



Study of efficiency and spectral resolution for mathematical filtration technique using novel unlimited derivative ratio and classical univariate spectrophotometric methods for the multicomponent determination-stability analysis



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ABSTRACT

Six simple, sensitive and selective spectrophotometric methods based on mathematical filtration technique are presented for concurrent determination of aceclofenac (ACE) and paracetamol (PAR) in presence of their degradation products, namely; diclofenac sodium (DIC) and 4-aminophenol (4-AP), respectively without preliminary physical separation procedures. This technique consists of several consecutive steps applied on built-in spectrophotometer software utilizing zero and/or derivative and/or ratio spectra of the studied components. These methods, namely, dual wavelength (DW), induced dual wavelength (IDW), derivative subtraction (DS) coupled with constant multiplication (CM), ratio difference method (RD), constant center method (CC) and the novel introduced unlimited derivative ratio method (UDD). This novel method has a very powerful competence for the analysis of the challengeable mixtures lacking zero crossing point. The linearity, accuracy and precision ranges of these methods were determined and validated as per ICH guidelines. Moreover, the specificity was checked by analyzing synthetic mixtures of both drugs. These methods were applied for the determination of the cited drugs in pharmaceutical formulation and a statistical comparison of the obtained results was made with each other and with those of reported spectrophotometric method. The comparison of the results of pure powder form showed that there is no significant difference between the proposed methods and the reported method regarding both accuracy and precision.

1. Introduction

Aceclofenac (ACE), Fig. 1(a) is an anti-inflammatory non-steroidal drug with convenient anti-rheumatic and analgesic properties. It is chemically [[2-[(2, 6-Dichlorophenyl) amino] phenyl] acetyl] oxy] acetic acid, utilized in different rheumatoid arthritis, osteoarthritis and ankylosing spondylitis conditions [1, 2].

Paracetamol (PAR), Fig. 1(b), is N-(4- hydroxyphenyl) acetamide possessing analgesic and antipyretic efficacy.

4-aminophenol (4-AP), Fig. 1(c) is the official degradation product and synthesis impurity of paracetamol which can be produced by the hydrolysis of paracetamol in high temperature, acidic or basic media [3, 4] conditions. It was reported that 4-aminophenol may cause

nephrotoxicity and teratogenicity; therefore, its amount should be strictly controlled [5, 6, 7]. Its concentration is reported to be limited to 0.1% of the PAR [8].

Diclofenac sodium (DIC), Fig. 1 (d) is the reported degradation product and an active metabolite of aceclofenac. It was reported that DIC is less potent in controlling pain, has less $t_{1/2}$ and more gastric side effects if compared to aceclofenac [9, 10], therefore, its presence should be avoided since it affects the potency of the administered drug.

Literature survey revealed that ACE has been determined in pure and pharmaceutical formulation using different quantitative methods including titrimetric [11, 12], spectrophotometric [2, 13] and colorimetric [14, 15] methods. Aceclofenac's determination was also achieved in occurrence of its degradation product; diclofenac (DIC) using

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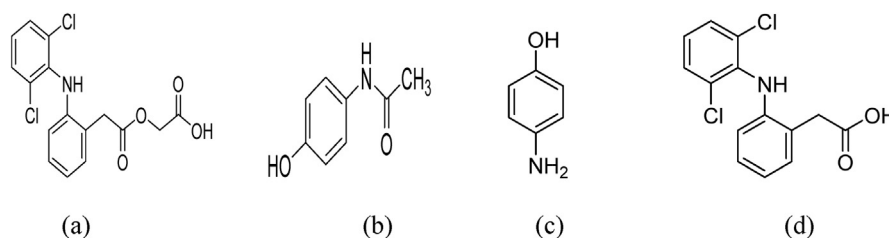


Fig. 1. Structural formula for (a) Aceclofenac, (b) Paracetamol, (c) 4-aminophenol and (d) Diclofenac sodium.

spectrophotometric [16, 17, 18], densitometric [14, 16, 17] and RP-HPLC [14, 17, 19, 20] methods. While PAR's pure determination was accomplished via titrimetric [21], spectrophotometric [22, 23, 24] and fluorimetric [25] methods. Stability indicating study for PAR was done using spectrophotometric [26], densitometric [27] and RP-HPLC [7, 28] methods.

Hifenac- P[®] tablet containing these studied drugs; ACE and PAR is used for joint pain, toothache, headache, fever, ear pain, ankylosing spondylitis, osteoarthritis, arthralgia, rheumatoid arthritis and other conditions, Where, this mixture has been analyzed by spectrophotometric [29, 30, 31, 32, 33], densitometric [34] and RP-HPLC [31, 35].

No Spectrophotometric methods have been previously studied for the concurrent determination of the cited drugs in the occurrence of their degradation products. While, literature survey revealed their stability study viz HPTLC [36], HPLC [37, 38] and UPLC [39]. But unfortunately, these stated methods were limited for mixtures containing minute amount of degradation products.

The UV spectrophotometric determination is one of the most widely used methods for quantification of drugs in their dosage forms due to its simplicity, low cost of implementation and wide availability in laboratories for quality control. The advantage of UV method over HPLC method is that the proposed UV method does not require the elaborate treatment and step up procedures usually associated with chromatographic method. HPLC technique is a typical laboratory separation technique involving developing method of separation and then implementing that assay to separate individual compounds from a solution. However, this usually results in multiple solutions that also need to undergo procedures leading to an exponential increase in complexity. Although HPLC can often simplify and speed up this process, the cost of developing an HPLC apparatus can become tremendous. Developing an HPLC apparatus, although much more efficient, is much more costly than UV assays for separating compounds. This makes it not financially viable for many small privately-owned laboratories. However, the higher selectivity and specificity of LC methods may be considered a negative factor for complex mixtures that need several trials for ensure the separation of each drug with accepted system suitability parameters. In addition, HPLC technique always requires a highly skilled technician to monitor the column and apparatus setup and make sure that the process is running exactly as planned. Thus, UV spectrophotometric methods are a good alternative for HPLC methods in quality control laboratories lacking HPLC apparatus as in the developing countries. Moreover, spectrophotometric assisted chemometric is a complex technique and involves high mathematical level that requires a statistical program to analyze the data. The results of multivariate analysis are not always easy to interpret and tend to be based on assumptions that may be difficult to assess.

In order to meet the challenges of simultaneous determination of multicomponent separation with high degree of selectivity, analytical methods based on analytical instrumentation must be employed to quantitate drug components without any interference of interfering substances. Thus, many analysts have concentrated to use built-in spectrophotometer software and innovative algorithmic mathematical equations. Resolving mixtures with overlapping spectra was accomplished by using various spectrophotometric quantitative methods such as

derivative spectrophotometry [40] and induced dual wavelength method [41]. Lotfy [42] instituted constant center method to attain the zero-order absorption spectra of both components. Two innovative methods; extended ratio subtraction methods and ratio difference were introduced [43, 44] where a well-established ratio subtraction method [45] was coupled with the former method for determination of binary mixtures. Derivative ratio spectrophotometry was developed for the concurrent determination of two components in binary mixtures [46]. Nevado *et al.* [47] presented a method for ternary mixtures' analysis using derivative ratio spectra zero-crossing based on measuring the amplitude at zero-crossing points in derivative ratio spectra [48, 49, 50, 51]. Dinc *et al.* [50, 52] presented double divisor-ratio spectra derivative method using a "double divisor" method for ternary mixtures' analysis. Lotfy *et al.* [53, 54] presented derivative subtraction method coupled with constant multiplication to obtain the derivative spectrum of each component in binary mixture.

Due to the importance of the studied dosage form comprising the proposed drugs and its widely recommendation as pain-killer and the degradation products' undesirable effects, this work is focused on developing simple, accurate, selective UV stability indicating spectrophotometric methods with optimum efficiency and maximum resolution power enabling the selective determination of ACE and PAR in their mixture and in presence of wider range of degradation products using built-in spectrophotometric software. These spectrophotometric methods and the scheme of their application were summarized as schematic diagram mentioned in Fig. 2. The present work couples both resolution (eliminating the overlapped spectra to get less complicated mixture) and quantitative methods (quantifying the active ingredients). Thus, a novel spectrophotometric method namely; unlimited derivative ratio was presented for multicomponent mixtures without searching for zero-crossing points. In addition, the other proposed spectrophotometric methods were centered on exploiting the absorbance difference of zero order absorption spectra and constants of the ratio spectra.

