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Ibrahim A. Naguib
Pharmaceutical Analytical Chemistry Department, Faculty of Pharmacy, Beni-Suef University, 62111 Beni-Suef, Egypt.
Email: ibrahim.yaseeen@pharm.bsu.edu.eg

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Spectrophotometric Methods for Quantitative Determination of Binary Mixture of Hydrochlorothiazide and Amiloride Hydrochloride without Prior Separation

Eglal A. Abdelaleem[a] , Ibrahim A. Naguib[a*], Hala E. Zaazaa[b] and Mohammed E. Draz[c]

[a]Pharmaceutical Analytical Chemistry Department, Faculty of Pharmacy, Beni-Suef University, 62111 Beni-Suef, Egypt.
[b]Pharmaceutical Analytical Chemistry Department, Faculty of Pharmacy, Cairo University, Kasr El-Aini St., 11562 Cairo, Egypt.
[c] Analytical Chemistry Department, Faculty of Pharmacy, Nahda University, Beni-Suef, Egypt.

Abstract

Simple, accurate, sensitive and precise UV spectrophotometric methods were developed and validated for quantitative determination of binary mixture of hydrochlorothiazide (HCZ) and amiloride hydrochloride (AM) in their bulk powder and pharmaceutical dosage forms. AM was determined in presence of HCZ by direct spectrophtometry at λ_{max} 361 nm. Three spectrophotometric methods, namely; isoabsorptive point (I), ratio subtraction (II) and ratio difference (III) were developed for the spectral resolution of HCZ when present in mixture with AM without preliminary separation. In method (I), the isoabsorptive point (A_{iso}) at 277.2 nm was chosen for determination of HCZ while in method (II), HCZ was determined at λ_{max} 270.4 nm after subtraction of interference exerted by AM. In method (III), absorption spectra of HCZ were recorded, divided by suitable divisor of AM then measuring the absorption difference at 267.6 and 290.2 nm to obtain the corresponding concentrations of HCZ. The developed methods were validated according to ICH guidelines demonstrating good accuracy and precision. The results were statistically compared with those obtained by reported ratio spectra derivative spectroscopic method indicting no significant difference and ability of methods to be used for routine analysis of proposed drugs.

Keywords: Hydrochlorothiazide; amiloride hydrochloride; isoabsorptive point; ratio subtraction; ratio difference spectrophotometry

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1.INTRODUCTION

Hydrochlorothiazide (HCZ); 6-Chloro-3,4-dihydro-2H-1,2,4-benzothiadiazine-7-sulfonamide1,1-dioxide[1] is the prototype of thiazides diuretics which reduces blood pressure by inhibition of NaCl reabsorption through blocking the Na^+/Cl^-transporter leading to decrease in extracellular volume and cardiac output [2].Amiloride hydrochloride (AM); 3, 5-Diamino-6-chloro-N-(diaminomethylidene) pyrazine-2-carboxamide[1] is potassium-sparing diuretic which reduces blood pressure by inhibition of Na^+ influx through ion channels in the luminal membrane of nephron. Formulation of HCZ with AM attenuates thiazides induced loss of potassium and also has synergetic effect on reduction of blood pressure [2]. The literature survey revealed different methods for simultaneous determination of HCZ and AM as binary mixture such as spectrophotometric[3-7], reversed phase high performance liquid chromatography (RP-HPLC)[7-15], thin layer chromatographic (TLC)-densitometric [4,16] ,chemometric [17-19] , capillary electrophoresis [20]and differential pulse polarographic [21] methods.

The present work is concerned with development and validation of simple, rapid and selective three spectrophotometric methods which don't need any special program, so they can be easily applied as alternative to reported LC method which requires time, experience, expensive instruments and solvents. They also show advantages over previously published spectrophotmetric methods which require sophisticated dervitization steps in quality control laboratories.

2. EXPERIMENTAL

2.1. Instruments

A double beam UV-visible spectrophotometer (SHIMADZU, Japan) model UV-1601 PC with quartz cell of 1 cm and UV-PC personal software version 3.7 was used. The spectral band width is 2 nm and wavelength-scanning speed 2800 nm/min.

2.2. Materials

2.2.1. Authentic samples

Standard HCZ and AM were kindly supplied by Sigma Pharmaceuticals Industries (El Monofeya, Egypt) with certified purities of 99.6 % and 99.7 % respectively.

