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Determination of two ternary mixtures containing phenobarbitone by second derivative of the ratio spectrum-zero-crossing and HPLC methods

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Abstract

A spectrophotometric method is developed for the determination of ternary mixtures with overlapping spectra. The method is based on the use of the second derivative of the ratio spectrum with a zero-crossing technique. The ratio spectrum was obtained by dividing the absorption spectrum of the mixture by that of one of the components. The concentration of the other components are then determined from their respective calibration graphs treated similarly. The method is accurate, non-destructive and do not require resolutions of equations. The method has been applied for the resolution of two ternary mixtures, namely, phenobarbitone, methylphenobarbitone and phenytoin (1), and phenobarbitone, papaverine HCl and piperazine acefyllinate (2). Also, a HPLC method was developed for determination of phenobarbitone, papaverine and HCL and piperazine acefyllinate. The HPLC method depends upon using ODS column with a mobile phase consisting of acetonitrile–5 mM aqueous heptane sulfonic acid sodium salt (50:50, v/v) and adjusted to apparent pH 4 using acetic acid. Quantitation was achieved with UV detection at 220 nm based on peak area. The proposed methods were applied for the determination of the two ternary combinations in synthetic mixtures and in commercial pharmaceutical products. The results obtained were precise and accurate.

Keywords: Second derivative of the ratio spectrum; LC; Phenobarbitone; Ternary mixture

1. Introduction

The analysis of drug mixtures without separation of the constituents is a rather difficult task. Derivative spectrophotometry has been used for the analysis of binary mixtures of compound with overlapping spectra by zero-crossing measurement [1,2]. However, sometimes the derivative technique cannot cope with the level of interference specially when the spectra are strongly overlapped or in case of ternary mixtures [3]. Salinas et al. [4] introduced a spectrophotometric method for resolving binary mixtures. The method is based on the use of the first derivative of the ratio spectra. The absorption spectrum of the mixture is recorded and divided (amplitudes at each wavelength) by the absorption spectrum of a standard solution of one of the components, then the first derivative of the ratio spectra is obtained. The concentration of another component is then determined from a calibration graph. Berzas Nevado et al. [5] developed a method and discussed the theory for resolving ternary mixtures based on the use of first derivative of the ratio spectra of mixtures, followed by measurements at the zero-crossing wavelength of first derivative of ratio spectra of single components. The above method is used for simultaneous determination of salicylaldehyde, 3-hydroxy benzaldehyde and 4-hydroxy benzaldehyde [5]; penicillin-G sodium, penicillin-G procain and dihydrostreptomycin sulfate [6]; dipyridamole, aspirin and salicylic acid; dipyridamole, oxazepam and 2- amino-5-chlorobenzophenone [7]; atenolol and nifedipine in presence of degradation products of nifedipine [3].

As a further development of our researches for the determination of drug mixtures by derivative spectrophotometry, [8–13], in this paper we present the

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application of the second derivative of the ratio spectrum-zero-crossing spectrophotometry (^{2}DD) to the determination of two ternary mixtures, namely, phenobarbitone (PH), methylphenobarbitone (MP) and phenytoin (PT) (mixture 1), and PH, papaverine HCl (PP) and piperazine acefyllinate (PA) (mixture 2).

The combination of PH, MP and PT as a tablet preparation is used for controlling of different types of epilepsy. Various spectrophotometric methods have been reported for the determination of this ternary mixture, including partial least-squares, principal component regression [14], least-squares and delta absorbance with Vierordt's method [15] ^{1}H NMR has been used for determination of PH and MP while PT was determine by a spectrophotometric procedure after oxidation with alkaline $KMnO_4$ [16]. The chromatographic techniques of analysis, TLC–densitometry [16] and HPLC [17,18] have been employed for the determination of this combination.

The other combination of PH, PP and PA as a suppository preparation is used for treating of bronchial asthma and chronic bronchitis. Two methods have been reported for the simultaneous determination of this ternary mixture using direct spectrophotometry after separation procedure [19] and first derivative spectrophotometry with solving of three simultaneous equations [20].

The primary aim of this paper is to verify the ratio spectra zero-crossing first [3,5–7] and third[6] derivative methods could be successfully extended to second derivative. The another aim is to develop of a spectrophotometric method for simultaneous determination of the two studied ternary mixtures without separation of their constituents or solving of simultaneous equations. The proposed spectrophotometric method is fast and economical in comparison to the more time-consuming HPLC method. The proposed spectrophotometric method has been validated to be precise and accurate, and is demonstrated to be excellent alternative to HPLC method for the determination of the constituents of the two studied ternary mixtures.

2. Experimental

2.1. Instrumentation

A double-beam shimadzu (Japan) UV–Visible spectrophotometer, model UV-1601 PC connected to an IBM compatible computer and a HP 600 inkjet printer was used. The bundled software was UVPC personal spectroscopy software version 3.7 (Shimadzu). The spectral bandwidth was 2 nm and the wavelength scanning speed was 2800 nm/min. The absorption spectra of test and reference solutions were recorded in 1-cm quartz cell over the range 200–280 and 200–380 nm for mixture (1) and (2), respectively. The second derivative of the ratio spectra was obtained using the accompanying software with $\Delta\lambda = 4$ nm and scaling factor of 20.

The HPLC (Bischoff, Germany) instrument was equipped with model series 2250 LC pump, Rheodyne 7125 injector with a 20 μl loop and a LC lambda1010 variable-wavelength spectrophotometric detector (Bischoff). Separation and quantitation were made on a 150×4.6 mm (i.d.) TSK-Gel 5 μm ODS-80 TM column (Tosoh, Japan).The detector was set at 220 nm for mixture (2). Data acquisition was performed on a model MCDACq data acquisition system (version 1.3x) and Hewlett Packed laser Jet 6L printer.

2.2. Materials and reagents

Pharmaceutical grade of PH, MP, PT, PP and PA were used and certified to contain 99.9, 99.7, 99.8, 99.7, 99.8%, respectively. The water for HPLC was prepared by double glass distillation and filteration through 0.45 μm membrane filter. Acetonitrile used was HPLC grade (BDH, Poole, UK). Heptane sulfonic acid sodium salt, hydrochloric acid, acetic acid, methanol, ethanol, borax and boric acid used were analytical grade.

Borax buffer (pH 9.2) was prepared by dissolving 19.07 gm of borax in 1 l of distilled water and the pH was adjusted to 9.2 ± 0.1 using 0.1 M boric acid solution.

One of the pharmaceutical combination of PH, MP and PT {Comital-L tablet, Batch No. 7408028} was manufactured by Alexandria pharmaceutical company under license from Bayer (Leverkusen, Germany). Each tablet contains 50 mg of each of PH, MP and PT. The other pharmaceutical combination of PH, PP and PA {Etaphylline phenobarbital papaverine suppositories, Batch No. 800159} was manufactured by Memphis company for pharmaceuticals and chemical industries (Cairo, Egypt). Each suppository contains 100 mg of PH, 100 mg of PP and 400 mg of PA.

