Research Article

Determination of 2-amino-4-chlorophenol (related substance) in marketed formulations of Chlorzoxazone by RP-HPLC

PRAFUL P. DEDHIYA[1*], NIPUNA G. PATEL[1], RUCHI H. VYAS[2], DINESH R. SHAH[1], SHAILESH A. SHAH[1]

[1]Maliba Pharmacy College, Bardoli – Mahuva Road, Tarsadi. District: Surat-394350 Gujarat, India, [2]Dr. Dayaram Patel Pharmacy College, Bardoli, Surat, Gujarat, India – 394 601.

ABSTRACT

Chlorzoxazone is one of the most frequently prescribed drugs in the treatment of muscle spasm in combination with NSAIDs. It is reported to undergo hydrolysis in alkaline medium to form 2-amino-4-chlorophenol, which is considered a significant related substance as per USP. Various regulatory authorities like ICH, USFDA, Canadian Drug and Health Agency are emphasizing on the purity requirements and the identification of impurities in active pharmaceutical ingredient (API) as well as in pharmaceutical formulations. To this aim, various marketed formulations were assessed with special attention to identification and quantification of 2-amino-4-chlorophenol by using compendial reversed phase high performance liquid chromatography method. The use of a 250 × 4.6 mm, 5 µm particle size, C_{18} column with 70:30:1 %, v/v/v water: acetonitrile: acetic acid as isocratic mobile phase at flow rate 1.5 ml/min enabled separation of the drug from its related substance. UV detection was performed at 280 nm. The method was verified for specificity, linearity, precision and accuracy. The related substance peak was well resolved from drug peak. The linearity of the method was satisfactory over the range 400-2000 ng (correlation coefficient 0.9993). The limits of detection for 2-amino-4-chlorophenol and Chlorzoxazone were 5 and 10.94 ng respectively. The limits of quantitation for 2-amino-4-chlorophenol and Chlorzoxazone were 20 and 33.14 ng respectively. Recovery of Chlorzoxazone ranged from 99.90-100.67%. The method was successfully applied to marketed formulations of Chlorzoxazone for quantitative analysis of Chlorzoxazone and 2-amino-4-chlorophenol.

Keywords: High Performance Liquid Chromatography, Chlorzoxazone, related substance, 2-amino-4-chlorophenol

1. INTRODUCTION

The determination of impurities in bulk drug substances and pharmaceutical formulations is one of the most important fields of activity in contemporary industrial analysis. Impurity is anything that is not the drug substance or an excipient in the drug product. According to ICH [1], impurity profile of a drug material is "A description of the identified and unidentified impurities, present in a new drug substance." Impurity profiling is considered to be the common name of analytical activities, the aim of which is the detection, identification/structure elucidation and quantitative determination of organic and inorganic impurities as well as residual solvents in bulk drugs and pharmaceutical formulations. The importance of drug impurity profiling is that it

[*]**Corresponding Author – Praful P. Dedhiya**, Maliba Pharmacy College, Bardoli-Mahuva Road, Tarsadi, Mahuva, Surat, Gujarat, India – 394 350. E. mail: praful.dedhiya@utu.ac.in
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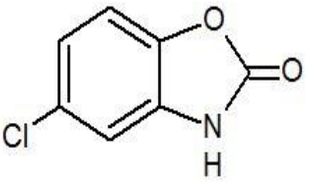
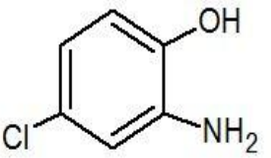
affords data which can directly contribute to the safety and efficacy of drug therapy by minimizing the impurity-related adverse effects of drug materials and the preparations made thereof. In recent years, the importance of assay methods for characterising the quality of bulk drug materials has decreased considerably. At the same time the importance of impurity profiling is continuously increasing [2]. Various regulatory authorities like ICH, USFDA, Canadian Drug and Health Agency have emphasized on the purity requirements of drug substances and products. Regulatory authorities have to ensure the quality of pharmaceutical formulations existing in the market.

In present research work, quality of marketed formulations of Chlorzoxazone has been assessed by determining 2-amino-4-chlorophenol (a significant related substance and degradation product of Chlorzoxazone) content using compendial reversed phase high performance liquid chromatography method [3].

