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SPECTROPHOTOMETRIC DETERMINATION OF 2- AMINO- 4- CHLOROPHENOL AS A POTENTIAL IMPURITY IN CHLORZOXAZONE BULK DRUG

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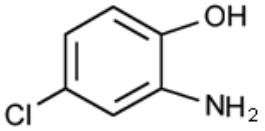
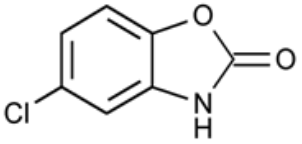
ABSTRACT

2-amino-4-chlorophenol (ACP) is a synthetic precursor of the skeletal muscle relaxant; chlorzoxazone. A rapid simple colorimetric method was proposed for the determination of 2-amino-4-chlorophenol as a potential impurity in chlorzoxazone bulk powder via its reaction with 4-aminoantipyrine in presence of alkaline oxidizing agent ($K_3[Fe(CN)_6]$ / NH_3) and measuring the produced red color at 520 nm. Different experimental parameters affecting the formation of the reaction product were studied. The study included the effect of volumes of the used reagents as well as the effect of time on the formation and stability of the reaction product. The formation constant (K_f) of the reaction product was found to be 1.2×10^4 indicating a very stable reaction product. Moreover, the Gibbs free energy change of the reaction (ΔG) was found to be -2.3×10^4 K.J/mole pointing out to the spontaneous nature of the reaction. The method was successfully applied for the trace analysis of the studied compound in spiked chlorzoxazone powder. The calibration graph was linear over the range of 1-20 µg/ml with average recovery of 100.58±0.89, detection limit of 0.2 µg/ml, and quantitation limit of 0.6 µg/ml. The obtained results were statistically analyzed and were in good agreement with those obtained by the official TLC method.

INTRODUCTION: Chlorzoxazone (5-Chloro-2(3*H*)-benzoxazolone) is a centrally active muscle relaxant. It is a colorless or white to creamy white crystalline powder, very slightly soluble in water [1]. 2-amino-4-chlorophenol (ACP) is a synthetic precursor of chlorzoxazone and so it is considered as a potential impurity in chlorzoxazone bulk drug. ACP is a grey to beige to brown powder, crystals, crystalline powder, and/or chunks [2], soluble in water [3]. The degradation of chlorzoxazone into ACP is very difficult and needs highly drastic conditions [1]. From the above mentioned information it is clear that ACP is important in the field of pharmaceutical analysis mainly as a manufacturing impurity in chlorzoxazone bulk powder not as a degradation product. The maximum allowed limit of ACP in chlorzoxazone is 0.5% according to the USP [4]. ACP is also considered by the United States Environmental Protection Agency [5] as a toxic environmental water pollutant.



2-AMINO-4-CHLOROPHENOL OR 5-CHLORO-2-HYDROXYANILINE



CHLORZOXAZONE

Pharmacologically, ACP is labeled in Sigma-Aldrich Catalogue as a harmful substance irritating to eyes, respiratory system, and skin [2]. The New Jersey Department of Health and Senior Services classifies ACP as a hazardous substance because acute health effects may occur immediately or shortly after exposure to it including the interference with the ability of the blood to carry oxygen causing headache, dizziness, and a blue color of the skin and lips (methemoglobinemia). Higher levels can cause trouble breathing, collapse and even death [6]. Few analytical methods were published for the determination of ACP. These methods were applied for the analysis of this compound as a related substance of chlorzoxazone or as an environmental water pollutant. Few colorimetric methods were described for the determination of ACP through coupling with diazotized sulfanilic acid [7] or through oxidative coupling with 3- methyl- 2-benzothia zolinone hydrazone (MBTH) in the presence of Fe(III), N, N-dimethyl-p-phenylene diamine (DMPD) in the presence of periodate (IO_4^-), or 2, 6-dichloroquinone chlorimide (DCQC) [8]. Fluorometric determination of ACP was investigated by Stewart and Chan [9] based on chemical derivatization of ACP with fluorescamine at pH 4.2 and measuring the fluorescence at 495 nm after excitation at 395 nm. Fluorescence immunoassay was also utilized for the analysis of ACP as an environmental pollutant [10]. Different electrochemical methods were developed for the determination of ACP [11-16].

