pournima *et al.*, 2011; Rote A.R and Bari P.D, 2010; Neela MB *et al.*, 2009), HPTLC (Solanki TB *et al.*, 2014; Shah N *et al.*, 2007; Bari PD and Rote A.R, 2009). Ravisankar P and Devala Rao G performed an improved rapid HPLC method for the separation of five anti-hypertensive agents using C₁₈ column: Application to Hydrochlorothiazide determination in bulk and tablet dosage form (Ravisankar P and Devala Rao G, 2013). Ravisankar P and Devala Rao G. Performed Isocratic separation of four beta blockers with Amlodipine by C₁₈ RP-HPLC: application to Amlodipine determination in pharmaceutical dosage form (Ravisankar P and Devala Rao G, 2013). Rao C *et al.*, 2012, Murakami T *et al.*, 2008. There are also methods available for the estimation of these drugs in binary combinations by HPLC (Gaurav P *et al.*, 2010, Patil P *et al.*, 2011, Chabukswa *et al.*, 2010, Amudhavalli V *et al.*, 2011, Patil KR *et al.*, 2010, Godse V *et al.*, 2010). Very few stability indicating methods have been published for the determination of ternary combination of these drugs using RP- HPLC (Jain Ps *et al.*, 2012) and UPLC (Kakumani KK *et al.*, 2012). Most of the reported methods are for individual drug or in combination with other drugs have limitations such as poor resolution of

peaks, more consumption of mobile phase, longer analysis time, low sensitivity and precision. In view of these, the author attempted to develop and validate simple, a quick, sensitive, accurate, economical and robust RP-HPLC method for analysis of tertiary combination contains OLM, AMD and HCTZ in recent tablet dosage formulation such as Tribenzor.

MATERIALS AND METHODS

Drugs and Chemicals used

The drug samples OLM, AMD and HCTZ were obtained as gift samples from Hetero Labs Ltd., Hyderabad, India. All the chemicals procured were analytical grade. Potassium dihydrogen orthophosphate and phosphoric acid from Rankem Ltd., Mumbai, India, while acetonitrile (HPLC grade) and triethylamine (HPLC grade) from Merck Pharmaceuticals Private Ltd., Mumbai, India. Ortho phosphoric acid utilized was of HPLC grade and purchased from Merck Specialties Private Ltd., Mumbai, India. HPLC grade distilled water was used throughout the analysis.

Instrumentation

Quantitative HPLC was performed on an isocratic high performance liquid chromatograph (Shimadzu LC-20AT Prominence Liquid Chromatograph) with a LC-20AT VP pump, manual injector with a loop volume of 20 μ L (Rheodyne), programmable variable wavelength Shimadzu SPD-20A Prominence UV-Vis detector and welchrom C₁₈ Column (4.6 X 250mm, 5 μ m particle size). The HPLC system was equipped with "Spinchrome" software. An electronic balance (Shimadzu TX223L), digital pH meter (Systronics model- 802), a sonicator (spectra lab, model UCB 40) and UV-Visible Spectrophotometer (Systronics model-2203) were utilised in this analysis.

Preparation of mobile phase

A 10 mM Phosphate buffer was prepared by dissolving 6.056 g of potassium dihydrogen orthophosphate in 445 mL of HPLC grade water. To this 55 mL of 0.1M phosphoric acid was added and pH was adjusted to 3.0 with triethylamine. The above prepared buffer and acetonitrile were mixed in the ratio of 50:50 v/v and was filtered through 0.45 μ m nylon membrane filter and degassed by sonication for 15 minutes.

Preparation of Standard stock solution

Standard stock solutions of 1000 μ g/mL of OLM, AMD and HCTZ pure drugs were prepared separately by transferring accurately weighed 10 mg of each pure drug into 10 mL volumetric flasks. These drugs were dissolved in 10 mL of mobile phase and then volume was made upto 10 mL to get a concentration of 1mg/mL for all three drugs. Aliquots from standard stock solutions were diluted

further with the mobile phase to obtain desired concentrations of working standard solutions.

Preparation of test sample solution

Ten tablets of Tribenzor were weighed and ground to get a fine powder. The powder equivalent to 40 mg of OLM, 10 mg of AMD, and 25 mg of HCTZ were accurately weighed and transferred into 25 mL volumetric flask containing 10 mL the mobile phase. The powder was dissolved with the mobile phase and sonicated for 20 minutes. Then the volume was filled up to the mark with the mobile phase and filtered through 0.45 μ m nylon filter. Further dilutions were made based on the required concentrations. Eventually it was injected and peak areas were recorded. As a matter of fact quantifications were done by keeping these values to the straight line equation of calibration plot.