2.3. HPLC conditions

The mobile phase for mixture (2) was prepared by mixing acetonitrile and 5 mM heptane sulfonic acid sodium salt in a ratio of 50:50 v/v and the apparent pH was adjusted to 4 using acetic acid. The mobile phase was filtered using 0.45 μm membrane filter (Millipore, Milford, MA) and degassed by vacuum prior to use. The samples were also filtered using 0.45 μm disposable filters. The flow rate was 1.5 ml/min. All determinations were performed at ambient temperature. The injection volume was 20 μl.

2.4. Standard solutions and calibration

2.4.1. For mixture (1)

Stock standard solutions of each PH, MP and PT were prepared separately by dissolving 30 mg of each drug in 100 ml methanol. The standard solutions were prepared by transferring various aliquots of each stock standard in the range of 1–3 ml into three sets of 50 ml volumetric flasks and the solutions were brought to constant volume (3 ml) with methanol and then completed to 50 ml with borax buffer to reach the concentration range of 6–18 μg/ml for each drug.

2.4.1.1. For determination of PH and PT. The UV absorption spectra of standard solutions of each PH and PT were divided by a normalized spectrum of MP (a spectrum of unit concentration). The second derivative was calculated for the obtained ratio spectra with $\Delta\lambda = 4$ nm. The second derivative of the ratio spectra obtained was smoothed with 8 experimental points and scaling factor of 20. The amplitudes at 231 and 223 nm were measured and found to be proportional to the concentration of PH and PT, respectively.

2.4.1.2. For determination of MP. The UV absorption spectra of standard solutions of MP were divided by a normalized spectrum of PH (a spectrum of unit concentration). The second derivative was calculated for the obtained ratio spectra with $\Delta\lambda = 4$ nm. The second derivative of the ratio spectra obtained was smoothed with 8 experimental points and scaling factor of 20. The amplitudes at 226.2 nm were measured and found to be proportional to the concentration of MP.

2.4.2. For mixture (2)

Stock standard solution of each PH, PP and PA were prepared separately by dissolving 20 mg of PH, 20 mg of PP and 80 mg of PA in 100 ml ethanol. The standard solutions were prepared by transferring various aliquots of each stock standard in the range of 1–2.5 ml into three sets of 100 ml volumetric flasks and the solutions were brought to constant volume (2.5 ml) with ethanol and then were completed to 100 ml with 0.1 N hydrochloric acid (for ^{2}DD method) or mobile phase (for HPLC method) to reach the concentration range of 2–5 μg/ml for PH and PP, and 8–20 μg/ml for PA.

2.4.3. For ^{2}DD method

2.4.3.1. For determination of PH and PP. The UV absorption spectra of standard solutions of each of PH and PP were divided by a normalized spectrum of PA)(a spectrum of unit concentration). The second derivative was calculated for the obtained ratio spectra with $\Delta\lambda = 4$ nm. The second derivative of the ratio spectra obtained was smoothed with 8 experimental points and scaling factor of 20. The amplitudes at 223 and 248 nm were measured and found to be proportional to the concentration of PH and PP, respectively.

2.4.3.2. For determination of PA. The UV absorption spectra of standard solutions of PA were divided by a normalized spectrum of PP. The second derivative was calculated for the obtained ratio spectra with $\Delta\lambda = 4$ nm. The second derivative of the ratio spectra obtained was smoothed with 8 experimental points and scaling factor of 20. The amplitudes at 272.2 nm were measured and found to be proportional to the concentration PA.

2.4.4. For HPLC method

Triplicate 20 μl injections were made for each concentration and chromatographed under the specified chromatographic conditions described previously. The peak area values were plotted against corresponding concentrations. Linear relationship was obtained.

2.5. Sample preparation

2.5.1. For mixture (1) tablet

Twenty tablets were weighed and finally powdered. A portion of the powder equivalent to about 30 mg of each of PH, MP and PT was accurately weighed and stirred for 30 min with 100 ml methanol. The methanolic extract was filtered. Further dilutions of the filterate were carried out with borax buffer to reach the calibration range. The general procedures described under calibration were followed and the concentration of PH, MP and PT were calculated.

2.5.2. For mixture (2) suppository

Twenty suppositories were accurately weighed and cut into small pieces. The suppositories mass was then transferred to a porcelain dish and melted on a hot water bath until complete homogeneity. An amount of the suppository mass equivalent to 20 mg of PH, 20 mg of PP and 80 mg of PA was dissolved in 100 ml hot ethanol by stirring for 30 min at 70 °C. The sample solution was cooled and filtered. Further dilutions of the filterate were made with 0.1 N hydrochloric acid (for ^{2}DD method) or mobile phase (for HPLC method) to reach the linearity range specified for each method. The general procedures for ^{2}DD and HPLC methods described under calibration were followed and the concentration of PH, PP and PA were calculated.

3. Results and discussion

3.1. For mixture (1)

The UV absorption spectra of PH, MP and PT in borax buffer pH 9.2 were produced in Fig. 1. The

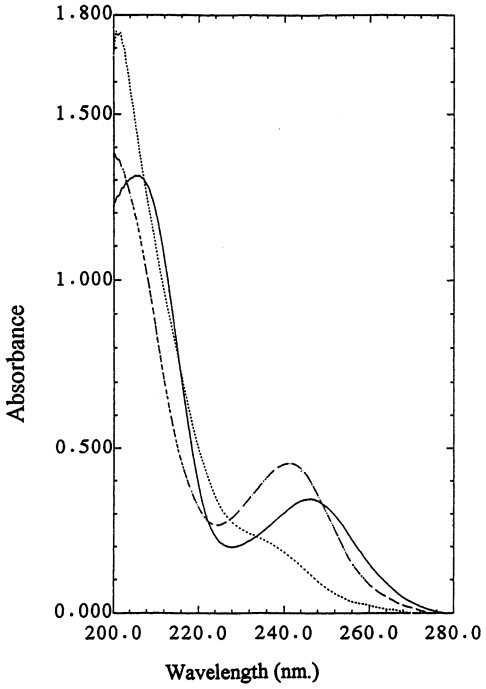

Fig. 1. UV absorption spectra of 10 μg/ml of each of PH (– – –), MP (—) and PT (--------) in borax buffer pH 9.2.

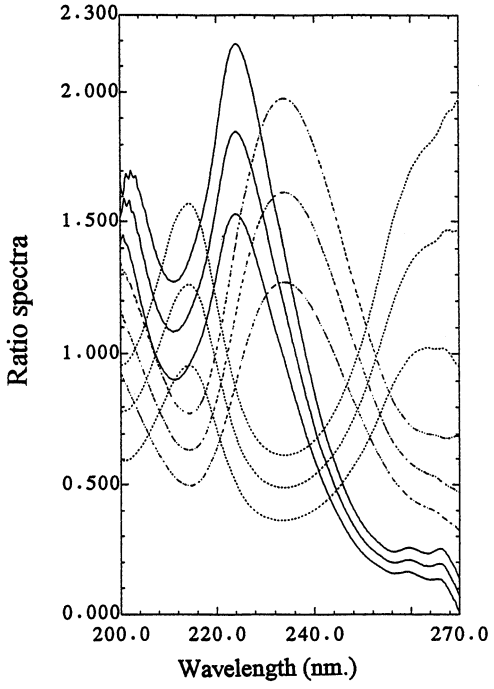

Fig. 2. Ratio spectra for different concentrations of PH (– – –)) (8, 10, 12 μg/ml) using normalized spectrum of MP as divisor, different concentrations of MP (-------)(6, 8, 10 μg/ml) using normalized spectrum of PH as divisor and different concentrations of PT (—) (10, 12, 14 μg/ml) using normalized spectrum of MP as divisor.

spectra of the three drugs display considerable overlap, that the application of the conventional spectrophotometry and the derivative technique failed to resolve it. However, this spectral overlapping was sufficiently enough to demonstrate the resolving power of the ^{2}DD method. The second derivative of the ratio spectra was preferred than the first derivative for a better resolution of the ratio spectra and more accurate and precise results. Three series of ratio spectra of PH/MP° and MP/PH° and PT/MP° (where PH° and MP° are normalized spectrum of PH and MP, respectively) are reported in Fig. 2.