The USP [4] recommended a simple TLC method for testing the presence of ACP as an impurity in chlorzoxazone bulk powder. A spectrodensitometric method was also reported for the determination of chlorzoxazone and ACP [17]. Different reversed-phase HPLC methods were investigated for the determination of ACP using UV-detector [18-20] or diode-array detector [21]. Various LC/MS methods were also utilized for the assay of ACP [22, 23]. Moreover, ACP was analyzed using GC/MS methods [24, 25]. Capillary electromigration separation techniques were also investigated for the assay of ACP with other phenolic compounds [26-28].

4-Aminoantipyrine is one of the most important reagents used for the colorimetric determination of phenolic compounds. Emerson [29] was the first to use this reagent to develop a color test for phenolic compounds based on an oxidative coupling reaction in presence of alkaline oxidizing agents to give quinone-imine dyes (antipyrine dyes). The mechanism of the reaction

and the reaction products were extensively studied by Fiamegos *et al*. [30, 31]. The 4-aminoantipyrine spectrophotometric method is still in common use in the procedures of analysis of many drugs either in batch or flow-injection analyses [32-38].

The objective of the present work was to develop a simple, rapid and accurate colorimetric method for the determination of ACP based on an oxidative coupling reaction with 4-aminoantipyrine in the presence of alkaline oxidizing agent to be applied for the determination of this compound as a potential impurity in chlorzoxazone bulk powder.

Experimental:

Materials and Reagents: All the chemicals used were of Analytical Reagents grade, and the solvents were of spectroscopic grade. 2-Amino-4-chlorophenol, 97% (Sigma-Aldrich Co Ltd., UK) was purchased and its purity was tested by determining its melting point and comparing it with the values cited in Sigma-Aldrich Catalogue (136-141°C) [2]. Chlorzoxazone, 99.8% was kindly provided by (GlaxoSmithKline, Cairo, Egypt) and was used as received without further purification. The chromatographic purity of the sample was tested according to the USP [4] method using TLC and the results indicated the absence of ACP. 4-Aminoantipyrine (Sigma-Aldrich Co Ltd., UK) was purchased and used as 0.2% w/v aqueous solution. Ammonium hydroxide 25% (El-Nasr Pharmaceutical Chemicals (ADWIC), Egypt) was used as 0.1 M aqueous solution and potassium ferricyanide (Winlab, UK) was used as 0.8% w/v aqueous solution.

Instrument: Spectrophotometric analyses were carried out on a Shimadzu (Kyoto, Japan) UV-1601 PC, UV-Visible double-beam spectrophotometer with matched 1 cm path-length quartz cells. Absorption spectrum of the reaction product was recorded on a fast scan speed between 350-700 nm setting slit width to be 1 nm and sampling interval to be auto.

Standard Solution: A stock solution (0.20 mg/ml) of ACP was prepared by transferring 0.020 g of this compound, accurately weighed, to a 100-ml volumetric flask, adding about 50 ml of distilled water, and dissolving by swirling and with the aid of sonication. The solution was then diluted with distilled water to volume, and mixed. The color of this solution was darkened by time so it should be freshly prepared and kept away from direct sunlight.

Procedure for the Study of Experimental Parameters: The different experimental parameters affecting the formation of the reaction product were studied. The study included the effects of volumes of reagents and the effect of time on the formation and stability of the reaction product. Variables were optimized by changing each in turn, while, keeping all others constant.

Construction of the Calibration Graph: Aliquot volumes of ACP standard solution were transferred into a series of 10-ml volumetric flasks so that the final concentration was in the range of 1-20 µg/ml. Then 0.6 ml of ammonia solution, 2.0 ml of 4-aminoantipyrine solution, and 1.0 ml of potassium ferricyanide solution were added to each flask. The solutions were mixed and then diluted to volume with distilled water. The absorbance values at 520 nm were measured against a reagent blank and then plotted against the final concentration to get the calibration graph. Alternatively, the corresponding regression equation was derived.

Application to the Analysis of the Studied Compound in Spiked Chlorzoxazone Powder: A pure chlorzoxazone powder was spiked with ACP, in a ratio lower than the maximum allowed limit by the USP [4], by mixing 8.00 g of chlorzoxazone with 0.02 g of ACP. The mixture was then transferred into a 250 ml beaker, mixed with about 60 ml of distilled water, and sonicated for 1 hr. The solution was filtered, quantitatively transferred to a 100-ml volumetric flask, diluted with distilled water to

volume, and then analyzed as described under *Construction of the calibration graph*. The concentration of ACP was determined using, either the calibration curve or the corresponding regression equation. The results obtained were compared to those given with the comparison method [9].

