Pharmaceutica Analytica Acta
ISSN: 2153-2435

Ramadan et al., Pharm Anal Acta 2018, 9:1
DOI: 10.4172/2153-2435.1000574

Research Article Open Access

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 (^{1}DD) at 282 and 306 nm. Method D Isoabsorptive Point (ISO) at 280 nm Coupled with Second Derivative (^{2}D). Method E depending on spectrofluorimetric determination of cyclobenzapirine HCl through quenching of uranyl acetate with λ_{exi} 228 nm and λ_{em} 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. The five proposed methods were successfully applied for the determination of CB in Multirelax tablets. Statistical comparison between the results obtained by the proposed methods and that obtained by the official one for the determination of the drug was done, founding that there were no significant differences between them.

Keywords: Cyclobenzapirine HCl (CB); Anthraquinone (AQ); Dual wavelength (DW); Ratio difference (RD); (^{1}DD) Derivative ratio; Isoabsorptive 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 muscloskeletal 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 [27-31].

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 (Agilant 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

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) (^{1}DD)

Then the first derivative of the ratio spectra ^{1}DD 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 (^{0}D) of each dilution was recorded against blank.

For the determination of AQ, the second derivative (^{2}D) 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 (A_{iso}) 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 and1 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 λ_{em} 458nm with λ_{ex} 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 λ_{em} 458 nm with λ_{ex} 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 was described in subsection of Construction of the calibration curves. The concentrations of CB were calculated from the corresponding regression equations.

-Methods (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:

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 differences of 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:

$\Delta P_{(283-306)}$ = 0.0067C+0.0067 r=0.9995

Where $\Delta P_{(283-306)}$ 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).

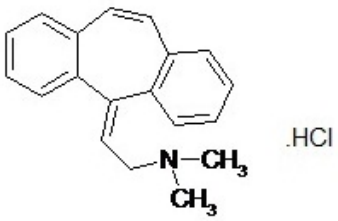

Figure 1: Chemical structure of CB.

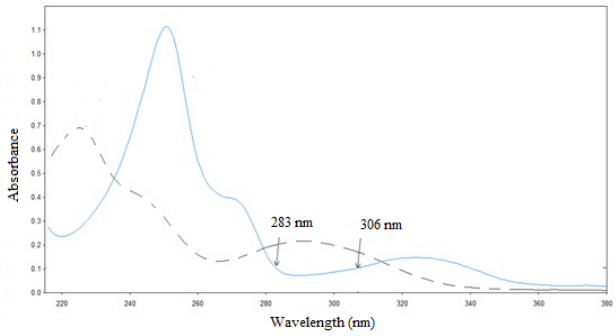

Figure 2: Zero order absorption spectra of 5.00 µg/ml cb (—) and 5.00 µg/ml of aq (- - -) in methanol.

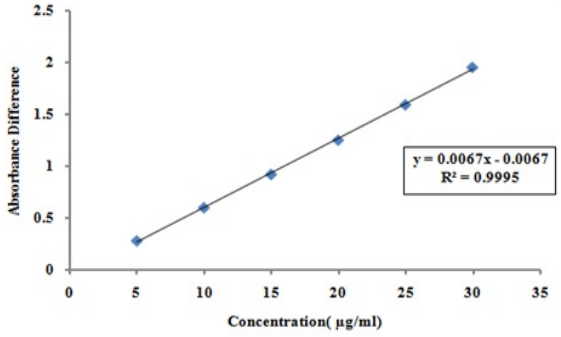

Figure 3: Linearity of the difference in absorbance at 283 and 306 nm to the corresponding concentrations of cb in methanol (5.00-30.00 µg/ml).

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 ^{1}D, ^{2}D and ^{3}D 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.

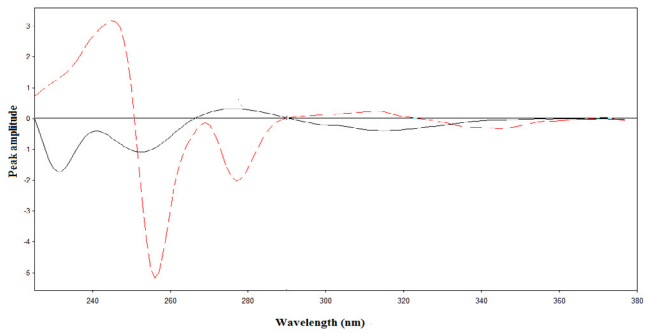

Figure 4: First derivative absorption spectra of 5.00µg/ml cb in methanol (—) and 5.00 µg /ml of aq (- - -).

