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QUANTITATIVE ASSAY OF BINARY AND TERNARY CARDIOVASCULAR MIXTURES USING ABSORPTION SUBTRACTION SPECTROPHOTOMETRIC METHOD

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Abstract

Objective: Development and validation of specific spectrophotometric analytical method for the simultaneous determination of co-administered cardiovascular mixtures consisting of Aspirine (ASP) and Prasugrel (PRA) binary mixture and Ticagrelor (TICA), Irbesartan (IRB) and Hydrochlorothazide (HCT) ternary mixture.

Methods: The proposed spectrophotometric method was based on Absorbance Subtraction, where, when a mixture of two drugs X and Y having overlapped spectra, intersecting at isoabsorptive point and Y spectrum is extended more than X, while X doesn't show any absorbance (A_2) at another wavelength (λ_2), X and Y could be determined in the mixture by simple mathematical manipulation

Results: This method obeyed Beer's law in the concentration range of 5-50 µg/ml for ASP, 5-25 µg/ml for PRAS, 10-30 µg/ml for TICA, 0.5-2 µg/ml for IRB, 4-10 µg/ml for HCT. The suggested method was applied on laboratory prepared mixtures and was successfully applied in quality control analysis of combined pharmaceutical dosage forms. The developed spectrophotometric method was validated statistically and recovery studies were carried out, where excellent percentage recoveries were obtained. The developed spectrophotometric method was also compared to reference HPLC methods.

Conclusion: The developed spectrophotometric method based on absorption subtraction is an easy, simple and time saving method, that was able to determine the active ingredients in the binary and ternary pharmaceutical formulation without any interference from then excipients.

Keywords

Binary, ternary, cardiovascular mixtures, absorption subtraction method, multicomponent Formulation, Validation

1. INTRODUCTION

Cardiovascular drugs often work best in combination, since multiple conditions are behind most cardiovascular problems. Moreover, multicomponent formulations are gaining interest due to greater patient acceptability, increased potency, multiple action, fewer side effects, and quicker relief. Antiplatelet therapy is the cornerstone of treatment for patients with acute coronary syndrome as it prevents ischemic events in patients undergoing Percutaneous coronary intervention (Wiviott et al., 2007). Cardiovascular diseases accompanied with high blood pressure are the largest risk factor for premature death. Some patients with hypertension require two or more antihypertensive and anti-platelet drugs with complementary mechanisms of action to control their cardiovascular condition. The angiotensin II type 1-receptor antagonist Irbesatran (IRB), the diuretic Hydrochlorothiazide (HCT) and the anti-platelet Ticargrelor (TICA) have recognized clinical efficacy and protective effect on the cardiovascular system. Therefore, the combination of the three drugs been found to be more effective than either drug alone in the treatment of cardiovascular diseases not adequately controlled by monotherapy. As well, PRA in combination with aspirin showed to have more potent antiplatelet effects and faster onset of activity (Angiolillo et al., 2008). IRB (IRB), chemically described as 2-butyl-3-[[2′-(1H-tetrazol-5-yl)[1,1′-biphenyl]-4-yl]-methyl1-3-diazaspiro-[4,4]-non-1-en-4-one, (Fig. 1) is an angiotensin II blocker, which acts mainly by selective blockade of AT1 receptors and reduces the effects of angiotensin II. IRB may be used alone or in combination with other antihypertensive or diuretic agents (O'Neil et al., 2001). Hydrochlorothiazide (HCT), chemically described as 6-chloro-3, 4-dihydro-2H-1, 2, 4-benzothiadiazine-7-sulphonamide-1, 1-dioxide, (Fig. 2), is a thiazide diuretic. It increases sodium and chloride excretion in distal convoluted tubule. Because of their synergistic anti-hypertensive action, IRB and Hydrochlorothiazide are available in the market as a combined dosage form (Bank, 2013).

TICA(TICA), chemically described as (1S,2S,3R,5S)-3-[7-{[(1R,2S)-2-(3,4-difluorophenyl)cyclopropyl]amino}-5-(propylthio)-3H-[1,2,3]-tria zolo [4,5-d]pyrimidin-3-yl]-5-(2-hydroxyethoxycyclo pentane-1,2-diol2, (Fig. 3), is an orally active antiplatelet agent indicated to reduce the rate of thrombotic cardiovascular events in patients with acute coronary syndrome. TICA and its major metabolite reversibly interact with the platelet P2Y12 ADP-receptor to prevent signal transduction and platelet activation, which inhibits platelet aggregation and thrombus formation in atherosclerotic disease. Aspirin (ASP), chemically described as 2-(Acetyloxy) benzoic acid (Fig. 4) is a nonsteroidal anti-inflammatory drug and platelet aggregation inhibitor. Acetylsalicylic acid binds to acetylates serine residues in cyclooxygenases, resulting in decreased synthesis of prostaglandin, platelet aggregation, and inflammation (Vemugunta et al., 2012) PRA (PRA), chemically described as [5-[2-cyclopropyl-1-(2-fluorophenyl)-2-oxoethyl]-6, 7-dihydro-4H-thieno [3, 2-c] pyridin-2-yl] acetate hydrochloride (Fig. 5) is a platelet activation and aggregation inhibitor. PRA is a prodrug that requires enzymatic transformation in the liver to its active metabolite. Ii irreversibly binds to ADP receptors on platelets thus preventing activation of the GPIIb/IIIa receptor complex.