RESULTS AND DISCUSSION: Trace analysis of impurities in bulk drugs requires sensitive and selective methods of analysis to give satisfactory and accepted results. Therefore, we resorted to colorimetry using 4-aminoantipyrine as a chromogenic reagent for the determination of ACP as a potential impurity in chlorzoxazone bulk drug. The molecule of ACP features a phenolic hydroxyl group so that it can be oxidatively coupled with 4-aminoantipyrine in presence of alkaline oxidizing agent to produce an intensely colored stable product. Typical absorption spectrum of the oxidative coupling reaction product is shown in **Fig. 1**. The results of the proposed method showed no significant differences with those obtained by the comparison method [9] as regards to accuracy and precision [39].

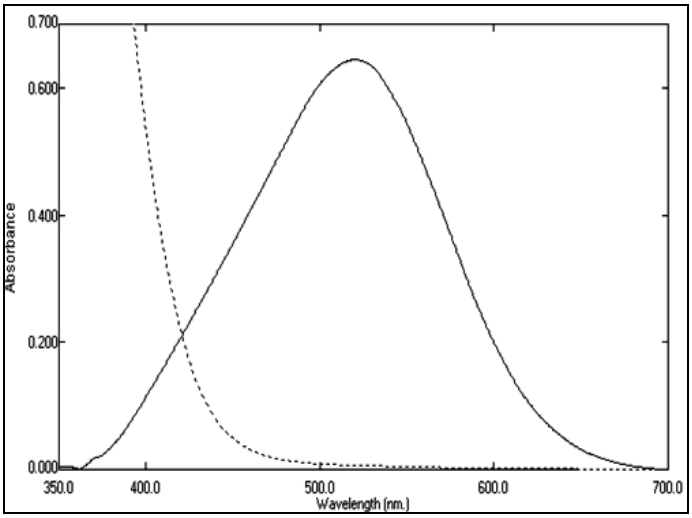


FIG. 1: ABSORPTION SPECTRUM OF THE REACTION PRODUCT OF 10 µg/ml ACP WITH 4-AMINOANTIPYRINE: (—) REACTION PRODUCT, (---) BLANK MEASURED AGAINST WATER

Optimization of the Reaction Conditions: The different experimental parameters affecting the reaction product were studied and optimized to give the maximum absorbance and hence the highest sensitivity. A study of the effect of the volume of 0.1 M aqueous ammonia solution was conducted. As shown in **Fig. 2**, the optimum volume was found to be 0.6 ml. Moreover, the study revealed that below 0.2 ml, no reaction product was formed and above 0.8 ml the reaction showed a marked decrease in the absorbance.

Different volumes of 0.2% aqueous 4-aminoantipyrine solution were tried and the corresponding absorbance values were measured. It was found that the optimum volume was 2.0 ml as shown in **Fig. 3**. The effect of different volumes of 0.8% aqueous potassium ferricyanide on the oxidative coupling reaction of ACP with 4-aminoantipyrine is illustrated in **Fig. 4**. The optimum volume was found to be 1.0 ml. Moreover, the effect of time on the formation and stability of the reaction product was studied. The oxidative coupling reaction was found to be instantaneous and the color was immediately developed. The formed product remained stable for more than 2 hours.

