

Simultaneous estimation of chlorthalidone and efonidipine hydrochloride in bulk and tablet dosage form

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ABSTRACT: Chlorthalidone and efonidipine hydrochloride are anti-hypertensive agents with different mechanisms of action. These drugs are generally used as monotherapy; however, recent study suggests their use in combination reduces the mortality rate. Based on this evidence, Zuventus Healthcare limited developed a combination dosage (Efnocar Ct) which has been recently approved for its use. Hence, a new, sensitive, precise and robust method was developed and validated to determine the purity of the compounds in bulk as well as tablet dosage forms. The HPLC analysis was achieved on Zobrax 150 column using acetonitrile and ammonium formate (60:40% v/v) as the mobile phase and the detection was achieved at a λ_{\max} of 248nm. The linearity was achieved between the concentration range of 3 μ g/ml – 18 μ g/ml for chlorthalidone and 10 μ g/ml – 60 μ g/ml concentration range for efonidipine hydrochloride. The % recovery for chlorthalidone was 100.03% and that for efonidipine was 100.24%, indicating the method to be accurate. The LOD and LOQ values for chlorthalidone was 0.01 μ g/ml and 0.02 μ g/ml and for efonidipine it was 0.26 μ g/ml and 0.88 μ g/ml. This indicates the sensitivity of the method. Robustness studies have shown no significant variations and degradation studies have shown acceptable results. The results of the proposed method for linearity, accuracy, sensitivity and robustness were within the acceptable limits.

KEYWORDS: Hypertension; combination dose; chlorthalidone; efonidipine hydrochloride; method development; validation

1. INTRODUCTION

Hypertension is a medical condition that increases the risk of heart, brain, kidney and other diseases. According to World Health Organization (WHO), there are circa 1.4 billion hypertensive patients among which 14% have it under control [1]. In a two-decade analysis, the number of deaths due to hypertension have been gradually increasing across the world. Among all, China had reported most of the deaths due to hypertension, followed by India (Figure 1) [2]. These deaths are reported either due to stroke or coronary heart disease which are a result of hypertension. Therefore, management of hypertension is essential to reduce the mortality rate. Several classes of antihypertensive drugs are available for the management. Among these, calcium channel blockers and diuretics are widely used as monotherapy for the treatment due to their greater tolerance and effectiveness in reducing cardiovascular diseases. However, these two classes of drugs are rarely explored together. A meta-analysis was conducted by *Rimoldi et al.*, in 2015 and demonstrated the use of calcium channel blockers in combination with diuretic reduced the risk of myocardial infarction and stroke [3]. Recently, a combination therapy of Chlorthalidone and efonidipine (12mg + 40mg) was approved by central drugs standard control organization (CDSCO), India, for the treatment of hypertension [4].

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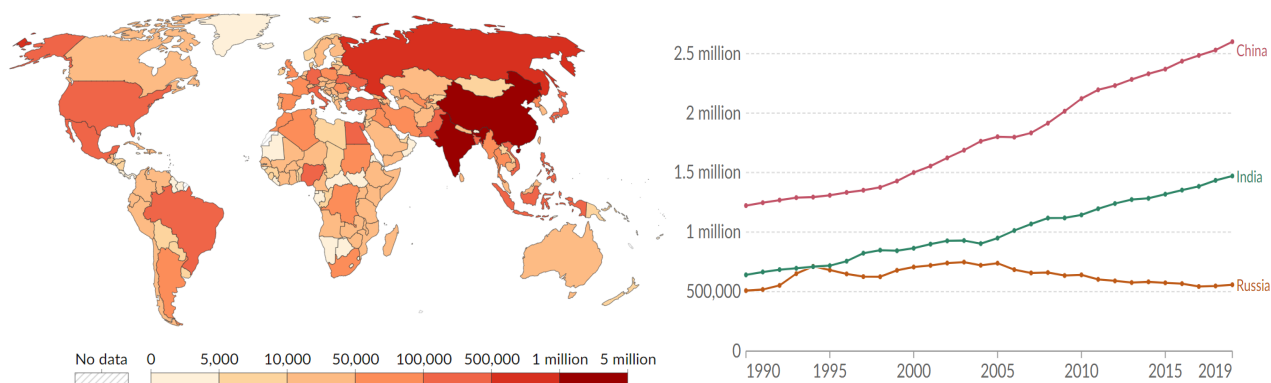


Figure 1. (a) Showing annual deaths caused due to hypertension across the world from 1990-2019 (b) showing the increase in number of deaths from 1990-2019 of countries with high mortality rate (source: <https://ourworldindata.org/grapher/deaths-due-to-high-blood-pressure?facet=None>)

Chlorthalidone is chemically 2-chloro-5-(1-hydroxy-3-oxo-2H-isoindol-1-yl)benzenesulfonamide (Figure 2a), and is currently used as thiazide diuretic in the treatment of hypertension caused by heart failure, renal failure, and hepatic cirrhosis. The mechanism of anti-hypertensive effect is achieved by inhibiting the Na⁺/Cl⁻ symporter in the ascending limb of Henle's loop preventing the reabsorption of sodium and chloride ions causing reduction in extracellular fluid and plasma volume [5,6]. Additionally, it also decreases platelet aggregation and vascular permeability as well as promote angiogenesis [7-9].