Chlorzoxazone is chemically 5-Chloro-2-benzoxazolinone. Chemical structure of Chlorzoxazone is shown figure 1(a). It is prone to alkaline hydrolysis and converted to 2-amino-4-chlorophenol [4]. Chemical structure of 2-amino-4-chlorophenol is shown in figure 1(b). 2-amino-4-chlorophenol is also a starting material for synthesis of Chlorzoxazone. It should not be present in marketed formulations above specified limit (< 0.5 % as per USP). USP have included the test for related substances in Chlorzoxazone and in dosage form. Chlorzoxazone is assayed by liquid chromatography as per USP. However, several chromatographic methods have been reported for the determination of Chlorzoxazone, in pharmaceutical formulations and/ or in biological fluids, including HPLC [5-8], stability indicating HPLC [9], LC-MS [10] and HPTLC [11, 12] for simultaneous determination of Chlorzoxazone with other drugs. Study for presence of related substances in marketed formulations of Chlorzoxazone is not reported. Therefore, various marketed formulations were analysed to determine 2-amino-4-chlorophenol.



(A)



(B)

Figure 1: Chemical structure of (A) Chlorzoxazone (B) 2-amino-4-chlorophenol

2. MATERIALS AND METHOD

2.1. Instrumentation

The HPLC system (LC-2010-HT, Shimadzu, Switzerland) consisting of UV-visible detector and autosampler was used. The chromatographic separation was accomplished on a Thermo C_{18} column (250 mm × 4.6 mm; 5 µ); protected by a guard column of the same phase. Degassing of mobile phase was done by ultrasonic bath sonicator. A Shimadzu analytical balance was used for weighing the materials.

2.2. Chemicals and reagents

Chlorzoxazone (99.8 % w/w) was received as gift sample from Vapi Care Pharma, Vapi. 2-amino-4-chlorophenol was synthesized at lab scale. Acetonitrile (HPLC grade) and acetic acid (AR grade) were purchased from sd fine-chem Ltd. (Mumbai, India). Double distilled water was prepared in laboratory. Formulations were procured from local pharmacy.

2.3. Chromatographic conditions

The mobile phase consisted of 70:30:1 %, v/v/v water: acetonitrile: acetic acid. Samples were analyzed using the following parameters: flow rate: 1.5 ml/min; run time: 10 min; temperature: 25 ± 2ºC; detection wavelength: 280 nm.

2.4. Characterization of Chlorzoxazone and 2-amino-4-chlorophenol

Chlorzoxazone and 2-amino-4-chlorophenol were characterized by melting point, IR spectrophotometry, TLC and UV-visible spectrophotometry.

2.5. Preparation of solutions

2.5.1. Stock solution of 2-amino-4-chlorophenol

Accurately weighed quantity of 2-amino-4-chlorophenol 25 mg was transferred into 100 ml volumetric flask, dissolved in and diluted to mark with methanol (250 µg/ml).

2.5.2. Working standard solution of 2-amino-4-chlorophenol

Five ml aliquot of 2-amino-4-chlorophenol stock solution was diluted to 25 ml with 1% acetic acid (50 µg/ml).

2.5.3. Stock solution of Chlorzoxazone

Accurately weighed quantity of Chlorzoxazone 20 mg was transferred into 10 ml volumetric flask, dissolved in and diluted to mark with methanol (2000 µg/ml).

2.5.4. Working standard solutions of Chlorzoxazone

Five ml aliquot of Chlorzoxazone stock solution was diluted to 100 ml with 1% acetic acid (100 µg/ml).

2.5.5. Mixture solution for system suitability test

Five ml aliquot of Chlorzoxazone stock solution (2000 µg/ml) and one ml aliquot of 2-amino-4-chlorophenol working standard solution (50 µg/ml) were transferred into a 10 ml volumetric flask and diluted to mark with 1% acetic acid to give a mixture solution having strength 1000 µg/ml Chlorzoxazone and 5 µg/ml 2-amino-4-chlorophenol.

2.6. Calibration curve of Chlorzoxazone

Working standard solution of Chlorzoxazone 4, 8, 12, 16 and 20 µl (100 µg/ ml) were injected to HPLC system and area was measured for each peak.

2.7. Sample solution for analysis of marketed formulations

Twenty tablets were weighed accurately, finely powdered and mixed. Powder equivalent to 100 mg of Chlorzoxazone was accurately weighed and transferred to a 100 ml volumetric flask. The flask was filled to about 80 % with methanol, sonicated for 10 minutes, diluted with methanol to mark, mixed well and filtered through Whatman filter paper (no. 42) (1000 µg/ml). Ten µl of this solution was injected to HPLC system for determination of 2-amino-4-chlorophenol. Further, 1 ml of filtrate was diluted to 10 ml with 1% acetic acid (100 µg/ml). Ten µl of this solution was injected to HPLC system for determination of Chlorzoxazone.