Selection of detection wavelength (λ max)

Appropriate concentrations of solution of each drug OLM, AMD and HCTZ were separately prepared with mobile phase. Each solution was scanned over a range of 200-400 nm in spectrum mode. The peaks of three drugs were over lined and absorbance was observed at 262 nm. The results showed that the isobestic point for the drugs was found to be 262 nm. Therefore this wavelength was selected after comparing the spectra to attain the highest sensitivity for detection of three selected drugs. Figure 4 shows the overlain Spectra of HCTZ, AMD and OLM.

Optimization and method development

In order to improve the separation, short run time and peak symmetry, several trials were carried out by varying the commonly used solvents, their compositions and flow rate. Mixtures of many different combinations of commonly utilized solvents such as water, methanol and acetonitrile with or without different buffers in different combinations were tested. This was undertaken by varying one parameter at a time and keeping all other conditions constant. Better resolution, short run time, minimal peak tailing and good reproducibility of the results were obtained when an analytical column of Welchome C₁₈ (250 mm X 4.6 mm, 5 μ m) column and a mobile phase containing a mixture of 10 mM Phosphate buffer (pH adjusted to 3.0) and acetonitrile in ratio of 50:50 v/v were utilized and found most suitable of all combinations. A mobile phase flow rate of 1mL/minute, 262 nm detection wavelength and injection volume was 20 μ L were proved to be suitable for the present study. Optimized chromatographic conditions for the developed method is shown in Table 1.

VALIDATION OF THE PROPOSED METHOD

The developed method was validated as per the ICH Q2 (R1) guide lines, for the parameters like system

suitability, specificity, linearity, precision, accuracy, robustness and limit of detection (LOD) and limit of quantitation (LOQ).

System suitability

System suitability tests are an integral part of chromatographic method which was utilized to verify reproducibility of the chromatographic system. To ascertain its effectiveness, certain system suitability test parameters were checked by repeatedly injecting the drug solution at the concentration level 10 μ g/ mL of the mixture. At first the HPLC system was stabilized for 40 minutes. One blank followed by six replicates of a single standard solution of mixture of OLM, AMD and HCTZ were injected to check the system suitability. To ascertain the system suitability for the proposed method, the parameters such as retention time, theoretical plates, peak asymmetry and resolution were carried out and the results are presented in Table 2.

Specificity

Specificity study was conducted to examine the interferences of the excipients which are available in the formulation during a chromatographic analysis. In present work, specificity for the analysis of the three combined drugs (OLM, AMD, HCTZ) was assessed by comparing the chromatograms obtained during the analysis of standard sample along with most commonly used excipients in tablet formulation such as microcrystalline cellulose, pregelatinized starch, sodium and magnesium stearate, polyvinyl alcohol, polyethylene glycol, titanium dioxide, talc, iron oxide and the blank solution. Drug to excipients ratio used were similar to that in the commercial formulations. The mixtures were filtered through 0.45 μ m membrane filter before injection. Figure 5 and Figure 6 shows the chromatograms of the test and blank solutions respectively. The results obtained for specificity study is shown in Table 3.

Linearity

10 mg OLM, 10 mg AMD and 10 mg of HCTZ of pure drug were prepared in 10 mL volumetric flask dissolved separately in mobile phase. For the linearity study, aliquots of the drug solutions were further diluted to get the final working standard of concentration ranges as OLM (8-40 μ g/mL), AMD (2-10 μ g/mL) and HCTZ (5-25 μ g/mL) respectively. The peak area of each drug was estimated by taking measurements of 5 concentration points. Linearity plots were constructed for OLM, AMD and HCTZ by plotting peak area against concentration range. The linearity data for OLM, AMD and HCTZ are tabulated in Table 4. The calibration plots for OLM, AMD and HCTZ were shown in Figure 7, Figure 8 and Figure 9 respectively. The representative chromatograms for standard drugs are shown in Figure 10 to Figure 12. Table 5 shows the regression analysis of the OLM, AMD and HCTZ.

Precision

HCTZ were conducted by determining the intra-day precision and inter day precision of the method. The intra-day precision was studied by repeating the assay six times on the same day where as the inter-day precision was studied on three different days for the concentration of 10 µg. The percent relative standard deviation (% RSD) was calculated which is within the acceptable criteria of not more than 2.0. The results for intraday and inter day precision studies are presented in Table 6.

Accuracy (Recovery studies)

The accuracy of the method was done by standard addition method. In this method the volume of the test drug solution was taken as constant and standard OLM solution was added in increasing amounts equivalent to 80 %, 100 % and 120 % level to each test solution. Known amount of standard OLM of 5 µg/mL concentrations were added to pre-analyzed sample for 8 µg/mL, 10 µg/mL and 12 µg/mL in triplicate. The percent recovery of the triplicate solutions was determined and average of the percent recovery was calculated. The results are presented in Table 7.

