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Selective Stability-Indicating Methods for the Determination of Clonidine Hydrochloride and/or Its Related Substance, 2,6-Dichloroaniline

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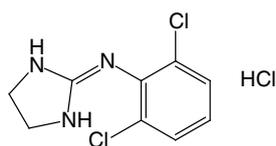
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ABSTRACT

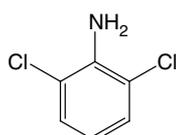
Three rapid, sensitive and selective analytical methods were developed for the determination of clonidine hydrochloride and its related substance: 2,6-dichloroaniline in a mixture of both. The first method depends on derivative-ratio spectrophotometry where the first derivative signals of the ratio spectra at 228.4 nm ($\Delta\lambda = 2$ nm) were selected to determine clonidine hydrochloride. The second method is based on measuring the first derivative response of 2,6-dichloroaniline at 300.8 nm with no interference from intact clonidine hydrochloride. In the third method, 2,6-dichloroaniline was determined via diazotization and coupling with N-(1-naphthyl) ethylenediamine to yield a colored azo dye which was measured at 498 nm. Various parameters affecting each method were studied and optimized. The proposed methods were validated according to USP guidelines concerning linearity, ranges, accuracy, precision, detection and quantification limits. The derivative-ratio spectrophotometric method was applied to the analysis of clonidine hydrochloride in tablets and the results were found in good agreement with those of the USP XXX HPLC procedure, while both the second and third methods permitted the selective analysis of 2,6-dichloroaniline in clonidine hydrochloride raw material.

Key words: clonidine hydrochloride, 2,6-dichloroaniline, derivative-ratio spectrophotometry, first derivative spectrophotometric method, n-(1-naphthyl) ethylenediamine

INTRODUCTION



Clonidine hydrochloride



2,6-Dichloroaniline

Clonidine hydrochloride (CH), 2-(2,6-dichloroanilino)-2-imidazoline is an imidazoline anti-hypertensive drug that appears to act centrally to reduce sympathetic tone. It is used in the management of hypertension including hypertensive crises, in the prophylactic treatment of migraine, in the treatment of menopausal flushing and in the management of cancer pain. It is also used as eye drops in case of glaucoma⁽¹⁾.

CH is official in both BP 2008⁽²⁾ and USP 30⁽³⁾. Each pharmacopoeia describes a potentiometric titration assay for

the drug determination. The USP includes an HPLC method for the assay of clonidine tablets while the BP describes a spectrophotometric method based on the reaction with bromothymol blue.

Clonidine has been determined colorimetrically in its pharmaceutical preparations by reacting with various reagents such as sodium nitroprusside in presence of sodium hydroxide^(4,5), 2,3-dichloro-5,6-dicyano-1,4-benzoquinone⁽⁶⁾, bromocresol green⁽⁷⁾, supracen violet 3B⁽⁸⁾, bromophenol blue⁽⁹⁾, p-NN-dimethylphenylenediamine and sodium metaperiodate⁽¹⁰⁾. A sensitive fluorimetric method based on the reaction of clonidine hydrochloride with dansyl chloride has also been published⁽⁶⁾.

Several gas chromatographic techniques have been applied to the determination of clonidine in biological fluids such as plasma^(11,12) and urine⁽¹³⁾. Clonidine has been analyzed in pharmaceutical preparations by HPLC^(14,15) and in serum by liquid chromatography-tandem mass spectrometry⁽¹⁶⁾. Other methods utilized for the quantitation of clonidine in human plasma and in urine include radio-immunoassay⁽¹⁷⁾.

CH is a weakly absorbing anti-hypertensive drug

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administered in tablet form at a low dosage level (150 µg/tablet). Its spectrophotometric determination in tablets faces problems of possible matrix interference in the short wavelength region in which it absorbs appreciably. 2,6-dichloroaniline (2,6-DCA) is a related substance to CH since it is one of the starting materials for its synthesis^(18,19). The USP 30 describes an HPLC method for the determination of CH. In this method, 2,6-DCA is added to the standard preparation to assess the system suitability of the assay to distinguish CH from 2,6-DCA. Therefore, the spectrophotometric analysis of CH in the presence of 2,6-DCA in the raw material and pharmaceutical tablets would be useful especially in the absence of a working HPLC apparatus.

Since in some circumstances the derivative technique cannot eliminate the present interference from formulation matrix, co-formulated drugs, degradation products and/or synthesis precursors, a derivative ratio spectrophotometric method (Salinas' method)^(20,21) could be used; in this method the absorption spectrum of the mixture is obtained and divided (amplitude by amplitude at appropriate wavelengths) by the absorption spectrum of a standard solution of one of the components, and the first derivative of the ratio spectrum is obtained.