2.8. System suitability

The resolution, column efficiency (no. of theoretical plates) and peak symmetry were calculated for the standard solution mixture and compared with USP specifications.

2.9. Solution stability

Sample solutions of Chlorzoxazone and 2-amino-4-chlorophenol were stored at room temperature for 24 hours, re-analyzed and assay/impurities were determined, compared against freshly prepared sample and % variation was calculated.

2.10. Method Validation

Validation of the method was carried out in terms of specificity, linearity, precision, accuracy, limit of detection and limit of quantitation as per ICH guidelines [13]. The linear responses of Chlorzoxazone in the range of 400-2000 ng were assessed in terms of slope, intercept and correlation coefficient values. The intraday and interday precision were assessed in terms of %RSD. The accuracy was determined by standard

addition method. To a fixed amount of pre-analyzed sample of Chlorzoxazone, increasing amount of standard Chlorzoxazone at three levels (i.e. 80 %, 100 % and 120 %) were added and analyzed.

2.11. Analysis of marketed formulations

2.11.1. Determination of % 2-amino-4-chlorophenol

The sample solution (10 µl) was injected into the chromatographic column and mean peak area of 2-amino-4-chlorophenol was noted. The % 2-amino-4-chlorophenol in the marketed formulations were estimated using following formula:

$$\% \text{ 2-amino-4-chlorophenol} = 100 * \frac{C_S}{C_T} * \frac{A_T}{A_S}$$

Where, C_S = conc. of 2-amino-4-chlorophenol in standard solution

= 5 µg/ml = 5 ng/µl = 50 ng/injection

C_T = conc. of Chlorzoxazone in test solution

= 1000 µg/ml = 1000 ng/µl = 10,000 ng/injection

A_T = peak area of 2-amino-4-chlorophenol obtained from test solution

A_S = peak area of 2-amino-4-chlorophenol obtained from standard solution

2.11.2. Determination of % Chlorzoxazone

The sample solution (10 µl) was injected into the chromatographic column and mean peak area of Chlorzoxazone was noted. The % Chlorzoxazone in the marketed formulations were estimated using following formula:

$$\% \text{ Chlorzoxazone} = 100 * \frac{C_S}{C_T} * \frac{A_T}{A_S}$$

Where, C_S = conc. of Chlorzoxazone in standard solution

= 100 µg/ml = 100 ng/µl = 1000 ng/injection

C_T = conc. of Chlorzoxazone in assay preparation

= 100 µg/ml = 100 ng/µl = 1000 ng/injection

A_T = peak area of Chlorzoxazone obtained from assay preparation

A_S = peak area of Chlorzoxazone obtained from standard solution

3. RESULTS AND DISCUSSION

3.1. System suitability test

Chromatogram for system suitability solution is shown in figure 2. System suitability data is shown in table 1. System suitability data complied with USP specifications.

Table 1: System suitability data

Parameters	Observed Values (n = 7)		USP 32 specification
	2-amino-4-chlorophenol	Chlorzoxazone	
Resolution (Rs)	8.12		NLT 2.0
Theoretical plates (N)	5974	4233	NLT 2000
Asymmetry factor (S)	1.13	0.61	NMT 2.0

Table 2: Linearity data for Chlorzoxazone

Amount of Chlorzoxazone injected (ng)	Area of peak (mV) Mean ± S.D. (n=5)	% R.S.D.
400	1237.84 ± 4.94	0.40
800	3200.38 ± 8.31	0.26
1200	5417.82 ± 17.33	0.32
1600	7733.22 ± 20.02	0.26
2000	9886.11 ± 35.02	0.35

3.2. Solution stability study

The results did not show much variation in peak area within the storage for 24 hours at room temperature indicated that Chlorzoxazone and 2-amino-4-chlorophenol solutions were stable for 24 hours at room temperature.

3.3. Validation of method

3.3.1. Specificity

Chromatograms of blank, mobile phase, 2-amino-4-chlorophenol standard (50 ng) and chlorzoxazone standard (2000 ng) are shown in figure 3. The retention time for standard and sample were identical i.e. 4.03 min for 2-amino-4-chlorophenol and 7.02 min for Chlorzoxazone. Since there was no interference of impurities and excipients observed, the method can be considered specific.