Procedure for the Preparation of Solution for 80% Recovery of OLM

From the working standard solution of OLM 5 mg was taken into a 10 mL volumetric flask. To this 8 mg of working test solution was added and mixed well. Volume was filled up to the mark with mobile phase.

Procedure for the Preparation of Solution for 100% Recovery of OLM

From the working standard solution of OLM 5 mg was taken into a 10 mL volumetric flask. To this 10 mg of working test solution was added, mixed well and volume was brought up to the mark with mobile phase.

Procedure for the Preparation of Solution for 120% Recovery of OLM

From the working standard solution of OLM 5 mg was taken into a 10 mL volumetric flask. To this 12 mg of working test solution was added, mixed well and the volume was filled up to the mark with mobile phase. The above said procedure was repeated for AMD and HCTZ. The results of recovery study are tabulated in Table 7.

Robustness

The robustness of a method was determined by slightly altering some of the basic experimental conditions deliberately and then by computing the changes in system

The precision experiments of OLM, AMD, and suitability parameters. In the present case, the parameters changed were flow rate (± 0.2 mL/min), detection wavelength (± 5 nm), and Mobile phase composition (± 5 %). The results of robustness study are summarized in Table 8.

LOD AND LOQ

Limit of Detection is the lowest concentration in a sample that can be detected, but not necessarily quantified under the stated experimental conditions. The limit of quantitation is the lowest concentration of analyte in a sample that can be determined with acceptable precision and accuracy. Limit of Detection and Limit of Quantitation were calculated using following formula $LOD = 3.3(SD)/S$ and $LOQ = 10 (SD)/S$, where SD = the standard deviation of response (peak area) and S = the slope of the calibration curve. The LOD and LOQ values are presented in Table 9.

Solution Stability

Stability of the sample solutions was evaluated by keeping the Sample solutions for 72 hours at room temperature and analyzed each sample by the end of the prescribed storage hour. The percent RSD and % assay difference from the freshly analyzed sample were computed. The percentage changes in the assay results after 72 hours storage were compared with freshly analyzed sample and % RSD values were also estimated. The % RSD values found to be 0.53, 0.25, and 0.43 for OLM, AMD and HCTZ, respectively. The stock solutions showed no significant change in analyte composition, retention time and peak areas of OLM, AMD and HCTZ. Similarly, the standard solutions of OLM, AMD and HCTZ solutions were also found to be stable at room temperature over a 72 hours period, which was sufficient for the whole analytical process.

Analysis of marketed formulation

As described in the experimental part, the prepared sample solution that contains the three drugs (OLM, AMD and HCTZ) in combined tablet formulation was injected in the optimized chromatographic system and peak areas were recorded. Infact the quantifications were carried out by keeping these values to the straight line equation of the linearity plot. Eventually the average values of the % assay for the triplicate samples were calculated. The result analysis of marketed formulation Tribenzor is tabulated in Table 10. The standard and sample chromatograms are shown in Figure 13 and Figure 14.

Table 1. Optimized chromatographic conditions for proposed HPLC method for the mixture of OLM, AMD, HCTZ

Parameter	Chromatographic conditions
Instrument	SHIMADZULC-20AT prominence liquid chromatograph
Column	WELCHROM C ₁₈ Column (4.6 X 250 mm, 5µm)
Detector	SHIMADZU SPD-20A prominence UV-Vis detector

Diluents	10 mM Phosphate Buffer (pH-3): Acetonitrile (50:50 v/v)
Mobile phase	10 mM Phosphate Buffer (pH-3): Acetonitrile (50 : 50 v/v)
Flow rate	1mL/minute
Detection wavelength	By UV at 262 nm.
Runtime	8 minutes
Column temperature	Ambient temperature (25 ± 1°C)
Volume of injection loop	20 µL

Table 2. System suitability parameters for OLM, AMD and HCTZ

Parameters	Obtained results (n=3)		
	OLM	AMD	HCTZ
Retention time (R _t)	5.410 minutes	4.067 minutes	3.353 minutes
Theoretical plates [th.pl] (Efficiency)	14251	12198	11584
Theoretical plates per meter [t.p/m]	285021	243957	231681
Tailing factor (asymmetry factor)	1.178	1.138	1.185
Resolution	8.199	5.261	-

Table 3. Specificity results

Name of the solution	Retention time (R _t) min.
Mobile phase	No peaks
Placebo	No peaks
Olmesartan 10 µg/mL	5.410
Amlodipine 10 µg/mL	4.067
Hydrochlorothiazide 10 µg/mL	3.353