3.1.1. For the determination of PH and PT

The UV absorption spectra of standard solutions of each of PH and PT or ternary mixture (1) were divided by a normalized spectrum [21] of MP (obtained by dividing the spectra of several standards of different concentrations by their corresponding concentrations and subsequently averaging them, in order to obtain a spectrum of unit concentration). The second derivative was calculated for the obtained ratio spectra as previously described in experimental (Fig. 3). The second derivative of the ratio spectrum obtained for the ternary mixture (1) consists only of PH and PT as the corresponding values of MP are equal to zero. Applying the zero-crossing method, PH can be determined in ternary mixture (1) by measuring the amplitude at 231 nm (zero-crossing point of PT), while PT can be determined by measuring the amplitude at 223 nm (zero-crossing point of PH) on using a normalized

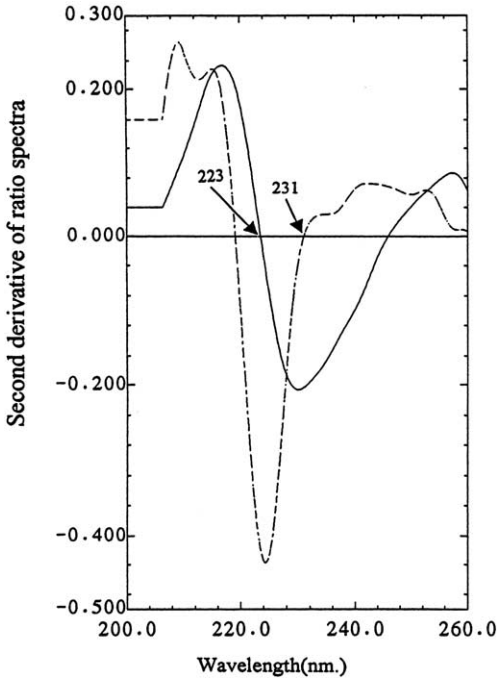

Fig. 3. Second derivative of the ratio spectra of 10 μg/ml of each of PT (– – –) and PH (—) using normalized spectrum of MP as divisor.

spectrum of MP as a divisor {the arrows indicate the zero-crossing wavelength for determination of PH and PT in ternary mixture (1)}.

On another hand, for the determination of MP, an analogous procedure was followed. The UV absorption of standard solutions of MP or ternary mixture (1) were divided by a normalized spectrum of PH. The second derivative was calculated for the obtained ratio spectra as previously described in experimental (Fig. 4). The second derivative of the ratio spectrum obtained for the ternary mixture (1) consists only of MP and PT as the corresponding values of PH are equal to zero. Applying the zero-crossing method, MP can be determined in ternary mixture (1) by measuring the amplitude at 226.2 nm (zero-crossing point of PT) without any contribution from PH or PT, on using a normalized spectrum of PH as a divisor {the arrow indicate the zero-crossing wavelength for determination of MP in ternary mixture (1)}.

The zero-crossing wavelengths which chosen exhibited the best linear response to the analyte concentration and were not affected by any other component (Fig. 5). The characteristic parameters for regression equations of the ^{2}DD methods are given in Table 1.

3.2. For mixture (2)

3.2.1. ^{2}DD method

The UV absorption spectra of PH, PP and PA in 0.1 N hydrochloric acid were overlapped (Fig. 6), that the application of the conventional spectrophotometry and its direct derivative technique was unable to resolve this interference. The use of zero-crossing technique and the second derivative of the ratio spectra helps greatly in the resolution of such ternary mixture. Three series of ratio spectra of PH/PA° and PP/PA° and PA/PP° (where PP° and PA° are normalized spectrum of PP and PA, respectively) are reported in Fig. 7.

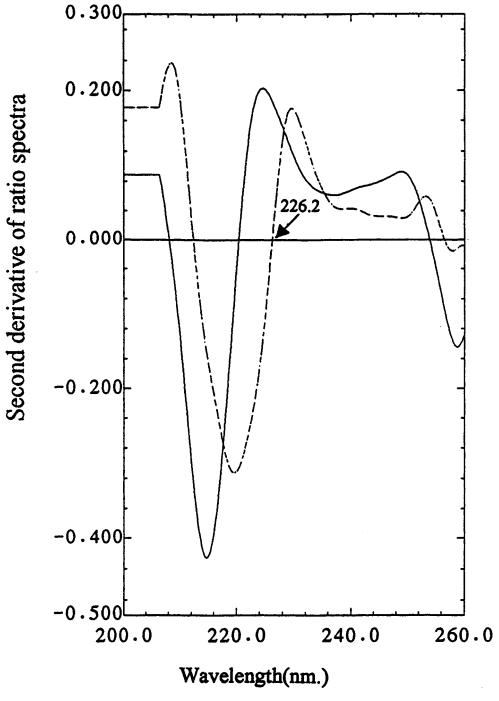

Fig. 4. Second derivative of the ratio spectra of 10 μg/ml of each of PT (– -- –) and MP (—) using normalized spectrum of PH as divisor.

3.2.1.1. For determination of PH and PP. The UV absorption spectra of standard solutions of each of PH and PP or ternary mixture (2) were divided by a normalized spectrum of PA. The second derivative was calculated for the obtained ratio spectra as previously described in experimental (Fig. 8). The second derivative of the ratio spectrum obtained for the ternary mixture (2) consists only of PH and PP as the corresponding values of PA are equal to zero. Applying the zero-crossing method, PH can be determined in ternary mixture (2) by measuring the amplitude at 223 nm (zero-crossing point of PP), while PP can be determined by measuring the amplitude at 248 nm (zero-crossing point of PH) on using a normalized spectrum of PA as a divisor.

On the another hand, for the determination of PA, an analogous procedure was followed. The UV absorption of standard solutions of PA or ternary mixture (2) were divided by a normalized spectrum of PP. The second derivative was calculated for the obtained ratio spectra as previously described in experimental (Fig. 9). The second derivative of the ratio spectrum obtained for the ternary mixture (2) consists of only PH and PA as the corresponding values of PP are equal to zero. Applying the zero-crossing method, PA can be determined in ternary mixture (2) by measuring the amplitude at 272.2 nm (zero-crossing point of PH) without any contribution from PH or PP, on using a normalized spectrum of PP as a divisor. The zero-crossing wavelengths which chosen exhibited the best linear response to the analyte concentration and were not affected by any other components (Fig. 10). The characteristic parameters for regression equations and correlation coefficients are given in Table 2.

