Development and Validation of Spectrophotometric and Spectrofluorimetric Methods for the Determination of Cyclobenzaprine HCl

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Abstract

Five simple and sensitive methods were developed for the determination of cyclobenzapirine hcl (CB) in presence of its degradation product anthraquinone (AQ).

Method A dual wavelength spectrophotometry (DW); where two wavelengths were selected for the drug 283 and 306 nm in such a way that the difference in absorbance was zero for its degradation. Method B ratio difference spectrophotometry (RD) was depended on measuring the ratio difference between 290 and 305 nm. Method C was depended on measuring the peak amplitude of the first derivative of the ratio spectra (1DD). Method D Isosorptive Point (ISO) at 280 nm Coupled with Second Derivative (2D). Method E depending on spectrofluorimetric determination of cyclobenzapirine HCl through quenching of uranyl acetate with λex at 228 nm and λem at 458 nm. Linearties were obtained in concentration range 5 µg/ml – 30 µg/ml in case of methods A, B, C and D, while in case of methods E linearity was obtained in concentration range of 1 µg/ml –10 µg/ml. The five methods were found to be specific for CB in presence of different concentration % of its degradation product.

Keywords: Cyclobenzaprin HC, Anthraquinone (AQ), Dual wavelength (DW), Ratio difference (RD), (1DD) Derivative ratio, Isosorptive point (ISO); Spectrofluorimetry; Uranyl acetate

Introduction

Cyclobenzaprine hydrochloride [3-(5H-dibenzo [a, d] cyclohepten-5-ylidene)-N-N-dimethyl-1-propanamine hydrochloride; (CB) is a centrally acting muscle relaxant and related to tricyclic antidepressent. It is used in the treatment of the musculoskeletal condition which associated with painful muscle spasms [1]. The determination of CB is described in the U.S.P. [2] by non-aqueous titration method. It is also determined by several methods involving spectrophotometry [3-6], TLC [7,8], different HPLC methods using several mobile phases [9-14], HPLC coupled with MS detection [15-22] and GC-MS [23-26]. Five stability-indicating methods were reported for the determination of CB in the presence of its degradation product [22-31].

The present study describes simple, sensitive and precise stability-indicating spectrophotometric and spectrofluorimetric procedures for the quantitative determination of CB in pure form, pharmaceutical formulation and in the presence of AQ (its degradation product) AQ. These methods are developed and validated according to ICH guidelines [32] for the determination of the chosen drug. There are many advantages of the proposed methods as low cost, rapidity and environmental protection which suitable for quality control laboratories where economy and time are essential.

Materials and Methods

Instruments

-UV-Visible spectrophotometer (Unicam UV 300, Kyoto, Japan).
- Spectrofluorimeter (Agilent Technologies, Cary Eclipse Fluorescence, Australia).

Sample

Reference sample: Pure sample CB was kindly supplied by Multi-Apex Pharma, Badr City, Cairo, Egypt (Batch No. mt4431014). According to the pharmacopeia method [2] its purity was found to be 99.50 ± 0.33%

Pharmaceutical formulation: Multi-Relax tablets, (Batch No CBP/1506004-M), claimed to contain 10 mg CB, manufactured by Multi-Apex Pharma Company for Pharmaceutical Industries, purchased from a local market.

Degraded sample: Anthraquinone (AQ), a degradation product of CB was purchased from Sigma-Aldrich (St. Louis, MO).

Reagents

-Methanol of analytical grade (Fischer scientific – UK)
-Distilled water
-Uranyl acetate (Fluka Chemie GmbH- Germany.) 0.25 × 10⁻³ mol freshly prepared in distilled water.

Stock and working solutions

CB Stock and working solutions:
- Methods (A), (B), (C) and (D)

CB stock standard solution of (1 mg/mL) in methanol was prepared

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Received January 06, 2018; Accepted January 20, 2018; Published January 29, 2018

Citation: Ramadan NK, Mohamed TA, Fouad RM, Moustafa AA (2018) Development and Validation of Spectrophotometric and Spectrofluorimetric Methods for the Determination of Cyclobenzaprine HCl. Pharm Anal Acta 9: 574. doi: 10.4172/2153-2435.1000574

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by dissolving 25 mg of CB in a 25-mL volumetric flask, and then the volume was completed to the mark with methanol. An aliquot of the prepared stock solution was further diluted with the methanol solvent to get a working solution with final concentration (0.25mg/mL).