In the present work, derivative ratio and derivative spectrophotometric methods were developed for the analysis of CH and 2,6-DCA, respectively, in a binary mixture. Moreover, 2,6-DCA was determined in CH raw material by chemical derivatization through diazotization and coupling with Bratton-Marshall reagent followed by spectrophotometric measurement.

MATERIALS AND METHODS

I. Apparatus

Measurements were performed on a Perkin Elmer, Lambda EZ 201 (Version 1.0) UV/VIS spectrophotometer equipped with 10 nm matched quartz cells and connected to a Panasonic Quiet KX-P 3626 printer. The spectral band width was 2.0 nm and the wavelength scanning speed was 200 nm/min. HPLC Shimadzu Model C-R7A Plus Chromatopac equipped with a UV detector SPD-10A was employed for chromatographic measurements.

II. Reagents and Materials

Authentic samples of CH (Boehringer Ingelheim, Pharma. KG for Boehringer Ingelheim International GmbH Ingelheim am Rhein, Germany) and 2,6-DCA (Sigma Chem. Co., USA) were used without any purification.

N-(1-naphthyl) ethylenediamine dihydrochloride (Bratton-Marshall Reagent, Aldrich Chemical Company, Inc.) was prepared as 0.1% aqueous solution⁽¹⁸⁾. Sodium nitrite (Fluka AG) was prepared as 0.1% aqueous solution. Urea was prepared as 0.5% aqueous solution. All solvents and chemicals utilized were of analytical grade.

III. Preparation of Standard Drug Solutions

(I) For the Derivative Spectrophotometric Methods

Fifteen mg of CH were accurately weighed and transferred into a 25-mL volumetric flask, the volume was completed to the mark with methanol. A 0.12 mg/mL working solution of CH was prepared by diluting 5 mL of this solution to 25 mL with methanol. For 2,6-DCA, a 0.12 mg/mL standard solution was prepared in methanol.

(II) For the Diazo Coupling Method

Accurately weighed 10 mg of 2,6-DCA was dissolved in 100 mL methanol.

IV. Spectrophotometric Derivative Measurements

Various aliquots of each standard solution (0.2 - 1.0 mL for CH and 0.1 - 0.7 mL for 2,6-DCA) within the concentration ranges stated in Table 2 were transferred into two separate sets of 25-mL volumetric flasks, the solutions were brought up to a constant volume of methanol and then were completed to volume with 0.1 N HCl.

(I) Derivative Spectrophotometric Method

The D_1 spectra of standard solutions were recorded within the wavelength range 320 - 280 nm against a blank solution. The D_1 values for each solution were recorded at 300.8 nm.

(II) Derivative Ratio Spectrum Method

The absorbances of the standard solutions were recorded within the wavelength range 240 - 200 nm against the same solvent and stored. The first derivative of the ratio spectrum was measured at 228.4 nm.

V. Diazo Coupling Method

Aliquots of the standard solution of 2,6-DCA, within the concentration range cited in Table 2 were transferred into a set of 25-mL volumetric flasks. Then the proper volume of 5M HCl (1.5 mL) and sodium nitrite (0.2 mL) were added to each flask. The contents of each flask were mixed well. 2 mL urea was added followed by 1.5 mL Bratton-Marshall Reagent. The flasks were completed to volume with distilled water. The absorbance of each solution was measured against a similarly treated blank at 498 nm.

VI. Procedure for Dosage Forms

An accurate weight of the mixed contents of 30 powdered tablets, equivalent to the weight of 10 tablets was transferred into a 25-mL volumetric flask. The powder was extracted

with 0.1 N HCl by sonication for 15 min, the volume was completed with the same solvent and the solution was filtered. Ten mL aliquot of the filtrate was transferred into a separating funnel, alkalinized with 5 mL 0.5 N NaOH and extracted with 3 successive portions of chloroform of 10 mL each. The chloroformic extracts were then evaporated to dryness; the residue was dissolved in methanol in a 10-mL volumetric flask. Aliquots of this solution, within the specified concentration ranges, were diluted as described under IV and V.

RESULTS AND DISCUSSION

Figure 1 shows the absorption spectra of CH with 2,6-DCA and a mixture of both of them at their nominal concentrations. As shown in the figure, the UV absorption bands of CH and 2,6-DCA are so overlapped that the conventional UV method for the assay of CH is susceptible to interference from 2,6-DCA. While the UV spectrum of the latter is not greatly affected in the region from 320-280 nm, this is a favorable condition so that conventional zero order or direct first derivative method could be applied for determination of 2,6-DCA in the studied binary mixture. In this context the first derivative method was found to be more advantageous by virtue of its better sensitivity.