2.2.2. Pharmaceutical dosage forms

Moduretic ® tablets batch No. (110530) were manufactured by EL Kahira CO. for Pharmaceutical and Chemical Industries (Cairo, Egypt) and Hydikal® tablets batch No. (210) were manufactured by Pharco Pharmaceuticals (Alexandria, Egypt). Each tablet is claimed to contain 5 mg of AM and 50 mg of HCZ.

2.2.3. Solvents

Methanol HPLC grade (CHROMASOLVE®, Sigma - Aldrich Chemie GmbH, Germany).

2.2.4. Standard solutions

a. Standard stock solution of HCZ and AM were prepared in methanol in the concentration of 1 mg ml^{-1}.

b. Standard working solutions of HCZ and AM were prepared in methanol in the concentration of 0.1 mg ml^{-1}.

2.2.5. Laboratory-prepared mixtures

Accurate aliquots equivalent to (40 -200 µg) and (20 - 160 µg) of HCZ and AM respectively were transferred from their working solutions into a series of 10 ml volumetric flasks and volumes were completed to the mark with methanol and mixed well.

2.3. Procedures

2.3.1. Isoabsorptive spectrophotometric method

Linearity: aliquots equivalent to 10-300 µg of HCZ and AM were separately transferred from their respective standard working solutions (0.1 mg ml^{-1}) into two separate series of 10-ml volumetric flasks and the volume was completed using methanol to obtain final concentrations ranges of 1-30 µg ml^{-1}. The zero order absorption spectra were recorded for both drugs using methanol as blank, then the absorbance was measured at 361 nm for AM and 277.2 nm (A_{iso}) for AM and HCZ. Two calibration curves were constructed for each drug relating the absorbance at the selected wavelength to the corresponding drug concentrations and the regression equations were computed.

Assay of laboratory-prepared mixtures:

Absorbance of the spectra of laboratory-prepared mixtures containing different ratios of AM and HCZ were measured at 361 nm corresponding to the contents of AM only, and at 277.2 nm (A_{iso}) corresponding to the total content of AM and HCZ in the mixture. The concentration of AM alone and the total concentration of the two drugs were

calculated from their corresponding regression equations; then by subtraction of AM concentration from the total mixture concentration, yielding the actual concentration of HCZ in the mixture.

2.3.2. Ratio subtraction spectrophotometric method

Linearity: Aliquots equivalent to 10–300µg from HCZ working solution (0.1mgml^{-1}) were transferred into a series of 10 ml volumetric flasks then completed to volume with methanol; and the spectra of the prepared standard solutions were scanned. A calibration curve was constructed relating the absorbance of zero order spectra of HCZ at λ_{max} 270.4 nm to the corresponding concentrations and the regression equation was computed.

Assay of laboratory-prepared mixtures: The absorption spectra of the laboratory-prepared mixtures containing different ratios of AM and HCZ were scanned and recorded then divided by the standard spectrum of 20 µg ml^{-1} of AM as suitable divisor to obtain ratio spectra and the absorbance in the plateau region (the constant) was subtracted. By multiplication of the obtained spectra by the spectrum of the divisor the original curves for direct determination of HCZ at 270.4 nm were obtained and the concentration was calculated from the corresponding regression equation.

2.3.3. Ratio difference spectrophotometric method

Linearity: Aliquots equivalent to 10–300 µg from HCZ working solution (0.1mgml^{-1}) were transferred into a series of 10 ml volumetric flasks then completed to volume with methanol. The zero order absorption spectra of each solution were recorded then divided by the standard spectrum of 20 µg ml^{-1} of AM as suitable divisor to obtain ratio spectra. Calibration curve was constructed relating the difference in absorbance of the resultant ratio spectra at 267.6 and 290.2 nm ($\Delta A_{267.6 - 290.2\ nm}$) to the corresponding HCZ concentrations and the regression equation was computed.

Laboratory-prepared mixtures were assayed by applying the procedure under linearity.

2.3.4. Analysis of pharmaceutical dosage forms

Twenty tablets of each of Moduretic® and Hydikal® tablets were powdered and mixed well. Accurately weighed amount of the powdered tablets equivalent to 100 mg of HCZ and AM were separately transferred into 100 ml volumetric flasks. 50 ml methanol was added and ultrasonicated for 30 min, cooled and flasks were completed to volume to obtain 1 mg ml^{-1} stock solution and then the solution was filtered. Appropriate dilutions of the prepared solution were made to prepare its working solution (0.1 mg ml^{-1}) and the procedures under linearity of each method were followed.