**Method (E)**

CB stock standard solution of (1 mg/mL) in water was prepared by dissolving 25 mg of CB in a 25-mL volumetric flask, and then the volume was completed to the mark with water. An aliquot of the prepared stock solution was further diluted with the suitable solvent to get a working solution with final concentration (0.1mg/mL).

**Stock and working solutions of AQ (degradation product of CB):**

- Methods (A), (B), (C) and (D)

AQ standard solution of (1 mg/mL) in methanol by dissolving 25 mg of AQ in a 25-mL volumetric flask, and then the volume was completed to the mark with methanol. An aliquot of the prepared stock solution was further diluted with methanol to get a working solution with final concentration (0.25mg/mL).

- Method (D)

AQ standard solution of (0.1 mg/mL) in water by dissolving 2.5 mg of AQ in a 25-mL volumetric flask, and then the volume was completed to the mark with water.

**Laboratory prepared mixtures containing different ratios of CB and AQ:**

- Methods (A), (B), (C) and (D):

Aliquots (1 – 0.2 mL) of CB were accurately transferred from its working solution (0.25 mg/mL) into a series of 10-mL volumetric flasks equivalent to (25 μg/mL – 5 μg/mL). Aliquots (0.2 – 1 mL) of AQ working solution (0.25 mg/mL) equivalent to (5 μg/mL – 25 μg/mL) were added, the volume was completed with methanol.

- Methods (E):

Aliquots (0.9 – 0.1 mL) of CB working standard solution (0.1mg/mL) equivalent to (90 – 10 μg) were accurately transferred into a series of 10-mL volumetric flasks. Aliquots (0.1 – 0.9 mL) of the degradation product working solution (0.1 mg/mL) equivalent to (10 μg – 90 μg) were added, followed by 2.5 mL of 0.25 × 10⁻³ mol uranyl acetate solution then the volumes were completed with water.

**Procedures**

**Construction of the calibration curve for method (A) (DW)**

Into a series of 10-mL volumetric flasks aliquots (0.2, 0.4, 0.6, 0.8, 1 and 1.2) of the CB stock standard solution (1 mg/mL) were transferred. The volume was then completed to mark with methanol. The zero-order spectra were recorded in the range of 200 nm–400 nm. Absorbance values at 283 and 306 were measured. The difference in absorbance of CB at 283 nm and 306 nm (difference is zero for AQ) was plotted against CB concentration. Then calibration curve was constructed and the regression equation was computed.

**Construction of the calibration curve for method (B) (RD)**

The ratio spectra were obtained by dividing the zero order spectra of the prepared solutions on the spectrum of 5 μg/mL of AQ (degradation product). The peak amplitudes of the ratio spectra were measured at 290 nm and 305 nm. The difference in amplitude of ratio spectra (ΔP290-305) was plotted against CB concentration. Then calibration curve was constructed and the regression equation was computed.

**Construction of the calibration curves for method (C) (1DD)**

Then the first derivative of the ratio spectra 1DD with Δλ=2 and scaling factor 10 were obtained. The peak amplitudes of the first derivative of the ratio spectra at 282 nm and 306 nm were measured. Linear calibration curves were constructed relating the peak amplitude of the first derivative of the ratio spectra at the specified wavelengths to CB concentrations and the regression equations were computed.

**Construction of the calibration curve for method (D) (ISO)**

Aliquots (0.2, 0.4, 0.6, 0.8, 1 and 1.2) of CB were separately transferred from its working standard solution (0.25 mg/mL) into a series of 10-mL volumetric flasks, diluted to volume with methanol. Then aliquots (0.1, 0.2, 0.4, 0.6, 0.8 and 1) of AQ were separately transferred from its working standard solution (0.25 mg/mL) into another series of 10-mL volumetric flasks, diluted to volume with methanol. The zero order absorption spectrum (1D) of each dilution was recorded against blank.

For the determination of AQ, the second derivative (2D) of the scanned spectra of AQ was obtained with Δλ=2 and scaling factor=10. The peak amplitudes at 250 nm were plotted against AQ concentrations and the calibration curve was constructed and the regression equation (1) was computed.