Table 3: Recovery data for Chlorzoxazone

% Recovery level	Amount of Chlorzoxazone drug sample taken (mg)	Amount of standard Chlorzoxazone spiked (mg)	Amount of Chlorzoxazone recovered (mg)	% Recovery	Mean % recovery
80 %	50	40	40.55	101.37	100.67
	50	40	39.85	99.63	
	50	40	40.40	101.00	
100 %	50	50	50.40	100.80	99.90
	50	50	49.75	99.50	
	50	50	49.70	99.40	
120 %	50	60	59.59	99.32	100.04
	50	60	60.09	100.15	
	50	60	60.39	100.65	

Table 4: Summary of validation results

Parameter	Results
Specificity	Specific
Linearity Range (ng) (n=5)	400-2000
Straight Line Equation	Y = 5.4573x – 1053.7
Correlation coefficient (R^2)	0.9993
Intraday precision (% R.S.D) (n=9)	0.19 - 0.31
Interday precision (% R.S.D) (n=9)	0.31 – 0.42
% Recovery (n=9)	99.90 – 100.67
LOD (ng)	10.94
LOQ (ng)	33.14

Table 5: Analysis of marketed formulations

	Strength		Results (n=3)
Formulation 1	250 mg	2-amino-4-chlorophenol (%)	0.12
		Assay (%)	98.66
Formulation 2	250 mg	2-amino-4-chlorophenol (%)	0.32
		Assay (%)	101.94
Formulation 3	250 mg	2-amino-4-chlorophenol (%)	0.09
		Assay (%)	99.78

3.3.2. Linearity

The calibration curve was prepared by plotting peak areas against respective concentration. The peak areas of Chlorzoxazone were linear with respect to concentrations over the range of 400-2000 ng. The overlain chromatogram is shown in figure 4. Data is shown in table 2 and calibration graph is shown in figure 5. The results show excellent correlation between peak area and concentrations. (R^2 = 0.9993)

3.3.3. Intraday and Interday precision

Percent R.S.D. for intraday and interday precision was found to be 0.19-0.31 % and 0.31-0.42 % respectively. From the data obtained, the method was found to be precise.

3.3.4. Accuracy

Percent recovery data for Chlorzoxazone obtained by the method are shown in table 3. The % recovery in all cases were within the acceptable limit (98 -102 %).

3.3.5. LOD and LOQ

For 2-amino-4-chlorophenol

The signal to noise ratio 3: 1 and 10:1 was considered for LOD and LOQ respectively. The LOD and LOQ were found to be 5 and 20 ng respectively for 2-amino-4-chlorophenol.

For Chlorzoxazone

Based on the calibration curve, the LOD and LOQ were calculated for Chlorzoxazone. The LOD and LOQ were found to be 10.94 and 33.14 ng respectively for Chlorzoxazone.

Validation summary

Validation summary is shown in table 4.

3.4. **Analysis of marketed formulations**

The method was successfully applied to marketed formulations of Chlorzoxazone for the determination of 2-amino-4-chlorophenol and Chlorzoxazone. Results are shown in table 5. Results show that all three formulations passes assay and test for related substance (less than 0.5 %).