Furthermore, this study is devoted to compare the accuracy and precision of the recommended methods in relation to the extent of overlapping of absorption bands and to check the possibility of determination of the individual components from its zero, ratio and derivative ratio manipulation. The applied spectrophotometric methods are labor, time saving and also considered a cheap substitute for the overpriced HPLC technique. On the other hand, sophisticated chemometric procedures are not required [52, 55, 56]. Subsequently, the effectiveness of the proposed methods had been confirmed through conducting a comparative study between them and the previously reported one. Validation of the developed methods was performed according to ICH guidelines [57] to assure their appropriateness for the intended use. In addition, the reliability and feasibility of these methods were evaluated, focusing on routine quality control analysis.

2. Theory

2.1. Unlimited derivative ratio (UDD)

The method is a novel method dealing with the resolution of ternary mixtures using minimum mathematical steps and minimum limitation

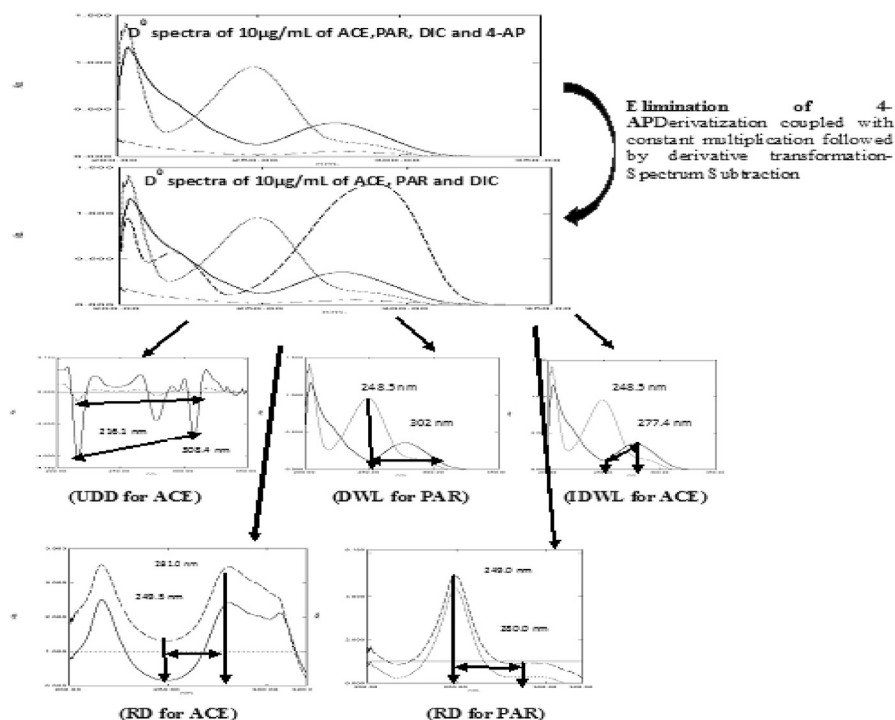


Fig. 2. Schematic diagram for the analysis of ACE and PAR in the presence of their degradation products using different spectrophotometric methods.

such as the presence of isosbestic point or extension of one of the components of the mixture. This method is considered as an extension to induced dual wavelength (IDW), Induced ratio difference (IRD) [58] and differential dual wavelength (DDWL) [59] methods for determination of severely overlapped binary mixtures in zero order, derivative and ratio spectra via difference between two wavelengths to ignore the contribution of one of interfering components in the mixtures lacking of equal response at these selected wavelengths. The proposed method was applied for simultaneous determination of ternary mixtures of X, Y and Z as summarized in the following equation;

$$[A_m] = [axCx] + [ayCy] + [azCz] \tag{1}$$

where, A_m is the absorbance of the mixture of X + Y + Z, a_x , a_y and a_z are the absorptivities of X, Y and Z respectively; C_x , C_y and C_z are their respective concentrations. The derivative ratio spectra obtained using component Z as a divisor

$$P_m = [axCx] / a_zC_z + [ay Cy] / a_zC_z + [azCz] / a_zC_z \tag{2}$$

$$P_m = P_X + P_Y + P_Z \tag{3}$$

where, (P_m) is the amplitude of derivative ratio spectrum of the mixture, P_X , P_Y and P_Z are the amplitude of component X, Y and Z respectively.

For determination of X, two wavelengths were selected in the derivative ratio spectra of the ternary mixture X, Y and Z (using any derivative order either first, second, third, fourth) A significant amplitude difference between the two selected wavelengths in the ratio spectra of pure X should be present while Z is zero (derivative of constant is zero).

$$P_{m1} = P_{X1} + P_{Y1} \text{ at } \lambda_1 \text{ where, } P_{z1} = \text{zero} \tag{4}$$

$$P_{m2} = P_{X2} + P_{Y2} \text{ at } \lambda_2 \text{ where, } P_{z2} = \text{zero} \tag{5}$$

To cancel the effect of Y at the two selected wavelengths, the equality factor of pure derivative ratio spectra of Y at these wavelengths (F_Y) is calculated which is the average of ratio between the two recorded amplitudes of different concentrations of Y at the chosen wavelength pair of X using the same derivative order of its ratio spectra using Z as a divisor.

$$F_Y = P_{Y1} / P_{Y2} \tag{6}$$

$$\therefore P_{Y1} = F_Y P_{Y2} \text{ (where } F \geq 1 \text{ or } \leq 1) \tag{7}$$

By substituting in Eq. (4)

$$P_{m1} = P_{X1} + F_Y P_{Y2} \tag{8}$$

By multiplying Eq. (5) by F_Y

$$F_Y P_{m2} = F_Y P_{X2} + F_Y P_{Y2} \tag{9}$$

And by calculating the difference, Equation(8) – (9), $F_Y P_{Y2}$ will be cancelled:

$$\Delta P (P_{m1} - F_Y P_{m2}) = P_{X1} - F_Y P_{X2} \tag{10}$$

Eq. (10) indicated that the response difference of the derivative ratio spectra of the ternary mixture X, Y and Z is dependent only on X and is independent on Y, thus the amplitude difference at wavelength pair are plotted against C_X . The concentration of X is calculated using the regression equation (obtained by plotting the amplitude difference values of the derivative ratio spectra of X obtained after division by the spectra of Z at the two chosen wavelengths ($\Delta P = P_1 - F_Y P_2$) against the corresponding concentrations X.

The concentration of Y is calculated using the same procedure after calculating the equality factor of pure X (F_X) at the two chosen wavelengths for Y.

Unlimited derivative ratio method (UDD) used for the resolution of ternary mixtures lacking of zero crossing is advantageous over derivative ratio method of these mixtures as it is more reliable with respect to utility and sensitivity without searching for the critical point either zero crossing point or coincident point [47] for the separated peaks, the maximum amplitude of the separated peaks could be measured. In addition, UDD beats the main drawbacks of successive derivative ratio spectrophotometry [60] for ternary mixture's analysis which requires the use of multiple divisors and several manipulating steps and it is also superior over differential derivative ratio [59] that was applied on derivative spectra thus enhancing signal to noise ratio.

3. Experimental

3.1. Apparatus and software

A double-beam UV/Visible spectrophotometer model Shimadzu (UV-1800), Japan was used. The absorption spectra of the standard and the tested solutions were scanned in 1.0 cm quartz cells over the range 200.0–400.0 nm at room temperature using spectra manager software.

3.2. Samples and solvents

Aceclofenac (ACE) and paracetamol (PAR) pure samples were supplied by Al-Amriya Pharmaceutical Industries, Al-Amriya, Alexandria. The purity was certified to be 99.76 ± 1.44 and 99.07 ± 1.33 , respectively; according to their reported method [29].

Diclofenac sodium (DIC) was supplied kindly by El Nasr pharmaceutical Co., Cairo, Egypt and its purity was certified to be 99.58 ± 1.25 , according to the reported method [61].

4- Aminophenol (4-AP) official impurity was kindly supplied by Glaxo Smithkline, Cairo, Egypt and its purity was certified to be 100.01 ± 0.20 .