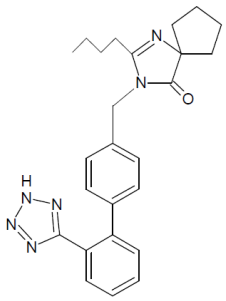

Fig.1: Irbesartan

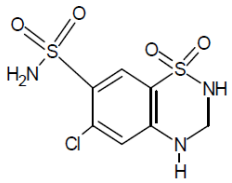

Fig.2: Hydrochlorobiazide

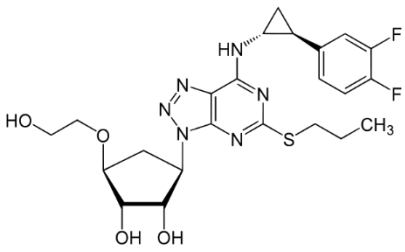

Fig.3: Ticagrelor

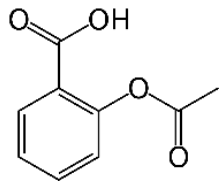

Fig.4: Aspirin

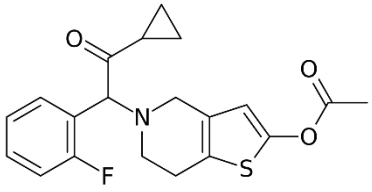

Fig.5: Prasugrel

Several analytical methods have been reported for the determination of IRB in pure and pharmaceutical dosage forms using spectrophotometry. Extractive and non-extractive (El Sutohy et al., 2013) spectrophotometry spectrofluorometry. In presence of hydrochlorothiazide, IRB has been determined by UV-Spectroscopy. Hydrochlorothiazide has been determined individually by spectrophotometry. In combination with many other drugs, Hydrochlorothiazide has been determined by spectrophotometric Chemometric analysis (Farouk et al., 2011; Patel et al., 2011). Sequential Spectrophotometry (Sridharan et al., 2010). Hydrochlorothiazide was assayed in combination with many other drugs using H-point standard addition method (Lakshmi et al., 2011; Sivasubramanian et al., 2015). TICA has been assayed by spectrophotometry (Pendaya et al., 2016). PRA has been determined individually by extractive spectrophotometry (22), colorimetric method(Harshini et al., 2011), HPLC (Mullangi et al., 2012; Prabahar et al., 2011) and HPTLC (Damle et al., 2017). In combination with ASP, PRA has been determined by HPLC (Patel et al., 2013; Martos et al., 2000), spectrophotometry (Chhonker et al., 2016), spectrofluorimetry (Porwal et al., 2015). ASP has been determined in presence of Clopidogrel in human plasma (Lotfy, 2014). ASP has been determined in presence of many other drugs by LC and HPLC. In this work, green analytical spectrophotometric method has been developed for the analysis of PRA and ASP binary combination and IRB, TICA and HCT ternary combination in synthetic mixtures and laboratory made tablets. The suggested spectrophotometric method is able to resolve the overlapped spectra by simple manipulation step, the absorbance values corresponding to the studied drugs could be easily obtained. The spectrophotometric method was validated according to ICH guide lines and the results were compared to reference RP-HPLC methods.

2. METHODOLOGY

2.1 Theoretical Background

The Absorbance Subtraction method is based on the same principle as the absorption factor method (Lotfy, 2014) and it depends on that, if you have a mixture of two drugs X and Y having overlapped spectra, intersecting at isoabsorptive point and Y is extended more than X, while X doesn't show any absorbance (A2) at another wavelength (λ2), X and Y could be determined in the mixture by simple mathematical manipulation. In the absorbance subtraction method (AS) the isoabsorptive point (λiso) could be used for the separate quantitative determination of each X & Y in their mixture (X+Y). The determination can be done using mathematically calculated factor of one of these components. So, the Concentration of each component could be obtained via the isoabsorptive point regression equation without any need for a complementary method. The absorbance values corresponding to X and Y at λiso were calculated by using absorbance factor {Aiso / A2} which is a constant for pure Y representing the average of the ratio between the absorbance values of different concentrations of pure Y at λiso divide be the absorbance values of pure Y at λ2.