Validity of the methods was assessed by spiking the pharmaceutical dosage forms by known amounts of standard drug powders (standard addition technique). The recovery of the added standards was then calculated after applying the proposed methods.

3. RESULTS AND DISCUSSION

The aim of this work is to develop three spectrophotometric methods for the determination of binary mixture without previous separation. AM can be determined by direct measurement of absorbance at 361nm, while the absorption spectra of HCZ and AM showed severe overlap; which makes the determination of HCZ concentration in the mixture more difficult (Fig. 1). In this work; three simple spectrophotometric methods; namely isoabsorptive, ratio subtraction and ratio difference methods have been described for analysis of HCZ in bulk powder and pharmaceutical dosage forms which have the advantage of no need to any derivatization or sophisticated manipulation steps like other spectrophotometric methods, and less costly than published chromatographic methods.

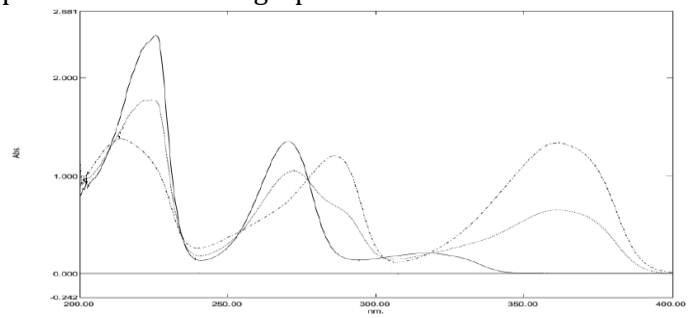

Figure 1: Zero order absorption spectra of 20 µg ml^{-1} of HCZ (___), 20 µg ml^{-1} of AM (-.-.-.-) and (1:1) mixture containing 10 µg ml^{-1} of each (……) using methanol as blank.

3.1. Isoabsorptive spectrophotometric method

The proposed method was developed by Erram and Tipnis[22] and is used for determination of HCZ in presence of AM in the presented work. At the isoabsorptive point the mixture of drugs acts as a single component and gives the same absorbance value as pure drug. Selection of suitable isoabsorptive point plays an important role with respect to selectivity and sensitivity; hence different isoabsorptive points were tried (as shown in Fig.1) but the best results regarding

selectivity and sensitivity were obtained by using the isoabsorptive point at 277.2 nm (A_{iso}). The total concentration of both drugs could be calculated at this isoabsorptive point, while the concentration of AM in the mixture could be calculated, without any interference, at 361nm. Accordingly, the concentration of HCZ could be calculated by subtraction.

A linear correlation was obtained between the absorbance values and the corresponding concentrations of both drugs at their corresponding wavelengths. The regression equations were:

A_{iso} = 0.0470 C + 0.0080 r = 0.9999 at 277.2 nm.

A = 0.0660 C + 0.0040 r = 0.9999 at 361.0 nm.

Where A is the absorbance, C is the concentration of the drug in µg ml-1 and r is the correlation coefficient.

3.2. Ratio subtraction spectrophotometric method

Following the theory of ratio subtraction [23]; HCZ could be determined in presence of AM in binary mixture. AM has extended spectrum than HCZ as shown in (Fig.1). Determination of HCZ could be achieved by dividing the mixtures' spectra containing HCZ and AM by suitable divisor of AM (20 µg ml^{-1}) to produce a new ratio spectra as shown in (Fig.2. a); then subtraction of the absorbance values of these constants in plateau as shown in (Fig.2. b); followed by multiplication of the obtained spectra by the divisor as shown in (Fig.2. c).; then finally the original spectra of HCZ which are used for direct determination of HCZ at 270.4 nm could be obtained and the concentrations from regression equation could be calculated. The correct choice of the divisor is fundamental, as, if the concentration of the divisor increases or decreases, the resulting constant value will be proportionally decreased or increased [24].

A linear correlation was obtained between the absorbance and the corresponding concentration of HCZ at its corresponding wavelength, and the regression equation was:

A= 0.0660 C +0.0170 r = 0.9999 at 270.4 nm.

Where A is the absorbance, C is the concentration of the drug in µg ml^{-1} and r is the correlation coefficient.