For CB determination, the absorbance of the scanned spectra of CB was measured at 280 nm (isoabsorptive point). The calibration curve relating the absorbances at 280 (Åiso) to CB concentrations was constructed and the regression equation (2) was computed.

**Construction of the calibration curve for method (E)**

Into a series of 10-mL volumetric flasks, aliquots (0.1, 0.2, 0.4, 0.6, 0.8 and 1 mL) of CB were separately transferred from its working standard solution (0.1 mg/mL) followed by 2.5 mL of 0.25 × 10⁻³ mol uranyl acetate solution then the volumes were completed with water. The fluorescence intensity was measured at λem 458nm with λex 228 nm at room temperature (25°C) against blank similarly prepared. The calibration curve was constructed relating the difference in fluorescence intensity (between blank and experiments) at λem 458 nm with λex 228 to CB concentrations and the regression equation was computed.

**Analysis of laboratory prepared mixtures of CB and AQ**

- Methods (A), (B) and (C)

The absorption spectra of the laboratory prepared mixtures were recorded. Then the procedures were completed as described in subsection of Construction of the calibration curves. The concentrations of CB were calculated from the corresponding regression equations.

- Method (D)

For AQ determination; the absorption spectra of the laboratory prepared mixtures were recorded. The procedure was performed as described under Construction of the calibration curves. The concentrations of AQ were calculated from regression equation (1).

For CB determination; the absorption spectra of the laboratory prepared mixtures were recorded at 280 nm (isoabsorptive point). Then the procedure was completed as described under Construction of the calibration curves. The total concentration of CB and AQ was calculated from the regression equation (2). The concentration of CB could be obtained after subtraction from the following equation:
ΔP \text{ (283 – 306)} = 0.0067C + 0.0067 \quad r = 0.9995

Where ΔP \text{ (283 – 306)} is the difference in absorbance at 283 and 306, C is the concentration of CB in µg/mL and r is the correlation coefficient.

The proposed DW method was successfully applied for the determination of the drug in pure powder form with mean percentage recovery of 99.84 ± 0.560.

Ratio difference (RD)

The most advantage of the ratio difference method (RD) is its simplicity, accuracy and reproducibility. It has the ability of solving severely overlapped spectra without prior separation so it doesn’t require any sophisticated apparatus or computer programs. This method depending on the amplitude difference between two points on the ratio spectra of a mixture is directly proportional to the concentration of the drug; independence of the interfering component (degradation product).

CB concentration = (total concentration – AQ concentration).

Methods (E)

The fluorescence intensity of the laboratory prepared mixtures was recorded. Then the procedures were completed was described in subsection of Construction of the calibration curve. The concentrations of CB were calculated from the regression equation.

Analysis of CB in pharmaceutical preparation

Five Multirelax tablets were weighed accurately and powdered. An amount of equivalent to 100 mg of CB was accurately weighed into 100-mL volumetric flask and extracted with 50-mL methanol using an ultrasonic bath for 30 minutes, diluted to volume with the same solvent and filtered. Into 50 mL of the filtered solution suitable dilutions were made using methanol to prepare tablet solution containing 1 mg/mL for methods (A), (B) (C) and (D). 2.5 mL of the solution was accurately transferred to a 10-mL volumetric flask and was diluted to volume with methanol to prepare tablet solution containing 0.25 mg/mL.

For method (D), the other 50 mL was evaporated to dryness, then dissolving the residue with 50 mL water and suitable dilutions were made using water to prepare tablet solution containing 0.1 mg/mL. Then 0.4 mL accurately transferred into 10 mL volumetric flask, then the procedures were completed was described under Construction of the calibration curves. The concentration of CB was calculated from the corresponding regression equations.

Results and Discussion

This work is concerned with the simultaneous determination of CB in the presence of one degradation product AQ using spectrophotometric and spectrofluorimetric techniques. There were 5 stability methods previously published concerning with degradation product; only one was a spectrophotometric method dealing with 2 degradation mixture dibenzocycloheptatrienone and anthraquinone [29]. This work has an advantage over the published methods in sensitivity and specificity.