Absorbance of Y in the mixture at λiso = Ay λiso/ Ay λ2 × A(x+y) λ2.

Absorbance of X in the mixture at λiso = A(x+y) λiso – (Ay λiso / Ay λ2) × A(x+y) λ2.

Where; Ay λiso and Ay λ2 is the absorbance value of pure Y at λiso and λ2; Ay λiso/ Ay λ2 is called the absorbance factor and abs λiso (X + Y) and abs λ2(X + Y) are the absorbance of the mixture at these wavelengths (λiso, λ2).

The concentration of each of X or Y is separately calculated using the isoabsorptive point regression equation that is obtained by plotting the absorbance values or the ratio signals of X or Y at isoabsorptive point (λiso) against their corresponding concentrations.

2.2 MATERIALS AND METHODS

Apparatus

The spectrophotometric measurements were carried out on a Jasco V-530 double beam UV-Vis Spectrophotometer connected to a computer loaded with Jasco UVPC software and HP Deskjet 5652 printer. The spectrophotometer is supported with Jasco Spectra Manager software for GULLIVER Ver. 1.53. The absorption spectra were recorded using 1 cm quartz cells.

Chemicals

Aspirin and PRA (supplied by SIGMA-ALDRICH) were used as working standards. TICA(supplied by Omnipharma, Astrazenica, Lebanon), IRB and Hydrochlorothiazide (supplied by Algorithm, Lebanon). Methanol (supplied by SIGMA-ALDRICH CHROMASOLV® FOR HPLC>99.9%) was used as solvent.

Standard stock solutions

The standard stock solutions were separately diluted with methanol to prepare working standards having the concentrations of 100 µg.mL-1 TICA, 10 µg.mL-1 IRB, 40µg.mL-1 HCT, 100 µg.mL-1 ASP and 100 µg.mL-1 PRA.

Working standard solutions

Standard solutions of TICA (1000 µg.mL-1), IRB (100 µg.mL-1), HCT(400 µg.mL-1), ASP (1000 µg.mL-1) and PRA (400 µg.mL-1) were separately prepared using methanol as solvent.

Calibration graphs

Into series of 10-mL measured flasks, volumes from the above prepared working standard solutions of ASP, PRA TICA, IRB or HCT, were separately transferred and diluted with methanol to give the final concentrations stated in Table 1.

Synthetic mixtures

Six validation synthetic mixtures from the binary and ternary mixtures were prepared by mixing appropriate volumes of the working standard solutions of ASP and PRA or TICA, IRB and HCT and diluting to volume with methanol. The combination of ASP and PRA or TICA, IRB and HCT are illustrated in Table 2.

The absorbance values for each solution of the calibration graph and the prepared synthetic mixtures were recorded at 1-nm intervals in the wavelength range 200-300 nm

For ASP and PRA determination, the A spectra of the standard solutions and their mixtures were recorded at 223 and 256 nm.

For TICA and HCT determination, the ratio spectra of the standard solutions and their mixtures were recorded at 214 and 240 nm, where the ratio spectra are obtained by dividing the absorption spectra of the three analyte and their mixtures by the spectra of 0.5 µg.mL-1 IRB standard solution).

For IRB and HCT determination, the ratio spectra of the standard solutions and their mixtures were recorded at 229 and 240 nm, where the ratio spectra are obtained by dividing the absorption spectra of the three analyte and their mixtures by the spectra of 12.5 µg.mL-1 TICA standard solution).

Regression equations of the signals obtained versus the standard concentration of analyte are derived to quantify each of the studied drugs in their binary or ternary mixtures.

Laboratory made tablets

Accurately two sets of ten laboratory made tablets; the first set containing 100 mg ASP and 10 mg PRA per tablet, and the second set containing 90 mg TICA, 15 mg IRB and 50 mg HCT per tablet (in addition to lactose, starch, talc and magnesium stearate as tablet fillers) were weighed and powdered. Accurate weight (equivalent to 10 mg ASP and 1 mg PRA) or (equivalent to 9 mg TICA, 1.5 mg IRB and 5 mg HCT) of the finely powdered tablets were transferred into two separate 100-mL calibrated flasks, 50 mL methanol were added and the flasks were shaken for 15 min, filtered and completed to volume with methanol. 1-mL of each of the prepared solutions, was transferred into two separate 10-mL calibrated flask and diluted to volume with methanol to prepare tablet solutions containing 5 µg.mL-1 PRA and 50 µg.mL-1 ASP or 9 µg.mL-1 TICA, 1.5 µg.mL-1 IRB and 5 µg.mL-1 HCT. The absorption spectra of the prepared tablet solutions were scanned from 200-300 nm against methanol as a blank.

