Original Article

RP-HPLC Method Development and Validation for Simultaneous Estimation of Benidipine Hydrochloride and Chlorthalidone in Pharmaceutical Dosage Form

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ABSTRACT

A simple, precise, rapid and accurate reverse phase High Performance Liquid Chromatography (HPLC) method was developed for the estimation of Benidipine Hydrochloride and Chlorthalidone in bulk and tablet dosage form. C18 C-18 Agilent Zorbax Bonus – RP (250 x 4.6 mm, Particle size -5 Micron) was used in this method. The mobile phase comprises of Methanol and 0.1% Orthophosphoric acid (45:55v/v) with flow rate 1 mL/min (Photodiode array Detector). The retention time for Benidipine Hydrochloride was 1.09 min and for Chlorthalidone was 3.52 min. The detection concentration was linear over 32-48 µg/mL for Benidipine Hydrochloride and 100-150 µg/mL for Chlorthalidone. The regression equation of Benidipine Hydrochloride and Chlorthalidone were found to be y = 50184x-30070 and y = 37102x-74642 respectively with regression coefficient of Benidipine Hydrochloride and Chlorthalidone were 0.999 and 0.999. So, the present work is aimed for development of simple, reproducible chromatographic RP-HPLC method for simultaneous estimation of Benidipine Hydrochloride and Chlorthalidone. The developed method was successfully validated in accordance to ICH guideline. Hence the method can be conveniently adopted for routine analysis in quality control laboratories.

Key words: Benidipine Hydrochloride, Chlorthalidone, RP-HPLC, Method Development, Validation

Benidipine Hydrochloride is a 5-*O*-[(3*R*)-1benzylpiperidin-3-yl] 3-*O*-methyl (4*R*)-2, 6dimethyl-4-(3-nitrophenyl)-1, 4-dihydropyridine3, 5-dicarboxylate; hydrochloride [1-2]. Benidipine Hydrochloride is a long acting anti-hypertensive drug class of Ca^{2+}channel blocker (Dihydropyridine Derivative) its longer duration of action to slow dissociation from the DHP receptor on smooth muscle cell [3].It is a Triple L, T and N channel blocker for treatment of Hypertension and Angina Pectoris mostly used in Japan and India [1-3]. Chlorthalidone is a 2-chloro-5-(1-hydroxy-3-oxo-2*H*isoindol-1-yl) benzenesulfonamide [4]. It is a long-acting thiazide, diuretic drug used in treatment of hypertension and certain kidney disorder [5].

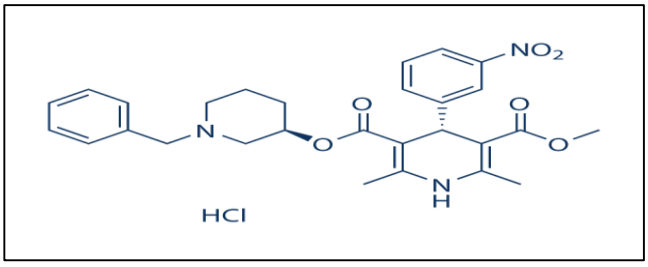

Fig. 1- Structure of Benidipine Hydrochloride

Molecular formula: - $C_{28}H_{32}ClN_3O_6$
Molecular Weight: - 542 g/mol

Chlorthalidone has longest duration of action but similar diuretic effect at maximum therapeutic doses. It reduces reabsorption of sodium and chloride primarily through inhibition of the Na^+ / Cl^- symporter in the apical membrane of distal convoluted tubule cells in kidney [6-7]. Chlorthalidone was first introduced in Switzerland in1959 it is also available as generic medicine [4].