Dual wavelength (DW)

The developed dual wavelength method provides a simple spectrophotometric method for selective determination of CB in the presence of its degradation product AQ using its zero order absorption spectra; depending on that the absorbance difference at two wavelengths on the spectra is directly proportional to the concentration of the drug, with no interference from its degradation product [33-38]. To apply this method, only two wavelengths should be carefully chosen on the basis that the interfering component shows the same absorbance value and the component of interest shows significant difference in absorbance with concentration. The zero order absorption spectra of CB and its degradate AQ revealed great spectral overlap was shown in Figures 1 and 2.

Several trials have been made to select the optimum wavelengths for the drug. The best results regarding selectivity and sensitivity were obtained by using the absorbance difference at 283 and 306 nm for determination of CB where AQ has zero absorbance difference so no interference from AQ has been founded. The concentration of CB was calculated from the regression equation representing the linear relationship between the absorbance at the two selected wavelengths versus the corresponding concentration of drug.

A linear relationship was obtained in the range of (5 µg/mL – 30 µg/mL) of CB and the regression equation was computed, (Figure 3) and found to be:

\[
\Delta P \text{ (283 – 306)} = 0.0067C + 0.0067 \quad r = 0.9995
\]

Where ΔP \text{ (283 – 306)} is the difference in absorbance at 283 and 306, C is the concentration of CB in µg/mL and r is the correlation coefficient.

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Ratio difference (RD)

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The RD method has the main basic advantage over the DW of complete elimination of the interfering component (AQ) in the form of constant and the difference at any two points will be equal to zero, so there is no need for critical measurements which leads to highly reproducible and robust results [39-43].

The zero-order spectra of CB and its degradation product AQ show an overlap, (Figure 2), that prevents the use of direct spectrophotometric analysis of the drug in the presence of its degradation product. In an attempt to resolve this overlap, derivative method was applied. The 1D, 2D and 3D failed to be determined CB in the presence of its degradate (Figures 4 to 6).

The spectra of the CB were divided by a certain concentration of AQ spectrum was a divisor. The obtained ratio spectra are shown in Figure 7.

The selected divisors should compromise between minimal noise and maximum sensitivity, so the divisors were chosen. The CB solutions (5 µg/mL – 30 µg/mL) spectra were divided by the spectrum of its deg (5µg/mL) and the concentration of CB was calculated by using the regression equation representing the linear relationship between the differences of these ratio spectra amplitudes at the two selected wavelengths versus the corresponding concentration of drug.

Linear relationship CB obtained in the range of (5 µg/mL – 30 µg/mL) of CB, and the regression equation was computed (Figure 8) and found to be:

\[ \Delta P_{(290 - 305)} = 0.0491C + 0.0593 \quad r=0.9997 \]

Where \( \Delta P_{(290 - 305)} \) is the difference in peak amplitudes at 290 and 305 nm, C is the concentration of CB in µg/mL and r is the correlation coefficient.

The proposed RD method was successfully applied for the determination of the drug in pure powder form with mean percentage recovery of 100.22 ± 0.509.

**Derivative ratio spectrophotometry (1DD)**

Derivative spectrophotometry has been first suggested during the last decades and soon become a well-established technique for the assay of drugs in mixtures and in pharmaceutical dosage forms [44-45].

The derivative ratio spectrophotometry (1DD) can also be applied to resolve the overlap between the drug and its degradation. The zero-order absorption spectra of CB were divided by the spectrum of 5 µg/mL of AQ and the obtained ratio spectra are shown in Figure 7.

The linearity of this method was found to be valid in the concentration range of 5 µg/mL – 30 µg/mL of CB and the regression equation was computed (Figure 8) and found to be:

\[ y = 0.6593x - 0.0593 \quad R^2=0.9997 \]

Where y is the absorbance and x is the concentration of CB in µg/mL.
mL of its degradation product. This gave the best compromise in terms of sensitivity, repeatability, and signal-to-noise ratio. The first derivative of the ratio spectra at 282 and 306 nm with $\Delta \lambda=2$ and scaling factor 10 were obtained. The peak amplitudes were measured at the selected wavelengths, (Figure 9).

