INTERNATIONAL JOURNAL OF RESEARCH IN PHARMACY AND CHEMISTRY

Available online at www.ijrpc.com

Research Article

ENANTIOMERIC SEPARATION AND QUANTITATIVE ESTIMATION OF CHLORTHALIDONE ENANTIOMERS BY CHIRAL ULTRA FAST LIQUID CHROMATOGRAPHY

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ABSTRACT

A chiral ultra fast liquid chromatographic method was developed and validated for separation of chlorthalidone enantiomers both in bulk and pharmaceutical formulations. Resolution was obtained in a normal phase mode on a Kromosil TBB chiral stationary phase (150 x 4.6mm, 5µm) with mobile phase composed of n-hexane, 2-proponol, acetic acid and triethylamine (92:8:0.3:0.01 v/v), at a flow rate of 1.2ml/min. The enantiomers and internal standard (2-nitro aniline) were detected at 260 nm wavelength. The internal standard and both the enantiomers were detected at 3.3min, 5.2min and 6.2 min, respectively. The correlation coefficient for linear regression curves of enantiomer 1 and enantiomer 2 were ≥0.999. The inter-day precision and intra-day precision, expressed as %RSD was less than 2. The accuracy determined by average recovery of enantiomer1 and enantiomer 2 were within the acceptable limits. Low levels of limit of detection (<0.1µg/mL) and limit of quantitation (<0.3µg/mL) for both enantiomers, makes the method sensitive. The method was useful for the determination of ratio of the two enantiomers in the bulk drug and also in the final formulated tablets.

Keywords: Chiral liquid chromatography, chlorthalidone enantiomers, resolution, internal standard.

INTRODUCTION

Chlorthalidone is a monosulfamyl diuretic, chemically known as (RS)-2-chloro-5-(1-hydroxy-3-oxo-2,3-dihydro-1H-isoindol-1-yl)benzene-1-sulfonamide. Chlorthalidone is a chiral drug administered clinically as a racemic (1:1) mixture of enantiomers. Chlorthalidone [Fig. 1] is a medium efficacy diuretic which inhibits Na+-Cl- symport. It is widely used in the management of increased blood pressure (hypertension). Chlorthalidone inhibits sodium reabsorption and promote sodium, chloride and water excretion in kidneys. It also decreases extracellular fluid volume, plasma volume and cardiac output which help in reducing blood pressure. It dilate arteries directly which helps in reducing the peripheral vascular resistance[1]

Examination of literature reveals that, several nonstereoselective analytical methods have been developed for the estimation of chlorthalidone in bulk and pharmaceutical dosage forms including UV-Visible[2],spectrophotometry using MBTH and FC reagents[3],HPLC[4].for the estimation of chlorthalidone in biological samples like blood, urine and red blood cells a GC with nitrogen detection[5] was developed also a method in GC was developed to estimate the chlorthalidone in nanogram level in plasma by using extractive alkylation technique[6]. However, the literature on stereoselective estimation of chlorthalidone describes that HPLC[7] and Super critical fluid chromatography[8](SFC) were developed for the separation of chlorthalidone enantiomers by using beta-cyclodextrin as chiral additive. but none of the

pharmacopeia or the journal described the separation of chlorthalidone enantiomers on a chiral stationary phase (CSP) which is simple, direct, reliable and accurate method for the seperation of enantiomers.

There are no HPLC reports available for the determination of chlorthalidone enantiomers by using a chiral stationary phase (CSP). This paper describes the resolution of racemic chlorthalidone using a TBB (0, 0'-bis (4-tert-butylbenzoyl)-N, N'-diallyl-Ltartardiamide) chiral stationary phase (Chiral-TBB) by using internal standard 2-nitro aniline and it also describes the quantitative determination of chlorthalidone enantiomers in their pharmaceutical formulation. The paper also adds the advantage of using UFLC than HPLC which pumps the mobile phase with more speed and pressure into the column and helps in eluting the enantiomers faster compared to HPLC. Several chromatographic parameters including the mobile phase composition were studied to optimize the proposed stereoselective analytical method.