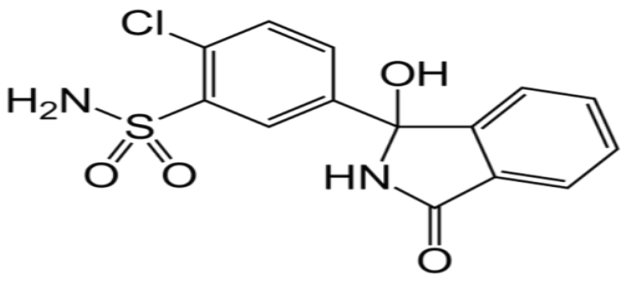

Fig. 2 – Structure of Chlorthalidone

Molecular Formula: - $C_{14}H_{11}C_lN_2O_4S$
Molecular Weight: - 338.8g/mol

MATERIALS AND METHODS

Chemicals used: A pure drug in powder form Benidipine Hydrochloride and Chlorthalidone received from Alkumentis Pharmaceuticals, Mumbai and Ipaca Pharmaceutical, Pvt. Ltd Mumbai, Methanol (AR grade) and Orthophosphoric acid utilized as diluent and received from Fine Chem laboratories, Mumbai and Loba Chemie Pvt. Ltd. and a commercial product manufactured by Alkumenties Pharmaceuticals "Benitowa-CH" which contain both the Benidipine Hydrochloride and Chlorthalidone received from market.

Instrument used: Agilent 1260 Infinity Quaternary HPLC device equipped with Photodiode array detector and the output signal was checked and processed using openLab EZChrome software.

Chromatographic conditions were as follows:
Oven Temperature: - 30^0 C,
Flow Rate was:-1mL/min,
Run Time was: - 10 min,
Injection Volume: - 10µL,
Wavelength: -238 nm,
Column: - C-18Agilent Zorbax Bonus –RP (250 x 4.6 mm, Particle size -5 Micron) and diluent: - Methanol: 0.1% Orthophosphoric acid (50:50).

Preparation of Mobile Phase: The mobile phase was prepared by mixing Methanol and 0.1% orthophosphoric acid in the ratio of 55:45 (v/v). The resultant solution filtered through Micron filter 0.45µm pore size by means of vacuum pump and then degassed by using Ultasonicator to remove gases in solvent.

Preparation of Standard solution: Standard solution of both the drug were prepared by weighing 5mg and 12.5mg of Benidipine Hydrochloride & Chlorthalidone and transfer in to 10mL of volumetric flask and make volume up to 10mL using mobile phase and prepared solutions concentration were 500µg/mL and 1250 µg/mL respectively.

Preparation of Working Standard solution: The Working Standard solution of both the drug were prepared by weighing 5mg and 12.5mg of Benidipine Hydrochloride & Chlorthalidone and transfer in to 10mL of volumetric flask and make volume up to 10mL using mobile phase then pipette out 0.8mL and 1.0mL respectively and add into another 10mL Volumetric flask and make up volume up to 10mL using mobile phase. The resultant solution was 40µg/mL and 125µg/mL, respectively.

Selection of Wavelength: The sample was scanned from 200-400 nm Photodiode array detectors. The wavelength selected for analysis chosen was 238 nm on basis of appropriate intensity of both the drug.

Analysis of Marketed Formulation

Take 5 tablets each tablet contains 8 mg of Benidipine Hydrochloride and 12.5 mg of Chlorthalidone and weighed and powdered from that transfer 5mg of Benidipine Hydrochloride and 12.5 mg of Chlorthalidone in 10 mL of volumetric flask and make up volume up to 10 mL with help of diluent and further dilution would make final concentration of solution were 40µg/mL and 125 µg/mL respectively. Then solution was filtered by 0.45 µm nylon membrane filter by using vacuum filter. Tablet formulation analysis was carried out as mentioned under section tablet formulation analysis. Procedure was repeated for 5 times. Sample solution was injected and area was recorded for each drug concentration and percentage purity was determined as shown in Table 1.

Analysis is the most important aspect of any drug development; a suitable method must be developed so as to ensure that any drug in dosage from. With help of method development ensure that the amount of particular drug can easily determine. The validation parameter confirms that the developed method is precise, accurate and reproducible and can be used for used for routine evaluation of Benidipine Hydrochloride and Chlorthalidone in combined dosage form [8-12]. In the present study suitable for RP-HPLC method was developed with the aim of making detection of Benidipine Hydrochloride and Chlorthalidone more accurate and precise with addition of validation parameters like Specificity, Linearity, Accuracy, and LOD & LOQ [8].