Efonidipine is chemically 2-(N-benzylanilino)ethyl 5-(5,5-dimethyl-2-oxo-1,3,2λ⁵-dioxaphosphinan-2-yl)-2,6-dimethyl-4-(3-nitrophenyl)-1,4-dihydropyridine-3-carboxylate (Figure 2b), and is a calcium channel blocker used in the treatment of hypertension. It belongs to dihydropyridine class and had been extensively studied in atherosclerosis and acute renal failure. It acts by inhibiting the L-type calcium channels and affects the cardiac function resulting in decreased blood pressure and heart rate [10, 11].

Literature studies have shown several reports for the estimation of chlorthalidone and efonidipine, either singly or in combination with other drugs [12,13,22-31,14-21]. There was only one report where in both the drugs were simultaneously estimated and validated the method using UV spectroscopic method [29]. Apart from these, there were no HPLC methods reported. As this is a newly approved combination, and needs a validated method for its estimation, we found it necessary to develop a method to estimate both the drugs and validate it according to the ICH guidelines [Q2 (R1)] [32]. Since it is a newly approved formulation, preliminary studies of drug estimation with HPLC helps in understanding the nature of the compound by providing valuable insights into its chromatographic behavior, separation characteristics, and interactions with the stationary phase. By establishing a reliable HPLC method, essential baseline data can be gathered for future comparison and confidently monitor the compound's behavior in various conditions, thus contributing to a comprehensive understanding of its pharmaceutical profile.

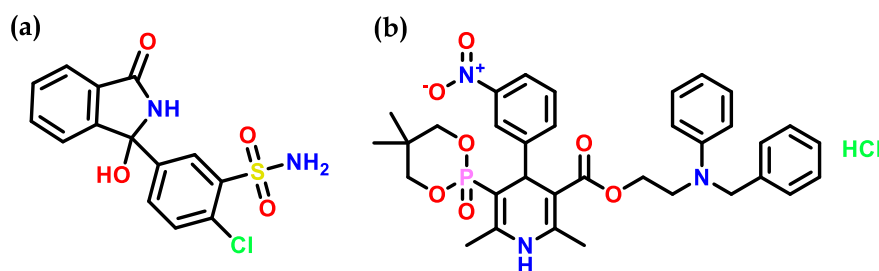


Figure 2. Structures of (a) Chlorthalidone (b) Efonidipine hydrochloride

2. RESULTS & DISCUSSION

2.1. Method optimization

Multiple trials were conducted with zobrax 150 column, using various concentrations of acetonitrile and ammonium formate. The acceptable peak separation was observed using the mobile phase containing ACN and ammonium formate in the ratio of 60:40% v/v on Zobrax 150 column with the dimensions 43.6x150mm having 3.5μm of particle size at 30°C temperature and detection wavelength of 248nm. The peak obtained was sharp with good resolution.

2.2. Study of retention time

A standard dilution of chlorthalidone and efonidipine was prepared and injected into the HPLC system. The mean retention time for chlorthalidone was found to be 2.26min and for efonidipine, it was 2.992mins. The typical chromatograms of standard and sample solution are shown in Figure 3.

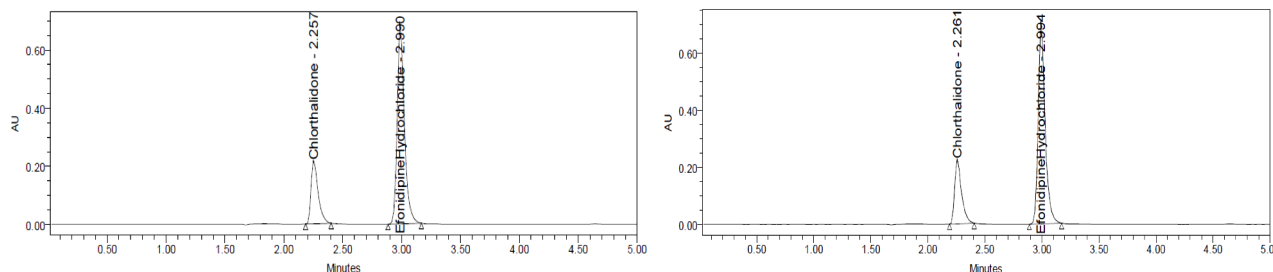


Figure 3. Typical chromatogram of chlorthalidone and efonidipine hydrochloride showing standard solution (left) and sample solution (right)

2.3. System suitability

After column equilibrations, 20 μ L of standard solutions of chlorthalidone and efonidipine was prepared in five replicates and injected to the chromatographic system. The system suitability parameters were recorded and shown in Table 1.