I. First Derivative Method

2,6-DCA was determined in the laboratory-prepared mixtures by first derivative method. In Figure 2, the D_1 spectrum shows that the first derivative technique can be used to quantitate 2,6-DCA without any contribution from the intact CH. Therefore, 2,6-DCA could be determined in presence of CH by measuring its D_1 response at 300.8 nm. Meanwhile the D_1 to D_4 spectra of CH were found completely overlapped by

those of 2,6-DCA in the preliminary investigations. Hence, derivative ratio spectrum method was applied for the determination of CH in this mixture.

II. Derivative Ratio Spectrum Method

For the determination of CH, the stored absorption spectra of standard solutions of CH and solutions of CH and 2,6-DCA laboratory prepared mixtures were divided (amplitude by amplitude at appropriate wavelengths) by the absorption spectrum of a standard solution of 7.2 $\mu\text{g/mL}$ 2,6-DCA to obtain the absorbance ratio spectra (Figure 3A). Then the first derivative of the obtained ratio spectra were calculated with $\Delta\lambda = 2$ nm (Figure 3B). This figure shows that CH can be determined in these mixtures by measuring the amplitude at 228.4 nm where the derivative ratio spectrum of 2,6-DCA equals zero.

III. Optimization of the Derivative Ratio Spectrum Method

(I) Effect of $\Delta\lambda$

The influence of $\Delta\lambda$ on the first derivative of the ratio spectra was studied to obtain the best wavelength interval, thus the absorption spectra of CH and its corresponding mixtures with 2,6-DCA were obtained. The amplitude of each CH solution and its corresponding mixture were then divided at every wavelength by the amplitude of 2,6-DCA. The ratio spectra thus obtained are differentiated with respect to wavelength at different $\Delta\lambda$ intervals (2 nm, 4 nm and 6 nm) of which $\Delta\lambda = 2$ nm was found optimum (Table 1). Using $\Delta\lambda$ higher than 6 nm changed the shape of the curve.

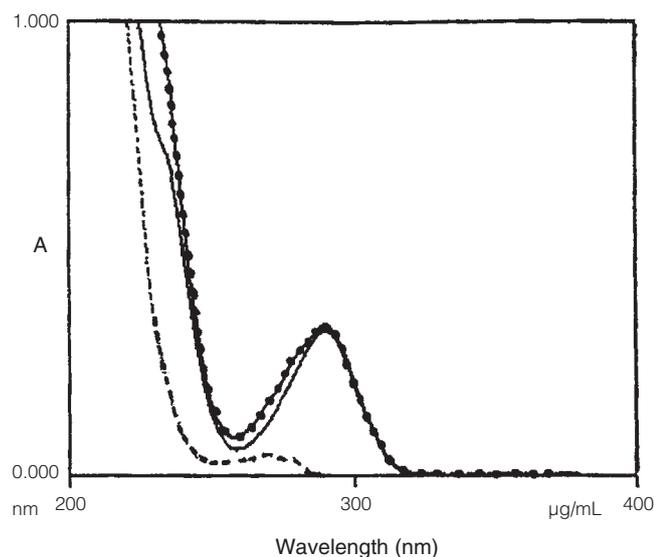


Figure 1. Absorption spectra of 24 $\mu\text{g/mL}$ CH(-----), 24 $\mu\text{g/mL}$ 2,6 DCA(—), and their mixture(—·—·—).

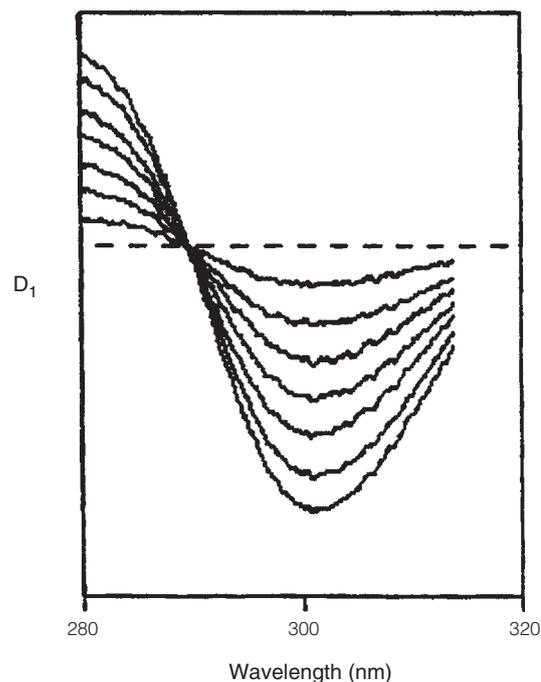


Figure 2. First derivative spectra of different concentrations of 2,6-DCA : (2.4, 4.8, 7.2, 9.6, 12, 14.4 and 16.8 $\mu\text{g/mL}$) at $\Delta\lambda = 2$ nm.