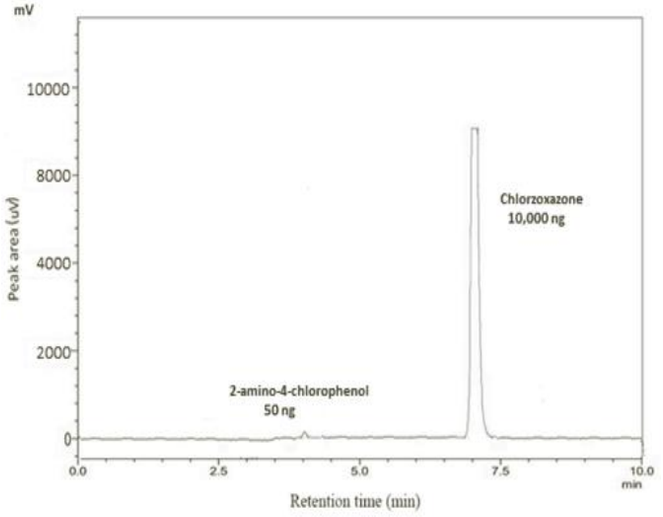


Figure 2: Chromatogram of system suitability test

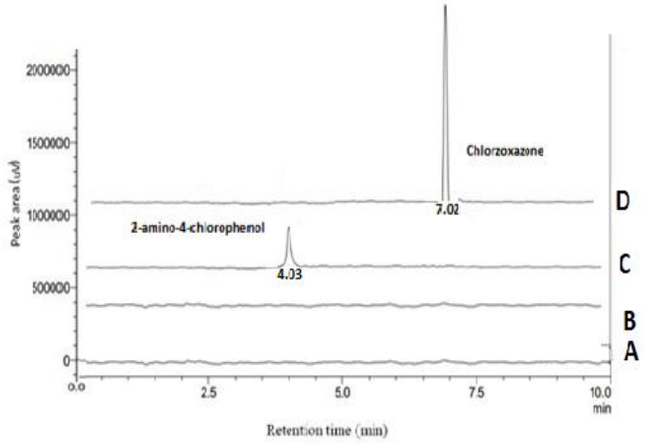


Figure 3: Chromatogram of (A) blank (B) mobile phase (C) 2-amino-4-chlorophenol (D) Chlorzoxazone

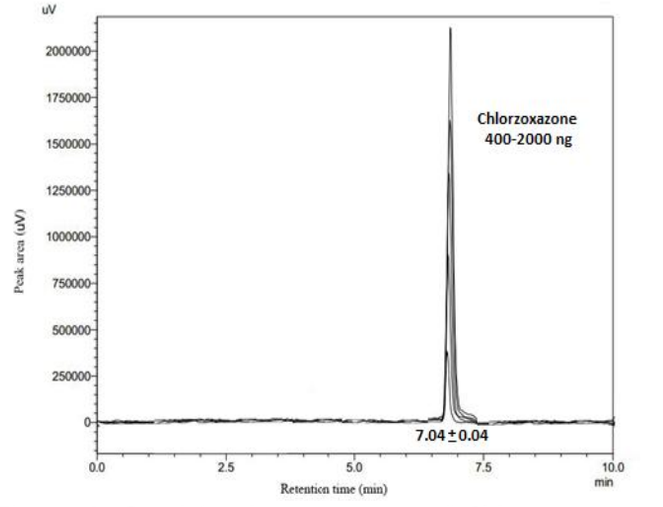


Figure 4: Overlain chromatogram of Chlorzoxazone (400-2000 ng)

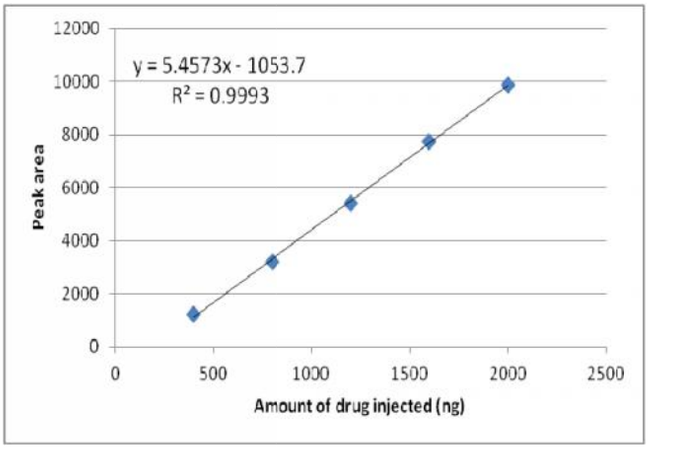


Figure 5: Calibration graph for Chlorzoxazone (400-2000 ng)

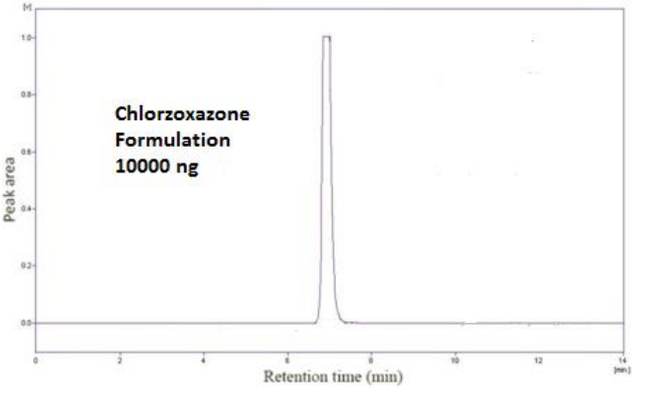


Figure 6: Chromatogram of marketed formulation

4. CONCLUSION:

The compendial reversed phase high performance liquid chromatography method was validated and successfully applied to marketed formulations of Chlorzoxazone for quantitative analysis of Chlorzoxazone and 2-amino-4-chlorophenol (related substance). Results of marketed formulation analysis show that all three formulations complied with the assay and test for related substance.

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