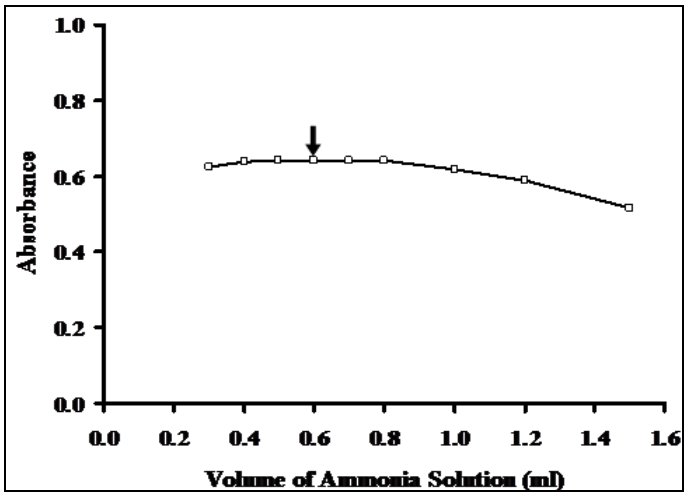


FIG. 2: EFFECT OF VOLUME OF 0.1 M AMMONIA SOLUTION ON THE REACTION OF 10 µg/ml ACP WITH 4-AMINOANTIPYRINE

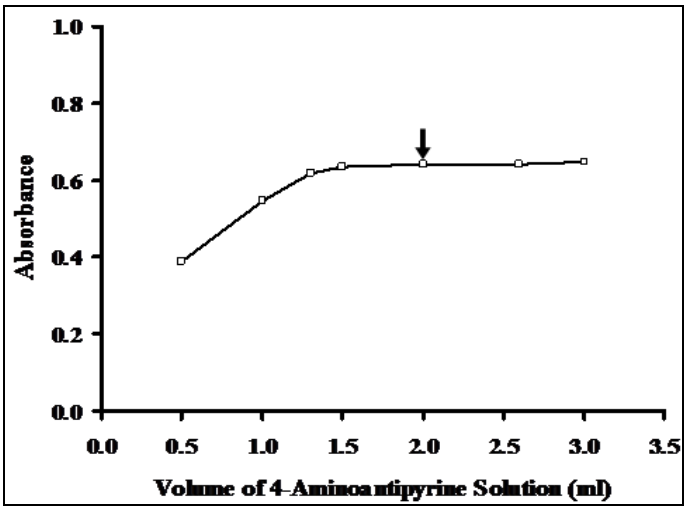


FIG. 3: EFFECT OF VOLUME OF 0.2% 4-AMINOANTIPYRINE SOLUTION ON THE REACTION OF 10 µg/ml ACP WITH 4-AMINOANTIPYRINE

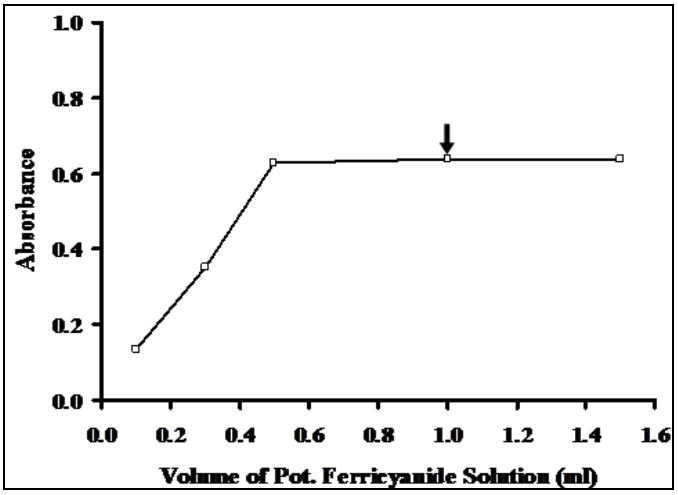
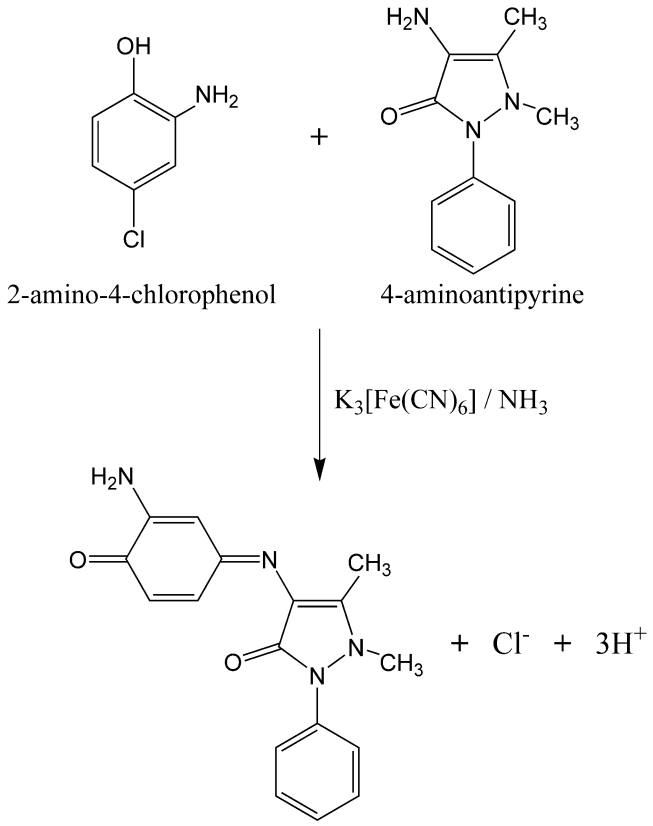