Hifenac-P® tablets dosage form, batch number: 737132, labeled to comprise 100.0 mg aceclofenac and 325.0 mg paracetamol, was manufactured by INTAS Life sciences, India. They were bought from the Indian market.

Spectroscopic analytical grade methanol was purchased from El Nasr pharmaceutical Co., Cairo, Egypt.

3.3. Procedure

3.3.1. Standard solutions

Stock standard solutions comprising 100.0 µg/mL of ACE, PAR, 4-AP and DIC were prepared separately in 100-mL volumetric flasks by dissolving 10.0 mg of each standard powder in methanol and then completed to the volume by the same solvent.

Working standard solutions (50.0 µg/mL) of ACE, PAR, 4-AP and (10.0 µg/mL) of DIC were prepared by suitable diluting the primary stock solutions with methanol.

3.3.2. Spectral characteristics and methods' selection

The zero-order absorption spectra (D^0) of 10.0 µg/mL for each of ACE, PAR, 4-AP and 1.2 µg/mL of DIC were prepared from their working stock solution and scanned against methanol as a blank at region 200.0–400.0 nm and overlaid on each other using UV probe spectrophotometer software to identify the extend of overlapping and suggest the proper methods for the cited drugs' analysis.

3.3.3. Analysis of synthetic mixtures in laboratory

Testing the specificity of the proposed spectrophotometric methods was attained by mixing accurate portions of ACE, PAR, 4-AP and DIC and transferring them to a series of 10- mL volumetric flasks using methanol as a solvent.

3.3.4. Preparation of pharmaceutical formulation solution

Ten Hifenac-P® tablets were powdered and a portion equivalent to 10.0 mg of ACE and 32.5 mg of PAR was weighed accurately and transferred to a 100-mL beaker. Then, 30 mL of methanol was added, sonicated using ultrasonic bath for 15 min and filtered through 0.5 µm Whatman filter into a 100-mL volumetric flask. The residue was washed three times each time with 15 mL of methanol and the solution was completed to the mark with methanol. Appropriate dilution was done using methanol obtaining a solution claimed to contain 10.0 µg/mL ACE and 32.5 µg/mL PAR. Finally, aliquots equivalent to 3 mL were then transferred accurately into 10 mL volumetric flask and completed to the mark with methanol.

3.4. Validation

Validation of the proposed methods was performed according to ICH guidelines [57].

3.4.1. Linearity and calibration graphs

Accurately measured aliquots of ACE and PAR were transferred from their working standard solutions into two separate series of 10-mL volumetric flasks and the volumes were adjusted to the mark with methanol. Calibration standards were prepared over concentration ranges 3.0–30.0 µg/mL of ACE and 1.0–14.0 µg/mL of PAR. Solutions scanned from 200.0 to 400.0 nm and the obtained D^0 spectra were saved on the computer.

Dual wavelength (DW): The calibration graph's regression equation was computed relating the absorbance differences at 248.5 nm and 302.0 nm of the stored spectra against PAR's corresponding concentrations.

Induced dual wavelength (IDW): The calibration graph's regression equation was computed relating the absorbance difference ($A_{277.4} - FA_{248.5}$) and ACE's corresponding concentrations, where (F) is the equality factor of pure PAR which was calculated by getting the ratio of absorbance of diverse PAR concentrations at 248.5 nm and 277.4 nm and found to be equal to 0.185.

Derivative subtraction (DS) coupled with constant multiplication (CM): The calibration graphs' regression equations were computed relating the first derivative spectra's peak amplitudes (peak to peak) of ACE and PAR at $P_{261.0-297.0}$ and $P_{236.0-262.0}$ versus their concentrations, respectively using $\Delta\lambda = 4$ and scaling factor = 10.

Ratio difference (RD): The calibration graphs' regression equations were computed relating the amplitude differences of the ratio spectra at 249.5 and 281.0 nm for ACE and 249.0 and 280.0 nm for PAR using standard solution of PAR (8.0 µg/mL) and ACE (15.0 µg/mL) as divisors, respectively versus their corresponding concentrations.

Constant center (CC): The calibration graphs' regression equations were computed relating amplitude difference at (249.0 nm and 280.0 nm) against amplitudes at 249.0 nm for ACE's determination and (249.5 nm and 281.0 nm) against amplitudes at 281.0 nm for PAR's determination. Another two regression equations of calibration graphs were computed relating the absorbance of the zero order spectra at maxima 277.2 nm for ACE and 248.6 nm for PAR were plotted versus their corresponding concentrations.

Unlimited derivative ratio (UDD): The calibration graph's regression equation was computed relating the amplitude difference at 216.1 nm and 308.4 nm ($P_{216.1} - FP_{308.4}$) of the second derivative (DD^2) of ratio spectra of ACE using PAR's standard solution (8.0 µg/mL) as a divisor at $\Delta\lambda = 8$ and scaling factor = 100 where (F) is the equality factor of pure DIC which was calculated by getting the ratio of amplitudes of different concentrations of pure DIC using PAR as a divisor at 216.1 nm and 308.4 nm and found to be equal 1.02.

3.4.2. Accuracy

Checking the accuracy of the developed methods was performed through analyzing three replicates of different ACE's and PAR's concentrations. The concentrations were obtained from the corresponding regression equation for each method, from which the percentage recoveries suggested good accuracy of the proposed methods.

3.4.3. Repeatability

Intra-daily analysis of three concentrations of ACE and PAR for three times was accomplished using the proposed methods. Calculation of relative standard deviations was performed.

3.4.4. Intermediate precision

The previous procedures were repeated inter-daily on three different days for the analysis of the three chosen concentrations. Calculation of relative standard deviations was performed.

3.4.5. Specificity

The prepared mixtures' spectra containing diverse ratios of ACE, PAR, 4-AP and DIC were scanned against methanol as blank over wavelength range 200.0–400.0 nm. The normalized zero order spectrum of 4-AP is obtained by dividing the spectrum of known concentration by its concentration value. The first derivative spectra of 4-AP and PAR (8.0 µg/mL) standard solution were obtained using $\Delta\lambda = 4$ and scaling factor = 10.

3.4.5.1. Resolution of proposed mixtures from PAR degradation product. For each prepared mixture, the derivative of the scanned spectrum was divided by standard solution's derivative spectrum of normalized 4-AP. From the obtained ratio spectra, the measured constant at plateau region at 324.0nm–325.0 nm was multiplied by the divisor's zero spectrum of normalized 4-AP to obtain the recovered zero order spectrum of 4-AP. Then, the resolved mixture's zero order spectra (D^0) (containing ACE, PAR and DIC) could be obtained after subtraction of the obtained zero order spectrum of 4-AP for each mixture from its corresponding mixture to apply data recommended methods on resolved zero order absorption spectrum of each mixture.

After data processing of the proposed resolved spectrum of each mixture, the concentrations of targeting drugs ACE and PAR were calculated from the corresponding regression equation constructed in linearity and calibration graph of each method.

Dual wavelength (DW): The stored resolved laboratory prepared mixtures' zero order spectra were computed and the differences in absorbance at 248.5 nm and 302.0 nm were recorded for PAR.

Induced Dual wavelength (IDW): The stored resolved laboratory prepared mixtures' zero order spectra were computed and the absorbance difference (ΔA) at 248.5 nm and 277.4 nm, after multiplying by F, was calculated ($A_{277.4} - FA_{248.5}$).

Derivative subtraction (DS) coupled with constant multiplication (DS-CM): For each resolved zero order mixture, derivative manipulation was applied using $\Delta\lambda = 4$ nm and scaling factor = 10, a ratio spectrum was obtained by dividing the resolved laboratory prepared mixtures' first derivative spectra by the first derivative spectrum of PAR' (8.0 µg/mL) standard solution, where, the obtained amplitude in the plateau region (the constant) at 319.5–323.0 nm was recorded and multiplied by the divisor PAR' (8.0 µg/mL) to get first order derivative spectrum of PAR with $P_{\max-\min}$ ($P_{236.0\text{ nm}-262.0\text{ nm}}$).

ACE's derivative spectrum could be then obtained through subtracting either the obtained PAR's spectrum from the mixture's first order derivative spectrum or the constant from the obtained ratio spectra then multiplying by PAR' (8.0 µg/mL) to possess $P_{\max-\min}$ ($P_{261.0\text{ nm}-297.0\text{ nm}}$).