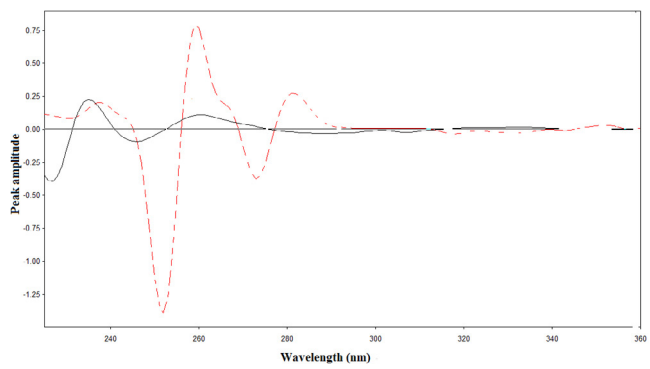

Figure 5: Second derivative absorption spectra 5.00 µg/ml cb in methanol (—) and 5.00 µg /ml of aq (- - -).

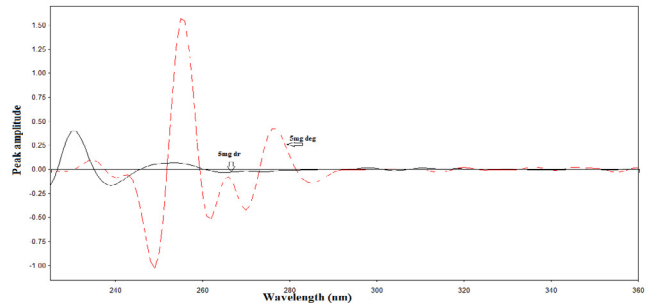

Figure 6: Third derivative absorption spectra 5.00µg/ml cb in methanol (—) and 5.00 µg /ml of aq (- - -).

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 (^{1}DD)

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 (^{1}DD) 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/

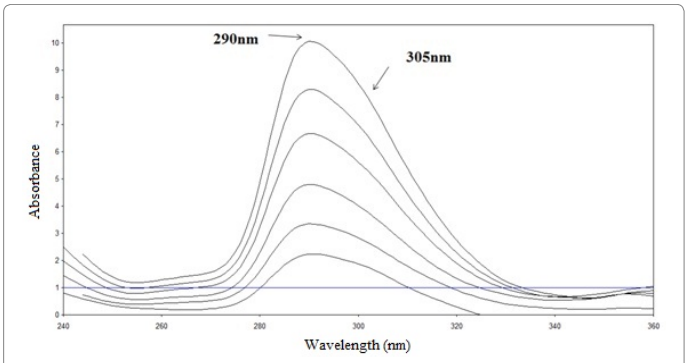

Figure 7: Zero order absorption spectra of ratio spectra of 5.00-30.00 µg/ml cb (—) and 5.00 µg /ml of aq (- - -) in methanol using 5.00 µg /ml of aq as a divisor.

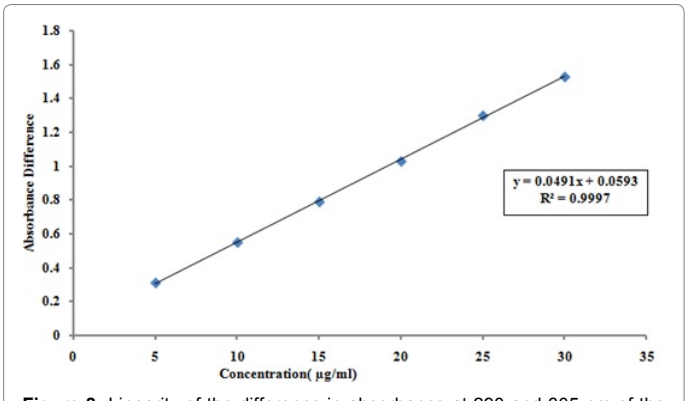

Figure 8: Linearity of the difference in absorbance at 290 and 305 nm of the ratio spectra to the corresponding concentrations of cb (5.00– 30.00 µg/ml) in methanol using 5.00µg/ml of aq as a divisor.

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 Δλ=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:

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

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

Where $^{1}DD_{282}$ and $^{1}DD_{303}$ 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 ^{1}DD 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 isoabsorptive 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].

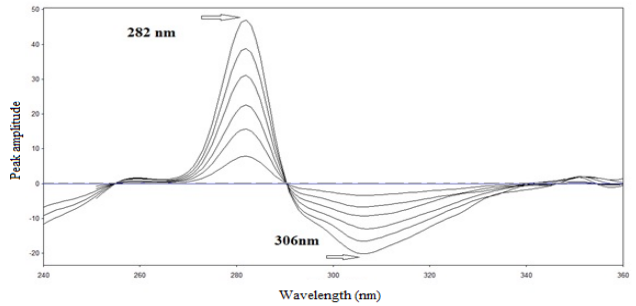

Figure 9: First derivative of ratio spectra (^{1}dd) of 5.00– 30 .00 µg/ml cb (—) and 5.00µg/ml of aq (- - -) in methanol using 5.00 µg/ml of aq as a divisor.