3.2.2. HPLC method

The developed HPLC method has been applied for the simultaneous determination of PH, PP and PA. To optimize the HPLC assay parameters, the mobile phase composition and pH were studied. A satisfactory separation was obtained with a mobile phase consisting of acetonitrile–5 mM heptane sulfonic acid sodium salt (50:50, v/v). Increasing acetonitrile concentration to more than 70% led to inadequate separation of the three drugs. At lower acetonitrile concentration ($<$ 30%) separation occurred but with excessive tailing for PH and PP peaks. Variation of apparent pH of the mobile phase resulted in maximum capacity factor (K′) value at apparent pH 7, with losses of peak symmetry for PP. At apparent pH 3.5–4.5 improved resolution for

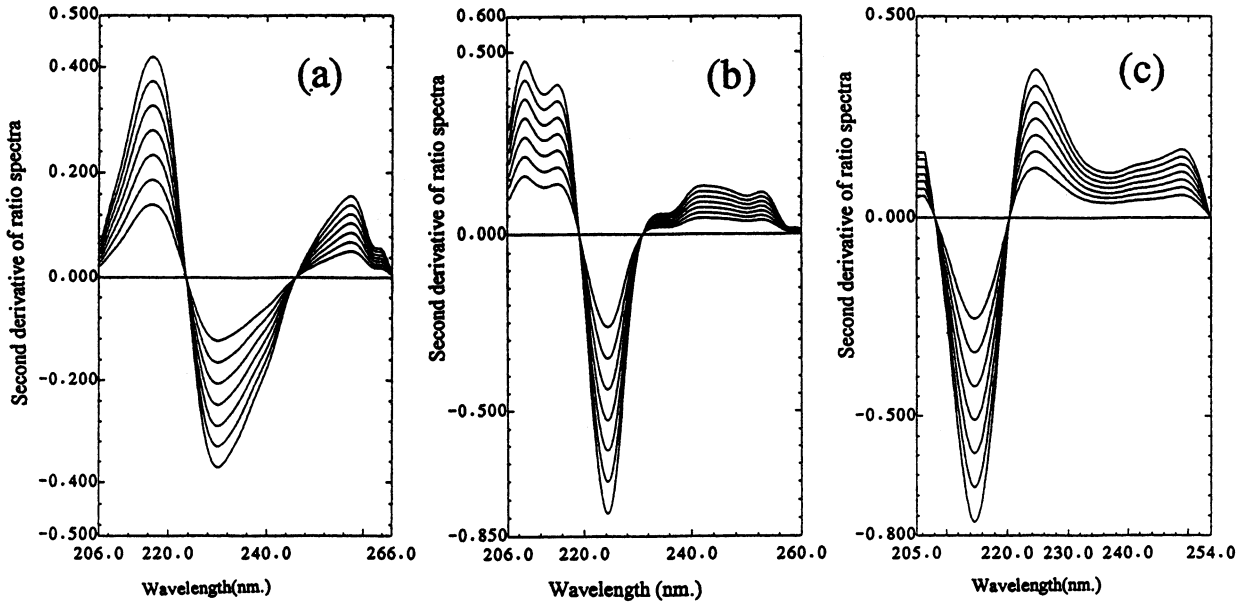

Fig. 5. Second derivative of ratio spectra of PH (a), PT (b) and MP (c) at various concentrations (6, 8, 10, 12, 14, 16, 18 μg/ml) using normalized spectrum of MP for (a) and (b); and normalized spectrum of PH for (c) as divisor.

the three drugs was observed. However at apparent pH 4 optimum resolution with reasonable retention time was observed. Quantitation was achieved with UV detection at 220 nm based on peak area. The specificity of the HPLC method is illustrated in Fig. 11 where complete separation of the three drugs was noticed. The average retention time ± standard deviation for PA, PH and PP were found to be 1.5 ± 0.013, 3.0 ± 0.023 and 4.5 ± 0.026 min, respectively, for ten replicates.

To determine the linearity of HPLC detector response, calibration standard solutions for the three drugs were prepared as described in text. Linear correlation was obtained between peak area versus concentration of each drug. Characteristic parameters for regression equations of the HPLC method and correlation coefficient obtained by least-squares treatment of the results were given in Table 2.

3.3. Analysis of pharmaceutical products

The proposed ^{2}DD method was applied to the simultaneous determination of PH, MP and PT in commercial tablets. Seven replicates determination were made. Satisfactory results were obtained in a good agreement with the label claims (Table 3). These results were compared with those of the published HPLC method using reversed-phase octadecylsilane column with a mobile phase consisting of 45% methanol, 55% water and 0.5 ml glacial acetic acid [17].

Table 1
Characteristic parameters of the calibration equations for the proposed second derivative of ratio spectra (^{2}DD) method for the determination of PH, MP and PT

Parameters	PH	MP	PT
Calibration range (μg/ml)	6–18	6–18	6–18
Detection limit (μg/ml)	1.29×10^{-2}	1.71×10^{-2}	2.77×10^{-3}
Quantitation limit (μg/ml)	4.3×10^{-2}	5.7×10^{-2}	9.23×10^{-3}
Regression equation (Y) [a]: Slope (b)	2.03×10^{-2}	1.84×10^{-2}	4.23×10^{-2}
Standard deviation of the slope (S_b)	1.12×10^{-4}	1.34×10^{-4}	1.32×10^{-4}
Relative standard deviation of the slope (%)	0.55	0.73	0.31
Confidence limit of the slope [b]	2.01×10^{-2} to 2.04×10^{-2}	1.84×10^{-2} to1.86×10^{-2}	4.21×10^{-2} to 4.24×10^{-2}
Intercept (a)	1.00×10^{-3}	1.30×10^{-3}	1.50×10^{-3}
Standard deviation of the intercept (S_a)	1.41×10^{-3}	1.69×10^{-3}	1.67×10^{-3}
Confidence limit of the intercept [b]	(-3.74×10^{-4})–2.37×10^{-3}	(-1.36×10^{-3})–1.93×10^{-3}	(-1.20×10^{-4})–3.12×10^{-3}
Correlation coefficient (r)	0.9999	0.9999	0.9999
Standard error of estimation	4.47×10^{-4}	5.35×10^{-4}	5.28×10^{-4}

[a] $Y = a + bC$, where C is the concentration of drug in μg/ml and Y is the ^{2}DD amplitude at the specified wavelength.
[b] 95% confidence limit.