Table 4. Linearity data of OLM, AMD and HCTZ

OLM		AMD		HCTZ	
Conc.(µg/mL)	Peak area	Conc.(µg/mL)	Peak area	Conc.(µg/mL)	Peak area
8	214.546	2	112.567	5	176.765
16	448.897	4	227.106	10	397.897
24	667.456	6	334.158	15	577.456
32	887.987	8	447.158	20	724.543
40	1098.865	10	559.125	25	956.678

Table 5. Regression data of the OLM, AMD and HCTZ

PARAMETER	OLM	AMD	HCTZ
Linearity range	8 - 40 µg/mL	2 - 10 µg/mL	5 - 25 µg/mL
Regression equation	y=27.61x + 0.5866	y = 55.80x + 0.9885	y = 37.75x + 0.345
Slope (b)	27.61	55.80	37.75
Intercept (a)	0.5866	0.9885	0.345
Standard deviation of slope (S _b)	0.0416	0.0547	0.0678
Standard deviation of intercept (S _a)	1.2615	1.3457	1.456
Standard error of estimation (Se)	1.7430	1.8976	1.987
Correlation coefficient	0.9998	0.9999	0.999
Percentage range of errors* (Confidence limits)			
0.05 significance level	0.033286	0.043768	0.05425
0.01 significance level	0.043746	0.057521	0.071297

* Average of 6 determinations; Acceptance criteria < 2.0.

Table 6. Precision study

PRECISION STUDY	OLM (n=6)	AMD (n=6)	HCTZ (n=6)
	%RSD	%RSD	%RSD
INTER- DAY	0.092852	0.138455	0.1046
INTRA -DAY	0.079756	0.12774	0.2347

Table 7. Recovery study

Drugs	Level (%)	Amount of sample taken(mg)	Amount Standard added(mg)	Total amount(mg)	% Recovery	Mean % Recovery	% RSD
OLM	80	8	5	13	12.95	99.61	0.26
	100	10	5	15	14.98	99.86	0.17
	120	12	5	17	16.96	99.76	0.060
AMD	80	8	5	13	12.98	99.84	0.26
	100	10	5	15	14.98	99.86	0.17
	120	12	5	17	16.98	99.88	0.060
HCTZ	80	8	5	13	12.97	99.76	0.26
	100	10	5	15	14.99	99.93	0.17
	120	12	5	17	16.98	99.76	0.060

Table 8. Robustness results

S.No	Parameters	Optimized	Used	Retention time (Rt)			Peak symmetry			Remark
				OLM	AMD	HCTZ	OLM	AMD	HCTZ	
1	Flow rate	1.0 mL.min ⁻¹	0.8	5.415	4.082	3.358	1.187	1.134	1.174	Robust*
			1.0	5.410	4.067	3.353	1.178	1.138	1.185	
			1.2	5.396	4.062	3.350	1.196	1.140	1.180	
2	Detection Wavelength (± 5 nm)	262 nm	260	5.412	4.068	3.355	1.178	1.138	1.185	Robust
			262	5.410	4.067	3.353	1.178	1.138	1.185	
			267	5.410	4.068	3.354	1.178	1.138	1.185	
3	Mobile phase composition (± 5%)	50:50 v/v	55:45	5.415	4.081	3.356	1.184	1.140	1.180	Robust*
			50:50	5.410	4.067	3.353	1.178	1.138	1.185	
			45:55	5.399	4.057	3.343	1.180	1.135	1.174	

* Significant changes in parameter values

Table 9. LOD AND LOQ

Name of the analyte	Limit of detection	Limit of Quantitation
OLM	0.150 µg/mL	0.456 µg/mL
AMD	0.0795 µg/mL	0.2410 µg/mL
HCTZ	0.1272 µg/mL	0.3856 µg/mL

Table 10. Assay results of OLM, AMD and HCTZ Formulation

S.No	Formulation	Drug	Label claim (mg)	Amount found (mg)	% Assay ±RSD*
1	Tribenzor	OLM	40	39.657	99.564 ± 0.234
		AMD	10	9.785	99.786 ± 0.134
		HCTZ	12.5	12.432	99.567 ± 0.456

* = Mean of three determinations.