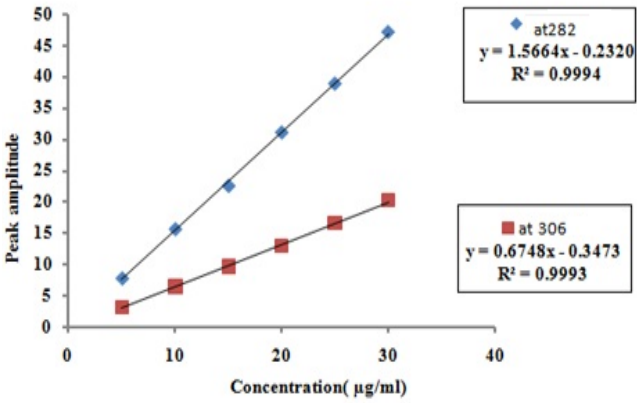

Figure 10: Linearities of the peak amplitude of the first derivative of ratio spectra to the corresponding concentrations of cb in methanol (5.00– 30.00 µg/ml) using 5.00 µg/ml of aq as a divisor.

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 isoabsorptive point at 280 nm. Meanwhile; their mixture has the same absorbance at their isoabsorptive 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 isoabsorptive point.

The total content of the mixture could be calculated as explained by the theory above by measuring the absorbance value at the chosen isoabsorptive point. The content of AQ alone could be calculated from its ^{2}D 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 (^{1}D) 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, ^{2}D 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:

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

Where, $^{2}D_{250}$ 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.

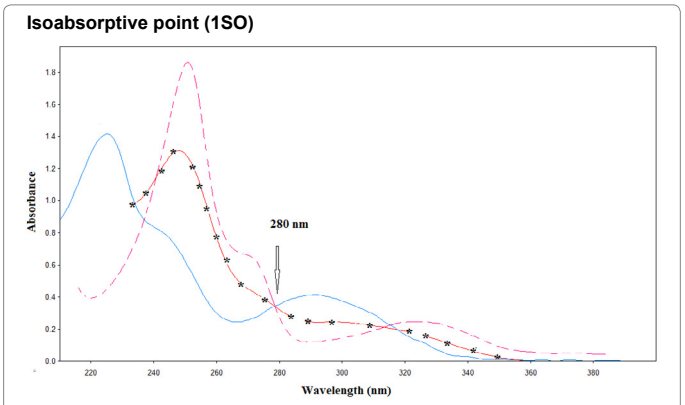

Figure 11: Zero order (^{0}d) absorption spectra of 10.00 µg/ml of cb (—), 10.00 µg/ml aq (- - -) and mixture containing 5.00µg/ml of cb and 5 µg/ml aq(*—*) showing isoabsorptive point at 280 nm using methanol as blank.

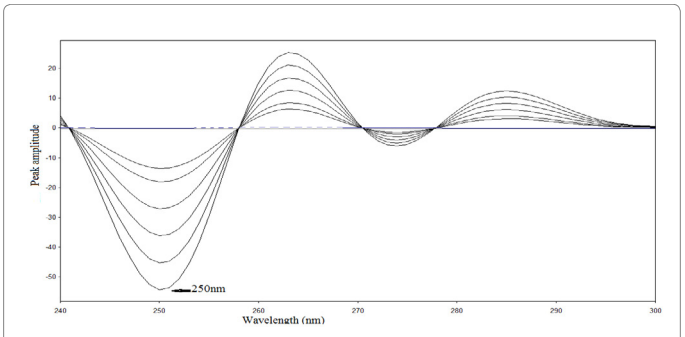

Figure 12: Second derivative absorption spectra of 2.50-25.00 µg/ml of aq (—) and 2.50 µg/ml of cb (- - -) using methanol as blank.

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:

$A_{iso=}0.0367C_T-0.0367$ r=0.9997 (2)

Where, A_{iso} is the absorbance at 280 nm, C_T is the total concentration of the mixture in µg/mL and r is the correlation coefficient.