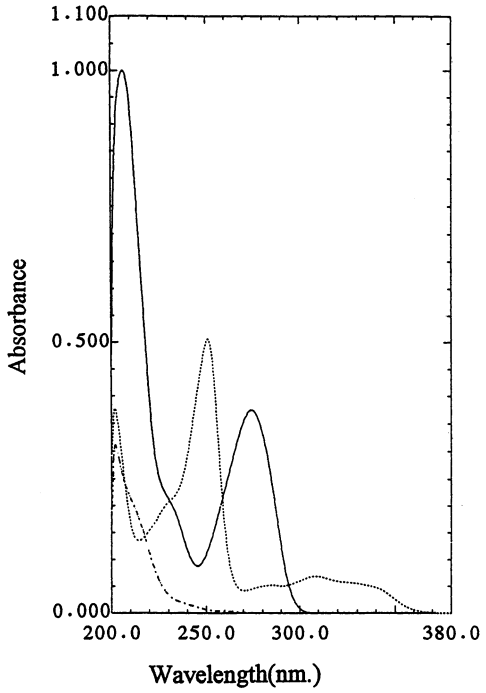

Fig. 6. UV absorption spectra of 3 μg/ml of each of PH (– – - – – - – –) and PP (--------); and 12 μg/ml PA (—) in 0.1 N hydrochloric acid.

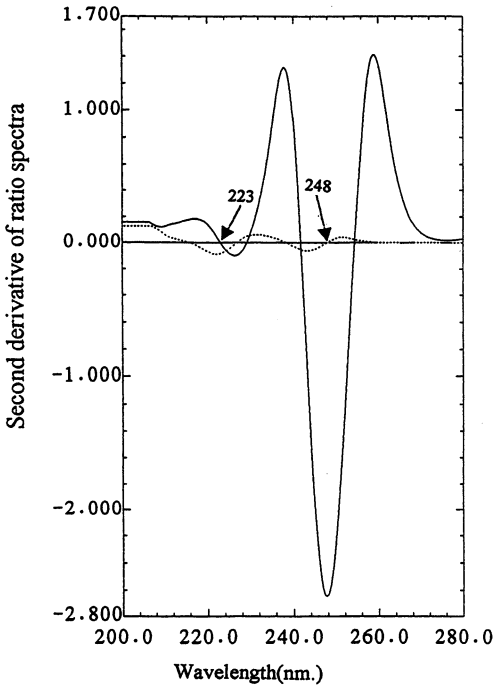

Fig. 8. Second derivative of the ratio spectra of 3 μg/ml of each of PH (-----) and PP (– – – – –) using normalized spectrum of PA as divisor.

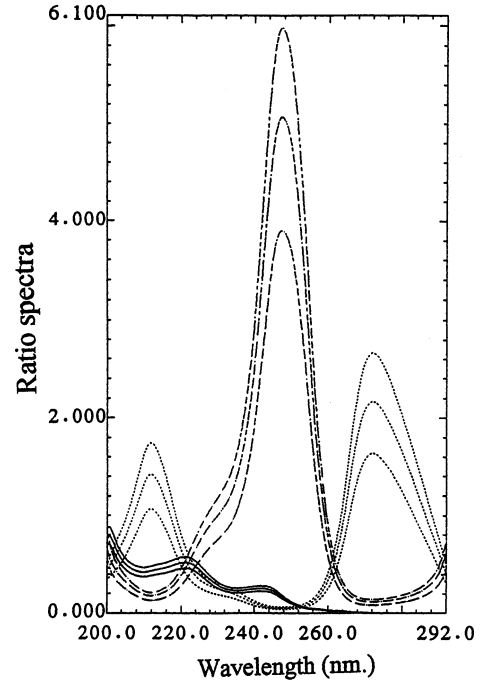

Fig. 7. Ratio spectra for different concentrations of PH (– – – – –) (4, 4.5, 5 μg/ml) using normalized spectrum of PA as divisor; different concentrations of PP (-------) (2.5, 3, 3.5 μg/ml) using normalized spectrum of PP as divisor; and different concentrations of PA (– – – – – – – – –) (8, 10, 12 μg/ml) using normalized spectrum of PA as divisor.

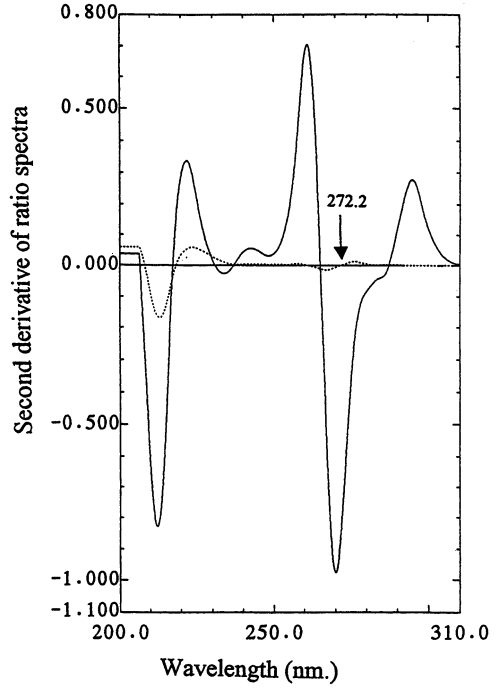

Fig. 9. Second derivative of the ratio spectra of 3 μg/ml of PH (-----) and 12 μg/ml PA (—) using normalized spectrum of PP as divisor.

The proposed ^{2}DD and HPLC methods were applied to the simultaneous determination of PH, PP and PA in commercial suppositories. Seven replicates determinations were made. Satisfactory results were obtained for the three drugs in a good agreement with the label claims (Table 3). These results were compared with those of the published first derivative spectrophoto-

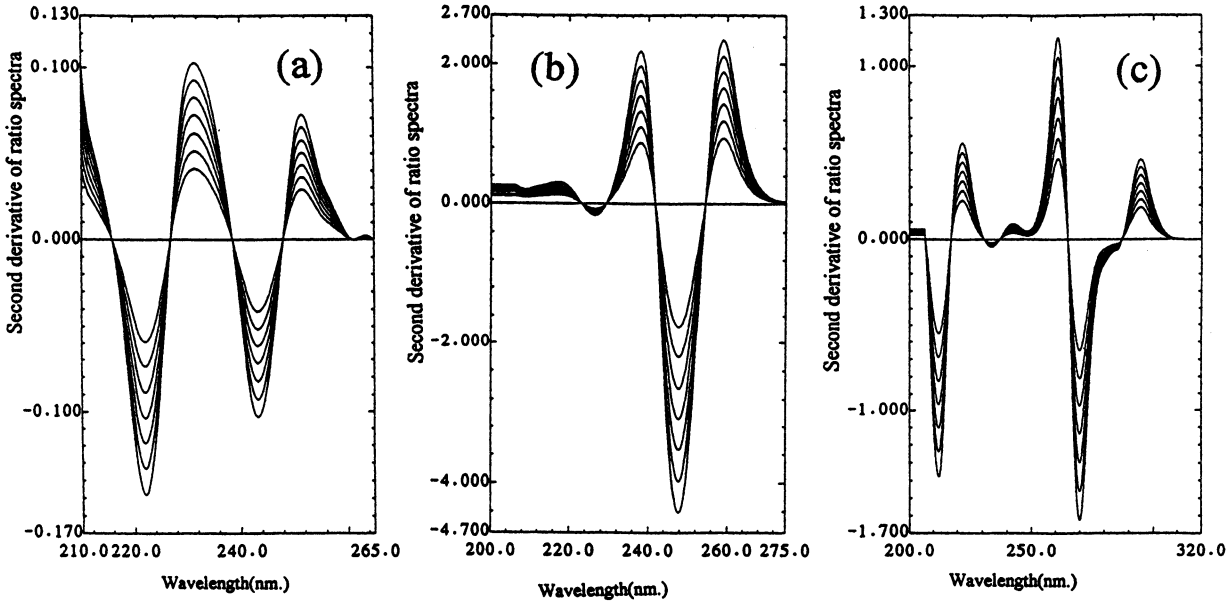

Fig. 10. Second derivative of ratio spectra of PH (a) and PP (b) at various concentrations (2, 2.5, 3, 3.5, 4, 4.5, 5 μg/ml); and PA (c) at various concentrations (8, 10, 12, 14, 16, 18, 20 μg/ml) using normalized spectrum of PA for (a) and (b); and normalized spectrum of PP for (c) as divisor.

metric method with solving of three simultaneous equations [20].