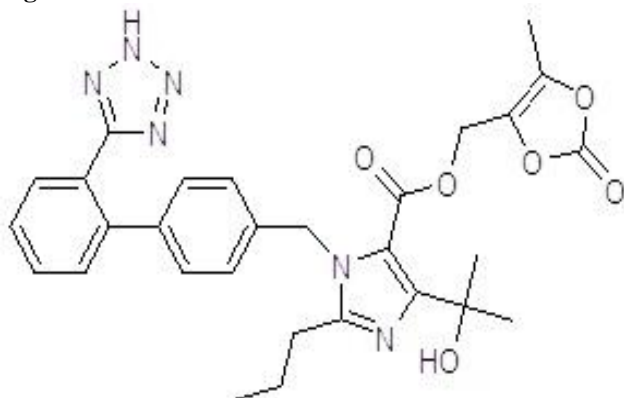
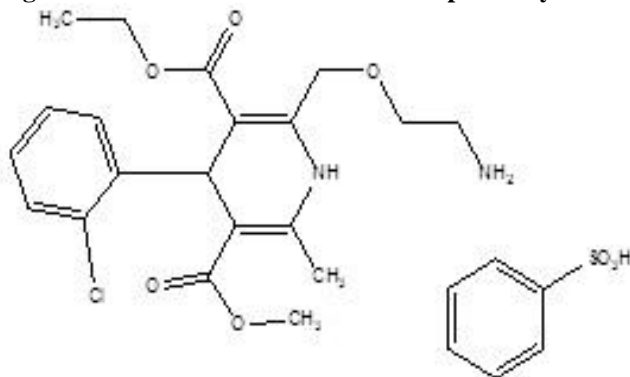
Figure 1. Chemical structure of Olmesartan medoxomil**Figure 2. Chemical structure of Amlodipine besylate**