3. RESULTS

Spectral characteristics

Quantitative analysis often involves the spectrophotometric resolution of mixtures of two or more components with partly overlapping spectra. The extensive overlapping between the absorption spectra of ASP and PRA and TICA, IRB and HCT in Fig. 6 and Fig. 7 respectively is sufficiently clear, making the resolution using the direct UV-Vis spectrophotometry more difficult. Thus, the need for the development of mathematical treatment of the data. Absorption subtraction method is one of the easily applied method in which minimal treatment of the data is done. This method could be used if there is existence of an isoabsorptive point for the drugs in mixture and extension of the spectra of the other component.

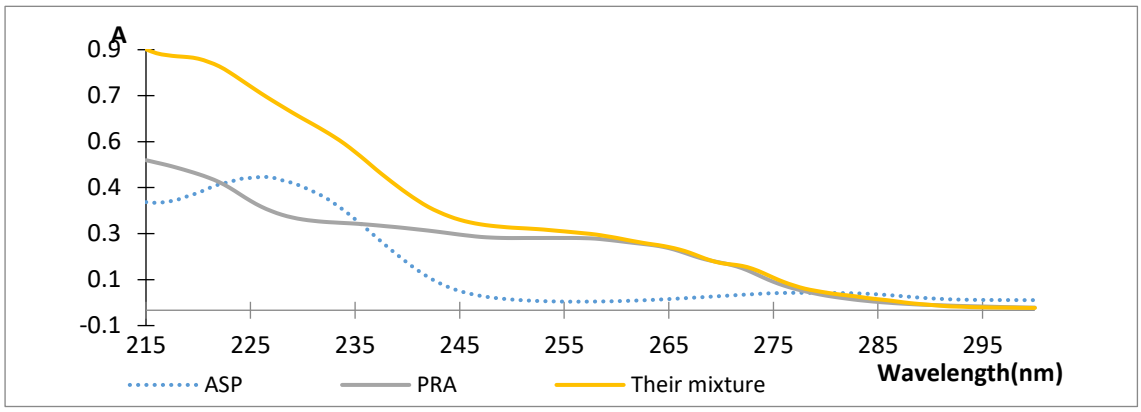

Fig.6: Absorption curves of 10 µg.mL-1 ASP and 15 µg.mL-1 of PRA in methanol

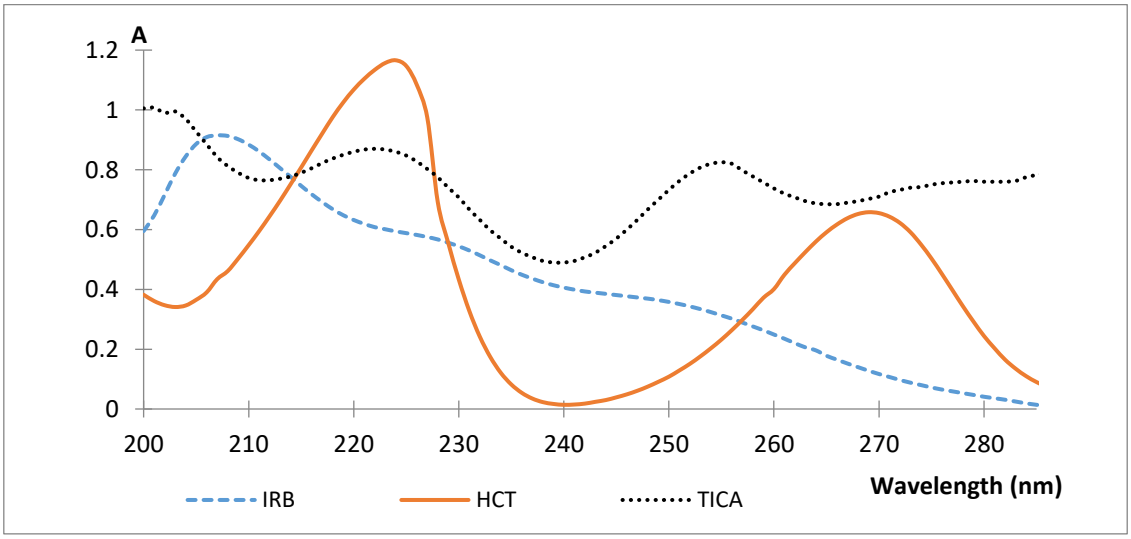

Fig.7: Absorption curves of 12.5 µg.mL-1 TICA, 0.5 µg.mL-1 IRB and 4 µg.mL-1 of HCT in methanol