Statistical comparison between the results of the proposed and published methods was performed with regard to accuracy and precision using Student's t-test and the F-ratio at 95% confidence level (Table 3). There is no significant difference between the proposed and published methods.

3.4. Validation of the methods

3.4.1. Linearity

The linearity of the proposed methods was evaluated by analysing a series of different concentrations of each drug. According to the International Conference on Harmonization [22], at least five concentrations must be used. In this study seven concentrations were chosen,

Table 2
Characteristic parameters of the calibration equations for the proposed HPLC and second derivative of ratio spectra (^{2}DD) methods for the determination of PH, PP and PA

Parameters	HPLC			^{2}DD		
	(PH)	(PP)	(PA)	(PH)	(PP)	(PA)
Calibration range (μg/ml)	2–5	2–5	8–20	2–5	2–5	8–20
Detection limit (μg/ml)	4.10×10^{-2}	2.90×10^{-2}	4.30×10^{-2}	2.73×10^{-2}	8.74×10^{-4}	6.48×10^{-3}
Quantitation limit (μg/ml)	1.37×10^{-1}	9.60×10^{-2}	1.43×10^{-1}	9.10×10^{-2}	2.91×10^{-3}	2.16×10^{-2}
Regression equation(Y) [a]:Slope (b)	26.48×10^{3}	40.57×10^{3}	29.29×10^{3}	2.82×10^{-2}	8.80×10^{-1}	6.44×10^{-2}
Standard deviation of the slope (S_b)	4.61×10^{2}	4.99×10^{2}	5.37×10^{2}	3.28×10^{-4}	3.28×10^{-4}	1.78×10^{-4}
Relative standard deviation of the slope (%)	1.74	1.23	1.83	1.16	3.73×10^{-2}	0.28
Confidence limit of the slope [b]	26.03×10^{3}– 26.92×10^{3}	40.08×10^{3}– 41.05×10^{3}	28.76×10^{3}– 29.81×10^{3}	2.79×10^{-2}– 2.85×10^{-2}	8.79×10^{-1}– 8.80×10^{-1}	6.42×10^{-2}– 6.46×10^{-2}
Intercept (a)	-1.05×10^{3}	-1.45×10^{4}	-3.64×10^{3}	4.0×10^{-4}	3.60×10^{-5}	-4.6×10^{-4}
Standard deviation of the intercept (S_a)	1.68×10^{3}	1.82×10^{3}	7.82×10^{3}	1.19×10^{-3}	1.19×10^{-3}	2.59×10^{-3}
Confidence limit of the intercept [b]	(-2.68×10^{3}) to 5.80×10^{2}	(-1.63×10^{4}) to (-1.28×10^{4})	(-1.12×10^{4}) to 3.96×10^{3}	(-7.60×10^{-4}) to 1.55×10^{-3}	(-1.2×10^{-3}) to 1.1×10^{-3}	(-3.0×10^{-3}) to 2.1×10^{-3}
Correlation coefficient (r)	0.9999	0.9999	0.9999	0.9999	0.9999	0.9999
Standard error of estimation	4.61×10^{2}	4.99×10^{-2}	2.15×10^{3}	3.27×10^{-4}	3.27×10^{-4}	7.12×10^{-4}

[a] $Y = a + bC$, where C is the concentration of drug in μg/ml and Y is the peak area or ^{2} DD amplitude for HPLC and ^{2} DD method, respectively.
[b] 95% confidence limit.

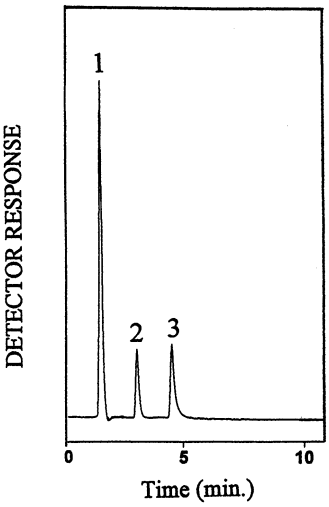

Fig. 11. HPLC chromatogram of 20 μl injection of synthetic mixture of 14μg/ml of PA (1), 3.5 μg/ml of PH (2) and 3.5 μg/ml of PP (3).

ranging between 6–18 μg/ml for PH, MP, PT in ternary mixture (1); and 2–5 μg/ml for PH and PP; and 8–20 μg/ml for PA in ternary mixture (2). Each concentration was repeated three times, the repeated runs were genuine repeats and not just repetitions at the same reading; this approach will provide information on the variation in peak area and ^{2}DD values between samples of same concentration. The assay was performed according to experimental conditions previously established. The linearity of the calibration graphs and adherence of the system to beer's law were validated by the high value of the correlation coefficient and the intercept value which was not statistically ($P < 0.05$) different from zero (Tables 1 and 2).

In order to test the mutual independence of the ^{2}DD value for each drug, the following experiments were performed for each drug, four calibration graphs were constructed from the second derivative ratio signals for the standard of each drug in absence and in presence of different concentrations of other two coexistings drugs (Table 4). The experiments showed that the amplitudes height of ^{2}DD signal at 231.0, 226.2 and 223.0 nm were proportional to the concentrations of PH, MP and PT, respectively in ternary mixture (1); and the amplitudes height of ^{2}DD signal at 223.0, 248.0 and 272.2 nm were proportional to the concentrations of PH, PP and PA, respectively in ternary mixture (2) in absence or presence of different concentration of the other two coexisting drugs. Table 4 summarizes the statistical analysis of the experimental data. The slopes of the calibration graphs of each drug were virtually independent of the other two coexisting drug concentration. Therefor, it can be deduced that amplitudes of the second derivative ratio signals measured at the selected wavelength were functionally of the drug under determination. In order to verify if the intercept, a, of the lines of regression were not significantly different from the theoretically expected value ($a = 0$), the Student's t-test at 95% confidence level and five degrees of freedom was applied. The calculated t-values do not exceed theoretical value and hence the intercept is negligible in all instances and not significantly different from zero (Table 4).