Table 1 - Assay result with Benidipine Hydrochloride & Chlorthalidone

S.no	Benidipine Hydrochloride			Chlorthalidone		
	Peak area	Amt. recovered in µg/mL	% Recovery	Peak area	Amt. recovered in µg/mL	% Recovery
1	1612593	32	98.54	3800032	100	100.41
2	1612895	31.97	98.56	3765482	99.81	99.67
3	1623583	31.82	99.22	3768221	99.98	99.57
4	1624896	32.03	99.31	3798523	100	97.67
5	1628369	32	99.52	3799213	100	100.39
Mean	1620467	31.96	99.03	3766294	99.95	99.54
% RSD	0.44	0.23	0.45	1.09	0.08	1.12

Table 2 - Concentration and Area of Benidipine Hydrochloride

% Level	Conc (µg/ml)	Area
80	32	1584314
90	36	1762564
100	40	1979466
110	44	2181734
120	48	2378413

Table 3 - Concentration and Area of Chlorthalidone

Chlorthalidone		
% Level	**Conc (µg/ml)**	**Area**
80	100	3800057
90	112.5	4214430
100	125	4721514
110	137.5	5199512
120	150	5626385

Table 4- Linearity values of Benidipine Hydrochloride and Chlorthalidone

Parameter	Benidipine Hydrochloride	Chlorthalidone
Range	32-48 µg/mL	100-150µg/mL
Slope	50184	37102
Intercept	30070	74642
Correlation Coefficient	0.999	0.999

Method Validation

1. Linearity: To get desired analyte concentrations the standard solution was created by adding 5 mg of Benidipine Hydrochloride and 12.5mg of Chlorthalidone in 10 mL of volumetric flask and make up volume up to 10 mL using diluent then further dilution was made to get 32-48µg/mL of Benidipine Hydrochloride and 100-150µg/mL respectively. The correlation coefficient for calibration curve Benidipine Hydrochloride and Chlorthalidone was found to be 0.999 and 0.999 respectively (Table 4).

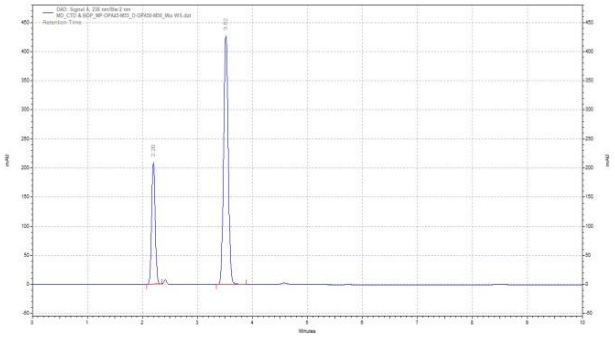

Fig. 3 - Chromatogram of Standard mixture of Benidipine Hydrochloride & Chlorthalidone

2. Precision: Precision of an analytical procedure express the closeness of agreement between a series of measurement obtained from multiple sampling of same homologous sample under précised condition. Intraday precision for Benidipine hydrochloride and Chlorthalidone are shown in Table no. (5&6). The %RSD for Benidipine Hydrochloride and Chlorthalidone was found to be 0.161and 0.390, respectively. Interday precision for Benidipine hydrochloride and Chlorthalidone are shown in Table no. (7&8). The %RSD or Benidipine Hydrochloride and Chlorthalidone was found to be 0.27and 0.41, respectively.