FIG. 4: EFFECT OF VOLUME OF 0.8% POTASSIUM FERRICYANIDE SOLUTION ON THE REACTION OF 10 µg/ml ACP WITH 4-AMINOANTIPYRINE

Mechanism of the Reaction: ACP can react with 4-aminoantipyrine through an oxidative coupling reaction in the presence of alkaline oxidizing agent ($K_3[Fe(CN)_6]/NH_3$) to form an antipyrine dye having the quinonoid structure shown in **Scheme 1**. It is reported that in this reaction, the *para* position to the phenolic group should be either free or substituted with a group that can be expelled during the reaction like halogen, carboxyl, sulfonic acid, hydroxyl, and methoxyl groups [29-31]. The stoichiometry of the reaction was studied adopting Job's method of continuous variation [40]. The molar ratio was found to be 1:1 (**Fig. 5**). Thus, the proposed mechanism of the oxidative coupling reaction could be presented as shown in **Scheme 1**.



SCHEME (1): PROPOSAL OF THE REACTION PATHWAY OF ACP AND 4-AMINOANTIPYRINE

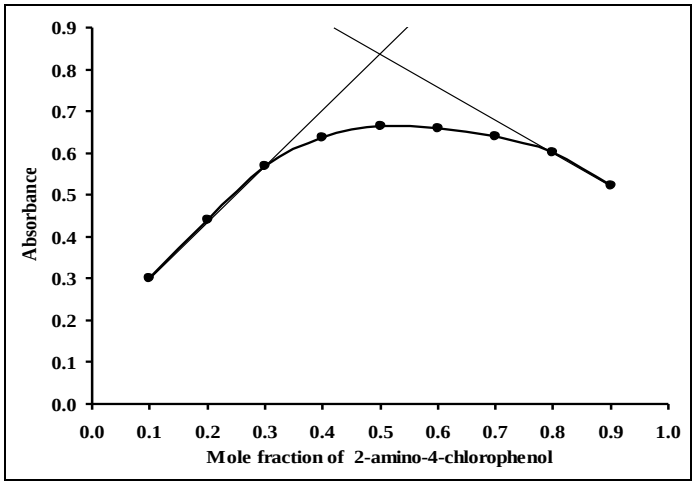


FIG. 5: CONTINUOUS VARIATION GRAPH FOR THE REACTION BETWEEN ACP AND 4-AMINOANTIPYRINE (0.001 M FOR EACH).

Formation Constant of the Reaction Product: The formation constant (K_f) of the reaction product was calculated adopting the following formula [41]:

$$K_f = \frac{\left(\frac{A}{A_m}\right)}{\left(\frac{1-A}{A_m}\right)^{n+1} C^n n^n}$$

Where;

K_f = the formation constant of the reaction product.
A = Maximum absorbance of the continuous variation curve (**Fig. 5**).
A_m = Absorbance corresponding to the intersection of the two tangents of the continuous variation curve (**Fig. 5**).
n = Number of molecules of the reagent in the reaction product.
C = Molar concentration of the drug corresponding to maximum absorbance.

The formation constant (K_f) of the reaction product was found to be 1.2 x 10^4. This high figure indicates a very stable reaction product. The Gibbs free energy change of the reaction (ΔG) was also calculated adopting the following equation:

$$\Delta G = -2.303\ RT \log K_f$$

Where;

ΔG = Gibbs free energy change of the reaction (K.J./mole).
R = Universal gas constant (8.314 joules).
T = Absolute temperature (273+25°C).
K_f = Formation constant of the reaction product.

The Gibbs free energy change of the reaction (ΔG) was found to be - 2.3 x 10^4 K.J./mole. The negative value of ΔG points out to the spontaneous nature of the reaction.