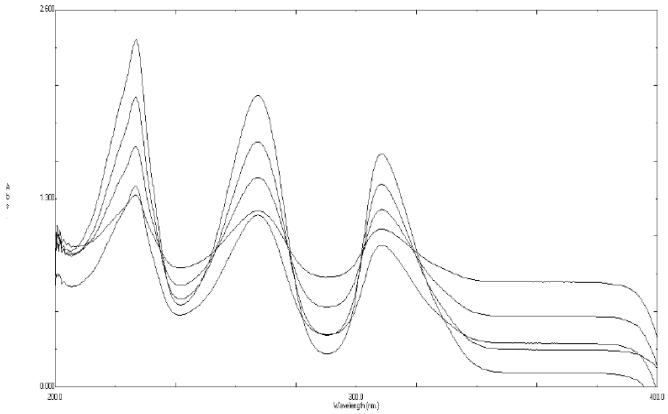

Figure 2 (a): Ratio spectra of laboratory prepared mixtures of HCZ and AM using 20 µg ml^{-1} of AM as a divisor and methanol as a blank.

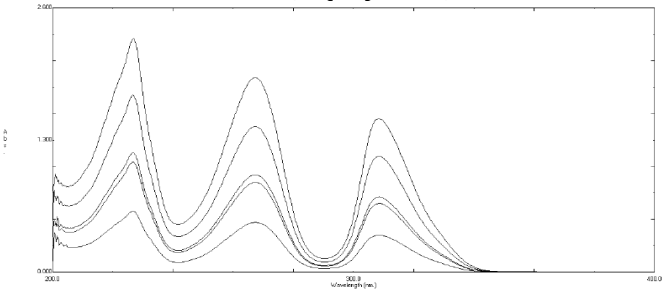

Figure 2 (b): Ratio spectra of laboratory prepared mixtures of HCZ and AM using 20 µg ml^{-1} of AM as a divisor and methanol as a blank after subtraction of the constant

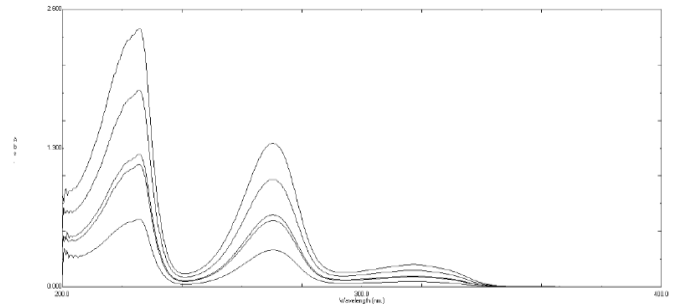

Figure 2 (c): The zero order absorption spectra of HCZ obtained by the proposed ratio subtraction method for the analysis of laboratory prepared mixtures after multiplication by 20 µg ml^{-1} of AM divisor

3.3. Ratio difference spectrophotometric method

The amplitude difference between two points on the ratio spectra of a mixture is directly proportional to the concentration of the component of interest. It is affected by two critical steps; the first is the choice of the suitable divisor where the selected divisor should be compromise between minimal noise and maximum sensitivity. The second one is the choice of the wavelengths at which measurements are recorded. Any two wavelengths can be chosen provided that they exhibit different absorbance in the ratio spectrum and a good linearity is present at each wavelength individually [25]. The mathematical explanation of the method was illustrated by *Lotfy et al*.[26]. Accordingly, to optimize the method, different concentrations of AM as divisors and wavelengths were tested, but the best result were obtained when using 20 µgml^{-1} of AM as a divisor and measuring absorbance difference between 267.6 and 290.2 nm ($\Delta A_{267.6 - 290.2\ nm}$) (Fig. 3).

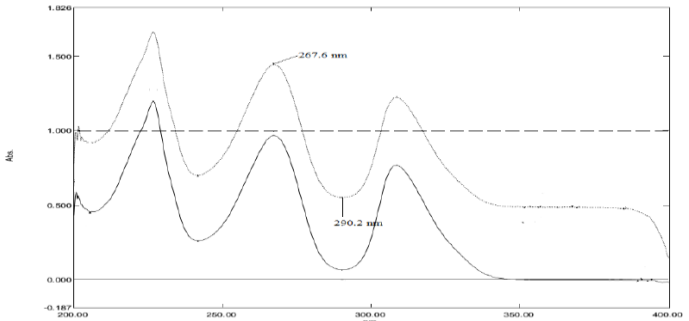

Figure 3: Ratio spectra of 20 µg ml^{-1} AM (-----) and 10 µg ml^{-1} HCZ(___) and (1:1) mixture containing 10 µg ml^{-1} of each (……) using 20 µg ml^{-1} AM as divisor.