Ratio difference (RD): The stored resolved laboratory prepared mixtures' zero order spectra was divided separately by the absorption spectra of standard ACE (15.0 µg/mL) and PAR (8.0 µg/mL). The amplitudes of the ratio spectra were recorded at [249.0 nm and 280.0 nm] for PAR and [249.5 nm and 281.0 nm] for ACE.

Constant center (CC): Using the previously manipulated ratio spectra in RD method. The obtained ratio spectrum of ACE was recorded at [249.5 nm and 281.0 nm]. The postulated amplitude at 281.0 nm was calculated using the corresponding regression equation, then after subtracting the mixtures' postulated amplitudes from their recorded amplitudes a constant value was obtained which was then multiplied by the spectra of 8.0 µg/ml standard PAR to obtain the original zero order absorption spectra of PAR. While the recovered D^0 of ACE in each mixture was obtained using analog procedures using the constructed regression equation of ratio spectra of PAR at [249.0 nm and 280.0 nm] versus 249.0 nm.

Unlimited derivative ratio method (UDD): The resolved zero order of each mixture were divided by the absorption spectra of standard solution of PAR' (8.0 µg/mL) and then the second derivative (DD^2) of the obtained spectra was recorded. The amplitude difference at 216.1 nm and 308.4 nm for ACE after multiplying by F, was calculated ($P_{216.1} - FP_{308.4}$).

3.4.6. Application to pharmaceutical formulation and its accuracy

The proposed methods were applied for the analysis of the pharmaceutical preparation solution previously prepared in 3.3.4. using the procedures mentioned under each method for the laboratory prepared mixtures' analysis for each method; the concentrations of the cited drugs were calculated from the corresponding regression equations. The accuracy of the results was confirmed by standard addition technique using tablets solution spiked by pure ACE and PAR, separately.

4. Results and discussion

Different applied spectrophotometric methods as ratio spectra spectrophotometry, derivative spectrophotometry, and chemometric techniques are found to be fundamental in the quality control laboratories and in regular marketable products' analysis. It was found out that these spectrophotometric methods are superior and advantageous over other analytical employed techniques such as GC-MS, LC-MS and LC-NMR which permanently necessitate former extraction, separation steps and other tiresome set up measures. Moreover, the related techniques employed to complex components always pass through many problems such as high expenses and time consumption. Considering all stated obstacles, the quantitative resolution of the complex mixtures possessing more than two compounds with spectral overlapping by spectrophotometric methods is a motivated issue for the analytical chemistry field. Moreover, the rapid, sensitive and easy application using the prevailed spectrophotometric methods made them very economical for mixtures' analysis.

No stability indicating spectrophotometric methods have been previously reported for the analysis of this proposed co-formulated drug. While only few publications implementing chromatographic methods have been reported for their concurrent analysis in the occurrence of 0.2% diclofenac and 0.5% 4-AP. Meanwhile the aim of this study is to develop stability indicating spectrophotometric methods able to determine the cited drugs with a high efficiency and good selectivity along with a higher existing percent of their degradates which have been summarized in Fig. 2.

4.1. Spectral characteristics and challenges

The absorption spectra of the studied compounds, ACE, PAR and their degradation products; DIC and 4-AP are overlapped in the UV region 200.0–350.0nm. While PAR's degradation product; 4-AP was found to be more extended over the rest of the components at 318.0–327.0 nm. In addition, the very close structure of ACE and DIC provides a very tiny dissimilarity of their zero order spectra with almost the same geometric features but with different absorptivities in the region 200.0–310.0 nm as presented in Fig. 3. For this reason, the determination of the above compounds without any interference from their degradation products wasn't possible by direct measurements of absorbance in the zero-order spectra. The coupling of resolution technique followed by data processing of the resolved less complicated spectra acts as pivotal strategy in spectrophotometric technique to analyze complex mixtures. Thus, the first derivative spectra of these compounds show that 4-AP has more extension region over the other components which augment its elimination via derivative subtraction technique. Resolution efficiency of the conventional, recently developed methods and novel unlimited derivative ratio method (UDD) was tested for simultaneous determination of these compounds in the same mixture.

4.2. Methods development

Mathematical derivatization of the recorded UV spectra leads to several maxima and minima and the obtained derivative curves of the studied drugs and their degradation products are more extended than the recorded zero order absorption spectrum, thus providing a wide extension between 4-AP and other spectra. The overlapped spectra can be

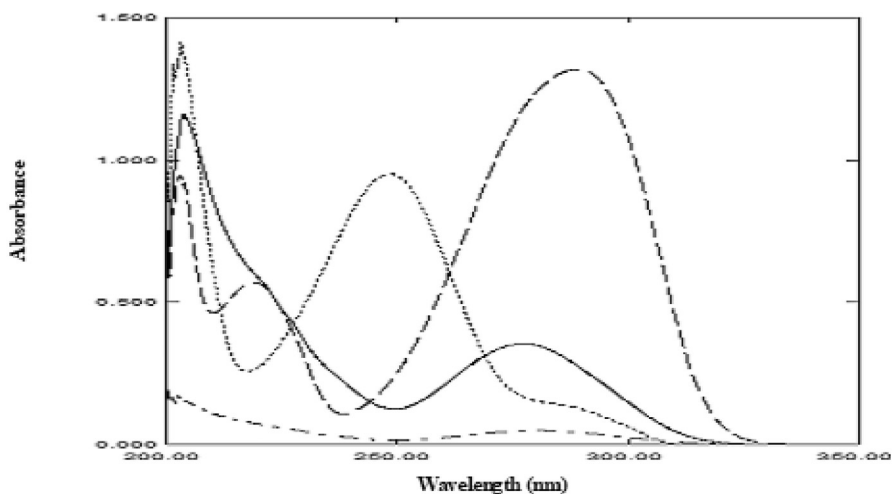


Fig. 3. Zero-order spectra of 10.0 µg/mL of ACE (—) PAR (....), and 4-aminophenol (---) and 1.2 µg/mL of DIC (-.-.-), separately in methanol.

resolved via elimination of 4-AP followed by the quantification of cited active ingredients using suitable recommended methods.

4.2.1. Elimination of 4-AP to get the resolved mixtures' spectra of ACE, PAR and DIC

For each prepared mixture, the derivative of the scanned spectrum was divided by normalized 4-AP standard solution's derivative spectrum (obtained via division of the derivative spectrum by its labelled concentration). The recorded constant obtained from the plateau region at 324.0–325.0 nm represents its concentration directly without the need of any equation according to concentration value method [62, 63]. This recorded concentration of the degradation product of PAR could be used as a confirmatory index for the obtained concentration of the estimated PAR in each mixture since both concentrations were complementary to each other.

This recorded constant value could be also multiplied by the divisor's zero spectrum (D^0) of normalized 4-AP to obtain the recovered zero order absorption spectrum of 4-AP.

Normalized spectrum of 4-AP was found to be the most suitable divisor for the method; thus the results are not affected by the choice of divisor and it gave large constant value even in presence of low concentration of 4-AP.

The resolved D^0 of each mixture of ACE, DIC and PAR shown in Fig. 4 can be obtained via subtracting the recovered D^0 spectrum of 4-AP (DEG_{PAR}) from its corresponding mixture then successively analyze the

resolved mixture by other methods.

The two cited drugs ACE and PAR could be simultaneously determined in the absence of DEG_{PAR} by dual wavelength, induced dual wavelength, derivative subtraction coupled with constant multiplication, ratio difference and constant center method. By substitution in the proposed regression equation of each method listed in Table 1, the concentrations of ACE and PAR are calculated.

4.3. Dual wavelength method (DW)

The dual wavelength spectrophotometric method was applied for the analysis of PAR in the resolved mixtures of cited drugs in presence of DIC by selecting two wavelengths (248.5 nm and 302.0 nm) on the resolved zero order spectrum (D^0) of mixture at which the difference in absorbance is zero for both ACE and DIC (Fig. 5). The difference in absorbance at the selected pair of wavelengths was calculated and plotted against the corresponding PAR's concentrations then its concentration could be determined via substitution in the proposed regression equation shown in Table 1. This method isn't applicable for determination of ACE in these mixtures with PAR due to lack of two wavelengths having equal absorbance values for PAR.