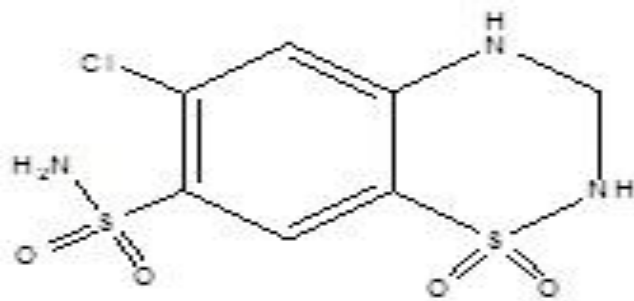
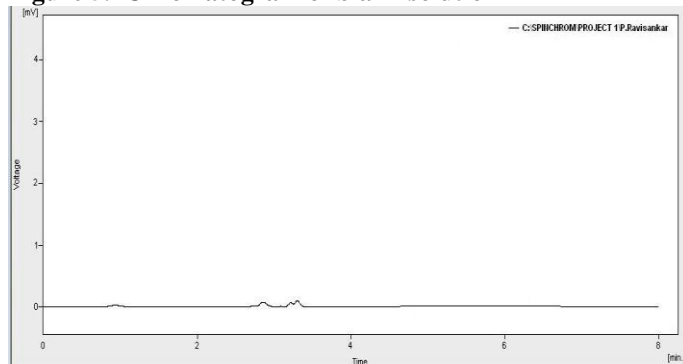
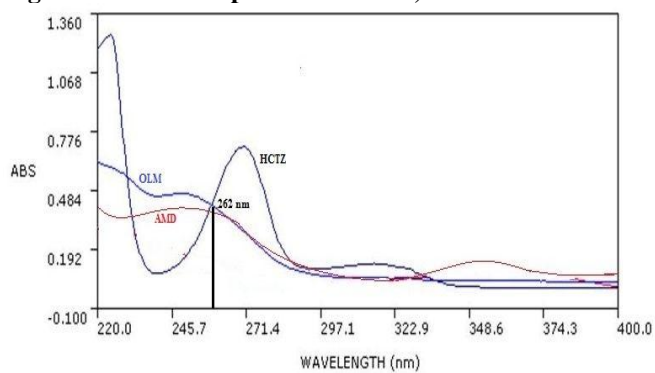
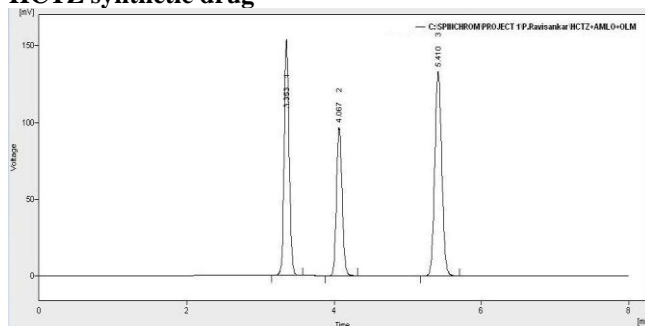
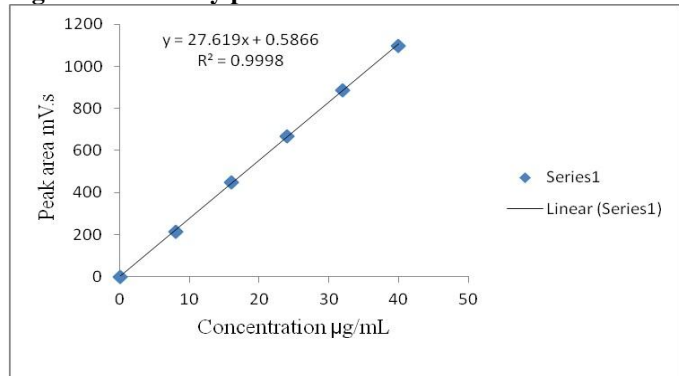
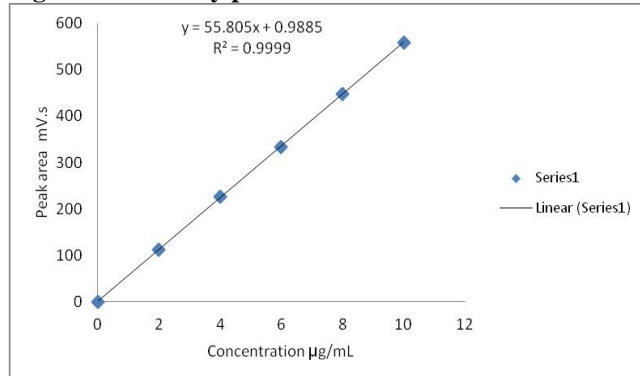
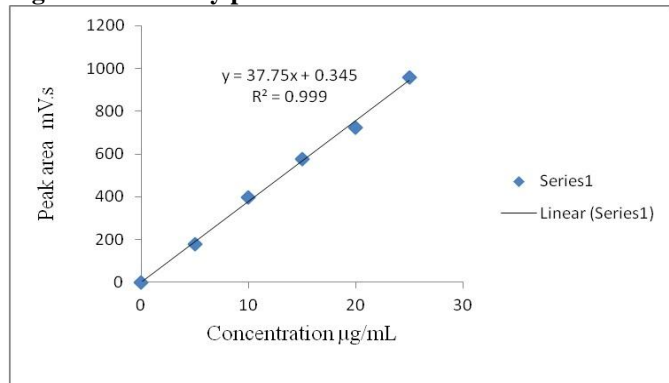
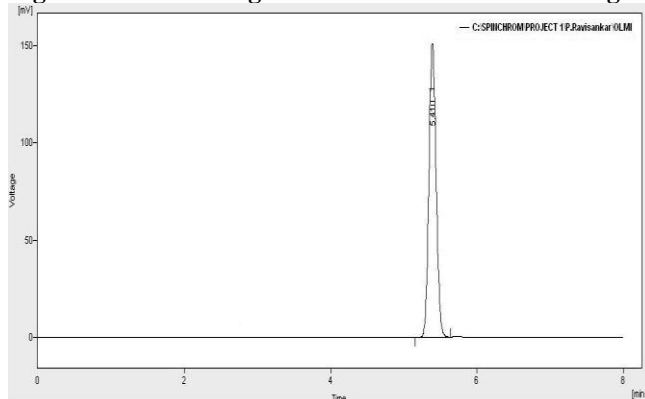
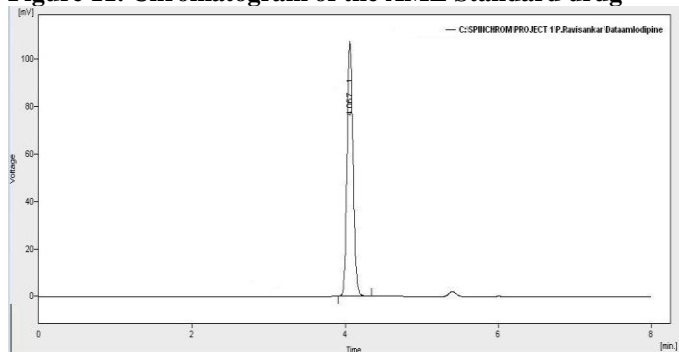
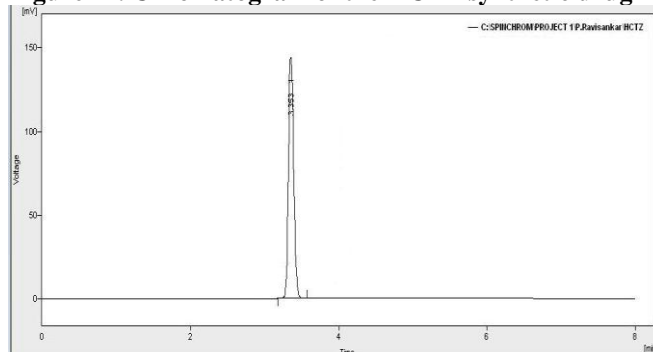
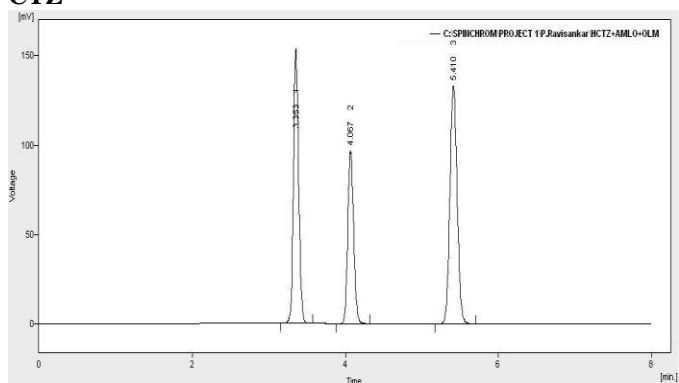
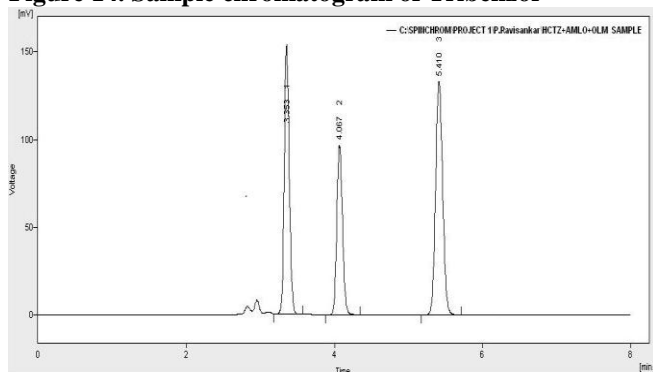
Figure 3. Chemical structure of Hydrochlorothiazide**Figure 5. Chromatogram of blank solution****Figure 4. Overlain spectra of HCTZ, AMD and OLM****Figure 6. Chromatogram of the OLM, AMD AND HCTZ synthetic drug****Figure 7. Linearity plot of OLM****Figure 8. Linearity plot of AMD****Figure 9. Linearity plot of HCTZ****Figure 10. Chromatogram of the OLM standard drug**