Validation:

Linearity and Range: The calibration graph for the determination of the studied compound (ACP) by the proposed method was constructed by plotting the absorbance *versus* the concentration as shown in **Fig. 6**. The graph was found to be rectilinear over the concentration range cited in **Table 1**.

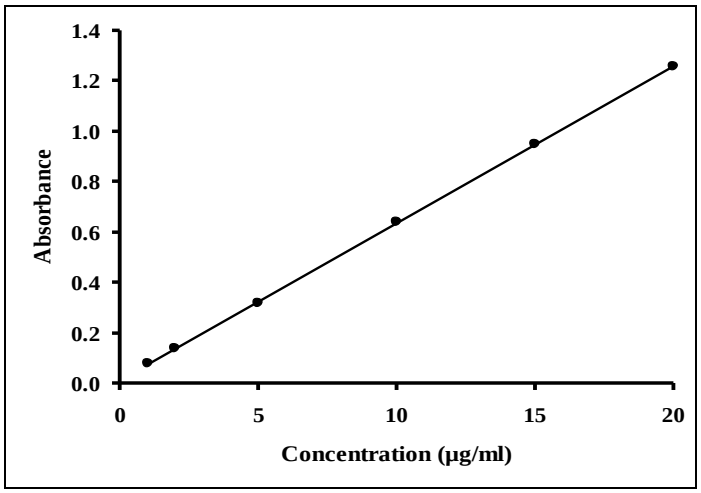


FIG. 6: CALIBRATION CURVE FOR THE COLORIMETRIC DETERMINATION OF ACP AT 520 nm

Statistical analysis [39] of the data gave high value of the correlation coefficient (*r*) of the regression equation, small values of the standard deviation of residuals ($S_{y/x}$), of intercept (S_a), and of slope (S_b), and small value of the percentage relative standard deviation and the percentage relative error (**Table 1**). These data proved the linearity of the calibration graph and the agreement of the measurements with Beer's law.

Accuracy and Precision: To prove the accuracy of the proposed method, the results of the assay of ACP in pure form were compared with those of the comparison method [9]. According to the USP [4] validation guidelines, the accuracy of the methods used for quantitative analysis of impurities should be assessed on samples spiked with known amounts of impurities. Therefore, the proposed method was applied to the analysis of ACP in spiked chlorzoxazone powder.

TABLE 1: ANALYTICAL PERFORMANCE DATA FOR THE COLORIMETRIC DETERMINATION OF ACP

Parameter	Value
Wavelength [λ_{max}] (nm)	520
Linearity range (µg/ml)	1 - 20
Intercept (*a*)	0.013
Slope (*b*)	0.062
Correlation coefficient (*r*)	0.9999
S.D. of residuals ($S_{y/x}$)	3.814×10^{-3}
S.D. of intercept (S_a)	2.526×10^{-3}
S.D. of slope (S_b)	2.252×10^{-4}
% RSD [a]	0.89
% Error [b]	0.36
LOD (µg/ml)[c]	0.2
LOQ (µg/ml)[d]	0.6
$A^{1\%}$ ($dl.g^{-1}.cm^{-1}$)[e]	620
ε ($L.mol^{-1}.cm^{-1}$)[f]	8901

[a] Percentage relative standard deviation for six replicate samples; [b] Percentage relative error for six replicate samples; [c] Limit of detection. [d] Limit of quantitation; [e] Specific absorbance; [f] Molar absorptivity

Statistical analysis [39] of the results obtained by the proposed and comparison methods using Student's *t*-test and variance ratio *F*-test showed no significant difference between them regarding accuracy and precision, respectively (**Tables 2, 3**). Intraday and interday precisions were assessed using three concentrations and three replicates of each concentration. The relative standard deviations were found to be very small indicating reasonable repeatability and intermediate precision of the proposed method (**Table 4**).

Specificity: According to the USP [4] validation guidelines, the specificity of the analytical procedures for impurities may be established by spiking the drug substance with appropriate levels of impurities and demonstrating that these impurities are determined with appropriate accuracy and precision. As shown in **Table 3**, the assay results of spiked chlorzoxazone powder and the corresponding statistical analysis [39] confirmed the specificity of the proposed method. That is due to the absence of phenolic group in chlorzoxazone molecule as well as the very slight solubility of chlorzoxazone in water [1] which is the solvent used through the present study.