The proposed method was found to be valid in the range of 2.5 µg/ml – 25 µg/mL for AQ and in the range of 5-30 µg/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 (λ_{ex}=228 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 (0.25×10^{-3} 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 significally 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 µg/mL – 10 µg/mL, and the regression equation was computed (Figure 19) and found to be:

I_f==50.9616C+453.5315 r=0.9995

Where I_f is the difference in fluorescence intensity, C is the concentration of CB in µg/mL and r is the correlation coefficient

The proposed spectrofluorimetric method was successfully applied for the determination of CB with mean percentages recovery of 100.55 ± 0.447%.

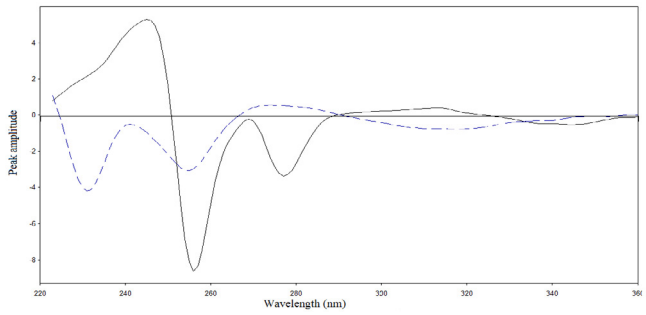

Figure 13: First order ('d) absorption spectra of aq (—) and cb(- - -) using methanol as blank (5.00µg/ml of each).

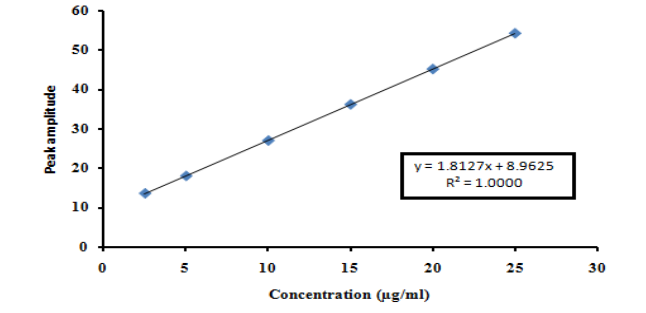

Figure 14: Linearity of the peak amplitudes of the second derivative spectra to the corresponding concentrations of aq (2.50-25.00 µg/ml) at λ 250 nm.

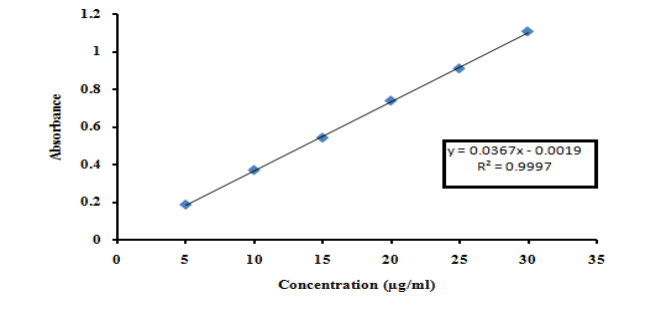

Figure 15: Linearity of absorbance at 280 nm (isoabsorptive point) of zero-order absorption spectra to the corresponding concentrations of cb (5.00-30.00 µg/ml).

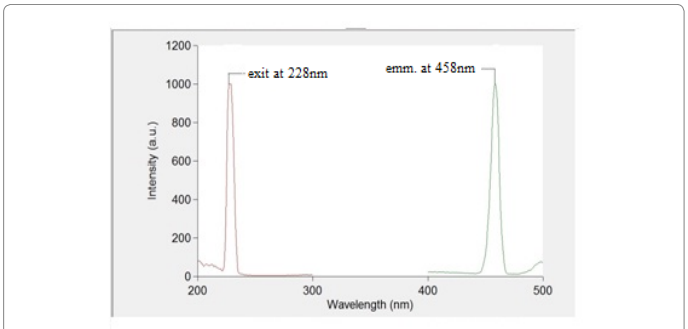

Figure 16: Excitation and emission spectra of uranyl acetate (blank) in water at λ_{em}458 nm with λ_{ex}228 nm.

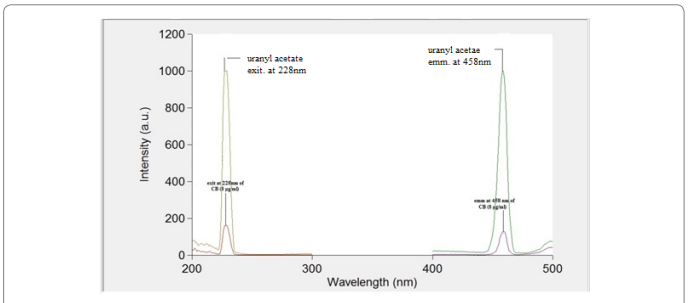

Figure 17: Excitation and emission spectra of the blank reagent (uranyl acetate) and the quenching effect of cb (8.00 µg/ml) on the blank reagent.