Figure 11. Chromatogram of the AML Standard drug**Figure 12. Chromatogram of the HCTZ synthetic drug****Figure 13. Standard chromatogram of OLM, AMD and CTZ****Figure 14. Sample chromatogram of Tribenzor**

RESULTS AND DISCUSSION

The present work is conducted in order to develop RP-HPLC method for the simultaneous estimation of OLM, AMD and HCTZ in tablet formulation. Several trials were undertaken prior to identifying the best operational and environmental conditions for optimizing suitable methods of separation. Before the development of this method, properties of drugs sample, their drug profiles and the available analytical methods for estimation either in combination or individual drugs were studied as the physical and chemical properties of a drug is the primary factor that should be considered while developing a method. The nature of samples, molecular weight, pKa value, stability, and other physical constant information about drugs are very useful to determine the initial optimum separation conditions.

An extensive literature search was carried out. However, only a single RP-HPLC method has been reported which has several limitations. This necessitated developing a relatively simple, fast and precise alternative method for the estimation of the three drugs in combined formulation.

Since all the three drugs are relatively polar in nature, a reversed phase HPLC was chosen for the initial better separation. As a result, welchrom-C₁₈ reversed phase HPLC column was chosen as stationary phase and a number of trials were carried out utilizing different buffer

solutions of different pH range with different compositions of mobile phases, variable flow rate and column temperature. Finally an optimum separation condition was attained with generally used HPLC grade solvents that consists of dihydrogen phosphate buffer (pH 3.0), acetonitrile in the ratio of 50:50 v/v. A mobile phase flow rate was adjusted at 1.0 mL/minute, a common detection wavelength was set at 262 nm for the three drugs and the column temperature was kept at ambient conditions. After the adjustment of such operational parameters at their corresponding optimum values, a chromatographic peak was obtained with characteristics of better resolution, gaussian peak shape and minimal peak tailing.

The developed method was then validated in pursuance of ICH Q2 (R1) guidelines with respect to linearity, specificity, detection and quantification limits, accuracy, precision and robustness. As per the ICH Q2 (R1) guidelines, system suitability study was conducted to ensure the suitability of complete testing system for the intended purpose. As a result system suitability parameters such as number of theoretical plates, tailing factor, and resolution of the peaks were computed for the optimized chromatographic condition for the proposed method. Accordingly 5.410, 4.067 and 3.353 minutes of retention time; 14251, 12198, and 11584 of plate number; 1.178, 1.138, and 1.185 tailing factors were obtained for OLM, AMD and HCTZ respectively. The resolutions between the

peaks were also computed and the results show that the separation between first and second eluted peak is 5.261 and third peaks is 8.199. All of these results were within the acceptable limit and hence the method is suitable for the intended purpose.