MATERIALS AND METHODS

Chemicals and reagents

Analytically pure reference standard of chlorthalidone was supplied by Hetero drugs Pvt. Ltd.
(Hyderabad, India), and was used as such without further purification. The formulation used
in this study was the CTD 12.5 tablet (Ipca Pharmaceuticals Ltd., Mumbai, India) labelled to
contain 12.5 mg of chlorthalidone. The chemicals like n-hexane (HPLC grade, LOBA Chemie Laboratories Pvt. Ltd., Mumbai, India), isoproponol, acetic acid (HPLC grade, Merck Specialities Pvt. Ltd., Mumbai, India), 2-nitro aniline and triethylamine (HPLC grade,
Spectrochem Pvt. Ltd, Mumbai,India) were used in the analysis.

Instrumentation

The ultra fast liquid chromatography (UFLC) used was of Shimadzu Prominence LC-20AD equipped with a 1260 binary pump VL (35MPa), Prominence SIL-20ACHT Autosampler, and Prominence SPD-M20A Diode array detector. All weighings for analysis were performed on a Shimadzu electronic analytical balance AY-220 (Shimadzu).Water used for analysis was prepared from Millipak Express 20 filter unit.

Preparation of Standard solutions

Chlorthalidone reference standard (10 mg) was accurately weighed, transferred to 100ml volumetric flask, and dissolved in isoproponol by sonicating for 15 minutes, and then completed to volume with isoproponol(final concentration 0.1mg/ml). Also, an accurately weighed 2-nitro aniline reference internal standard(10mg) was transferred to 100ml volumetric flask and dissolved in isoproponol by sonicating for 15 minutes, then completed to volume with isoproponol(final concentration 0.1mg/ml). Also solutions were freshly prepared.

For construction of calibration graph, aliquot proportions (1-6ml) of 0.1mg/ml chlorthalidone standard solution were taken into a series of 10ml volumetric flasks, 1 ml of 2-nitro aniline was added into each volumetric flask, and volume madeup to 10ml with isoproponol to get 10, 20, 30, 40, 50, 60 µg/ml of each enantiomer and 10µg/ml of 2-nitro aniline. All the dilutions were filtered through a 0.45µm pore size membrane filter and degassed by an ultrasonicator.

Preparation of sample solution

About 20 tablets were weighed and finely powdered. Powder weight equivalent to one tablet (claimed to contain 12.5mg of Chlorthalidone) was transferred into a 50ml volumetric flask. Then extracted with isoproponol and sonicated the solution for 30min and made up to volume with isoproponol. The solution was filtered by using 0.45µm millipore membrane filter. From the filtered solution 0.8ml of filtrate was pipetted into a 10ml volumetric flask and 1mL of (0.1mg/mL) internal standard was added and made up the volume with isoproponol. Concentration of chlorthalidone enantiomers was determined by peak area ratio of enantiomer to that of internal standard. Concentration of unknown sample could be derived from the calibration graph or calculated from regression equation.

Chromatographic conditions

The filtered dilutions were chromatographed by the set of conditions on Shimadzu LC-20AD Prominence series. The mixture of n-Hexane, isoproponol, acetic acid and triethylamine in the ratio of (92:8:0.3:0.01v/v/v/v) was used as mobile phase for the elution of the drug on KR100-5CHI-TBB column (150 mm × 4.6 mm, 5µm) at 1.2 ml/min of flow rate. 2-nitro aniline was used as an internal standard and it was eluted at 3.312 min. Chlorthalidone enantiomers were successfully eluted at 5.293 min and 6.246min with a run time of 15 min and detection was performed by diode array detector at 260 nm.

Method Validation

Validation of the method was done according to the ICH guidelines[9] contains system suitability, linearity, Precision, Accuracy, Detection limit, Quantification limit, Robustness and solution stability.

RESULTS AND DISCUSSION

Optimization of the method

The method was optimized to separate the two enantiomers of chlorthalidone. The main target of the chromatographic method is to get the separation between the closely eluting enantiomers which have the same physico-chemical properties. The separation was done using different chiral stationary phases like lux cellulose-4 and Kromosil chiral TBB and mobile phases containing organic solvents like hexane and isoproponol with different proportions, and using organic modifiers like diethylamine, tri-ethylamine and acetic acid in the mobile phase. But the separation was better in the adopted chromatographic conditions only. It indicated that the mixture of n-Hexane, isoproponol, acetic acid and tri-ethylamine (92:8:0.3:0.01v/v/v/v) as a mobile phase with flow rate of 1.2ml/min through KR100-5CHITBB column (150 mm × 4.6 mm, 5 µm) was successful in separating both the enantiomers and the internal standard. The optimized chromatogram was shown in [Fig. 2].The following chromatographic parameters were analysed for the chlorthalidone enantiomers. The capacity factors(K1 and K2) which gives the information about the interaction of enantiomers with the stationary phase were found to be 1.39 and 1.63 respectively,. Selectivity factor(α) which is defined as a ratio of capacity factors was found to be 1.170. Resolution factor (Rs) which shows the degree of separation between the two enantiomers on a chromatography system was found to be 1.97. The formulas[10] of the corresponding chromatographic parameters were listed in Table 1.