3.4.2. Precision

For evaluation of the precision estimates, repeatability and intermediate precision were performed at three concentration levels for each drug. The data for each concentration levels were evaluated by one-way ANOVA. A 8 days × 2 replicates design was performed. Statistical comparison of the results was performed using the P-value of the F-test. Table 5 shows three univariate analyses of variance for each concentration level. It can be seen from the table, that since the P-value of the F-test is always greater than 0.05, there is no statistically significant difference between the mean results obtained from one level of day to another at the 95% confidence level.

3.4.3. Range

The calibration range was established through consideration of the practical range necessary, according to each drug concentration present in pharmaceutical product, to give accurate, precise and linear results. The calibration range of the proposed methods are given in Tables 1 and 2.

3.4.4. Detection and quantitation limits

According to ICH recommendations [22] the approach based on the SD of the response and the slope was used for determining the detection and quantitation limits. The theoretical values were assessed practically and given in Tables 1 and 2.

3.4.5. Selectivity

Methods selectivity was achieved by preparing different mixtures of the studied drugs at various concentrations within the linearity range. The synthetic mixtures were analysed according to the previous procedures described under the proposed methods. Satisfactory results were obtained (Table 3), indicating the high selectivity of the proposed methods for determination of the studied drugs in their mixture.

3.4.6. Accuracy

This study was performed by addition of known amounts of the studied drugs to a known concentration of the commercial pharmaceutical products (standard addition method). The resulting mixtures were assayed and results obtained were compared with the expected results.

Table 3
Determination of PH, MP, PT, PP and PA in synthetic mixtures and commercial pharmaceutical products using the proposed methods

		Mean found ± SD [a]		
		^{2}DD	HPLC	Published method
Ternary mixture (1)				
	Synthetic mixtures			
	For PH	99.7 ± 0.28		
	For MP	99.8 ± 0.41		
	For PT	100.0 ± 0.37		
	Commercial tablet			
	For PH	99.8 ± 0.35		100.1 ± 0.47
	t	1.35		(2.18) [b]
	F	1.80		(4.28) [b]
	For MP	100.0 ± 0.53		99.7 ± 0.72
	t	0.89		(2.18) [b]
	F	1.85		(4.28) [b]
	For PT	100.0 ± 0.50		99.9 ± 0.81
	t	0.28		(2.18) [b]
	F	2.62		(4.28) [b]
	Recovery [c]			
	For PH	99.8 ± 0.45		
	For MP	99.9 ± 0.55		
	For PT	99.9 ± 0.52		
Ternary mixture (2)				
	Synthetic mixtures			
	For PH	100.2 ± 0.40	100.2 ± 0.26	
	For PP	100.0 ± 0.22	100.1 ± 0.38	
	For PA	100.2 ± 0.29	100.0 ± 0.43	
	Commercial suppositories			
	For PH	99.8 ± 0.55	100.1 ± 0.31	99.9 ± 0.47
	t	0.37	0.94	(2.18) [b]
	F	1.37	2.30	(4.28) [b]
	For PP	99.7 ± 0.44	99.9 ± 0.47	100.1 ± 0.54
	t	1.52	0.74	(2.18) [b]
	F	1.51	1.32	(4.28) [b]
	For PA	100.0 ± 0.46	100.0 ± 0.56	99.8 ± 0.51
	t	0.77	0.70	(2.18) [b]
	F	1.23	1.21	(4.28) [b]
	Recovery [c]			
	For PH	100.1 ± 0.63	100.2 ± 0.42	
	For PP	100.2 ± 0.59	100.0 ± 0.54	
	For PA	100.0 ± 0.51	99.9 ± 0.58	

[a] Mean and SD for seven determinations; percentage recovery from the label claim amount.
[b] Theoretical values for *t* and *F*.
[c] For standard addition of 50% of the nominal content.

Also, spiked placebos were prepared according to the manufacturing formula of each pharmaceutical product. The spiked placebos were tested at five levels: 50, 75, 100, 125 and 150% of label claim for each individual drug. Assays were performed in duplicate on two samples at five levels. This was repeated with a second standard, sample preparation and analyst on different days. The recoveries ranging from 99.6 to 100.5% of the amount of active ingredient spiked into the placebos. The bias showed only minor variation in recovery at each level with 0.6% the maximum variation observed.

The excellent recoveries of standard addition method (Table 3) and spiked placebos suggest that good accuracy of the proposed methods and there is no interference from the excipients present in pharmaceutical product.

Table 4
Statistical analysis of the calibration graphs [a] of PH, MP, PT, PP and PA using second derivative of the ratio spectra-zero-crossing method for $n = 7$ standard specimens

Drug determined	Conc. Of the drug determined	Coexisting drug 1	Conc. of coextisting drug1 (μg/ml)	Coexisting drug 2	Conc. of coextisting drug 2 (μg/ml)	Selected λ (nm)	Intercept (a)	Standard deviation of intercept $S_{(a)}$	Slope (b)	Standard deviation of slope $S_{(b)}$	Correlation coefficient (r)	Calculated t [b]
Ternary mixture 1												
PH	6–18					231.0	0.0010	1.41×10^{-3}	0.0203	1.12×10^{-4}	0.9999	0.71
PH	6–18	PT	6	MP	18	231.0	0.0017	3.57×10^{-3}	0.0206	3.97×10^{-4}	0.9999	0.48
PH	6–18	PT	12	MP	12	231.0	0.0030	5.01×10^{-3}	0.0203	3.97×10^{-4}	0.9998	0.60
PH	6–18	PT	18	MP	6	231.0	0.0036	5.50×10^{-3}	0.0201	4.34×10^{-4}	0.9998	0.65
MP	6–18					226.2	0.0013	1.69×10^{-3}	0.0184	1.34×10^{-4}	0.9999	0.77
MP	6–18	PT	6	PH	18	226.2	0.0039	4.27×10^{-3}	0.0182	3.39×10^{-4}	0.9999	0.91
MP	6–18	PT	12	PH	12	226.2	0.0029	4.65×10^{-3}	0.0184	3.68×10^{-4}	0.9998	0.62
MP	6–18	PT	18	PH	6	226.2	0.0018	4.46×10^{-3}	0.0183	2.52×10^{-4}	0.9998	0.40
PT	6–18					223.0	0.0015	1.67×10^{-3}	0.0423	1.32×10^{-4}	0.9999	0.90
PT	6–18	PH	6	MP	18	223.0	0.0039	7.09×10^{-3}	0.0421	5.61×10^{-5}	0.9999	0.55
PT	6–18	PH	12	MP	12	223.0	0.0022	5.43×10^{-3}	0.0422	4.29×10^{-4}	0.9999	0.41
PT	6–18	PH	18	MP	6	223.0	0.0015	7.18×10^{-3}	0.0425	5.69×10^{-4}	0.9999	0.21
Ternary mixture 2												
PH	2–5					223.0	0.0004	1.191×10^{-3}	0.0282	3.28×10^{-4}	0.9999	0.33
PH	2–5	PP	2	PA	20	223.0	0.0002	2.558×10^{-3}	0.0282	7.038×10^{-4}	0.9998	0.07
PH	2–5	PP	3.5	PA	14	223.0	0.0017	2.672×10^{-3}	0.0282	7.355×10^{-4}	0.9997	0.64
PH	2–5	PP	5	PA	8	223.0	0.0008	2.884×10^{-3}	0.0281	7.408×10^{-4}	0.9997	0.28
PP	2–5					248.0	0.000036	1.19×10^{-3}	0.8801	3.28×10^{-4}	0.9999	0.70
PP	2–5	PH	2	PA	20	248.0	0.0260	5.9×10^{-2}	0.8741	1.63×10^{-2}	0.9999	0.44
PP	2–5	PH	3.5	PA	14	248.0	0.0100	1.68×10^{-2}	0.8829	4.62×10^{-3}	0.9999	0.59
PP	2–5	PH	5	PA	8	248.0	0.0324	5.3×10^{-2}	0.8720	1.45×10^{-2}	0.9999	0.61
PA	8–20					272.2	−0.00046	2.59×10^{-3}	0.0644	1.78×10^{-4}	0.9999	0.19
PA	8–20	PH	2	PP	5	272.2	0.0053	1.36×10^{-2}	0.0639	9.35×10^{-4}	0.9999	0.39
PA	8–20	PH	3.5	PP	3.5	272.2	0.0048	8×10^{-3}	0.0648	5.50×10^{-4}	0.9999	0.60
PA	8-20	PH	5	PP	2	272.2	0.0014	1.62×10^{-2}	0.0642	1.11×10^{-3}	0.9999	0.08