(II) Effect of Divisor Concentration

A study was carried out to test for the effect of the divisor concentration 2,6-DCA on the obtained calibration graphs, hence the absorption spectra of standard solutions of CH in the concentration range stated in Table 2 were divided by the corresponding amplitudes of standard solutions of 2,6-DCA (2.4, 4.8 and 7.2 $\mu\text{g}/\text{mL}$). The resultant ratio spectra were then differentiated with respect to wavelength with $\Delta\lambda = 2$ nm. The derivative ratio value of each solution was measured at 228.4 nm, and plotted versus its concentration. The statistical analysis of these graphs by least squares method (Table 3) shows high values of the correlation coefficients and small values of the intercepts thus indicating good linearity.

The results obtained (Table 3) indicate that the divisor concentration has no effect on the assay.

IV. Diazo Coupling Method

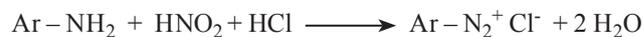
2,6-DCA was determined on the basis of diazotization and coupling with N-(1-naphthyl) ethylenediamine to yield a colored azo dye. The color produced in this reaction develops rapidly and reaches its full intensity within 1-2 min⁽²²⁾. The

Table 1. Effect of $\Delta\lambda$ on the determination of 4.8 $\mu\text{g}/\text{mL}$ clonidine hydrochloride (using 7.2 $\mu\text{g}/\text{mL}$ 2,6-DCA as divisor) at 228.4 nm

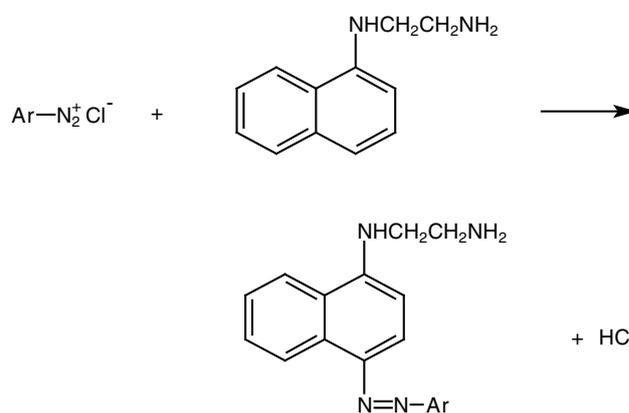
$\Delta\lambda$	Average % recovery*
1	98.71
2	99.32
3	99.47
4	98.31
5	97.49

* Average of three separate determinations.

reaction proceeds through electrophilic aromatic substitution, where the aromatic primary amino group in 2,6-DCA is converted to diazonium compound by the action of nitrous acid.



The diazonium compound is then coupled with an appropriate aromatic substrate (Bratton-Marshall) to produce a chromophore that usually absorbs light in the visible region of the spectrum. N-(1-naphthyl) ethylenediamine was the most satisfactory in terms of color yield and stability⁽²³⁾.



Bratton-Marshall is preferred for quantitative work because the product is usually soluble and has high molar absorptivities⁽²⁴⁾.

V. Optimization of the Diazo Coupling Procedure

The different parameters affecting the diazo coupling reaction and the color development were extensively studied to determine the optimal conditions for the assay procedure:

Increasing the volume of sodium nitrite was found to

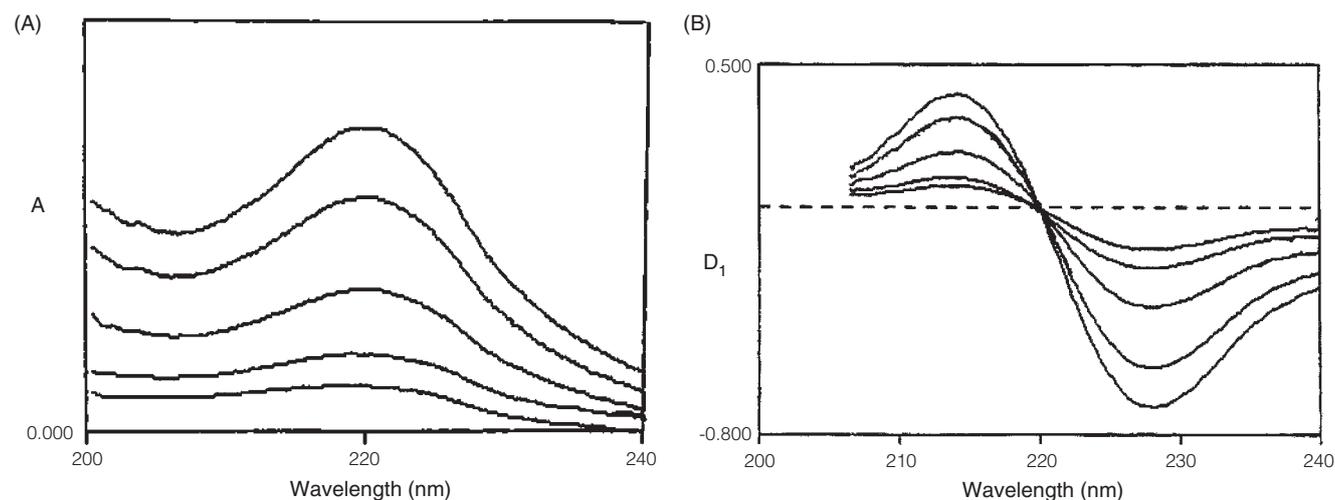


Figure 3. (A) Absorbance ratio spectra and (B) first derivative ratio spectra of different concentrations of CH: 0.96, 1.44, 2.4, 3.84 and 4.8 $\mu\text{g}/\text{mL}$, using 7.2 $\mu\text{g}/\text{mL}$ 2,6-DCA as divisor at $\Delta\lambda = 2$ nm.

Table 2. Assay parameters for the determination of clonidine hydrochloride and 2,6-dichloroaniline by the proposed spectrophotometric methods

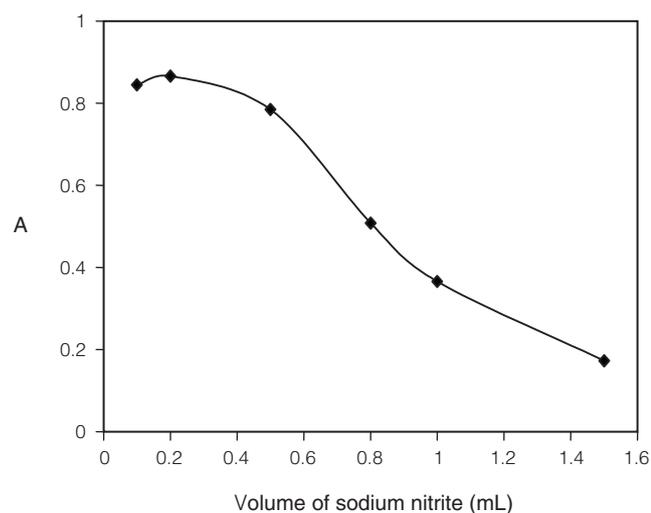
Item	D ₁ R method	D ₁ method	Diazo coupling method
$\Delta\lambda$ (nm)	2	2	-
Selected λ (nm)	228.4	300.8	498
Concentration range ($\mu\text{g/mL}$)	0.96 - 4.8	2.4 - 16.8	0.8 - 4.0
Regression equation			
Intercept (a)	-0.014	2.43×10^{-3}	-9.71×10^{-3}
Variance around intercept (S_a^2)	2.55×10^{-5}	1.35×10^{-6}	2.79×10^{-5}
Slope (b)	0.15	0.013	0.23
Variance around slope (S_b^2)	2.28×10^{-6}	1.17×10^{-8}	4.16×10^{-6}
Correlation coefficient (r)	0.9998	0.9998	0.9998
Variance ($S^2_{y,x}$)	1.96×10^{-5}	1.89×10^{-6}	2.79×10^{-5}
Accuracy (Mean \pm SD)	100.27 ± 1.43	99.80 ± 1.14	100.00 ± 0.90
Precision (RSD %)	1.43	1.14	0.90

D₁R method : Derivative ratio spectrum method.

D₁ method : First derivative method.