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.

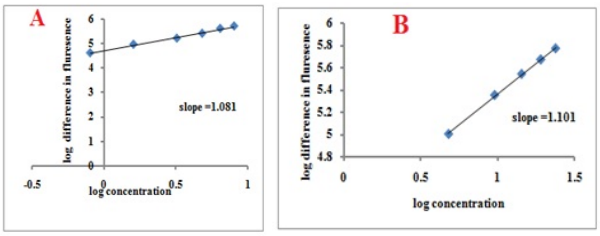

Figure 18: Stoichiometry of the Reaction Between CB and Uranyl Acetate Adopting Limiting Logarithmic Method; A-Variable CB Concentrations and Constant Uranyl Acetate Concentration B-Variable Uranyl Acetate Concentrations and Constant CB Concentration (6.00µg/mL).

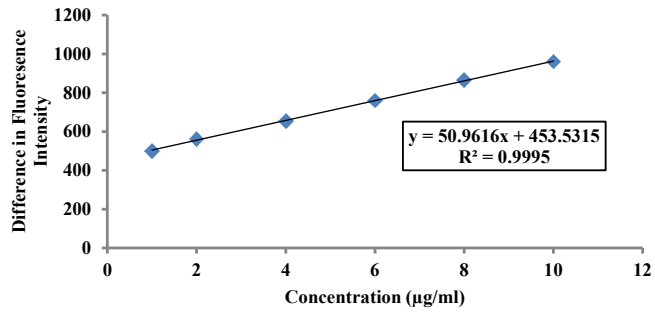

Figure 19: Linearity of the fluorescence intensity to the corresponding concentration of cb (1.00 – 10.00 µg/ml) in water (at λ_{em} 458 nm with λ_{ex} 228 nm).

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.

Parameters	Method A	Method B	Method C		Method D	Method E
	DW	RD	DD[1]		ISO	Fluorimetry
	ΔP (283 − 306)	ΔP (290− 305)	At 282 nm	At 306 nm	At 280 nm	
Validation of response						
Linearity range (µg /ml)	5.00-30.00	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	0.280
LOQ (µg /ml)	3.360	4.000	2.270	2.790	1.230	0.850
Accuracy[*] Mean ± S.D%	99.84 ± 0.598	100.22 ± 0.510	98.70 ± 0.612	100.27 ± 0.529	99.20 ± 0.612	100.55 ± 0.449
Precision (Mean ± S.D%)						
Repeatability [**]	99.35 ± 0.315	100.35 ± 0.115	99.87 ± 0.312	100.43 ± 0.434	100.53 ± 0.153	99.87 ± 0.324
Intermediate precision [***]	99.52 ± 0.543	100.72 ± 0.213	99.73 ± 0.322	99.65 ± 0.456	99.87 ± 0.654	99.23 ± 0.832
Validation of regression equation						
Slope	0.007	0.049	1.566	0.675	0.037	50.962
SE of slope	0.001	0.001	0.020	0.009	0.003	0.578
Intercept	0.007	0.059	-0.232	0.347	-0.002	453.532
SE of intercept	0.015	0.009	0.389	0.176	0.007	3.506
Correlation coefficients	0.9995	0.9997	0.9994	0.9993	0.9997	0.9995
SE of estimation	0.016	0.010	0.417	0.189	0.007	4.506

[*]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

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

Three different concentrations of CB were analyzed in different lab using Shimadzu 1260 instead of Unicam and Shimadzu RF – 1501 instead of Agilant 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.

Degree %	Methods (A), (B), (C) and (D)							Method (E) *Fluorimetry*		
	CB (µg/mL)	AQ (µg/mL)	Recovery* *% of DW method*	Recovery*% of RD method	Recovery*% of *DD*[1] *method*		*Recovery*% of ISO method*	CB (µg/mL)	AQ (µg/mL)	*Recovery* *%
			ΔP (283 − 306)	ΔP (290 − 305)	At 262 nm	At 306 nm	At 280 nm			
10	25.00	5.00	99.6	99.73	98.98	99.63	99.72	9.00	1.00	99.9
30	20.00	10.00	99.85	99.76	99.63	100.37	99.35	7.00	3.00	100.43
50	15.00	15.00	100.33	100.05	98.71	100.96	100.47	5.00	5.00	100.99
70	10.00	20.00	100.9	100.34	98.93	100.52	100.8	3.00	7.00	105**
90	5.00	25.00	106***	100.86	98.37	99.87	110***	1.00	9.00	
Mean			100.17	100.15	98.924	100.27	100.09			100.44
± SD%			0.572	0.468	0.467	0.528	0.666			0.543