TABLE 2: ASSAY RESULTS FOR THE DETERMINATION OF ACP IN PURE FORM BY THE COLORIMETRIC AND COMPARISON METHODS

Parameter	Proposed method	Comparison method [9]
% Found [a]	101.60	101.40
	100.80	100.70
	99.04	99.17
	101.13	99.33
	100.75	100.28
	100.16	
Mean ± S.D.	100.58 ± 0.89	100.18 ± 0.94
t	0.73 (2.26)[b]	
F	1.106 (5.192)	

[a] The average of three separate determinations; [b] The figures between parentheses are the tabulated values of *t* and *F* at *P* = 0.05.

TABLE 3: ASSAY RESULTS FOR THE DETERMINATION OF ACP IN SPIKED CHLORZOXAZONE POWDER BY THE COLORIMETRIC AND COMPARISON METHODS

Parameter	Proposed method	Comparison method [9]
% Found [a]	98.41	99.18
	100.00	100.00
	100.00	99.12
	99.42	99.84
	99.08	98.91
	100.36	
Mean ± S.D.	99.55 ± 0.72	99.41 ± 0.48
t	0.36 (2.26)[b]	
F	2.259 (6.256)	

[a] The average of three separate determinations; b The figures between parentheses are the tabulated values of *t* and *F* at *P* = 0.05

TABLE 4: ACCURACY AND PRECISION DATA FOR THE DETERMINATION OF ACP BY THE COLORIMETRIC METHOD

	Parameter	ACP concentration (µg/ml)		
		2.0	10.0	15.0
Intraday	% Found	100.80	101.13	100.75
		100.39	100.09	99.66
		100.46	100.29	100.98
	Mean	100.55	100.50	100.46
	S.D.	0.22	0.55	0.71
	% RSD	0.22	0.55	0.70
	% Error	0.13	0.32	0.41
Interday	% Found	100.80	101.13	100.75
		99.14	100.02	100.47
		99.28	100.00	99.69
	Mean	99.74	100.38	100.30
	S.D.	0.92	0.65	0.55
	% RSD	0.92	0.64	0.55
	% Error	0.53	0.37	0.32

N.B.; Each result is the average of three separate determinations

Limit of Detection (LOD) and Limit of Quantitation (LOQ): LOD and LOQ were determined according to the USP [4] guidelines. LOD was determined by establishing the minimum level at which the analyte can reliably be detected (signal-to-noise ratio is 3:1) while LOQ was determined by establishing the lowest concentration of analyte that can be determined with acceptable precision and accuracy (signal-to-noise ratio is 10:1). The results are shown in **Table 1**.

Ruggedness: To examine the ruggedness of the procedure, the intraday and interday precisions were evaluated as shown in **Table 4**. The precision of the proposed method was fairly high, as indicated by the low values of the percentage relative standard deviation (% RSD).

Application to the Analysis of the Studied Compound in Spiked Chlorzoxazone Powder: The analysis of ACP is cited in the USP [4] monograph of chlorzoxazone bulk drug and not repeated in the monograph of the finished product (chlorzoxazone tablets) and according to the USP [4] guidelines for testing impurities, the pharmacopoeia does not repeat impurity tests in subsequent preparations where these appear in the monographs of bulk pharmaceutical chemicals and where these impurities are not expected to increase.

Moreover, the ICH [42] guideline Q3B states that, by-products of synthesis have been controlled already during examination of the substance before formulation and thus further testing for these impurities is unnecessary. For these reasons, the proposed method was not applied on chlorzoxazone tablets and applied only on spiked chlorzoxazone powder. The proposed method was successfully applied to the assay of ACP in spiked chlorzoxazone powder as shown in **Table 3**. The average percent recoveries were based on the average of three replicate determinations. The results obtained were in good agreement with those obtained with the comparison method [9].

CONCLUSION: The proposed colorimetric method could be successfully applied for the determination of ACP in chlorzoxazone bulk drug. The proposed procedure is simple, sensitive, reliable, accurate, and rapid. The good validation criteria of the propose method allow its use in quality control laboratories as an alternative to the official TLC method. The detection limit of the proposed method was found to be 0.2 µg/ml while the quantitation limit was 0.6 µg/ml.

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