Table 3. Effect of divisor concentration on the determination of clonidine hydrochloride by the proposed first derivative ratio spectrum method

Conc. Range ($\mu\text{g/mL}$)	Selected λ (nm)	Divisor Conc. ($\mu\text{g/mL}$)	Regression Equation			RSD%
			Intercept (a)	Slope (b)	Correlation Coefficient (r)	
0.96 - 4.8	228.4	2.4	2.05×10^{-3}	0.45	0.9999	0.53
0.96 - 4.8	228.4	4.8	6.90×10^{-4}	0.20	0.9999	0.57
0.96 - 4.8	228.4	7.2	5.09×10^{-4}	0.13	0.9999	0.94

**Figure 4.** Effect of sodium nitrite volume (0.1%) on the diazotization of 2,6-dichloroaniline (3.85 $\mu\text{g/mL}$).

cause an effective decrease in the absorbance value of the color formed. 0.2 mL of 0.1 % sodium nitrite solution was found to be quite suitable (Figure 4).

The acidity of the reaction medium was adjusted using 1.5 mL 5 M HCl (Figure 5).

The volume of urea needed to get rid of the excess nitrite after diazotization and before coupling with the Bratton-Marshall Reagent was studied, and 1 mL of urea 0.5% was found to be enough.

The reaction was studied as a function of the volume of the coupling reagent (Bratton-Marshall); the maximum absorbance was attained upon using 1.5 mL of 0.1% of Bratton-Marshall Reagent solution (Figure 6).

The reaction time course was followed by the color development at different time intervals at room temperature. The subsequent addition of sodium nitrite in acidic medium and Bratton-Marshall solution resulted in the formation of the orange-red diazo coupling product instantaneously

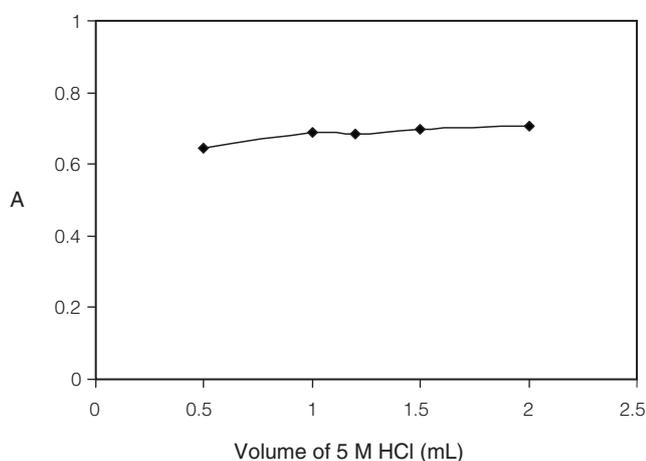


Figure 5. Effect of the volume of 5 M HCl on the diazotization of 2,6-dichloroaniline (3.11 $\mu\text{g/mL}$).

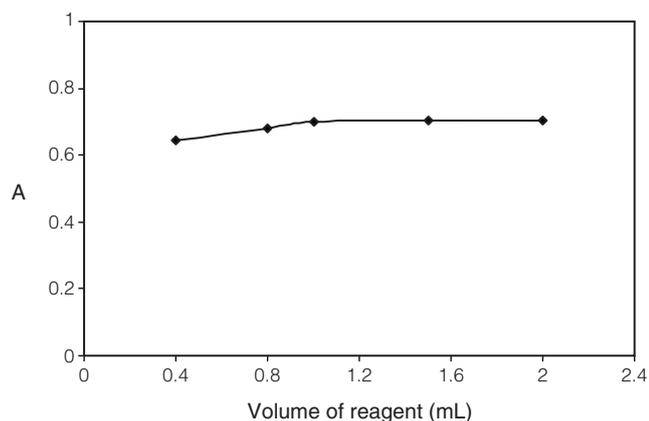


Figure 6. Effect of the volume of (N-(1-naphthyl) ethylenediamine) dihydrochloride (0.1%) on coupling with the diazo derivative of 2,6-dichloroaniline (3.14 $\mu\text{g/mL}$).