The linear regression equation was calculated:
$A = 0.0890\ C + 0.0090$. $r = 0.9999$.
Where A is the absorbance, C is the concentration of the drug in µg ml^{-1} and r is the correlation coefficient.

The specificity of the proposed methods was proved by the analysis of different laboratory prepared mixtures containing different ratios of the suggested drugs, where satisfactory results were obtained, (Table 1).

Parameters	Direct determination of AM at 361 nm	Isoabsorptive method	Ratio subtraction method	Ratio difference method
Calibration range µg ml^{-1}	1-30	1-30	1-30	1-30
Slope	0.0660	0.0470	0.0660	0.0890
Intercept	0.0040	0.0080	0.0170	0.0090
Correlation coefficient	0.9999	0.9999	0.9999	0.9999
Accuracy (%) ± SD	101.85 ± 1.01	101.99 ± 0.92	100.60 ± 1.69	100.95 ± 1.01
Specificity (%) ± SD	99.57 ± 1.77	100.93 ± 0.79	100.92 ± 0.67	100.48 ± 0.78
Repeatability (RSD %)[a]*	0.77	0.74	1.04	0.87
Intermediate precision (RSD%)[b]*	1.46	1.82	2.10	1.68
LOD**	0.25	0.30	0.25	0.24
LOQ**	0.75	0.90	0.75	0.72

Table 1: Regression and validation parameters of the proposed methods for determination of HCZ in presence of AM
*a. The intraday precision (n=3), average of three different concentrations repeated three times within day. b. The interday precision (n=3), average of three different concentrations repeated three times in three successive days.
** Limit of detection and quantitation are determined via calculations LOD = (SD of the response/slope) × 3.3; LOQ = (SD of the response/slope) × 10

The developed spectrophotometric methods were successfully applied for the determination of HCZ and AM in Moduretic® and Hydikal® tablets without interferences from tablets excipients and satisfactory results were obtained. The validity of the methods was further assessed by applying standard addition technique which also confirmed the accuracy of the proposed methods (Table 2). The results obtained by applying the proposed methods were statistically compared to reported ratio spectra derivative spectroscopic method [7] (Table 3) and the values of the calculated t and F are less than the tabulated ones, which reveals that there is no significant difference with respect to accuracy and precision between the proposed methods and the reported one. Furthermore, statistical analysis of the results obtained by the proposed methods and reported method were carried out using one way ANOVA at ($P < 0.05$). Calculated F-value was found to be less than tabulated F-value (Table 4). The test ascertains that the proposed methods are as precise and accurate as the reported ratio spectra derivative spectroscopic method [7] and are comparable to one another.

Methods	Pharmaceutical formulations	Taken (µg ml^{-1})	Found[a] (%) ± SD	Added (µg ml^{-1})	% recovery[b]	Mean (%) ± SD
Direct determination of AM at 361 nm	**Moduretic®**	10.00	100.70 ±0.76	8.00	100.38	100.97 ± 0.57
				5.00	101.52	
				3.00	101.01	
	Hydikal	10.00	100.77 ±1.01	10.00	100.15	101.11 ±1.18
				8.00	100.76	
				5.00	102.42	
Isoabsorptive method	**Moduretic®**	10.00	98.87 ±1.80	8.00	97.61	99.72 ±1.83
				5.00	100.85	
				3.00	100.71	
	Hydikal®	10.00	100.45 ±1.10	10.00	100.64	100.80 ±0.23
				8.00	101.06	
				3.00	100.71	
Ratio subtraction method	**Moduretic®**	10.00	98.99 ±1.11	10.00	100.61	99.56 ±0.98
				8.00	98.67	
				5.00	99.39	
	Hydikal®	10.00	99.82 ±1.03	10.00	99.20	99.58 ±1.60
				8.00	101.33	
				5.00	98.18	
Ratio difference method	**Moduretic®**	10.00	99.06 ±1.02	8.00	97.47	98.96 ±1.66
				5.00	98.65	
				3.00	100.75	
	Hydikal®	10.00	100.29 ±1.04	10.00	100.79	100.39 ±1.00
				8.00	101.12	
				3.00	99.25	

Table 2: Quantitative determination of HCZ and AM in Moduretic® and Hydikal® tablets by the proposed methods and application of standard addition technique [a]: average of six experiments [b]: average of three experiments