The linearities between the concentrations of the drug and the peak amplitudes at the selected wavelengths were studied. Linear relationship obtained in the range of (5 µg/ml – 30 µg/mL) of CB, and the regression equations were computed (Figure 10) and found to be:

$$1DD_{282} = 1.5664C - 0.2320 \quad r=0.9994$$

$$1DD_{306} = 0.6748C - 0.3473 \quad r=0.9993$$

Where $1DD_{282}$ and $1DD_{306}$ are the peak amplitudes at 282 and 306 respectively, $C$ is the concentration of CB in µg/mL and $r$ is the correlation coefficient.

The proposed $1DD$ method CB was successfully applied for the determination of the drug in pure powder form with mean percentage recovery of 98.70 ± 0.620 and 100.27 ± 0.528% at the 282 nm and 306 nm.

The isosbestic point is used for the analysis of two drugs in their binary formulation, by having the total concentration of both drugs, and then by the means of other method if the concentration of either drug could be determined separately, the concentration of the second one can be calculated by subtraction [46-51].

The theory of this method could be confirmed experimentally by recording the absorbance spectra of CB and AQ each having a concentration of 10 µg/mL and the spectrum of a mixture having a total concentration of 10 µg/mL (5 µg/mL CB and 5 µg/mL AQ), (Figure 11).

In this figure, one can observe that the pure CB and AQ have their absorption spectra with an isosbestic point at 280 nm. Meanwhile; their mixture has the same absorbance at their isosbestic point. According to the theory, that the mixture of CB and AQ act as a single component and give the same absorbance value as pure drug at their isosbestic point.

The total content of the mixture could be calculated as explained by the theory above by measuring the absorbance value at the chosen isosbestic point. The content of AQ alone could be calculated from its $1D$ spectra at 250 nm without any interference from CB, (Figure 12). Then the content of CB could be calculated by subtraction.

It was found that, the first ($1D$) derivative, (Figure 13) failed to determine AQ in presence of CB; upon applying the second derivatives, (Figure 12), zero crossing of CB was observed at 250 nm. Therefore, $2D$ was applied for the determination of AQ in presence of CB.

The calibration curve relating the peak amplitudes of the second derivative absorption spectra of AQ at 250 nm to AQ concentrations was constructed, (Figure 14) and regression equation (1) was computed and found to be:

$$2D_{250} = 1.8127C_{AQ} + 8.9625 \quad r=1 (1)$$

Where, $2D_{250}$ is the peak amplitude of the second derivative absorption spectra of AQ at 250 nm, $C_{AQ}$ is the concentration of AQ in µg/mL and $r$ is the correlation coefficient.
Also the calibration curve relating the absorbance at 280 nm (isoabsorptive point) of the zero-order absorption spectra of CB to the corresponding concentrations was constructed, (Figure 15) and regression equation (2) was computed and found to be:

\[ A_{\text{iso}} = 0.0367C_t - 0.0367 \quad r=0.9997 \quad (2) \]

Where, \( A_{\text{iso}} \) is the absorbance at 280 nm, \( C_t \) is the total concentration of the mixture in \( \mu g/mL \) and \( r \) is the correlation coefficient.

The proposed method was found to be valid in the range of 2.5 \( \mu g/mL \) – 25 \( \mu g/mL \) for AQ and in the range of 5-30 \( \mu g/mL \) for CB.

**Fluorimetric method**

Uranyl acetate has been used was a fluorogenic reagent; upon reacting with its fluorescence was quenched. This derivatization reaction [52-55] has been used for the determination of the cited drug by measuring the quenching of uranyl acetate fluorescence at 458 nm (\( \lambda_{ex}=228 \text{ nm} \)). The uranyl acetate solution has relative fluorescence intensity (Figure 16); upon the addition of CB the fluorescence intensity of the solution decreased significantly and the magnitude of the decrease was proportional to the concentration of CB, (Figure 17).

This would permit the determination of intact CB in presence its degradation product. The fluorescence intensity of different concentrations of CB in water was recorded against water as a blank, using 228 and 458 nm as excitation and emission wavelengths, respectively.