Table 1. System suitability results of chlorthalidone and efonidipine hydrochloride

S. no	Chlorthalidone			Efonidipine Hydrochloride		
	RT (min)	Plate count (N)	Tailing	RT (min)	Plate count (N)	Tailing
1.	2.236	6987	1.44	2.950	11895	1.25
2.	2.244	6695	1.44	2.954	12458	1.25
3.	2.256	6270	1.46	2.989	11467	1.35
4.	2.258	7138	1.45	2.990	12069	1.30
5.	2.257	6124	1.45	2.994	12504	1.30

2.4. Method validation

The developed method was validated in terms of specificity, linearity, robustness, and limit of detection & quantitation. The validation parameters were verified only after the technique satisfy the acceptable limits as per ICH guidelines [32].

2.5. Specificity

Specificity is carried out to determine the presence of excipients. For this, a blank solution, followed by the standard and sample solutions are injected into the chromatographic systems and their respective chromatograms are recorded. There were no excipient interactions observed as the correlation between standard and sample were good.

2.6. Linearity

Linearity of the method was carried out between the concentration range of 3.125 μ g/ml to 15.62 μ g/ml. Primarily, the stock solution was taken and suitable dilutions were made to obtain the required concentrations. Each concentration was injected in triplicates and the average of them are shown in Table 2. A calibration curve is plotted between concentration (on x-axis) and peak area (on y-axis). The correlation coefficient between the concentration and peak area for chlorthalidone and efonidipine were >0.999, indicating that the method has a good linear correlation. The results of linearity are shown in Table 2 and the respective calibration plots are shown in Figure 4.

Table 2. Linearity results of chlorthalidone and efonidipine hydrochloride

S. no	Chlorthalidone		Efonidipine Hydrochloride	
	Conc (µg/mL)	Peak area	Conc (µg/mL)	Peak area
1.	0	0	0	0
2.	3.125	222748	10	725974
3.	6.25	455228	20	1498543
4.	9.375	685971	30	2231989
5.	12.5	914677	40	2920963
6.	15.62	1104866	50	3653441
7.	18.75	1348484	60	4424450
Slope		71656		73395
Intercept		4274.0		6047.4
Correlation co-efficient		0.9998		0.9998

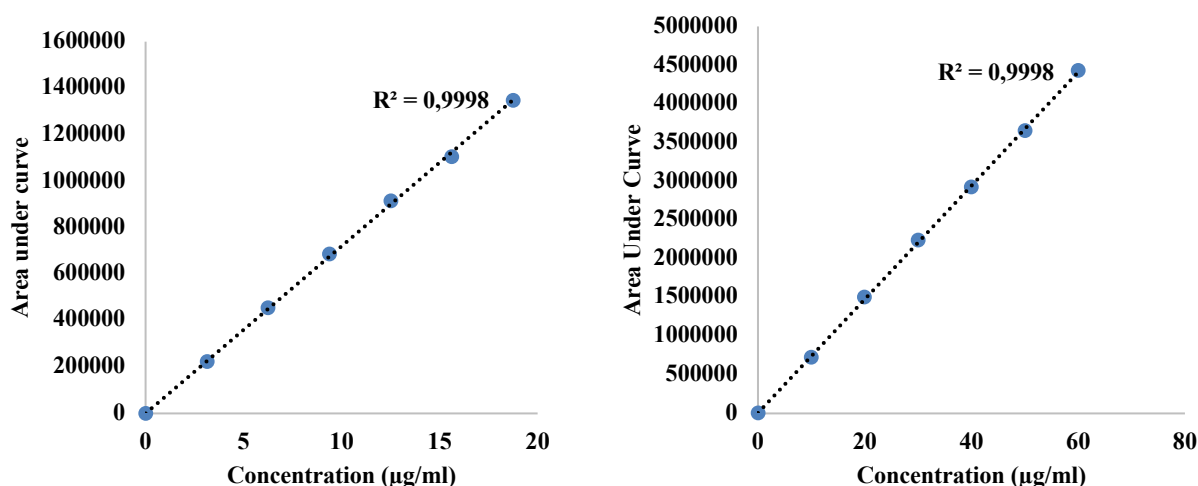


Figure 4. Calibration curve obtained from linearity study of chlorthalidone (left) and efonidipine hydrochloride (right)