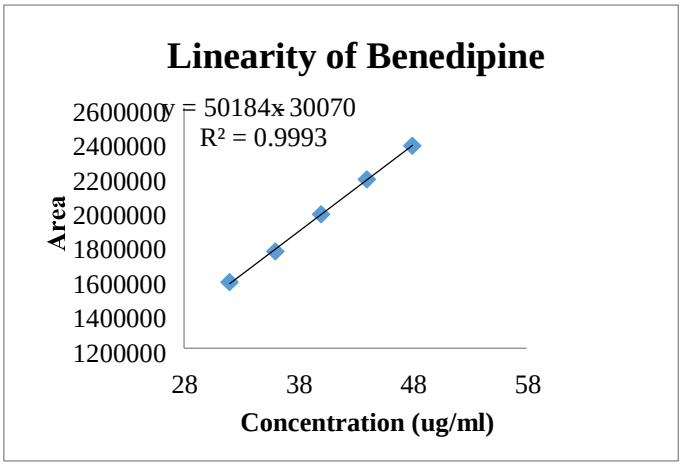

Fig. 4 - Linearity graph of Benidipine Hydrochloride

3. LOD & LOQ: Limit of Detection (LOD) means the detection of an individual analytical procedure express the closeness of agreement between a series of measurement obtained from multiple sampling of same homologous sample under précised condition and Limit of Quantitation (LOQ) means the quantitation of an analytical procedure is the lowest amount of an analyte in a sample which can be quantitatively determine with suitable precision and accuracy. It can be calculated using formula 3.3 σ/S and 10 σ/S, respectively. Where σ is SD of y- intercepts of regression line and S is slope of calibration curve (Table 13).

4. Robustness: This was done by minute changes in the Chromatographic condition and found to be unchanged by minute changes like ±2% changes in volume of organic solution of mobile phase.

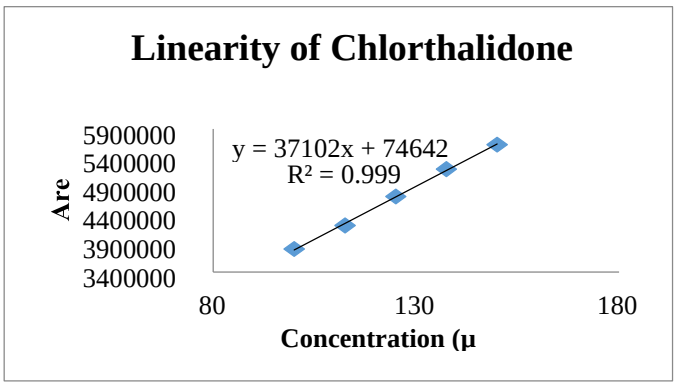

Fig. 5 - Linearity graph of Chlorthalidone

5. Accuracy: Accuracy is the closeness of agreement between the values found. The value accepted as the conventional true value or the accepted reference value. The accuracy of the

method was confirmed by recovery study from the marketed formulation at three level of standard addition (Table 9 & 10).

6. System suitability parameters:

7. Specificity: Specificity is ability to assess the analyte for the presence of various components that may be present. Specificity of the RP_HPLC method was determined by entire separation of Benidipine Hydrochloride and Chlorthalidone with parameter like Retention Time (RT), resolution (Rs) and Tailing factor (T). The peak obtained for Benidipine Hydrochloride and Chlorthalidone were sharp and have a clear baseline separation. The results are shown in Table 12.

Table 5 - Intraday Precision study of Benidipine Hydrochloride

Concentration in µg/mL	Area	% Recovery	Mean % Recovery ± SD	Mean % Recovery ±RSD
	1612694	**98.55**		
32 µg/mL	**1613005**	**98.57**	**98.59±0.064**	
	1614658	**98.67**		
	1825799	**99.39**		**99.21±0.161**
36 µg/mL	**1836154**	**99.97**	**99.79±0.34**	
	1837001	**100.01**		
	2023149	**99.28**		
40 µg/mL	**2024153**	**99.33**	**99.25±0.08**	
	2020746	**99.16**		

Table 6 - Intraday Precision study of Chlorthalidone

Concentration in µg/mL	Area	% Recovery	Mean % Recovery ± SD	Mean % Recovery ±RSD
	3794817	**100.26**		
100 µg/mL	**3745628**	**98.94**	**99.53±0.66**	
	3767898	**99.41**		
	4256897	**100.19**		
122.5 µg/mL	**4215783**	**99.21**	**99.75±0.49**	**99.75±0.39**
	4243162	**99.86**		
	4712539	**100**		
125 µg/mL	**4710986**	**99.96**	**99.98±0.02**	
	4711656	**99.98**		