4.4. Induced dual wavelength method (IDW)

The aim of developing this method was to enhance the specificity of

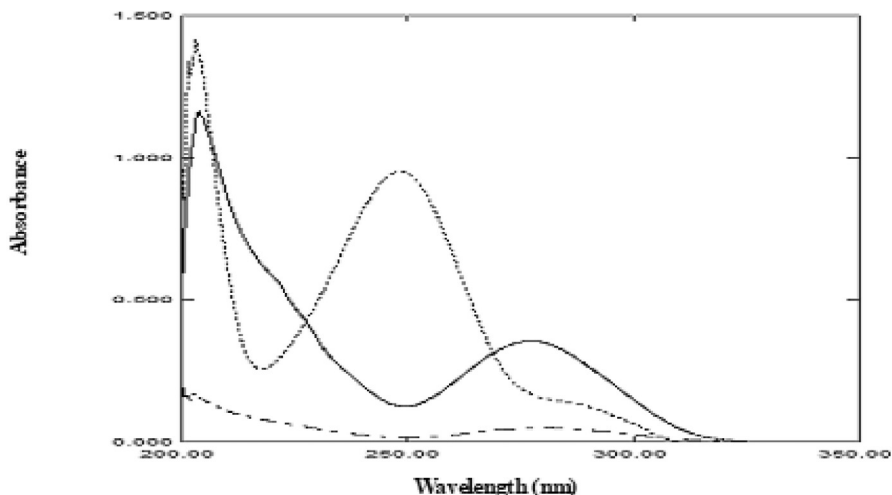


Fig. 4. Resolved zero-order spectra of 10.0 µg/mL of ACE (—) PAR (....) and 1.2 µg/mL of DIC (-.-.-), separately in methanol.

Table 1

Assay parameters and results of determination of pure samples of ACE and PAR by the proposed methods.

	ACE					PAR			
	RD	CC	IDW	DS-CM	UDD	RD	CC	DW	DS-CM
Linearity (µg/ml)	3.0–30.0	3.0–30.0	3.0–30.0	3.0–30.0	3.0–30.0	1.0–14.0	1.0–14.0	1.0–14.0	1.0–14.0
Slope	0.232	0.0365	0.0345	0.0025	0.0365	0.4089	0.0946	0.09	0.0076
Intercept	0.017	0.0095	-0.0042	0.0003	0.238	0.0614	0.016	0.0101	0.0006
Correlation coefficient (r)	0.9999	0.9997	1.0000	1.0000	0.9998	0.9997	0.9997	0.9997	0.9998
Accuracy(Mean ± SD)	100.23 ± 1.43	99.91 ± 0.17	100.41 ± 1.38	99.88 ± 1.28	100.56 ± 0.92	101.42 ± 0.52	99.74 ± 0.26	101.54 ± 0.28	100.16 ± 1.20
*RSD% ^a	1.383	0.158	1.257	1.447	1.232	0.666	0.081	0.979	0.284
**RSD% ^b	1.410	0.250	1.521	1.232	1.405	0.684	0.051	0.948	0.289

* RSD%^a, **RSD%^b: the intra-day & inter-day respectively (n = 3) relative standard deviation of concentrations (9.0, 15.0, 24.0 µg/mL for ACE and 6.0, 10.0, 12.0 µg/mL for PAR).

ACE compared to the conventional DW method which is suitable only for determining PAR. Two wavelengths, 248.5 nm and 277.4 nm were selected on ACE absorption spectrum. The equality factor (F) was calculated from the spectrum of pure PAR at two selected wavelengths ($F = [A_{248.5}/A_{277.4}] = 0.185$). This factor succeeded to equalize the

absorbance of the interfering substance (PAR) at the two selected wavelengths, while the absorbance of ACE was different. The ΔA of the mixture's zero order spectra at 248.5 nm and 277.4 nm, after multiplying the later by F was calculated and was related to ACE, while PAR was eliminated, as shown in (Fig. 6). By substitution in the specified

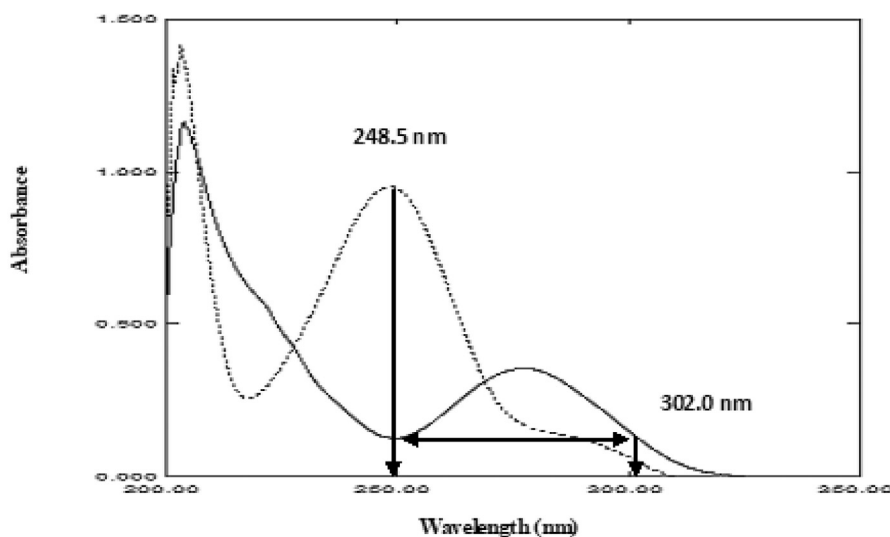


Fig. 5. Zero-order spectra of 10.0 µg/mL of ACE (—) and PAR (....), separately in methanol.

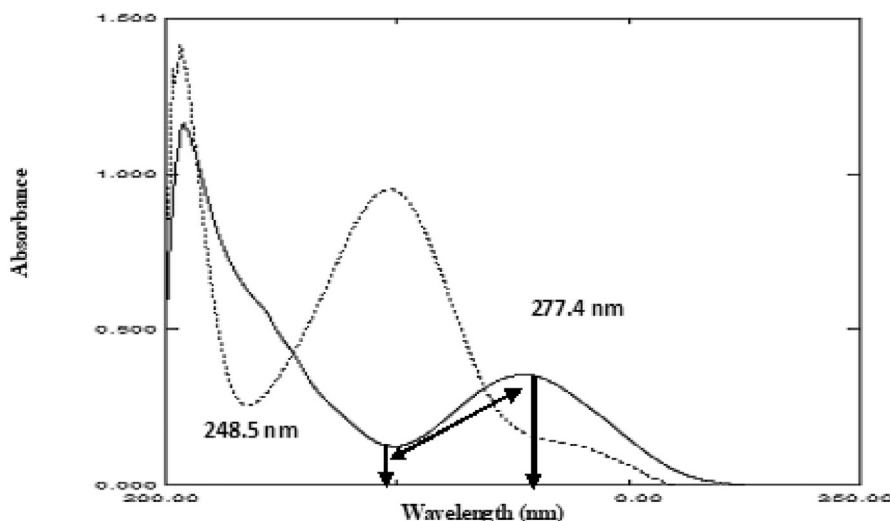


Fig. 6. Zero-order spectra of 10.0 µg/mL of ACE (—) and PAR (....), separately in methanol.

regression equation shown in Table 1, the concentration of ACE was calculated.

4.5. Derivative subtraction method (DS) coupled with constant multiplication (CM)

This method has been applied at first order derivative spectrum of the resolved mixtures. DS-CM has the advantage of being more selective as the extended part of PAR in the mixture becomes more obvious within a wavelength range of 319.5–323.0 nm shown in Fig. 7 and the concentration of the extended component is calculated using $P_{\text{maxima-minima}}$ of first derivative spectra, thus decreasing the error and increasing the sensitivity of the method. ACE's concentration was calculated by substitution in its regression equation which represents a linear relationship between the peak amplitudes at 261.0 nm and 297.0 nm ($P_{261.0-297.0}$) and ACE's corresponding concentrations, Table 1. While, the concentration of PAR was calculated by substitution in its regression equation representing the linear relationship between peak amplitudes at 236.0 nm and 262.0 nm ($P_{236.0-262.0}$) against PAR's corresponding concentrations,

Table 1.