2.7. Accuracy

Accuracy of the method was evaluated by preparing 50%, 100% and 150% concentration of the drug solution and injected in triplicates to the chromatographic system. From the amount spiked, the amount recovered is measured and the percentage recovery is calculated. The % recovery of both the drugs were greater than 100% (100.03% for chlorthalidone, and 100.24% for efonidipine). The accuracy results of both the drugs are shown in Table 3.

Table 3. Accuracy results of chlorthalidone and efonidipine hydrochloride

S. No	Level	Amount Spiked (µg/ml)	Amount recovered (µg/ml)	% Recovery	Mean % Recovery
Chlorthalidone					
1.	50%	6.25	6.0 ± 0.06	100.2 ± 0.66	100.03%
2.	100%	12.5	12.5 ± 0.10	100.1 ± 0.76	
3.	150%	18.75	18.6 ± 0.15	99.8 ± 0.66	
Efonidipine					
1.	50%	20	20.0 ± 0.16	100.07 ± 0.79	100.24%
2.	100%	40	40.16 ± 0.37	100.39 ± 0.93	
3.	150%	60	60.15 ± 0.43	100.26 ± 0.71	

2.8. Precision

To demonstrate the preciseness of the developed method, repeatability and intermediate precision studies were carried out. For repeatability studies, six working sample solutions were injected into the chromatographic system and its area under curve is measured. The relative standard deviation (RSD) for both the drugs were 0.3% (chlorthalidone) and 0.6% (efonidipine). In the case of intermediate precision, six sample solutions were prepared and injected into the chromatographic system in the next day and their respective area under curve were measured. From the results obtained. The RSD for chlorthalidone was 0.3%

and for efonidipine was 0.7%. As the % RSD for both drugs were below 2.5, it indicates the developed method is precise. Results for repeatability and intermediate precision are shown in Table 4 and 5 respectively.

Table 4. Repeatability results of chlorthalidone and efonidipine hydrochloride

S. No	Chlorthalidone		Efonidipine	
	Retention time	Area under curve	Retention time	Area under curve
1.	2.994	911935	2.261	2946511
2.	2.995	915288	2.258	2931530
3.	3.001	919085	2.263	2969980
4.	2.998	916789	2.265	2976947
5.	2.998	918028	2.262	2970082
6.	3.001	918997	2.266	2948685
Mean		916687		2957289
S. D		2736.2		17691.1
%RSD		0.3		0.6

Table 5. Intermediate precision results of chlorthalidone and efonidipine hydrochloride

S. No	Chlorthalidone		Efonidipine	
	Retention time	Area under curve	Retention time	Area under curve
1.	2.253	910183	2.972	2903615
2.	2.236	916515	2.950	2962069
3.	2.261	916387	2.994	2923202
4.	2.254	915175	2.997	2940748
5.	2.197	915755	2.903	2936590
6.	2.210	911303	2.913	2937287
Mean		914220		2933919
S. D		2758.2		19445.9
%RSD		0.3		0.7

2.9. Limit of detection and quantitation

The sensitivity of the method is determined by calculating the lowest limit of detection (LOD) and quantitation (LOQ) based on signal-noise ratio. This provides information about the sensitivity and reliability of the developed analytical method. To achieve this, 0.25ml of standard stock solution was dissolved in 10ml volumetric flask. From this 0.25ml and 0.9ml solutions were injected into the chromatographic system to determine LOD and LOQ respectively. The lowest detection limit for chlorthalidone was 0.01 μ g/ml and efonidipine was 0.02 μ g/ml, while the lowest quantitation limit for chlorthalidone was 0.29 μ g/ml and efonidipine was 0.88 μ g/ml. These results suggest sensitive detection of both the drugs. The respective chromatograms of LOD and LOQ are shown in Figure 5.