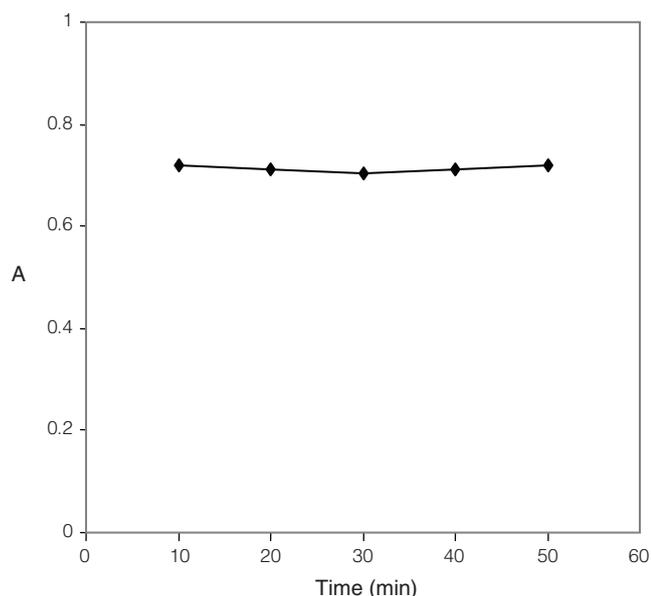


Figure 7. Effect of the reaction time at room temperature on the coupling of 2,6-dichloroaniline (3.2 $\mu\text{g/mL}$) with N-(1-naphthyl) ethylenediamine dihydrochloride.

without increase in intensity of color by time (Figure 7).

The reaction product was found to be stable for at least one hour under the studied experimental conditions.

Figure 8 shows the absorption spectrum of the formed orange red azo dye which absorbs maximally at 498 nm.

VI. Validation of the Proposed Methods

(I) Linearity

The linearity of the proposed methods was evaluated for each compound by analyzing a series of different concentrations of each component within the concentration ranges cited in Table 2 in the absence and presence of a certain concentration of the other component in the mixture. The assays were applied according to the established experimental conditions. The derivative and the absorbance values for each component were recorded and plotted against the concentration. A straight line was obtained in each case. Regression equations, variances, variances around slopes and intercepts are given in Table 2.

The good values of correlation coefficients and the small values of intercepts validated the compliance with Beer's law, and showed excellent linearity.

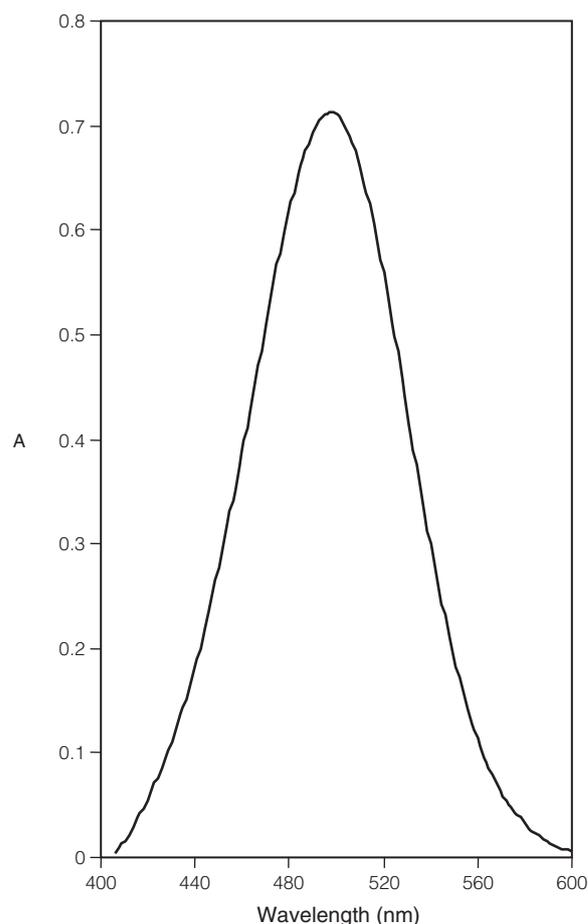


Figure 8. Absorption curve of the orange red azodye of 2,6-dichloroaniline (3.2 $\mu\text{g/mL}$) with Bratton-Marshall Reagent.

Furthermore, in the derivative ratio spectrum method, three different concentrations of the divisor were used in the analysis of standard solutions of CH (Table 3). A straight line was obtained in each case. The statistical analysis using least squares method for these assays showed high correlation coefficient values and small values of intercepts, which proved good linearity.

(II) Accuracy

The accuracy of the proposed methods was assessed by calculating the recovery of the drugs spiked in regular tablet excipients. The results are presented in Table 2. The accuracy was moreover confirmed by comparing the results of the assay of pharmaceutical preparation and laboratory prepared mixtures by the official pharmacopoeial HPLC method. The good recoveries obtained suggest the good accuracy of the proposed methods. There was no interference from the excipients present in the pharmaceutical preparation, which indicates the high selectivity of the method (Tables 4 and 6).