4.6. Ratio difference method (RD)

Ratio difference method (RD) was applied for the analysis of mixtures of ACE and PAR in presence of official impurity of PAR (4-AP) only. This method is simple, accurate, economic and time saving with few applied manipulation steps. RD method is efficient in solving the completely overlapped spectra without former separation; meanwhile it does not require any complicated apparatus or particular computer programs. The chief principle of the RD spectrophotometry is that the mixture's absorption spectrum was obtained and divided by the absorption spectrum of the standard solution of one of the components obtaining its corresponding ratio spectrum. Using the difference between two selected wavelengths cancelling the interfering substance thus consequently showing no interference. The amplitude difference value will be corresponding to the drug of interest.

The selected divisors; PAR (8.0 $\mu\text{g/mL}$) and ACE (15.0 $\mu\text{g/mL}$) compromise between minimal noise and maximum sensitivity. Two

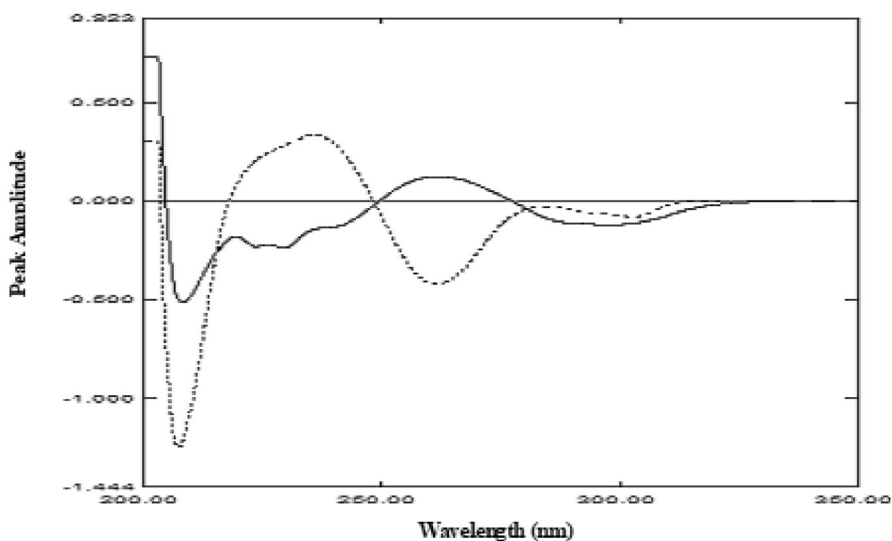


Fig. 7. First derivative (D^1) spectra of 10.0 $\mu\text{g/mL}$ of ACE (—) and PAR (....), separately in methanol.

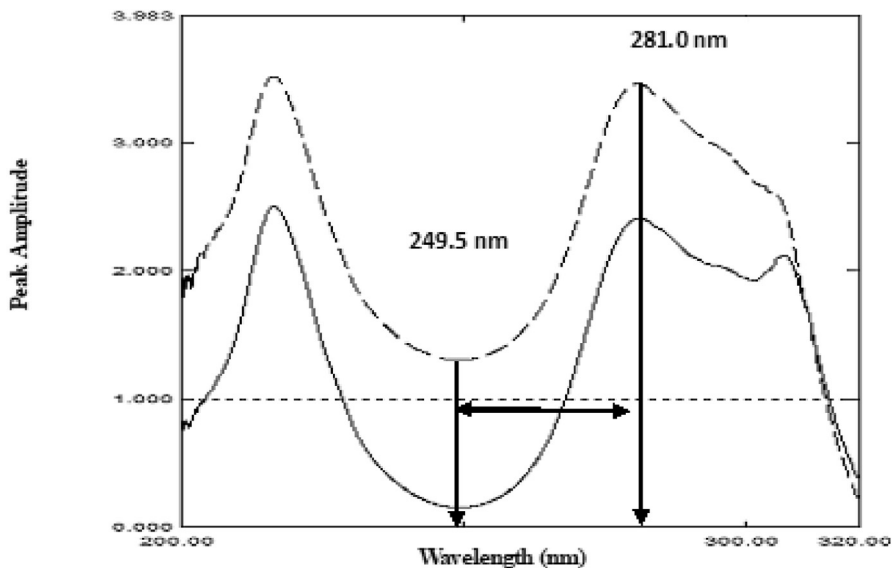


Fig. 8. Ratio spectra of 10.0 $\mu\text{g/mL}$ of ACE (—) and PAR (....) and their binary mixture (---), separately in methanol, using spectrum of PAR (8.0 $\mu\text{g/mL}$) as a divisor showing the two selected wavelengths (249.5 nm and 281.0 nm).

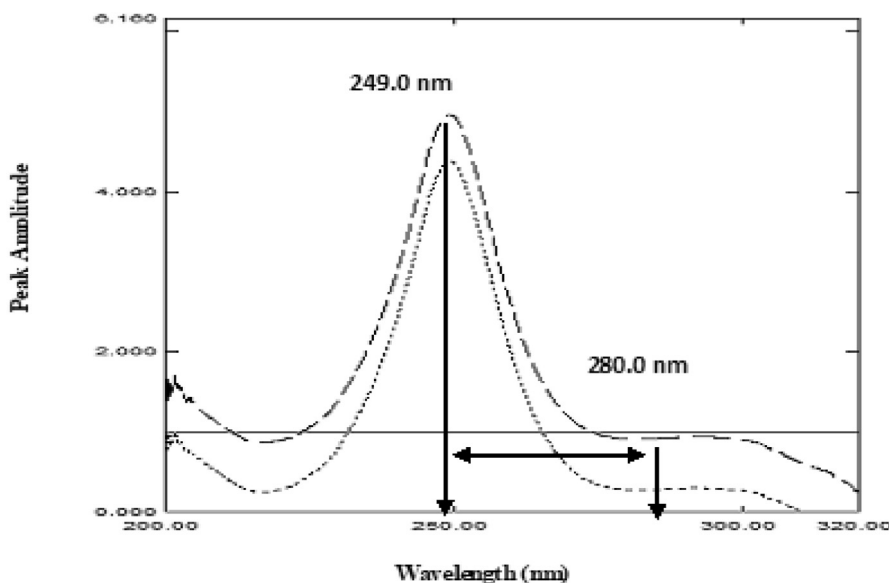


Fig. 9. Ratio spectra of 10.0 µg/mL of ACE (—) and PAR (....) and their binary mixture (---), separately in methanol, using spectrum of ACE (15.0 µg/mL) as a divisor showing the two selected wavelengths (249.0 nm and 280.0 nm).

points are selected on the ratio spectrum of a mixture at which the difference in amplitude is significant for the component of interest and eliminating the contribution of the interfering component. Two pairs of wavelengths, 249.5 nm and 281.0 nm were selected for ACE determination in the mixture using PAR (8.0 µg/mL) as a divisor (Fig. 8). Similarly, the amplitudes at 249.0 nm and 280.0 nm were selected for PAR estimation in the mixture using ACE (15.0 µg/mL) as a divisor (Fig. 9). Calculation of the concentration of the cited drugs was achieved by substituting in their respective regression equations presented in Table 1.

4.7. Constant center method (CC)

This method was applied on the ratio spectra using the drug of

interest as a divisor. Two pairs of wavelengths were selected. Thus, for ACE, the absorption spectrum of ACE' (15.0 µg/mL) was used as a divisor using 249.0 nm and 280.0 nm. By substituting in the equation representing the linear relationship between the ratio difference of ratio spectra at 249.0 nm and 280.0 nm versus the corresponding amplitudes at 249.0 nm for different concentrations of PAR, the postulated ratio amplitude value of (PAR/ACE') was obtained.

$$\Delta P = 0.938P_1 - 0.0073 \quad (r^2 = 1) \tag{11}$$

where; ΔP is the difference between the amplitudes at 249.0 nm and 280.0 nm of the ratio spectra of different concentrations of PAR (1.0–14.0 µg/mL) using 15.0 µg/mL ACE as a divisor.