Various parameters affecting the reaction process, the reaction was found to be sensitive to volume of uranyl acetate (0.25 \( \times 10^{-3} \text{ mol} \)); 2.5 mL was found to be sufficient to produce maximum difference in fluorescence intensity. Also the stability of the difference in fluorescence intensity was studied and it was found that the intensity of the fluorescence difference was stable up to 60 min. The fluorescence intensity was affecting significantly by any organic solvent so water is the ideal solvent.

The stoichiometry of the reaction between CB and uranyl acetate was studied using the adopting limiting logarithmic [56] method as shown in Figure 18 and found one to one.

The difference in the fluorescence intensity was linear in the range of 1 \( \mu g/mL \) – 10 \( \mu g/mL \), and the regression equation was computed (Figure 19) and found to be:

\[ I_f = 50.9616C + 453.5315 \quad r=0.9995 \]

Where \( I_f \) is the difference in fluorescence intensity, \( C \) is the concentration of CB in \( \mu g/mL \) and \( r \) is the correlation coefficient.

The proposed spectrofluorimetric method was successfully applied for the determination of CB with mean recoveries of 100.55 ± 0.447%.
Methods Validation

Methods validation was performed according to ICH guideline [32] for the proposed methods as follows:

Range and linearity

The linearity of the proposed methods was evaluated by processing the different calibration curves on three different days. Each concentration was repeated in triplicate. The statistical parameters for all methods are listed in Table 1.

Accuracy

To study the accuracy of the proposed methods, the procedures under linearity for CB, were repeated three times for the determination of three different concentrations of pure form. The accuracy expressed as percentage recoveries as shown in Table 1.

Precision

The intra-day and inter-day precisions of the proposed methods were determined by the analysis of three different concentrations of CB three replicate on a single day and on three consecutive days, the results are illustrated in Table 1.

Detection and quantification limits

Limit of detections (LOD=3.3 × SD/b) and the limit of quantifications (LOQ=10 × SD/b) were calculated for CB using the proposed methods and the results are included in Table 1.

Specificity and selectivity

The specificity of the proposed methods was assessed by the analysis of a laboratory prepared mixture containing different percentages of AQ. Method (A) and (D) was found to be specific for CB in the presence of up to 70% of its degradate. Method (B) and (C) were found to be specific for CB till 90% in the presence of AQ. The specificity of method (E) was achieved in the presence of up to 50% of its degradate (Table 2).

Robustness and ruggedness

Small variations of the UV conditions and Fluorimetry were applied in order to determine the robustness of the methods. Analysis of CB at 25 ± 3°C (different temperature) and using methanol from Sigma Aldrich (different company) did not have a significant effect on the UV spectra, illustrating excellent robustness of the methods.

Table 1: Results of validation parameters obtained by the proposed methods for the determination of CB.

| Parameters | Method A | Method B | Method C | Method D | Method E |
|------------|----------|----------|----------|----------|----------|
|            | DW       | RD       | DD       | ISO      | Fluorimetry |
| Linearity range (µg /ml) | 5.00-30.00 | 5.00-30.00 | 5.00-30.00 | 5.00-30.00 | 1.00-10.00 |
| LOD (µg /ml) | 1.110 | 1.230 | 0.750 | 0.920 | 3.277 |
| LOQ (µg /ml) | 3.360 | 4.000 | 2.270 | 2.790 | 1.230 |
| Accuracy Mean ± S.D% | 100.22 ± 0.510 | 100.22 ± 0.510 | 100.27 ± 0.529 | 100.27 ± 0.529 | 100.55 ± 0.449 |
| Precision Mean ± S.D% | 100.35 ± 0.115 | 100.35 ± 0.115 | 100.43 ± 0.434 | 100.43 ± 0.434 | 100.53 ± 0.153 |
| Repeatability Intermediate precision | 100.72 ± 0.213 | 100.72 ± 0.213 | 100.65 ± 0.456 | 100.65 ± 0.456 | 100.65 ± 0.654 |
| Validation of regression equation | 100.55 ± 0.449 | 100.55 ± 0.449 | 100.55 ± 0.449 | 100.55 ± 0.449 | 100.55 ± 0.449 |
| Slope | 0.007 | 0.049 | 1.566 | 0.675 | 0.037 |
| SE of slope | 0.001 | 0.001 | 0.020 | 0.009 | 0.003 |
| Intercept | 0.007 | 0.059 | -0.232 | 0.347 | -0.002 |
| SE of intercept | 0.015 | 0.009 | 0.389 | 0.176 | 0.007 |
| Correlation coefficients | 0.9995 | 0.9997 | 0.9994 | 0.9993 | 0.9997 |
| SE of estimation | 0.016 | 0.010 | 0.417 | 0.189 | 0.007 |

Average of six determinations.