Parameters		Direct determination of AM at 361nm	Isoabsorptive method	Ratio subtraction method	Ratio difference method	Reported ratio spectra derivative spectroscopic method	
						AM	HCZ
Moduretic® tablets	Mean %	100.70	98.87	98.99	99.06	99.80	99.53
	SD	0.76	1.80	1.11	1.02	1.31	1.03
	n	6	6	6	6	6	6
	Student's *t*-test	1.394 (2.228)*	0.783 (2.228)*	0.874 (2.228)*	0.787 (2.228)*	----	----
	F – value	2.952 (5.050)*	3.079 (5.050)*	1.162 (5.050)*	1.012 (5.050)*	----	----
Hydikal® tablets	Mean %	100.77	100.45	99.82	100.29	100.47	99.87
	SD	1.01	1.10	1.03	1.04	1.10	1.55
	n	6	6	6	6	6	6
	Student's *t*-test	0.502 (2.228)*	0.751 (2.228)*	0.059 (2.228)*	0.553 (2.228)*	----	----
	F – value	1.175 (5.050)*	1.980 (5.050)*	2.248 (5.050)*	2.211 (5.050)*	----	----

Table 3: Statistical analysis of the proposed methods and reported ratio spectra derivative spectroscopic method for determination of HCZ and AM in their pharmaceutical formulations. *Figures in parentheses represent the corresponding tabulated of *t* and *F* at *P* = 0.05.

Methods	Moduretic® tablets		Hydikal® tablets	
	n	Mean (%) ± RSD	n	Mean (%) ± RSD
Isoabsorptive	6	98.87 ±1.82	6	100.45 ±1.10
Ratio subtraction	6	98.99 ±1.12	6	99.82 ±1.03
Ratio difference	6	99.06 ± 1.03	6	100.29 ±1.04
Reported ratio spectra derivative	6	99.53 ± 1.03	6	99.87 ±1.55
F- value	0.307 (3.098)*		0.402 (3.098)*	
P-value	0.820		0.753	

Table 4: Statistical analysis of the results obtained by applying the proposed and reported methods on Moduretic® and Hydikal ® tablets for determination of HCZ using one way ANOVA
*Figures in parentheses represent the corresponding tabulated of F at P < 0.05

3.4. Method validation

Method validation of the proposed methods was performed according to ICH guidelines [27].

3.4.1. Linearity and range

The calibration range for HCZ and AM was established through considerations of the practical range necessary according to adherence to Beer-lambert's law to give accurate, precise and linear results. Linearity ranges of HCZ and AM are shown in (Table. 1).

3.4.2. Accuracy

Accuracy of the proposed methods was calculated as the percentage recoveries of blind pure samples of the studied drugs. The concentrations were calculated from the corresponding regression equations and the results are shown in (Table. 1). Accuracy was further assessed by applying the standard addition technique to Moduretic® and Hydikal® tablets, where good recoveries were obtained revealing no interference from excipients and good accuracy (Table. 2).

3.4.3. Precision

3.4.3.1. Repeatability. Three concentrations (5, 10 and 15 µg ml^{-1}) of both HCZ and AM were analyzed three times intra-daily using the proposed methods. Good results and acceptable relative standard deviations (RSDs) were obtained, (Table. 1).

3.4.3.2. Intermediate precision. The previous procedures were repeated inter-daily on three different days for the analysis of the chosen concentrations. Good results and acceptable RSDs were obtained, (Table. 1).

3.4.4. Specificity

Specificity of the proposed methods was assessed by the analysis of different synthetic laboratory prepared mixtures containing different ratios of HCZ and AM within their linearity ranges. Satisfactory results are shown in (Table. 1).

3.4.5. LOD and LOQ

ICH recommendations [27] were followed to calculate the LOD and LOQ values of HCZ and AM. Low LOD and LOQ values indicate the high sensitivity of the proposed methods (Table 1).

4. CONCLUSION

The developed methods have advantages over the published methods in being more simple, rapid, cost effective and data processing steps are not time consuming. Spectrophotometric methods can be regarded as a useful alternative to chromatographic techniques in the routine quality control analysis of pharmaceutical formulations allowing rapid determination at relatively low cost. The developed isoabsorptive, ratio subtraction and ratio difference spectrophotometric methods were successfully applied for simultaneous determination of HCZ and AM in their combined marketed dosage forms. Furthermore, the proposed methods considered simpler and don't

require any derivatization steps or complex algorithms rather than published spectrophtometric methods. Accordingly, they can be used in routine quality control analysis of HCZ and AM in pharmaceutical formulations.

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