The constant value (C.V.) was calculated by measuring the difference

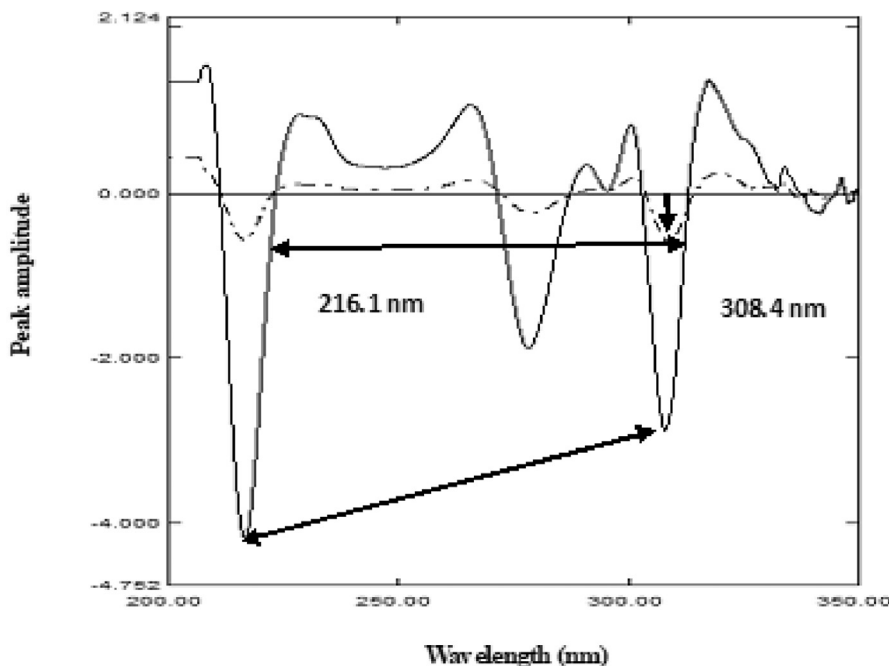


Fig. 10. ²DDspectra of 10.0 µg/mL of ACE (—) PAR (....) and 1.2 µg/mL of DIC (-.-.-), separately in methanol using spectrum of PAR (8.0 µg/mL) as a divisor showing the two selected wavelengths (216.1 nm and 308.4 nm).

between the recorded and postulated amplitudes at 249.0 nm; $[P_{\text{recorded}}] - [P_{\text{postulated}}]$.

Where; P_{recorded} is the recorded amplitude of the ratio spectrum of the laboratory prepared mixture using 15.0 $\mu\text{g/mL}$ ACE' as a divisor at 249.0 nm and $P_{\text{postulated}}$ is the calculated amplitude using the specified regression equation.

The recovered zero order absorption spectrum (D^0) of ACE in the mixture could be obtained by multiplying the obtained constant (ACE/ACE') of each mixture by ACE' (the divisor), which is used for direct determination of ACE at its maxima 277.2 nm, Table 1.

Determination of PAR was done by performing the same previous steps but by using a spectrum of 8.0 $\mu\text{g/mL}$ PAR as a divisor at (249.5 nm and 281.0 nm) versus 281.0 nm to calculate the constant value of PAR via amplitude difference.

$$\Delta P = 0.9334P_1 + 0.0029 \quad (r^2 = 1) \quad (12)$$

where; ΔP is the difference between the amplitudes at 281.0 nm and 249.5 nm of the ratio spectra of different concentrations of ACE (3.0–30.0 $\mu\text{g/mL}$) using 8.0 $\mu\text{g/mL}$ PAR as a divisor.

The recovered zero order absorption spectrum (D^0) of PAR in the mixture could be then determined at 248.6 nm, Table 1.

4.8. Unlimited derivative ratio method (UDD)

This is a novel approach for the analysis of challengeable ternary mixtures or resolved ternary mixture (quaternary mixture after eliminating the extended component). The target of developing this method was to enhance the specificity of the quantification of ACE in presence of its degradation products (DIC) relative to the conventional derivative ratio method which isn't suitable for determination of ACE in presence of PAR and DIC by using PAR (8.0 $\mu\text{g/mL}$) as a divisor. Apply derivatization using optimum studied parameters those second derivative ratio, $\Delta\lambda = 8$ nm and scaling factor = 10 two wavelengths, at 216.1 nm and 308.4 nm and found to be equal 1.02; ($F = [P_{216.1}/P_{308.4}] = 1.02$). This factor succeeded to equalize the amplitude of the degradation product (DIC) at the two selected wavelengths, while the amplitude of ACE was different. The ΔP of the mixture's second order derivative spectra at 216.1 nm and 308.4 nm, after multiplying the later by F was calculated and was related to DIC, while PAR was eliminated since it is a constant. By substitution in the specified regression equation shown in Table 1, the concentration of ACE was calculated.

For all the following measurements, spectra of 8.0 $\mu\text{g/mL}$ PAR were used as standard divisor. These guaranteed the best concession in terms of sensitivity, repeatability and signal to noise ratio. The effect of $\Delta\lambda$ for plotting the derivative of the ratio spectra was tried out to attain the wavelength's best interval. $\Delta\lambda$ value highly affects the shape and position of peaks of the analyzed compound as well as the position of the two equal amplitudes points of the other compound in the mixture. In some cases, this effect is remarkable in that only a single $\Delta\lambda$ value can be successful while others give poor resolution; therefore, we can conclude that the effect of divisor concentration is chiefly a matter of sensitivity, while the effect of $\Delta\lambda$ is chiefly a matter of resolution. In this studied mixture, $\Delta\lambda = 8$ nm was the optimum for all determinations. For each compound, 2 points could be successfully used for the specific determination of ACE where the other compounds showed no interference as shown in Fig. 10.

Using higher derivatives gives a great opportunity for selecting more appropriate wavelengths from a larger range of possibilities; thus, the ^2DD mode was useful for the resolution of this mixture. The influence of the diverse parameters was considered to augment the signal of the derivative ratio spectra; i.e., to provide good selectivity and higher sensitivity in the determination. Testing the effect of the concentration's divisor on the calibration graphs was studied. When the divisor's concentration is decreased or increased, the resulting derivative values are proportionately increased or decreased, respectively, although the

Table 2
Determination of the studied drugs in laboratory prepared mixtures by the proposed methods.

ACE: PAR	Recovery%									
	Concentration ($\mu\text{g/mL}$)					Recovery%				
	ACE	PAR	CC	IDW	DS-CM	UDD	RD	CC	DW	DS-CM
1:3.25*	4.0	13.0	99.10	100.33	100.00	100.10	100.29	100.90	99.40	100.30
1:4	3.0	12.0	99.25	98.00	101.20	99.95	99.42	100.50	99.23	101.25
2:3.25	6.0	9.75	99.66	100.66	98.99	99.05	100.16	101.40	100.35	101.74
1:2	6.0	12.0	100.95	101.00	98.58	100.23	100.35	101.71	100.38	101.25
Mean \pm SD			99.70 \pm 1.38	99.99 \pm 1.35	99.69 \pm 1.16	99.83 \pm 0.53	100.05 \pm 0.43	101.12 \pm 0.53	99.84 \pm 0.61	101.13 \pm 0.60
Acetofenac and paracetamol in presence of their degradation products										
% Degradation	Recovery%									
	ACE:DIC:PAR:4-AP					Paracetamol (PAR)				
%DIC	%4-AP	RD	CC	IDW	DS-CM	UDD	RD	CC	DW	DS-CM
6.25	23	-	-	-	-	100.21	99.00	-	101.75	102.00
12.5	50	-	-	-	-	99.98	98.32	-	101.80	101.99
25	92.3	-	-	-	-	100.02	101.90	-	99.95	98.95
Mean \pm SD		-	-	-	-	100.07 \pm 0.12	99.74 \pm 1.90	-	101.16 \pm 1.05	100.98 \pm 1.75

* Ratio found in pharmaceutical dosage form.

maxima and minima remain at the same wavelengths.

By comparing the developed methods, successive resolution technique by eliminating one or more of the interfering components in the mixtures by the mathematic filtration using the spectrophotometer software has a great power in the analysis of the complicated mixture with overlapped spectra to get more simple resolved mixture which can be successively analyzed by the simple spectrophotometric methods. The efficiency and spectral resolution power of the spectrophotometric methods on zero, derivative, ratio or derivative ratio spectra based on either the response difference between two wavelengths points (directly or induced) or measuring the constant representing the undesirable component followed by further manipulation on the spectrophotometer software to get the proposed component. It was found that PAR in its mixture with ACE was successfully determined by dual wavelength, derivative subtraction coupled with constant multiplication, ratio difference and constant center method. Meanwhile, an effective determination of ACE in these mixtures was performed by induced dual wavelength, derivative subtraction coupled with constant multiplication, ratio difference, constant center and unlimited derivative ratio method where the selectivity of the proposed methods for both cited drugs in the mixtures containing the degradation product of PAR weren't affected by the presence of up to 92.3% 4-AP. In case of mixtures containing the degradation product of ACE, PAR was estimated by all the proposed methods except constant center method while, ACE was determined by the novel unlimited derivative ratio method in presence of up to 25% DIC.