Method validation

System suitability

The system suitability parameters measured were retention time, theoretical plates and tailing factor. The average retention times of enantiomers were 5.293 and 6.246 for enantiomer 1 and 2, respectively. Theoretical plates, calculated from the equation 5 were more than 2,000 which indicate good resolution of enantiomers on chiral-TBB column in described chromatographic conditions. Tailing factor were less than 2 indicating a perfectly symmetrical peak. The results were summarized in Table 2.

Linearity

The calibration curves for both the enantiomers were linear over the concentration range of 10-60 µg/mL The data for the peak area ratio of the drug to the internal standard corresponding to the concentration was treated by linear regression analysis and the regression equation for the calibration curve were found to be y = 0.0654x + 0.0743, with a correlation coefficient of 0.999 for enantiomer 1, and y = 0.0669 + 0.055, with a correlation coefficient of 0.9993 which is nearly equal to unity. Calibration curves were shown in [Fig. 3] and [Fig. 4], and results were summarized in Table 3.

Precision

In precision six sample solutions of 20µg/ml were injected and %RSD was calculated. The repeatability (intra-day precision) of the analytical method was found to be reliable based on %RSD (<2). Intermediate precision (inter-day precision) was demonstrated on different days. The %RSD values were less than 2, confirming that the method is sufficiently precise. The results were summarized in Table 4.

Accuracy

In the preparation of 50% spiked solution, 2ml was pipette from standard stock solution and transferred to 10ml volumetric flask, to that 0.4ml of sample stock solution and 1ml (0.1mg/ml) of internal standard were added and diluted with diluent. For 100% spiked solution, 2ml was pipette from standard stock solution and transferred to 10ml volumetric flask, to that 0.8ml of sample stock solution and 1ml (0.1mg/ml) of internal standard was added and diluted with diluent and for 150% spiked solution, 2ml was pipette from standard stock solution and transferred to 10ml volumetric flask, to that 1.2ml of sample stock solution and 1ml (0.1mg/ml) of internal standard was added and diluted with diluent. The accuracy of the method was confirmed by determining the average recoveries from the samples by applying the standard addition method. The mean percentage recoveries of chlorthalidone enantiomers at three different levels (50%, 100% and 150%) were found in accordance with the fixed limits of 98 to 102%, indicating the suitability of the developed method in quantifying the concentration of chlorthalidone enantiomers in pharmaceutical tablets. The results were summarized in Table 5.

LOD and LOQ

The LOD and LOQ were obtained using the slope and standard deviation of the intercept from three calibration curves.The results of the LOD and LOQ were 0.091µg/ml and 0.278µg/ml for enantiomer-1 and 0.0812µg/ml and 0.246µg/ml for enantiomer-2 respectively for 12.5mg chlorthalidone tablets. The values are low which indicate the sensitivity of the method. The results were summarized in Table 3.

Robustness

The robustness of the method was evaluated by the small but deliberate changes in chromatographic conditions such as the flow rate (± 0.02 ml/min), wavelength (± 1 nm), and mobile phase composition (± 2%). It was observed that there were no marked changes in the chromatograms, which demonstrated that the normal phase-UFLC method developed is robust. The results were summarized in Table 6.

Solution stability and mobile phase stability

The sample and standard solutions were injected at 0 hr (comparison sample),24hr and after 48 hr (stability sample) at ambient room temperature 30^{O}C. The %RSD for 0 hr, 24hr and 48 hr. for sample and standard solutions were <2. And hence, the sample and standard stock are stable for 24 h in ambient room temperature.

Assay of pharmaceutical product

The validated method was applied for the determination of chlorthalidone enantiomers in tablets. Samples from 12.5 mg CTD tablet were analyzed. The results were expressed as % amount found. The results were summarized in Table 7.