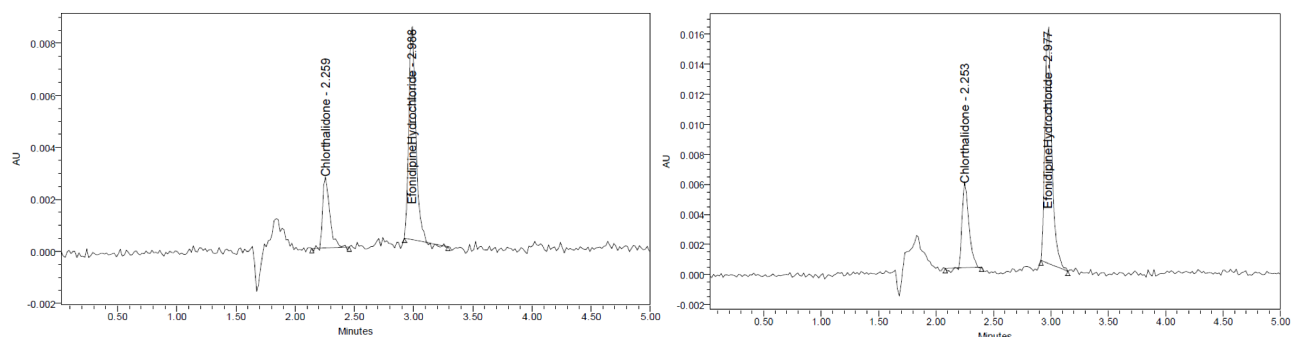


Figure 5. Typical chromatograms of chlorthalidone and efonidipine hydrochloride representing LOD (on the left) and LOQ (to the right)

2.10. Robustness

Robustness is a measure that shows the ability of a system to work efficiently despite having disturbances. To evaluate this parameter, modifications are done on the chromatographic conditions by varying the flow rate, mobile phase composition and temperature. There were no significant changes with change in the chromatographic conditions. The results at varying parameters are shown in Table 6.

Table 6. Robustness results of chlorthalidone and efonidipine hydrochloride

Parameters	Chlorthalidone		Efonidipine	
	Retention time	% RSD	Retention time	% RSD
F (-) 0.9ml/min	2.403 ± 0.002	0.4	3.177 ± 0.009	0.3
F (+) 1.1ml/min	2.091 ± 0.045	0.3	2.755 ± 0.058	0.6
MP (-) 55B:45A	2.176 ± 0.003	0.3	2.780 ± 0.01	0.4
MP (+) 65B:35A	2.350 ± 0.003	0.4	3.216 ± 0.006	0.8
Temp (-) 27°C	2.297 ± 0.001	0.4	3.083 ± 0.003	0.4
Temp (+) 33°C	2.206 ± 0.005	0.3	2.862 ± 0.001	0.3

2.11. Degradation studies

To determine stability of the developed method, degradation studies were carried out by exposing the stock solutions to different stress condition. For oxidative stress, 1ml of the stock solution was mixed with 1ml of 20% hydrogen peroxide and kept aside for 30 mins at 60°C where as for acid and alkali degradation, the stock solutions were mixed with 1ml of 2N hydrochloric acid and 2N sodium hydroxide respectively. For dry heat, the standard drug solution was placed in oven at 105°C for 1 h, and for photo stability the drug solution was placed in UV Chamber for 1days or 200-Watt hours/m² in photo stability chamber. Additionally, Stress testing under neutral conditions was studied by refluxing the drug in water for 1hrs at a temperature of 60°C. All the samples were diluted with the diluent to obtain the concentrations of 40µg/ml & 12.5µg/ml of chlorthalidone and efonidipine respectively, and 10µl of each sample was subjected for HPLC analysis. The results have shown that the % degradation of the drug in all conditions were less than 10% indicating the stability of the method. Degradation results are shown in Table 7.

Table 7. Degradation results of chlorthalidone and efonidipine hydrochloride

Degradation condition	Area		% Recovery		% Drug degradation	
	Chlorthalidone	Efonidipine	Chlorthalidone	Efonidipine	Chlorthalidone	Efonidipine
Acid	855055	2747823	93.29	93.10	6.71	6.90
Alkali	889993	2861033	97.11	96.93	2.89	3.07
Oxidation	886397	2857164	96.71	96.80	3.29	3.20
Thermal	892484	2869402	97.38	97.21	2.62	2.79
Photo	902505	2922098	98.47	99.00	1.53	1.00
Water	912463	2940480	99.56	99.62	0.44	0.38

3. CONCLUSION

A simple, precise, and robust HPLC method was developed for the estimation of chlorthalidone and efonidipine hydrochloride in bulk and tablet dosage form. The method was validated based on various parameters such as specificity, linearity, accuracy, precision, and robustness. All the results for these parameters were within acceptable limits. Results from the degradation studies also show the stability of the method. The established HPLC method offers a straightforward, accurate, and resilient approach for quantifying chlorthalidone and efonidipine hydrochloride in both bulk samples as well as tablet formulations, and therefore, emerges as a valuable asset for ensuring the quality control of these drugs in the pharmaceutical industries.