An influence of the overlapping of absorption bands on the precision and accuracy of determinations can be deduced from a comparison of the results for all proposed methods. Markedly the results of induced dual wavelength improved the results of dual wavelength method, which is

the least precise and accurate due to critical measurement of the chosen wavelengths. When overlapping is larger as in case of analysis of the cited drugs in presence of both degradation products, the methods based on the ratio and derivative ratio are more advantageous with the increase in resolution efficiency via division by standard solution of one of interfering component. Smaller overlapping in case of analysis of ACE and PAR in absence of both degradates or in the presence of 4-AP only was found that induced dual wavelength method and constant center method (CC) show poor mathematic filtration efficiency and fail to give satisfactory results due to geometric features similarity of ACE and DIC leading to error in the data processing. The main advantage of CC method in mixture analysis is obtaining the D^0 spectra of each drug separately which acts as spectral profile of ACE and PAR as well as maximum accuracy in their quantification at their λ_{max} .

Based on the achieved results, it can be concluded that derivative subtraction coupled with constant multiplication (DS-CM) increases substantially the precision and accuracy of the analysis of mixtures with overlapping absorption bands as in case of PAR in presence of ACE and DIC as an official degradation product of ACE. The advantages of RD method over the dual wavelength method that the interfering component is constant which is a straight line all over the curve thus the difference at any two wavelengths will be equal to zero thus no critical measurements at certain wavelengths.

When the absorption bands of the individual components overlap to a large extent as in case of ACE and DIC in presence of PAR, the classical methods are not efficient enough and the determined concentrations of components are imprecise and often inaccurate. Better results can be obtained by novel UDD method which is successfully applied for the determination of ACE overcoming the complete overlapping between ACE and its degradation product (DIC). Furthermore, even the

Table 3

Determination of the studied drugs in tablet dosage form by the proposed methods and application of standard addition technique.

Sample	Aceclofenac (*mean% ± SD)				
	RD	CC	IDW	DS-CM	UDD
Hifenac-P® Batch No.737132 Standard addition	90.60 ± 1.69	99.60 ± 0.99	98.70 ± 1.86	106.00 ± 2.22	96.59 ± 1.45
Sample	Paracetamol (*mean ± SD %)				
Method	RD	CC	DW	DS-CM	
Hifenac-P® Batch No.737132 Standard addition	100.81 ± 1.24	100.37 ± 1.20	99.67 ± 1.98	98.43 ± 0.40	98.04 ± 0.99
Sample	Paracetamol (*mean ± SD %)				
Method	RD	CC	DW	DS-CM	
Hifenac-P® Batch No.737132 Standard addition	100.90 ± 1.23	102.00 ± 1.12	101.80 ± 0.99	101.00 ± 1.35	
	98.60 ± 0.98	99.25 ± 0.90	98.23 ± 0.66	99.63 ± 1.09	

* Found% ± SD in case of pharmaceutical preparation and Recovery % ± SD in case of standard addition.

Table 4

Statistical analysis of the proposed methods and the reported method of ACE and PAR in their pure powdered forms.

Parameter	Aceclofenac (ACE)					
	RD	CC	IDW	DS-CM	UDD	Reported method** [29]
Mean	100.01	99.73	99.84	99.67	100.37	99.76
SD	1.16	1.43	0.78	1.54	0.70	1.44
n	6	6	6	6	6	5
Variance	1.3456	2.0449	0.6084	2.3716	0.4900	2.0736
Student's t test* (2.262)	0.759	0.976	0.911	0.924	0.420	
F*	1.54 (5.19)	1.01 (5.19)	3.40 (5.19)	1.14 (5.39)	4.23 (5.19)	
Parameter	Paracetamol (PAR)					
	RD	CC	DW	DS-CM	Reported method** [29]	
Mean	99.32	99.40	99.25	99.95	99.07	
SD	1.84	1.99	2.20	1.88	1.33	
n	5	5	5	5	5	
Variance	3.3856	3.9601	4.8400	3.5344	1.7689	
Student's t test* (2.306)	0.809	0.764	0.880	0.420		
F* (5.53)	1.91	2.23	2.73	1.99		

* The figures in parenthesis are the corresponding theoretical values at P = 0.05.

** The reported method used are the first order derivative spectrophotometry at 243.6 nm for ACE and 273.4 nm for PAR, using methanol as a blank.

Table 5

Results of ANOVA (single factor) for the comparison of the proposed methods and the reported method of ACE and PAR in their pure form.

Aceclofenac						
Source of Variation	SS	df	MS	F	P-value	F crit
Between Groups	2.029367143	5	0.405873	0.274089	0.923577	2.545386
Within Groups	42.94345	29	1.480809			
Total	44.97281714	34				
Paracetamol						
Source of Variation	SS	df	MS	F	P-value	F crit
Between Groups	2.20948	4	0.55237	0.157128	0.95747	2.866081
Within Groups	70.30812	20	3.515406			
Total	72.5176	24				

amplitudes measured at the sides of overlapping obtained bands can be used in the analysis due to great numbers of peaks of derivative of ratio spectrum with high amplitude values and they are measured with sufficient precision.

Validation of the proposed spectrophotometric methods was accomplished according to ICH guidelines [57] with respect to methods' sensitivity, linearity range, accuracy, precision, LOD and LOQ as shown in Table 1. Table 2 shows the specificity results obtained from the analysis of laboratory prepared mixtures containing diverse ratios of the drugs ensuring the specificity of the proposed methods where satisfactory results were obtained over the calibration range. It was found that UDD is the only method proving its ability to assess unequivocally the ACE in the presence of PAR as co-formulated drug and DIC as degradation product. The proposed methods were also functioned for the determination of the drugs in Hifenac-P® tablets and the validity of the proposed methods was been further assessed by applying the standard addition technique as presented in Table 3.

5. Calculation

The results obtained by the proposed methods and the reported spectrophotometric method [29] were statistically compared where, the calculated *t* and *F* values were found to be less than the theoretical ones demonstrating that there was no considerable difference between the proposed and reported method regarding both accuracy and precision, Table 4.

Statistical analysis using one way ANOVA test was also performed on the results obtained by applying the proposed methods and those obtained by the reported method, where calculated *F* (F_{cal}) values were always less than tabulated *F* (F_{tab}) values for both studied analytes proving that there is no significant difference between the proposed methods and the reported one, Table 5.

6. Conclusion

This work has introduced the first quantitative stability indicating spectrophotometric methods for the selective determination of ACE and PAR in synthetic mixtures with their degradation products and pharmaceutical formulation using built-in spectrophotometer software. The UV spectrophotometric methods used in this study are more adaptable and easier to apply than the other analytical methods. The proposed methods did not necessitate any sophisticated instrumentation, such as HPLC, which requires organic solvents and time consumption or advanced methodologies (like chemometric methods). An optimization procedure is applied to choose the optimal analytical manipulation steps and the corresponding response of each method is used for calculation of calibration data or concentrations of components in the samples. The application of the proposed methods confirms that the novel introduced unlimited derivative ratio method has a very high selectivity and acts as rapid, simple stability indicating method with optimum mathematic filtration power to resolve the severe overlapping geometrically similar spectra of ACE and its degradation product in presence of co-formulated

drug PAR.

In conclusion, the mathematic filtration technique for all the proposed methods either separate or after coupling with each other via successive resolution steps are suitable and valid for the analysis of multi-component mixtures in laboratories lacking liquid chromatographic instruments.

Declarations

Author contribution statement

Mohamed Abd El-Rahman, Soheir Weshahy: Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data.

Hayam Lotfy: Conceived and designed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data.

Dina Ahmed: Performed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data; Wrote the paper.

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Competing interest statement

The authors declare no conflict of interest.

Additional information

No additional information is available for this paper.

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