Linearity of the proposed method was studied by taking five points concentration. Then the linearity range of OLM, AMD and HCTZ were found to be 8 – 40 µg/mL, 2 – 10 µg/mL, and 5 - 25 µg/mL respectively. The response of each drug was found to be linear in the specified concentration range and a linearity plot was constructed for each drug between these ranges. Linear regression equations were found to be $y = 27.61x + 0.586$ ($R^2=0.9998$), $y = 55.80x + 0.988$ ($R^2=0.9999$), and $y = 37.75x + 0.345$ ($R^2=0.999$) for OLM, AMD, and HCTZ respectively (where X is the concentration in µg/mL) and y is the peak area in absorbance unit). In all the above cases, the calculated results are within the acceptable limit indicating the strong and positive relationship between concentrations of each drug and their peak areas.

As per the guidelines, a mixture of commonly utilized excipients and pure drug samples were injected to the system in order to test specificity of the proposed method for quantifying the three drugs (OLM, AMD, and HCTZ) without the interferences of the excipients. A blank solution was also prepared in similar way like the drugs and excipients mixture and used for comparison. The drug sample and the blank solutions were injected separately. The peaks were well resolved with no co-eluting peak along with the normal peaks of the drugs and no interference due to the commonly utilized excipients. Thus the method is specific to quantify the drugs simultaneously in tablet dosage form.

Precision of the method was evaluated by analyzing inter-day and intra-day precision studies. Then, the % RSD values of each drug were calculated. In both the intra-day and inter-day precision, the % RSD for all three drugs were found to be less than 2 % which indicates that the developed method is precise. The inter-day and intra-day precision of OLM, AMD and HCTZ were found to be 0.092852, 0.138455, 0.1046 and 0.079756, 0.12774, 0.2347. The accuracy of the proposed method was evaluated by adding known amount of standard drugs to pre-analyzed samples at three levels 80 %, 100 %, 120 % and the recovery was studied. All solutions were prepared and analyzed in triplicate. The above procedure was adopted for all three drugs and a high recovery values were obtained (99.74, 99.86 and 99.81 for OLM, AMD and HCTZ respectively). The relative standard deviations (% RSD) were also found to be less than 2% for each analyte. The developed analytical method had good accuracy and values show that the proposed method was to be highly accurate and suitable for intended use. The robustness was validated by assessing the effect of slightly small changes in chromatographic parameters such as slight variation in mobile phase composition, different flow rate, buffer

composition, and detector wavelength. It was evaluated by varying one parameter at a time and keeping other two constant. Tailing factor, number of theoretical plates, detection wavelength were determined and the values were compared with the results of the normal optimized conditions. The result shows that all of the above parameters are within the acceptable limit. In fact no obvious effect on chromatographic parameters was observed which showed that the developed method was robust in nature.

The limit of detection (LOD) and limit of quantification (LOQ) were determined. For each drug six replicates of the analyte at lowest concentration within the calibration range were measured and quantified. The result of LOD and LOQ for OLM, AMD and HCTZ were found to be 0.150 µg/mL, 0.456 µg/mL; 0.0795 µg/mL and 0.2410 µg/mL; 0.1272 µg/mL and 0.3856 µg/mL respectively. These results show that the method had relatively lower LOD and LOQ values indicating that this method is highly sensitive.

Application of the developed method was finally tested for quantification of commercially available marketed formulation and the percentage assay of OLM, AMD, and HCTZ were found to be 99.564, 99.786 and 99.567 respectively. A study to established bench top stability of drugs was conducted and analysed. The standard solutions of OLM, AMD and HCTZ were also found to be stable at room temperature over a period of 72 hours, which was sufficient for the whole analytical process. Hence, as none of the excipients interfered with the analytes of interest, the method was found to be suitable for analyzing the commercial formulations.

CONCLUSION

An efficient and rapid RP-HPLC method has been developed for the separation and determination of OLM, AMD and HCTZ in pharmaceutical formulation. The developed method is simple, accurate, specific, precise, sensitive, linear, robust and cost effective based on method validation. Considering all the results of validation parameters satisfactory results were obtained. It can be concluded that this RP-HPLC method for separation and quantification of these drugs for simultaneous estimation is preferable than the other existing methods reported hitherto. Thus, the method can be successfully be feasible for routine analysis in quality control and validation studies of OLM, AMD and HCTZ in combined tablet dosage forms.

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