*Average of three determinations
SD: Standard Deviation

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[1])`		Method D (ISO)	`Method E *Fluorimetry*	
		Recovery**%	Recovery**%	Recovery**% at 282nm	Recovery**% at 306nm	Recovery**%	Standard added (µg/mL)	Recovery**%
Multi-relax tablets, (B. No CBP/1506004-M)	5.00	99.40	100.83	99.76	100.13	99.90	2.00	100.54
	10.00	98.90	100.62	99.83	100.75	100.90	4.00	100.67
	20.00	99.95	100.55	100.72	100.93	99.60	6.00	100.31
	Mean ± SD%	99.42 ± 0.832	100.67 ± 0.118	100.10 ± 0.534	100.60 ± 0.417	100.13 ± 0.555	Mean ± SD%	100.51 ± 0.181
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	

Claimed amount 10.00(µg/mL) in method A, B, C and D while 4.00 (µg/mL) in method E
* Average of five determination
**Average of three determination

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*[1]		Method (D) ISO	Method (E) *Fluorimetry*	Official Method (*)
	ΔP (283 − 306)	(ΔP (290 − 305)	At 282 nm	At 306 nm	At 280nm		
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

Table 4: Statistical comparison between the results obtained for the determination of CB in pure samples by the proposed methods and that obtained by the official method (*).

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 ^{1}DD methods were more specific than the other methods.