4. DISCUSSION

Fig. 6 shows that the A spectra of ASP and PRA exhibit an isoabsorptive point at 223 nm and it is also clear that the A spectrum of ASP is more extended in the spectral region between 245-260 nm, Thus the AS method could be applied for the determination of ASP and PRA in presence of each other. By the analysis of the recorded A values for ASP, PRA and their mixture at 223 nm and 256 nm, the ratio signals of ASP and PRA could be separately calculated from the following equations:

Absorbance of PRA in the mixture at λ223 = APRAS λ223/ APRAS λ256 × A(ASP+PRAS) λ256.

Absorbance of ASP in the mixture at λ223 = A(ASP+PRA) λ223 – (APRA λ223 / APRA λ256)× A(ASP+PRA) λ256,

Where, the APRA λ223 / APRA λ256 is the absorbance factor of pure PRA at 223 and 256 nm.

To be able to quantify TICA or HCT, in their ternary mixture, AS method, has been developed on the ratio spectra (Fig. 8); where the effect of IRB has been eliminated by dividing the zero absorption spectrum of the mixture by the zero order spectrum of a standard IRB solution. Upon this division, constant spectrum equivalent to the contribution of IRB in the mixture is generated (AIRB/ A IRB0). Subtracting the value of this constant from the ratio spectrum, will give ratio signals equivalent to TICA and IRB only. On the obtained ratio signals, SA method has been applied to determine the concentration TICA and HCT.

Fig. 8 shows that the ratio spectra of TICA and HCT, exhibit an isoabsorptive point at 214 nm and it is also clear that the ratio spectrum of HCT is more extended in the spectral region between 235-245 nm, Thus the AS method could be applied for the determination of TICA and HCT in presence of each other. By the analysis of the recorded ratio signals for TICA, HCT and their mixture at 214 nm and 240 nm, the ratio signals of TICA and HCT could be separately calculated from the following equations:

Ratio signal of TICA in the mixture at λ214 = RTICA λ214/ RPTICA λ240 × R(TICA+HCT) λ240.

Ratio signal of HCT in the mixture at λ214= R(TICA+HCT) λ214 – (RTICAλ214 / RTICA λ240) × R(TICA+HCT) λ240,

Where, the RTICA λ214 / RTICA λ240 is the absorbance factor of pure TICA at 214 and 240 nm.

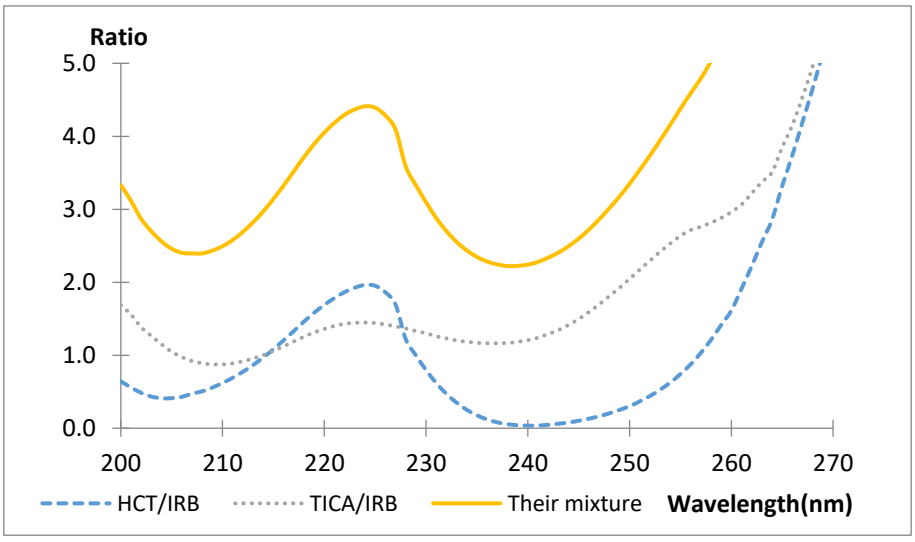

Fig.8: The ratio spectra of 4 µg/mL HCT and 12.5 µg/mL TICA, divided by the spectra of 0.5 µg/mL IRB

To be able to quantify IRB in its ternary mixture, AS method, has been developed on the ratio spectra (Fig. 9); where the effect of TICA has been removed by dividing the zero absorption spectrum of the mixture by the zero order spectrum of a standard TICA solution. Upon this division, constant spectrum equivalent to the contribution of TICA in the mixture is generated (ATICA/ A TICA0). Subtracting the value of this constant from the ratio spectrum, will give ratio signals equivalent to TICA and IRB only. On the obtained ratio signals, SA method has been applied to determine the concentration IRB and HCT.