4. MATERIALS AND METHODS

4.1. Chemical and reagents

Chlorthalidone and Efonidipine hydrochloride pure drugs were received as gift sample from spectrum labs. The other reagents such as acetonitrile, phosphate buffer, methanol, and potassium dihydrogen ortho phosphate were obtained from Rankem. The pharmaceutical dosage for of chlorthalidone

and efonidipine (Efnocar Ct) with the dosage of 40mg of efonidipine hydrochloride and 12.5mg of chlorthalidone was purchased from local pharmacy.

4.2. Instrumentation

The analytical balance used for weighing the samples was of Denver make, the sonication of sample and pH was monitoring was carried out by ultra sonicator and pH metere, respectively, purchased from BVK enterprise. The HPLC system used for the analysis of compounds was of WATERS HPLC 2695 SYSTEM.

4.3. Preparation of buffer

In a 1000 mL volumetric flask, 0.63g of Ammonium formate is weighed and added to 900 mL milli-Q water and degassed by sonicating. The final volume is diluted to 999ml. 1 mL of o-phosphoric acid is added to adjust the pH to 4.2.

4.4. Preparation of the mobile phase

600 mL (60%) of the acetonitrile was mixed with 40 mL (40%) of ammonium formate. It was then degassed in a sonicator for around 10 minutes and subjected to filtration through 0.45 μ filter under vacuum filtration. The same solution was used as diluent.

4.5. Method Development

The column Zobrax 150 column (4.6 x 150mm, with particle size of 3.5 μ m) was used to develop the method. The mobbile phase used was the mixture of acetonitrile and ammonium formate (60:40 v/v). The flow rate was maintained at 1 ml/min at a temperature of 30°C and injection volume was set to 10 μ L for 10 mins run time. The ultraviolet (UV) detection was achieved at λ_{max} of 248nm. With the above mentioned chromatographic conditions, a clear and satisfactory peak was obtained. Using these conditions, validation parameters were further evaluated.

4.6. Standard Solution Preparation:

6.25mg of chlorthalidone and 20mg of efonidipine was added to 50ml volumetric flask, and dissolved in small quantity of diluent. It was sonicated for 10 minutes for the residue to dissolve and the final volume was made to 50.0mL Further, 1.0 ml of the prepared standard stock solution was pipetted into a 10ml VF and diluted up to the mark with diluent.

4.7. Sample Solution Preparation:

Ten tablets of Efnocar Ct was crushed and the tablet power containing equivalent weight of 12.5mg chlorthalidone and 40 mg of efonidipine hydrochloride was transferred to 100 ml volumetric flask. To this, 50ml of diluent was added and sonicated for 25 minutes, and then volume was made to 100.0 ml. From this, 1 ml was pipetted into a 10ml VF and diluted up to the mark with diluent.

4.8. Procedure:

A mobile phase containing acetonitrile and ammonium formate buffer in the ratio of 60:40% v/v was found to be the most suitable one. The column was equilibrated by injecting mobile phase at a rate of 1ml/min prior to sample injection and ambient temperature was maintained. 20 μ l of the standard as well as sample were injected into the HPLC system for 10 mins of run time, and measured the areas for chlorthalidone and efonidipine peaks. The detection was carried out at 248nm wavelength. Under these conditions, peaks corresponding to chlorthalidone was obtained at a run time of 2.263 mins and efonidipine was obtained at 2.994 mins. Typical chromatograms of peak separation is shown in Figure 3. From the obtained peaks of both the drugs, the % assay was calculated.