Intra-day (n=3), average of three concentrations of CB repeated 3 times within the same day

Inter-day (n=3), average of three concentrations of CB repeated 3 times in three consecutive days

LOD: Limit of Detection; LOQ: Limit of Quantification, SD: Standard Deviation, SE: Standard Error
Three different concentrations of CB were analyzed in different labs using Shimadzu 1260 instead of Unicam and Shimadzu RF – 1501 instead of Agilent did not affect the UV and fluorescence spectra proved good ruggedness of the methods.

The proposed methods were also applied for the determination of CB in Multirelax tablet. The validity of the methods was assessed by applying the standard addition technique, as shown in Table 3. From data obtained the mean percentage recoveries revealed that any excipients have no interference in the analysis of the pharmaceutical dosage forms. Statistical comparison between the results obtained by applying the proposed methods and that obtained by the official method [2] and there is no significant differences were found, as shown in Table 4.

### Table 2: Results of analysis of CB in laboratory prepared mixtures containing different ratios of CB and AQ by the proposed methods.

| Pharmaceutical formulation | Standard added (µg/mL) | Method A (DW) | Method B (RD) | Method C (DD) | Method D (ISO) | Method E Fluorimetry |
|-----------------------------|------------------------|---------------|---------------|---------------|---------------|----------------------|
| Multi-relax tablets, (B. No CBP/1506004-M) | 5.00 | 99.40 | 100.83 | 99.76 | 100.13 | 99.90 |
| | 10.00 | 98.90 | 100.62 | 99.83 | 100.75 | 100.90 |
| | 20.00 | 99.95 | 100.55 | 100.72 | 100.93 | 99.60 |
| Mean ± SD% | 99.42 ± 0.632 | 100.67 ± 0.118 | 100.10 ± 0.534 | 100.60 ± 0.417 | 100.13 ± 0.555 | Mean ± SD% |
| Found% ± of Claimed amount ± S.D | 99.60 ± 0.610 | 100.13 ± 0.538 | 100.11 ± 0.559 | 100.22 ± 0.246 | 99.38 ± 0.828 | 100.66 ± 0.215 |

### Table 3: Quantitative determination of CB in pharmaceutical formulations by the proposed methods and results of application of standard addition technique.

| Item | Method (A) DW | Method (B) RD | Method (C) DD | Method (D) ISO | Method (E) Fluorimetry | Official Method (*) |
|------|---------------|---------------|---------------|---------------|-----------------------|-------------------|
| Mean | 99.84 | 100.22 | 98.70 | 100.27 | 99.20 | 100.55 | 99.50 |
| S.D. | 0.598 | 0.510 | 0.612 | 0.529 | 0.612 | 0.449 | 0.328 |
| R.S.D.% | 0.560 | 0.509 | 0.620 | 0.528 | 0.617 | 0.448 | 0.330 |
| Variance | 0.358 | 0.260 | 0.375 | 0.280 | 0.375 | 0.202 | 0.108 |
| n | 6 | 6 | 6 | 6 | 6 | 6 | 6 |
| Student’s t (2.228) | 0.344 | 0.756 | 0.805 | 0.802 | 0.781 | 1.133 |
| F test (6.260) | 3.315 | 2.407 | 3.472 | 2.593 | 3.468 | 1.870 |

* Official method non aqueous titration [2]
** SD: Standard Deviation , RSD: Relative Standard Deviation
† Figures in parentheses are the corresponding tabulated values at p = 0.05
Conclusion

The five suggested methods have many advantages of being simple, accurate, precise, sensitive and inexpensive. These methods could be applied in quality control laboratories for quantitative determination of CB, in pure form, in its formulation and in the presence of AQ (its degradation product). The presented fluorimetric method could provide a highly sensitive method for the determination of CB than spectrophotometric methods; even the RD and 1DD methods were more specific than the other methods.

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