(III) Precision

The precision of the methods was evaluated by calculating the relative standard deviation of the assay results of three different concentrations of each drug either alone or

Table 4. Assay results for the determination of clonidine hydrochloride in commercial tablets using the first derivative ratio spectrum method

Item	Proposed D ₁ R method	Reference HPLC method (USP XXX)
Catapres tablets		
Mean recovery %	96.07	97.65
SD	0.98	1.98
t		1.60
F		4.05

Theoretical values for t- and F- at $p = 0.05$ are 2.31 and 6.39, respectively.

Table 5. Assay results for the determination of clonidine hydrochloride in laboratory prepared mixtures using the proposed first derivative ratio spectrum method

Replicate No.	Amount Added ($\mu\text{g}/\text{mL}$)		Amount of CH found ($\mu\text{g}/\text{mL}$)	% Recovery
	CH	2, 6- DCA		
1	4.8	0.48	4.83	100.52
2	4.8	0.96	4.87	101.37
3	4.8	1.44	4.88	101.65
4	4.8	1.68	4.88	101.65
5	4.8	2.4	4.88	101.65

in the presence of certain concentration of the other component of the mixture in three replicates. The mean relative standard deviations are tabulated (Table 2) and are considered satisfactory indicating high degree of precision.

(IV) Selectivity

The selectivity of the methods was studied by preparing different mixtures of CH and 2,6-DCA within the linearity ranges of each method. The laboratory prepared mixtures were analyzed according to the proposed procedures. Statistical analysis of the data, (Tables 3, 5 and 6) showed that the slope of the calibration graph for each component was independent of the concentration of the other component of the mixture. This means that either the D₁ or D₁R amplitudes or absorbances of the color were only a function of the concentration of the component at the specified wavelengths.

Hence, the results tabulated (Tables 5 and 6) were satisfactory indicating the selectivity of the methods.

(V) Limit of Detection and Limit of Quantitation

In accordance with the official compendial method (USP XXX) the limits of detection and quantitation were defined as $3 s.b^{-1}$ and $10 s.b^{-1}$, respectively where s is the standard deviation of blank response. Using such formulae the limits of detection and quantification for 2,6-DCA by the proposed diazo coupling procedure were 0.08 and 0.26 $\mu\text{g}/\text{mL}$ respectively.

VII. Application to the Commercial Tablets and Laboratory Prepared Mixtures

The proposed procedures were applied for the determination of CH and its major related substance 2,6-DCA in pharmaceutical tablets and laboratory prepared mixtures (Tables 4 and 6). The results were compared statistically with those obtained by the USP XXX HPLC procedure. The calculated t- and F- values did not exceed the theoretical ones, indicating good agreement between the proposed and the referee method (Tables 4 and 6).

Table 6. Assay results for the determination of 2,6-dichloroaniline in laboratory prepared mixtures using the proposed D1 and diazo coupling methods.

Replicate No.	D ₁ method				HPLC	Diazo coupling method			
	Amount Added (µg/mL)		Amount Found 2,6-DCA (µg/ mL)	% Recovery	reference method (USP XXX) % Recovery	Amount Added (µg/mL)		Amount Found 2,6-DCA (µg /mL)	% Recovery
	CH	2,6-DCA				CH	2,6-DCA		
1	24	2.4	2.357	98.22	100.03	8	4	4.00	100.03
2	24	4.8	4.746	98.89	99.91	12.8	3.2	3.20	99.91
3	24	7.2	7.285	101.19	99.44	10	2	2.03	101.42
4	24	9.6	9.749	101.56	101.42	20	2	1.99	99.44
5	24	12	11.989	99.91	100.03	40	2	2.03	101.42
Mean			99.95		100.16			100.44	
SD			1.44		0.74			0.92	
t			0.29					0.53	
F			3.74					1.53	

Each value is the mean of five measurements.

Theoretical values for t- and F- at $p = 0.05$ are 2.31 and 6.39, respectively.

CONCLUSIONS

In this work clonidine hydrochloride was successfully determined in presence of its synthetic precursor 2,6-dichloroaniline by the derivative ratio spectrophotometric method.

Although this method seems not to be the best regarding sensitivity, however, it holds up as an alternative to the currently available HPLC and spectrofluorimetric methods and it also offers some advantages over these latter methods as it is simpler, more economical, environment-friendly (since no chemical reagents or organic solvents are used). Moreover, it is a cost effective non separation stability-indicating method.

2,6-Dichloroaniline was analyzed by a first-derivative method and a diazo coupling colorimetric method which have both the advantage of being simple, direct and selective for its determination even in the presence of a large excess of clonidine without the need for a previous separation step.

Finally, the three proposed methods were found to be accurate and precise thus making them convenient for quality control purposes.

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