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REFERENCES

- [1] World Health Organization. Guideline for the pharmacological treatment of hypertension in adults: summary. <https://www.who.int/publications/i/item/9789240050969> (accessed April 14, 2023).
- [2] Deaths due to high blood pressure, 2019. <https://ourworldindata.org/grapher/deaths-due-to-high-blood-pressure?country=CHN~RUS~IND~USA~BRA~ARG> (accessed April 24, 2023).
- [3] Rimoldi SF, Messerli FH, Chavez P, Stefanini GG, Scherrer U. Efficacy and safety of calcium channel blocker/diuretics combination therapy in hypertensive patients: A meta-analysis. *J Clin Hypertens.* 2015;17(3):193–9. <https://doi.org/10.1111/jch.12462>.
- [4] Central Drugs Standard Control Organization. Recommendations of the SEC (Cardiovascular & Renal). 2020. [https://www.cdsc.gov.in/opencms/resources/UploadCDSCOWeb/2018/UploadCommitteeFiles/73rdSEC Cardiovascular & Renal6fe20.pdf](https://www.cdsc.gov.in/opencms/resources/UploadCDSCOWeb/2018/UploadCommitteeFiles/73rdSEC%20Cardiovascular%20&%20Renal6fe20.pdf) (accessed April 24, 2023).
- [5] Shahin MH, Johnson JA. Mechanisms and pharmacogenetic signals underlying thiazide diuretics blood pressure response. *Curr Opin Pharmacol.* 2016; 27 :31–37. <https://doi.org/10.1016/j.coph.2016.01.005>.
- [6] Agarwal R, Sinha AD, Cramer AE, Balmes-Fenwick M, Dickinson JH, Ouyang F, Tu W. Chlorthalidone for hypertension in advanced chronic kidney disease. *N Engl J Med.* 2021;385(27):2507-2519. <https://doi.org/10.1056/nejmoa2110730>.
- [7] Woodman R, Brown C, Lockette W. Chlorthalidone decreases platelet aggregation and vascular permeability and promotes angiogenesis. *Hypertension.* 2010;56(3):463–470. <https://doi.org/10.1161/HYPERTENSIONAHA.110.154476>
- [8] Rapoport RM, Soleimani M. Mechanism of thiazide diuretic arterial pressure reduction: The search continues. *Front Pharmacol.* 2019; 10: 815. <https://doi.org/10.3389/fphar.2019.00815>.
- [9] De la Espriella R, Cobo M, Núñez J. Thiazides in chronic kidney disease: “back to the future.” *Clin Kidney J.* 2023;16(1):1–4. <https://doi.org/10.1093/ckj/sfac228>.
- [10] Oh IY, Seo MK, Lee HY, Kim SG, Kim KS, Kim WH, Hyon MS, Han KR, Lim SJ, Kim JH. Beneficial effect of efonidipine, an L- and T-type dual calcium channel blocker, on heart rate and blood pressure in patients with mild-to-moderate essential hypertension. *Korean Circ J.* 2010;40(10):514–519. <https://doi.org/10.4070/kcj.2010.40.10.514>.
- [11] Jariwala P, Jadhav K. The safety and efficacy of efonidipine, an L- and T-Type dual calcium channel blocker, on heart rate and blood pressure in indian patients with mild-to-moderate essential hypertension. *Eur Heart J.* 2022;43. <https://doi.org/10.1093/eurheartj/ehab849.143>.
- [12] Wang N, Ye L, Ku S, Zhao BQ, Yu JX. Spectroscopic studies on the interaction of efonidipine with bovine serum albumin. *Braz J Med Biol Res.* 2008;41(7):589-595. <https://doi.org/10.1590/S0100-879X2008000700007>
- [13] Kumar GS, Ramya V, Mondal S, Kumar SP. Development and validation of RP-HPLC method for simultaneous estimation of Atenolol and Chlorthalidone in pharmaceutical formulation. *Int Res J Pharm.* 2012; 3(10): 215-219.
- [14] Liu M, Zhao H, Tong Y, Zhang D, Wang X, Zhang L, Han J, Liu H. Determination of efonidipine in human plasma by LC-MS/MS for pharmacokinetic applications. *J Pharm Biomed Anal.* 2015; 103: 1–6. <https://doi.org/10.1016/j.jpba.2014.11.001>.
- [15] Aneesh TP, Radhakrishnan R, Aravind PM, Sasidharan A, Choyal M. RP-HPLC method for simultaneous determination of Losartan and Chlorthalidone in pharmaceutical dosage form. *Int Res J Pharm.* 2015;6(7):453–457. <https://doi.org/10.7897/2230-8407.06793>.
- [16] Dangre P, Sawale V, Meshram S, Gunde M. Development and validation of RP-HPLC method for the simultaneous estimation of eprosartan mesylate and chlorthalidone in tablet dosage form. *Int J PharmTech Res.* 2015;8(2):163–168.
- [17] Aishwarya J. Method development and validation for the estimation of Telmisartan and Chlorthalidone in bulk and pharmaceutical dosage form by HPTLC method. *Res J Pharm Technol.* 2015;8(4):376–381. <https://doi.org/10.5958/0974-360X.2015.00063.3>.