Table 7 - Interday Precision study of Benidipine Hydrochloride

Concentration in µg/mL	Area	% Recovery	Mean % Recovery ± SD	Mean % Recovery ±RSD
	1611936	98.50		
32 µg/mL	1611127	98.45	98.45±0.04	
	1610456	98.41		
	1796321	97.76		
36 µg/mL	1795854	97.73	97.48±0.44	97.93±0.270
	1789500	96.97		
	1999120	98.04		
40 µg/mL	1887664	97.49	97.88±0.33	
	1998664	98.06		

Table 8 - Interday Precision study of Chlorthalidone

Concentration in µg/mL	Area	% Recovery	Mean % Recovery ± SD	Mean % Recovery ±RSD
	3689541	97.48		
100 µg/mL	3698627	97.73	97.79±0.354	
	3715496	98.18		
	4158946	97.89		
122.5 µg/mL	42011993	98.93	98.62±0.637	98.67±0.421
	4207234	99.05		
	4699137	99.75		
125 µg/mL	4678789	99.31	99.62±0.273	
	4701856	99.81		

Table 9 - Recovery study of Benidipine Hydrochloride

Std Area			1968846					
%Level	Reps	Spiked Conc. (µg/ml)	Area	Amount Recovered µg/ml)	%Recovery	AVG	STDEV	RSD
	Rep 1	32	1584314	32.19	100.59			
80%	Rep 2	32	1572215	31.94	99.82	99.86	0.702637	0.70
	Rep 3	32	1562213	31.74	99.18			
	Rep 1	40	1979466	40.22	100.54			
100%	Rep 2	40	1957783	39.78	99.44	99.72	0.726323	0.73
	Rep 3	40	1952473	39.67	99.17			
	Rep 1	48	2378413	48.32	100.67			
120%	Rep 2	48	2324579	47.23	98.39	99.62	1.149973	1.15
	Rep 3	48	2357896	47.90	99.80			

Table 10 - Recovery study of Chlorthalidone

Std Area			4735952					
% Level	Reps	Spiked Conc. µg/ml)	Area	Amount Recovered (µg/ml)	% Recovery	AVG	STDEV	RSD
	Rep 1	100	3800057	100.30	100.30			
80%	Rep 2	100	3821454	100.86	100.86	100.58	0.282482	0.28
	Rep 3	100	3810244	100.57	100.57			
	Rep 1	125	4721514	124.62	99.70			
100%	Rep 2	125	4732156	124.90	99.92	100.06	0.444878	0.44
	Rep 3	125	4762145	125.69	100.55			
	Rep 1	150	5626385	148.50	99.00			
120%	Rep 2	150	5621020	148.36	98.91	98.96	0.047238	0.05
	Rep 3	150	5623887	148.44	98.96			

Table 11 - System suitability parameters

Sr.no	Parameters	Benidipine Hydrochloride	Chlorthalidone
1	**Retention time (RT)**	1.09	3.52
2	**Area**	1979466	4731515
3	**Theoretical plates**	5344	8346
4	**Resolution**	0.0	9.62

RESULTS AND DISCUSSION

In this method development 100 mL of mobile phase was prepared by mixing 550 Ml of 1% orthophosphoric acid and 450 mL of Methanol. The mobile phase filtered through 0.45µm filters to avoid the column clogging due to smaller particle size. The flow rate was found to be 1 mL/min resulting in short retention time, good baseline stability with low noise level. In this present developed RP-HPLC method, the standard and sample preparation required less me. By the use of proposed method the retention time of Benidipine Hydrochloride and Chlorthalidone was found to be 1.09 and 3.52 minutes respectively.

Table 12 - Specificity Parameter

Parameter	Benidipine Hydrochloride	Chlorthalidone
Tailing Factor	1.09	1.05
Resolution	0.00	9.62
Retention Time	1.09	3.52

The assay of Benidipine Hydrochloride and Chlorthalidone in bulk drug and combined dosage form was found to be 100.99% and 100.15% respectively. A good linear relationship, r2 = 0.999 for Benidipine Hydrochloride and r^2 = 0.999 for chlorthalidone was observed between the concentration range of 32-48µg/mL and 100-150µg/mL, respectively. The LOD and LOQ values for Benidipine Hydrochloride was found to be 2.05µg/mL and 6.22 µg/mL and for Chlorthalidone 7.71 µg/mL and 23.38 µg/mL, respectively.