[a] Regression equation, $^2DD = a + bC$ where C is the concentration of drug in μg/ml and ^2DD is the second derivative of the ratio amplitude at the selected wavelengths.
[b] Theoretical value of t at 95% level of confidence is 2.57.

Table 5
Analysis of variance for repeatability and intermediate precision for PH, MP, PT, PP and PA by the proposed methods

Drug	Conc. level (μg/ml)	Source of variation	Sum of squares		Degree of freedom	Mean squares		F-ratio [a]		P-value	
			^{2}DD	HPLC		^{2}DD	HPLC	^{2}DD	HPLC	^{2}DD	HPLC
Ternary mixture (1)											
PH	6	Between	0.78		7	0.11		0.47		0.83	
		Within	1.91		8	0.24					
		Total	2.69		15						
	14	Between	1.22		7	0.17		0.66		0.70	
		Within	2.10		8	0.26					
		Total	3.32		15						
	18	Between	0.89		7	0.13		0.57		0.76	
		Within	1.78		8	0.22					
		Total	2.67		15						
MP	6	Between	0.80		7	0.11		0.77		0.63	
		Within	1.18		8	0.14					
		Total	1.98		15						
	14	Between	0.64		7	0.09		0.44		0.86	
		Within	1.71		8	0.21					
		Total	2.35		15						
	18	Between	0.61		7	0.09		0.41		0.87	
		Within	1.71		8	0.21					
		Total	2.32		15						
PT	6	Between	1.50		7	0.21		1.13		0.43	
		Within	1.52		8	0.19					
		Total	3.02		15						
	14	Between	1.77		7	0.25		1.53		0.28	
		Within	1.33		8	0.17					
		Total	3.10		15						
	18	Between	1.43		7	0.20		1.17		0.41	
		Within	1.39		8	0.17					
		Total	2.82		15						
Ternary mixture (2)											
PH	2	Between	0.35	0.70	7	0.05	0.10	0.24	0.20	0.96	0.97
		within	1.64	4.01	8	0.21	0.50				
		Total	1.99	4.71	15						
	3.5	Between	0.28	1.27	7	0.04	0.18	0.08	0.31	0.99	0.93
		within	3.96	4.71	8	0.50	0.59				
		Total	4.24	5.98	15						
	5	Between	0.33	1.67	7	0.05	0.24	0.12	0.55	0.99	0.77
		within	3.01	3.47	8	0.38	0.43				
		Total	3.33	5.14	15						
PP	2	Between	0.26	1.48	7	0.04	0.21	0.11	0.44	0.99	0.85
		within	2.71	3.86	8	0.34	0.44				
		Total	2.97	5.34	15						
	3.5	Between	0.61	0.60	7	0.09	0.08	0.19	0.28	0.98	0.94

Table 5 (*Continued*)

Drug	Conc. level (μg/ml)	Source of variation	Sum of squares		Degree of freedom	Mean squares		*F*-ratio [a]		*P*-value	
			^{2}DD	HPLC		^{2}DD	HPLC	^{2}DD	HPLC	^{2}DD	HPLC
		within	3.66	2.41	8	0.46	0.30				
		Total	4.27	3.01	15						
	5	Between	0.68	0.51	7	0.10	0.07	0.22	0.13	0.97	0.99
		within	3.50	4.43	8	0.44	0.55				
		Total	4.18	4.94	15						
PA	8	Between	0.45	1.13	7	0.06	0.16	0.13	0.34	0.99	0.91
		within	3.89	3.73	8	0.49	0.47				
		Total	4.34	4.86	15						
	14	Between	0.58	2.16	7	0.08	0.31	0.21	0.86	0.97	0.57
		within	3.14	2.85	8	0.39	0.36				
		Total	3.72	5.01	15						
	20	Between	0.33	0.52	7	0.05	0.07	0.12	0.20	0.99	0.97
		within	3.21	2.89	8	0.40	0.36				
		Total	3.54	3.41	15						

[a] *F*-critical is 3.5.

3.4.7. Robustness

The robustness of a method is its ability to remain unaffected by small change in parameters. To determine robustness of the proposed methods, experimental conditions such as pH and percentage of organic strength of the mobile phase, pH of borax buffer and strength of hydrochloric acid were purposely altered and chromatographic, ^{2}DD characteristics were evaluated. Variation of pH of the mobile phase by ± 0.1 and its organic strength by ± 2 % did not have a significant effect on chromatographic resolution in HPLC method. Variation of pH of borax buffer by ± 0.1 and strength of hydrochloric acid by ± 0.05 N did not have significant effect on ^{2}DD amplitude of each drug in spectrophotometric method.

3.4.8. Stability

The stability of each drug standard and sample solutions were evaluated. The solutions were stored in tightly capped volumetric flasks, protected from light, on a laboratory bench and in the refrigerator. Recovery of these solutions was checked for 3 h in interval of 15 min against freshly prepared solutions. The solutions kept on the laboratory bench and in the refrigerator were found to be stable for this period.

The ^{2}DD and HPLC methods for analysis of mixture 2 were compared. Relative sensitivity, based on detection limit, was calculated. The ^{2}DD method was found to be more sensitive with lower quantitation limit than the HPLC method (Table 2). While the HPLC method was found to be more selective than the ^{2}DD method.

The proposed ^{2}DD and HPLC methods provide simple, accurate and reproducible quantitative analysis for determination of the studied drugs in pharmaceutical preparations, without any interference from the excipients. The proposed methods were found to be easier than the other methods previously reported for the determination of the two studied ternary mixture which require multistep procedures becoming, consequently, time-consuming. The proposed HPLC method was found to be more specific and selective than the ^{2}DD method. While the ^{2}DD method has the advantage of lower cost, rapid and environmental protecting. The proposed methods were completely validated and suitable for quality control laboratories.

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