References

1. Martindale (2002) The Complete Drug Reference. 3rd edn. Pharmaceutical Press, UK.
2. U.S. Pharmacopeia (2007) U.S. Pharmacopeial Convention, Inc., USA.
3. Moffat AC, Osselton MD, Widdop B (2004) Clarke's analysis of drugs and poisons: In pharmaceuticals, body and postmortem material. Pharmaceutical Press, UK.
4. Kaasem AM, Guesmi NE (2016) Sensitive kinetic spectrophotometric determination of cyclobenzaprine Hcl in pure form and pharmaceutical formulations. Anal Chem Lett 6: 657-668.
5. Kumar TV, Seethamma M, Venkateshwarlu G (2014) Quantitative determination of drugs & pharnaceuticalsby using iodine as analytical reagent: A spectrophotometric study. IOSR-JAC 7: 7-15.
6. Matsui F, French WN (1971) Analysis of binary mixtures of pharmaceutical amines by the acid dye technique. J Pharm Sci 60: 287-291.
7. Faber DB (1972) Separation of cyclobenzaprine from biological samples and its determination by thin-layer chromatography followed by densitometry. J Chromatog A 74: 85-98.
8. Hardea MT, Wankhedeb SB, Chaudharia PD (2016) Validated inherent stability indicating HPTLC method for estimation of cyclobenzaprine hydrochloride in tablets and use of MS–QTOF in characterization of its alkaline stress degradation product. Bull Facul Pharm 54: 145-156.
9. Heinitz ML (1982) Determination of cyclobenzaprine in tablets by high-performance liquid chromatography. J Pharm Sci 71: 656-658.
10. Constanzer M, Chavez C, Matuszewski BK (1995) Development and comparison of high-performance liquid chromatographic methods with tandem mass spectrometric and ultraviolet absorbance detection for the determination of cyclobenzaprine in human plasma and urine. J Chromatogr B 666: 117-126.
11. Holeman JA, Danielson ND (1995) Microbore liquid chromatography of tertiary amine anticholinergic pharmaceuticals with tris (2,2'-bipyridine) ruthenium(III) chemiluminescence detection. J Chromatogr Sci 33: 297-302.
12. Ryan TW (1993) Identification and quantification of tricyclic antidepressants by UV-photodiode array detection with multicomponent analysis. J Liq Chromatogr Rel Tech 16: 1545-1560.
13. Hwang PT, Young DA, Straughn AB, Meyer MC (1993) Quantitative determination of cyclobenzaprine in human plasma by high pressure liquid chromatography. J Liq Chromatogr 16: 1163-1171.
14. Roshni DP, Usmangani KC, Falgun AM, Dimal AS, Kashyap KB (2014) Liquid chromatographic estimation of cyclobenzaprine hydrochloride and aceclofenac in pharmaceutical formulation. RRJPPS 3: 37-44.
15. Yu W, Yang X, Sui W, Xu H, Luan X, et al. (2014) Rapid and sensitive analysis of cyclobenzaprine by LC–MS/MS: Application to a pharmacokinetic study of cyclobenzaprine in dog. Asian J Pharm Sci 9: 117-122.
16. Herrin GL, McCurdy HH, Wall WH (2005) Investigation of an LC-MS-MS (QTrap) method for the rapid screening and identification of drugs in postmortem toxicology whole blood samples. J Anal Toxicol 29: 599-606.
17. Wong EC, Koenig J, Turk J (1995) Potential interference of cyclobenzaprine and norcyclobenzaprine with HPLC measurement of amitriptyline and nortriptyline: Resolution by GC-MS analysis. J Anal Toxicol 19: 218-224.
18. Li JC, Chen FH, Zhang JD, Dong HJ, Gao S (2012) A sensitive, fast and accurate liquid chromatographyelectrospray ionization-tandem mass spectrometry (LCMS/MS) method for the pharmacokinetic study of cyclobenzaprine tablets. Afr J Pharm Pharmacol 6: 708-716.
19. Koves EM (1995) Use of high-performance liquid chromatography-diode array detection in forensic toxicology. J Chromatogr A 692: 103-119.
20. Xiang Y, Zhou L, Qian Z, Peng K, Li D, et al. (2012) Determination of cyclobenzaprine in human plasma by liquid chromatography electrospray ionization tandem mass spectrometry and its application in a pharmacokinetic study. Biomed Chromatogr 26: 1083-1088.
21. Liu Y, Zhao D, Zhongxi Z (2014) Study of degradation mechanisms of cyclobenzaprine by LC-MS/MS. Anal Methods 6: 2320-2330.
22. Coulter C, Taruc M, Tuyay J, Moore C (2010) Antidepressant drugs in oral fluid by liquid chromatography-tandem mass spectrometry. J Anal Toxicol 34: 64-72.
23. Constanzer ML, Vincek WC, Bayne WF (1985) Determination of cyclobenzaprine in plasma and urine using capillary gas chromatography with nitrogen-selective detection. J Chromatogr 339: 414-418.
24. Schember I, Bailey K, Kraner J, Richards-Waugh L (2007) Validation of a GC/MS method for the determination of alkaline drugs in whole blood. Forensic Sci Int 165: 216-224.
25. Hucker HB, Stauffer SC (1976) Gas-liquid chromatographic determination of nanogram amounts of cyclobenzaprine in plasma using a nitrogen detector. J Chromatogr 124: 164-168.
26. Hucker HB, Stauffer SC (1976) GLC determination of cyclobenzaprine in plasma and urine. J Pharma Sci 65: 1253-1255.
27. Ramadan NK, Zaazaa HE, Merey HA (2011) Microsized graphite sensors for potentiometric determination of cyclobenzaprine hydrochloride in pure powder, tablets, and plasma. J AOAC Int 94: 1807-1814.
28. Harde MT, Wankhede SB, Chaudhari PD (2016) A validated inherent stability indicating HPTLC method for estimation of cyclobenzaprine hydrochloride in tablets and use of MS–QTOF in characterization of its alkaline stress degradation product. Bull Faculty Pharm 54: 145-156.
29. Naguib IA, Abdelaleem EA, Abdallah FF, Ali NW (2016) Development and validation of three spectrophotometric methods for determination of Cyclobenzaprine Hcl in the presence of its two major degradation products. Anal Chem Lett 6: 24-34.
30. Cioroiu BI, Grigoriu IC, Cioroiu ME, Niculaua M, Lupuleasa R, et al. (2016) Determination of degradation products of Cyclobenzaprine Hydrochloride, Lidocaine and Piroxicam in a semi-topical formulation: MS-MS confirmation of unknown impurities. J Chromatogr Sci 54: 902-911.
31. Ramadan NK, Mohamed TA, Fouad RM, Moustafa AA (2017) Stability-indicating HPLC and TLC chromatographic methods for determination of cyclobenzaprine hydrochloride and asenapine maleate. JPC 30 : 313-322.
32. ICH, Q2 (R1) (2005) Validation of Analytical Procedures: Text and Methodology, ICH Harmonized Tripartite Guideline.
33. Kant RAVI, BODLA R, KAPOOR G, BHUTANI R (2016) Development and validation of novel spectrophotometric methods for simultaneous estimation of pioglitazone and metformin in bulk and fixed dosage forms by area under the curve and dual wavelength mode. Int J Appl Pharm 8: 48-53.
34. Topagi KS, Sinha PK, Jeswani RM, Damle MC (2009) Spectrophotometric methods for simultaneous estimation of diacerhein and aceclofenac. Int J Chem Tech Res 1: 991-995.
35. Abdelrahman MM (2013) Simultaneous determination of Cinnarizine and Domperidone by area under curve and dual wavelength spectrophotometric methods. Spectrochimica Acta Part A: Mol Biomol Spectroscopy 113: 291-296.
36. Abdelwahab NS, Ali NW, Abdelkawy MM (2014) Different spectrophotometric and TLC-densitometric methods for determination of two analgesic drugs. Asian J Biomed Pharm Sci 4: 26-33.
37. Abdelrahman MM, Naguib IA, El Ghobashy MR, Ali NA (2015) Quantitative determination of zopiclone and its impurity by four different spectrophotometric methods. Spectrochimica Acta Part A: Mol Biomol Spectroscopy 137: 617-624.
38. Lotfy HM, Hegazy MAM (2013) Simultaneous determination of some cholesterol-lowering drugs in their binary mixture by novel spectrophotometric methods. Spectrochimica Acta Part A: Mol Biomol Spectroscopy 113: 107-114.
39. Elzanfaly ES, Saad AS, Abd-Elaleem AEB (2012) Simultaneous determination of retinoic acid and hydroquinone in skin ointment using spectrophotometric technique (ratio difference method). Saudi Pharm J 20: 249-253.
40. Elzanfaly E, Saadand A, Abd-Elaleem A (2012) A novel simple method for