The low values of standard deviation of retention time and peak area indicates high precision of method from recovery study data it has found to be mean % recovery was within a limit, indicates high accuracy of proposed method. There are no additional peaks are observed in the chromatogram which indicates no interference of common ingredients used in formulation. No marked changes were observed in % assay of the optimized condition with that of alternate condition in the robustness study indicating that the method is robust.

Table 13 - LOD & LOQ Parameter

Name of Drug	LOD (µg/mL)	LOQ (µg/mL)
Benidipine Hydrochloride	2.05	6.22
Chlorthalidone	7.71	23.38

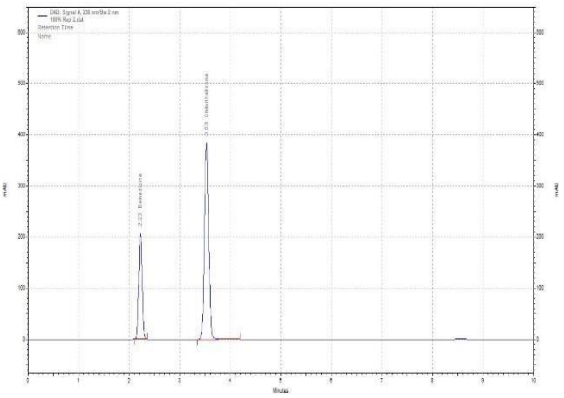

Fig. 6 - Chromatogram of 80% Accuracy

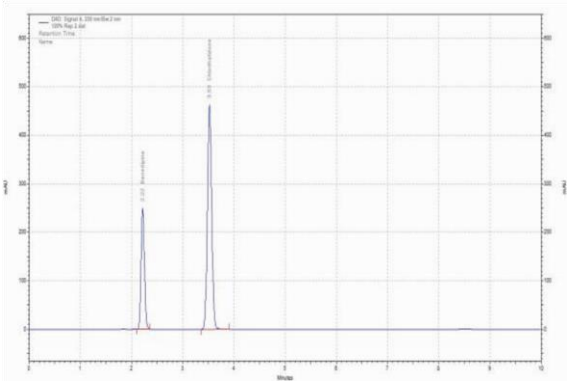

Fig. 7 - Chromatogram of 100% Accuracy.

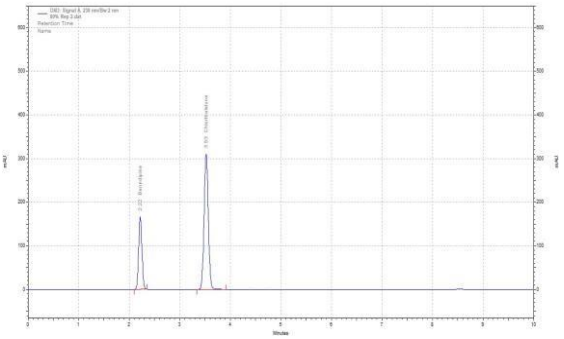

Fig .8 - Chromatogram of 120% Accuracy.

CONCLUSION

A simple competent method was developed for the simultaneous equation of Benidipine Hydrochloride and Chlorthalidone within a short analysis time with no interference of common additives present in tablet formulation.

The proposed method can be applied for routine quality control and applied for bulk and in tablet dosage form.

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How to cite this article: Shaikhmulani HI, Tamboli AM, Tambili NA, Kshirsagar RT, Suryawanshi HT, Khandare O. RP-HPLC Method Development and Validation for Simultaneous Estimation of Benidipine Hydrochloride and Chlorthalidone in Pharmaceutical Dosage Form. Indian J Pharm Drug Studies. 2022: 1(3) 102-107.

Funding: None *Conflict of Interest: None Stated*