- [18] Liu M, Deng M, Zhang D, Wang X, Ma J, Zhao H, Zhang L, Tong Y, Liu H. A chiral LC-MS/MS method for the stereospecific determination of efonidipine in human plasma. *J Pharm Biomed Anal.* 2016;122(15):35–41. <https://doi.org/10.1016/j.jpba.2016.01.039>.
- [19] Kumar A, Shoni SK, Dahiya M, Kumar R, Yadav AK, Kumar V, Chaudhary H. Development and validation of liquid chromatography (RP-HPLC) methodology for estimation of Efonidipine HCl Ethanolate (EFD). *Pharm Anal Acta.* 2017;8(5). <https://doi.org/10.4172/2153-2435.1000547>
- [20] Abdullah NS, Hassan MA, Hassan RO. Spectrophotometric determination of chlorthalidone in pharmaceutical formulations using different order derivative methods. *Arab J Chem.* 2017;10(2):3426–3433. <https://doi.org/10.1016/j.arabjc.2014.02.002>.
- [21] Rathod RH, Patil AS, Shirkhedkar AA. Novel NP and RP-HPTLC in praxis for simultaneous estimation of Chlorthalidone and Cilnidipine in bulk and pharmaceutical formulation. *Anal Chem Lett.* 2018;8(6):862–871. <https://doi.org/10.1080/22297928.2018.1527252>.
- [22] Kharat C, Shirsat VA, Kodgule YM, Kodgule M. A Validated RP-HPLC stability method for the estimation of Chlorthalidone and its process-related impurities in an API and tablet formulation. *Int J Anal Chem.* 2020: 1-11. <https://doi.org/10.1155/2020/3593805>.
- [23] Rajput AS, Jha DK, Gurram S, Shah DS, Amin PD. RP-HPLC method development and validation for the quantification of Efonidipine hydrochloride in HME processed solid dispersions. *Futur J Pharm Sci.* 2020;6(70):1-9. <https://doi.org/10.1186/s43094-020-00094-2>.
- [24] Pandya CP, Rajput SJ. Forced degradation study of efonidipine HCl ethanolate, characterization of degradation products by LC-Q-TOF-MS and NMR. *J Appl Pharm Sci.* 2020;10(4):75–99. <https://doi.org/10.7324/japs.2020.104012>.
- [25] Chaudhari SR, Shirkhedkar AA. Application of Plackett-Burman and central composite designs for screening and optimization of factor influencing the chromatographic conditions of HPTLC method for quantification of efonidipine hydrochloride. *J Anal Sci Technol.* 2020;11(48). <https://doi.org/10.1186/s40543-020-00246-2>
- [26] Prajapati P, Patel S, Mishra A. Simultaneous estimation of azilsartan medoxomil and chlorthalidone by chromatography method using design of experiment and quality risk management based quality by design approach. *J Planar Chromatogr - Mod TLC.* 2020;33:631–646. <https://doi.org/10.1007/s00764-020-00067-4>.
- [27] Vyas AJ, Gol DA, Usdad RG, Patel AI, Patel AB, Patel NK, Chudasama A. HPTLC-densitometric method for simultaneous estimation of Olmesartan medoxomil and Chlorthalidone in tablet dosage form. *Anal Chem Lett.* 2020;10(4):498–506. <https://doi.org/10.1080/2229792820201814859>.
- [28] Prajapati P, Patel M, Shah S. A robust high-performance thin-layer chromatography method for the simultaneous estimation of chlorthalidone and metoprolol succinate using quality risk assessment and design of experiments-based enhanced analytical quality by design approach. *J Planar Chromatogr - Mod TLC.* 2021;34(3):229–242. <https://doi.org/10.1007/s00764-021-00107-7>.
- [29] Solanki D, Patel D, Meshram D. Development and validation of UV Spectrophotometric method for simultaneous estimation of Efonidipine hydrochloride ethanolate and Chlorthalidone in their synthetic mixture. *Drug Anal Res.* 2022;6(1):27–34. <https://doi.org/10.22456/2527-2616.125170>.
- [30] Wang X, Sacramento CQ, Jockusch S, Chaves OA, Tao C, Fintelman-Rodrigues N, Chien M, Temerozo JR, Li X, Kumar S, Xie W, Patel DJ, Meyer C, Garzia A, Tuschl T, Bozza PT, Russo JJ, Souza TML, Ju J. Combination of antiviral drugs inhibits SARS-CoV-2 polymerase and exonuclease and demonstrates COVID-19 therapeutic potential in viral cell culture. *Commun Biol.* 2022;5(1):154. <https://doi.org/10.1038/s42003-022-03101-9>.
- [31] Patel GH, Adeshra SD, Meshram DB. RP-HPLC method development and validation for simultaneous estimation of efonidipine hydrochloride ethanolate and telmisartan in their synthetic mixture. *Int J Pharm Drug Anal.* 2021;9(3):190–195. <https://doi.org/10.47957/ijpda.v9i3.480>.
- [32] Rahi S, Rana A. Role of ICH guidelines in registration of pharmaceutical products. *Int J Drug Regul Aff.* 2019;7(4):14–27. <https://doi.org/10.22270/ijdra.v7i4.365>.