CONCLUSION

A new ultra fast liquid chromatographic method was developed and validated for the separation of chlorthalidone enantiomers in bulk and tablet dosage form on a chiral stationary phase. The method is fast and cost effective as both the enantiomers and the internal standard eluted within ten min. The resolution between two peaks was more than 1.5. The method is sensitive to determine the concentration of enantiomers below 1µg/ml. The method is accurate and precise as the %RSD of both interday and intraday were < 2. Hence, it can be employed for stereo-selective estimation of chlorthalidone. This chiral UFLC assay was used to determine the enantiomer ratio for the bulk drug and in formulated tablet. This assay method ensured that the enantiomers were in 1:1 ratio. The method is applicable for the chiral purity testing of chlorthalidone and also for the enantiomer analysis in quality control laboratories.

ACKNOWLEDGEMENT

The authors would like to thank the principal and management, JSS College of Pharmacy, Mysore for providing laboratory facilities to carry out this research work.

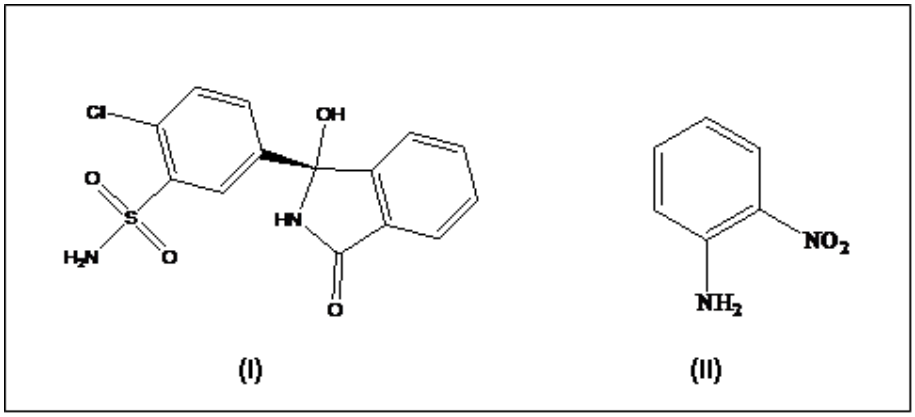


Fig. 1: Chemical structures of Chlorthalidone (I) and 2-nitro aniline (II)

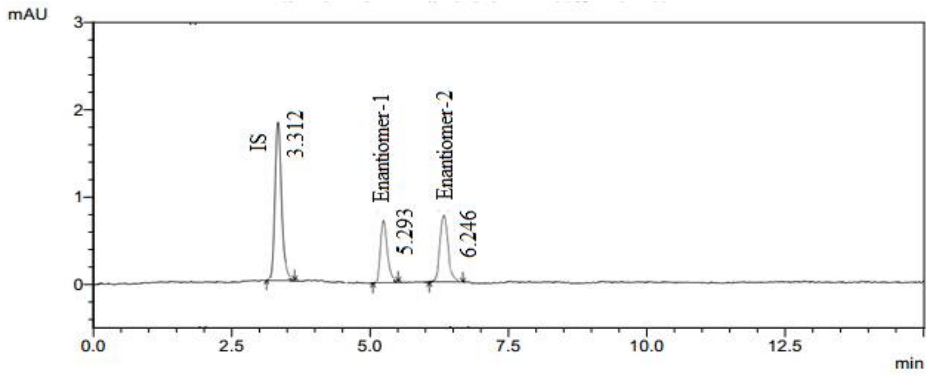


Fig. 2: Optimized chromatogram of chlorthalidone enantiomers on chiral TBB stationary phase