Fig. 9 shows that the ratio spectra of IRB and HCT, exhibit an isoabsorptive point at 229 nm and it is also clear that the ratio spectrum of HCT is more extended in the spectral region between 235-245 nm, Thus the AS method could be applied for the determination of IRB and HCT in presence of each other. By the analysis of the recorded ratio signals for IRB, HCT and their mixture at 229 nm and 240 nm, the ratio signals of IRB and HCT could be separately calculated from the following equations:

Ratio signal of IRB in the mixture at λ229 = RIRB λ229/ RIRB λ240 × R (IRB+HCT) λ240.
Ratio signal of HCT in the mixture at λ229= R (IRB+HCT) λ229 – (RTICAλ229 / RTICA λ240) × R(IRB+HCT) λ240,
Where, the RIRB λ229 / RIRB λ240 is the absorbance factor of pure IRB at 229 and 240 nm.

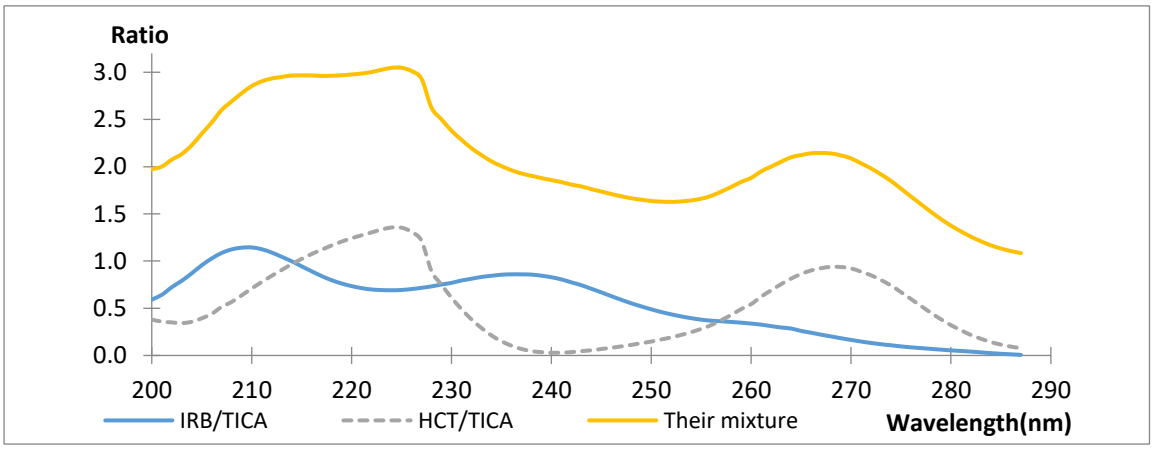

Fig.9: The ratio spectra of 0.5 µg/mL IRB and 4 µg/mL HCT, divided by the spectra of 12.5 µg/mL TICA

Method validation and statistical analysis

The proposed method was validated according to ICH guidelines. The validation parameters included: linearity, limits of detection and quantification, accuracy, precision and specificity. The applied specrophotometric analytical method was able to quantify the studied drugs in their double and ternary mixtures.

Linearity and concentration ranges

Under the described experimental conditions, the graphs obtained by plotting the signals of the proposed method versus concentration for ASP, PRA, TICA, IRB and HCT gave linear relationships over the concentration ranges stated in Table 1. Linearity data and statistical parameters for the proposed method were calculated, including linear regression equation parameters (intercepts, slopes, correlation coefficients, standard deviation of intercept and standard deviation of the slope (Table 1). Regression analysis confirmed good linearity as shown from correlation coefficient value (r> 0.999). The high F-value proved that the linear correlation between calculated signals (spectrophotometric method) and concentrations in significant to a high level of confidence. The high values of the correlation coefficient (r) with negligible intercepts indicate the good linearity of the calibration graphs. Standard deviation of residuals (Sy/x), of intercept (Sa) and of slope (Sb) are presented for each drug. (Sy/x) is a measure of the extent of deviation of the found (measured) y-values from the calculated ones. Also, the small degree of scatter of the experimental data point around the line of regressions was confirmed by the small values of the variances around the slopes (Sb2).