resolving overlapped spectral data with a wide range of applicability, and its application as a stability indicating method for determination of tazarotene. Pharm Anal Acta 3: 153-156.

41. Ankit S, Mukesh G, Vaishali T, Lalji B, Tejal G (2014) Simultaneous determination of moxifloxacin hydrochloride and difluprednate by ratio derivative spectrophotometry. IJPPs 6: 387-390

42. Lotfy HM, Hagazy MAM (2012) Comparative study of novel spectrophotometric methods manipulating ratio spectra: An application on pharmaceutical ternary mixture of omeprazole, tinidazole and clarithromycin. Spectrochim Acta A Mol Biomol Spectrosc 96: 259-270.

43. Samir A, Lotfy HM, Salem H, Abdelkawy M (2014) Development and validation of simultaneous spectrophotometric and TLC-spectrodensitometric methods for determination of beclomethasone dipropionate and salbutamol in combined dosage form. Spectro Acta Part A 128: 127-136.

44. Altinös S, Tekeli D (2001) Analysis of glimepiride by using derivative UV spectrophotometric method. J Pharm Biomed Anal 24: 507-515.

45. Salinas F, Nevado JJB, Espinosa MA (1990) A new spectrophotometric method for quantitative multicomponent analysis resolution of mixtures of salicylic and salicyluric acids. Talanta 37: 347-351.

46. Erram SV, Tipnis HP (1993) Simple spectrophotometric analysis of acebutanol hydrochloride and atenolol in combined pharmaceutical dosages with hydrochlorothiazide. Indian Drugs 30(7): 319-323.

47. Erram SV, Tipnis HP (1993) Simple spectrometric analysis of frusemide and amiloride hydrochloride from combined pharmaceutical dosages. Indian Drugs 30: 371-376.

48. Erram SV, Tipnis HP (1994) Simple spectrometric analysis of propranolol hydrochloride and hydrochlorothiazide from combined pharmaceutical dosages. Indian Drugs 31: 65-68.

49. Erram SV, Tipnis HP (1993) Simple spectrometric analysis of acebutolol hydrochloride and atenolol in combined pharmaceutical dosages with hydrochlorothiazide. Indian Drugs 30:462-467.

50. Connors AK (1967) Pharmaceutical Analysis. Wiley, USA.

51. Abd EL-Hafez MT, El-Ragehy NA, Ramadan NK , El-Zeany BE (2015) Simultaneous determination of a binary mixture of pantoprazole sodium and itopride hydrochloride by four spectrophotometric methods. Spectro Acta part A 137: 463-470.

52. El-Reis MA (1998) Determination of piroxicam in pharmaceutical preparations by continuous-flow chemiluminescence . J Anal Lett 31: 793.

53. Sah U, Sen AK, Das TK (1988) Spectrophotometric determination of clioquinol and diodoquin in pharmaceutical preparations using uranyl acetate as a chromogenic agent. Analyst 113: 1653-1655.

54. Sărbu C, Horn M, Hantz A (1985) Uranylsalze als fluoreszenzindikatoren für den dünnschichtchromatographischen Nachweis von komplexbildner. J Chromatogr A 322: 272-274.

55. Taha EA, Hassan NY, Aal FA, Abdel Fattah LE (2007) Fluorimetric determination of some sulfur containing compounds through complex formation with terbium (Tb^{+3}) and uranium (U^{+3}). J Fluoresc 17:293-300.

56. Rose J (1964) Advanced Physicochemical Experiments. Pitam, UK.