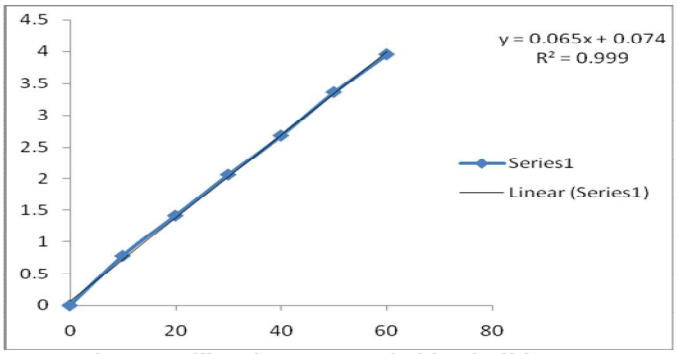
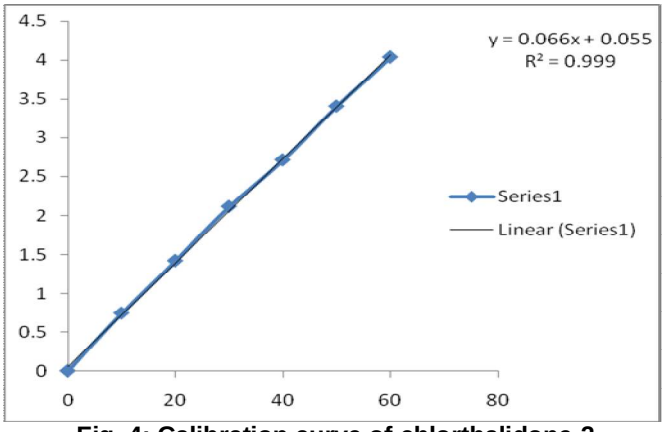


Fig. 3: Calibration curve of chlorthalidone-1



.Fig. 4: Calibration curve of chlorthalidone-2

Table 1: chromatographic data for chlorthalidone enantioseperation on chiral TBB column

S.No	Chromatographic parameter	Formula	Value obtained
1	Capacity factor of first eluted enantiomer (K_1)	$K_1 = \frac{t_1 - t_0}{t_0}$	1.39
2	Capacity factor of second eluted enantiomer (K_2)	$K_2 = \frac{t_2 - t_0}{t_0}$	1.63
3	Selectivity factor(α)	$\alpha = \frac{K_2}{K_1}$	1.170
4	Resolution factor(R_s)	$R_s = \frac{2(t_2 - t_1)}{w_1 + w_2}$	1.97
5	Theoritcal plates(N)	$N = 16\left(\frac{t_r}{w_h}\right)^2$	>2000

Where t_0 = retention time of unretained component; t_1 = retention time of enantiomer 1
t_2 = retention time of enantiomer 2; w_1= baseline band width of the enantiomer 1 peak
w_2= baseline band width of the enantiomer 2 peak; t_r = retention time of the enantiomer
w_b = band width of the enantiomer peak

Table 2: System suitability data of enantiomer-1 and enantiomer-2

Parameters	enantiomer-1	enantiomer-2
Retention time	5.2±0.3	6.2±0.3
Plate count	3674	4027
Tailing factor	0.8	1.1
Resolution	-	1.97

Table 3: Linearity data of enantiomer-1 and enantiomer-2

Parameters	enantiomer-1	enantiomer-2
Beer's range (ppm)	10-60ppm	10-60ppm
Slope (m)	0.0654	0.0669
Y Intercept	0.0743	0.055
Correlation coefficient (r^2)	0.999	0.9993
LOD (ppm)	0.091	0.0812
LOQ (ppm)	0.278	0.246

Table 4: Precision data of enantiomer-1 and enantiomer-2

Theoretical concentration	Intraday(n=6)		Inter day(n=6)	
	Found(µg/ml)	%RSD	Found(µg/ml)	%RSD
enantiomer-1				
10	10.108	1.003	10.052	0.276
20	20.247	0.293	20.259	1.766
30	30.522	0.595	30.480	0.904
enantiomer-2				
10	10.164	1.524	10.178	1.002
20	19.978	0.319	20.020	0.210
30	30.492	0.119	30.492	0.122

Table 5: Accuracy data of enantiomer-1 and enantiomer-2

Sample	Amount added	Amount found	%Recovery	%RSD
enantiomer-1	10	10.041	100.412	1.149
	20	19.964	99.824	0.405
	30	29.862	99.542	0.193
enantiomer-2	10	9.980	99.8	1.423
	20	19.890	99.451	1.983
	30	29.89	99.634	0.652

Table 6: Robustness data of enantiomer-1 and enantiomer-2

Parameters	%RSD
Flow Rate Minus (0.98ml/min) Plus (1.02ml/min)	1.45
Wavelength Minus (259) Plus (261)	0.838
Mobile phase Minus (90:10%) Plus (94:6%)	0.914

Table 7: Assay of CTD 12.5 tablet

Sample %RSD	Injected (µg/ml)	Amount found (µg/ml)	% Amount found
Enantiomer-1 0.286	20	20.247	101.3
Enantiomer-2 0.329	20	19.978	99.89

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