Limit of detection (LOD) and limit of qualification (LOQ)

Limits of detection (LOD) and quantification (LOQ) were calculated according to the ICH guidelines (33). LOD was defined as 10 Sa/b, where Sa is the standard deviation of the intercept and b is the slope of the calibration curve. The sensitivity of the proposed methods can be confirmed by the low LOD and LOQ values obtained in Table 1. The variance test for the regression lines revealed that, for equal degrees of freedom, the increase in the variance ratio (F-values) means an increase in the mean squares due to regression and a decrease in the mean squares due to residuals, (the less is the scatter of experimental points around the regression line). Consequently, regression lines with high F-values (low significance F) are much better than those with lower ones. Good regression lines show high values for both r and F statistical parameters (Armitage et al., 2008).

Accuracy

The applicability of the developed method was tested by the analysis of ASP-PRA and TICA-IRB-HCT in several synthetic mixtures of different proportions. Each solution was measured in triplicate, and the recoveries were calculated. Good accuracy, expressed as percentage relative error (Er %) were obtained. The results, summarized in Table 2 show that the calculated (Er %) values do not exceed the accepted limits, which demonstrate the high accuracy of the developed methods.

Precision

Intra-day precision was determined by analyzing the prepared mixtures for five times in the same day. Inter-day precision was determined by analyzing the prepared mixtures individually for three successive days. High precision expressed as % RSD were obtained. The results are summarised in Table 3 where the values of % RSD did not exceeded the accepted limits which demonstrate the precision of the method.

Laboratory made mixtures

Table 4 presents a statistical comparison between the proposed method and a reference RP-HPLC method for the assay of ASP and PRA binary combination, TICA, IRB and HCT ternary combination by using the student's t-test and the variance ratio F-test. Since the calculated t- and F- values for each drug did not exceed the theoretical ones, this indicated that there was no significant difference between the applied methods for determination of the two drugs in commercial tablets.

Table 1: Assay parameters for the determination of ASP, PRA, TICA, IRB or HCT in their binary or ternary mixtures using the Absorption subtraction method

	ASP	**PRA**	**PRA**	**TICA**	**TICA**	**IRB**	**IRB**	**HCT**
Conc. Range ($\mu g.mL^{-1}$)	5-50	5-25	5-25	10-30	10-30	0.5-3	0.5-3	4-10
λ (nm)	223	223	256	229	240	229	240	229
r	0.9995	0.9986	0.9998	0.999	0.9990	0.9995	0.9983	0.9991
$S_{y/x}$	0.16×10^{-2}	0.39×10^{-3}	1.56×10^{-5}	0.40×10^{-3}	0.19×10^{-3}	1.15×10^{-3}	1.81×10^{-3}	0.15×10^{-3}
F	3263	1120	11654	1642	1564	3110	892	1192
Significance F	1.18×10^{-5}	5.86×10^{-5}	1.75×10^{-6}	3.30×10^{-5}	3.55×10^{-5}	1.27×10^{-5}	8.23×10^{-5}	0.83×10^{-3}
a (intercept)	-0.11×10^{-1}	0.21×10^{-1}	0.23×10^{-2}	-0.10×10^{-1}	-0.15×10^{-1}	-0.18×10^{-1}	0.28×10^{-1}	0.15×10^{-1}
b (slope)	0.42×10^{-1}	0.24×10^{-1}	0.15×10^{-1}	0.29×10^{-1}	0.20×10^{-1}	0.56	0.38	0.66×10^{-1}
S_a	0.24×10^{-1}	0.12×10^{-1}	0.23×10^{-2}	0.15×10^{-1}	0.11×10^{-1}	0.18×10^{-1}	0.23×10^{-1}	0.14×10^{-1}
S_b	0.74×10^{-3}	0.72×10^{-3}	0.14×10^{-3}	0.73×10^{-3}	0.51×10^{-3}	0.11×10^{-1}	0.12×10^{-1}	0.19×10^{-2}
LOD (µg/mL)	1.74	1.48	0.46	1.57	1.60	0.97×10^{-1}	0.18	0.63
LOQ (µg/mL)	5.80	4.90	1.50	5.20	5.36	0.32	0.61	2.12
a/S_a	-0.43	1.71	0.96	-0.64	-1.37	-0.99	1.24	1.05
$(S_b)^2$	5.52×10^{-7}	5.24×10^{-7}	2.08×10^{-8}	5.33×10^{-7}	2.63×10^{-7}	0.10×10^{-3}	0.16×10^{-3}	3.75×10^{-6}
S_b %	0.74×10^{-1}	0.72×10^{-1}	0.14×10^{-1}	0.73×10^{-1}	0.51×10^{-1}	1.02	1.27	0.19

Table 2: Assay results for the determination of ASP-PRA and TICA-IRB-HCT and in synthetic mixtures using the Absorbance subtraction method.

Mean Recovery ± SD[a] **RSD %[b]** **Er %[c]**			
ASP:PRA **µg.mL^{-1}**		**TICA: IRB:HCT** **µg.mL^{-1}**	
50:5	100.00± 0.86 0.86 0.00	**10:0.5:10**	100.00± 0.86 0.86 0.00
5:25	100.79 ± 0.34 0.33 0.79	**15:3:6**	100.79 ± 0.34 0.33 0.79
10:20	100.91± 1.78 1.76 0.91	**20:1:4**	100.91± 1.78 1.76 0.91
20:15	98.76 ± 0.45 0.45 -1.01	**20:3:8**	98.76 ± 0.45 0.45 -1.01
30:10	98.00 ± 1.41 1.44 -2.00	**20:2.5:6**	96.62 ± 0.73 0.75 -3.38
40:5	101.24 ± 0.99 0.97 1.24	**30:3:10**	101.24 ± 0.99 0.97 1.24

[a] Mean ± SD for the three determinations
[b] % Relative standard deviation
[c] % Relative error

Table 3: Intra-day and inter-day precision for the simultaneous determination of ASP and PRA, TICA, IRB and HCT, in laboratory-made mixtures using the proposed RP-HPLC methods

ASP:PRA **µg.mL^{-1}**	**Intra-day precision** **Mean Recovery ± SD[a]** **RSD%[b]** **Er%[c]**		**Inter-day precision** **Mean Recovery ± SD[a]** **RSD%[b]** **Er%[c]**	
	ASP	**PRA**	**ASP**	**PRA**
50:5	98.21±1.93 1.96 -1.01	99.44 ± 1.38 1.38 -0.51	99.01 ± 0.98 0.99 -0.99	98.64 ± 0.77 0.78 -1.36

TICA: IRB: HCT µg.mL^{-1}	**Intra-day precision** **Mean Recovery ± SD[a]** **RSD%[b]** **Er%[c]**			**Inter-day precision** **Mean Recovery ± SD[a]** **RSD%[b]** **Er%[c]**		
	TICA	**IRB**	**HCT**	**TICA**	**IRB**	**HCT**
9 : 1.5 : 5	99.82 ± 0.83 0.83 -0.18	99.32 ± 1.07 1.07 -0.68	101.21 ± 0.86 0.85 1.21	100.26 ± 0.92 0.92 0.26	100.39 ± 0.86 0.86 0.39	98.92 ± 0.98 0.99 -0.58

[a] Mean ± SD for the five determinations
[b] % Relative standard deviation
[c] % Relative error

Table 4: Assay results for ASP-PRA and TICA-IRB-HCT in their laboratory made tablets using the proposed Absorption subtraction method

Ratio **ASP:PRA** **50:5** **µg/mL**	**Mean Recovery ± SD[a]** **RSD %[b]** **Er %[c]**	
	HPLC (28)	**AS**
ASP	99.58 ± 0.60 0.60 -0.42	98.63±0.85 0.86 -1.37
****t-test**	—	-2.04
****F-test**	—	2.00
PRA	99.48 ± 0.46 0.46 -0.52	98.56±0.89 0.90 -1.44
****t-test**	—	0.70
****F-test**	—	3.74
Ratio **TICA : IRB : HCT** **9 : 1.5 : 5** **µg/mL**	**Mean Recovery ± SD[a]** **RSD %[b]** **Er %[c]**	
	HPLC(37)	**AS**
TICA	98.76 ± 0.45 0.45 -1.24	98.99 ± 0.87 0.87 -1.01
****t-test**	—	0.53
****F-test**	—	3.73
IRB	98.99 ± 0.87 0.87 -1.01	99.30 ± 0.65 0.88 -1.24
****t-test**	—	0.64
****F-test**	—	0.56
HCT	99.08 ± 0.61 0.61 -0.92	98.71 ± 1.10 0.67 -0.29
****t-test**	—	0.56 3.25
****F-test**	—	

[a] Mean ± SD for the five determinations
[b]% Relative standard deviation
[c]% Relative error
**Theoretical values of t- and F- at P = 0.05 are 2.13 and 6.93, respectively

5. CONCLUSION

For analytical purposes, it is always important to establish methods capable of analysing co administered drugs in short period of time with acceptable accuracy and precision. The developed spectrophotometric method is simple, inexpensive, require easy treatment of the samples and is based on simple mathematical and statistical background. The Absorption subtraction method demonstrated its ability to resolve the binary and ternary co-administered mixtures and individually quantify each of their components. The Absorption subtraction method could be well adopted since it gave satisfactory results in the analysis of synthetic mixtures, and proved to be sensitive, selective, accurate and precise and can be applied for different concentration ratios.

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