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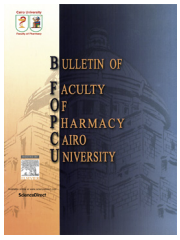

ORIGINAL ARTICLE

Univariate spectrophotometry and multivariate calibration: Stability-indicating analytical tools for the quantification of pimozide in bulk and pharmaceutical dosage form

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Abstract Simple, accurate sensitive and precise spectrophotometric and chemometric stability indicating techniques were adopted for the determination of Pimozide (PIM) in presence of its alkaline and acidic degradation products over a concentration range of 10–100 μg mL^{-1}. The proposed spectrophotometric technique includes first derivative (D^1) spectrophotometric one at 252 nm and 256.6 nm in presence of its acidic and alkaline degradates, respectively, first-derivative of the ratio spectra spectrophotometry (DR^1) at 292.5 nm, the Q-analysis (absorption ratio) method, which involves the formation of absorbance equation at 242.2 nm and 281.7 nm, dual wave length method at 270.1 nm and 284 nm, the H-point standard addition method (HPSAM) and the mean centering of the ratio spectra method. The second technique is chemometric methods which include determination of PIM in presence of both its acidic and alkaline degradates using multivariate calibration methods [the classical least squares (CLS), principle component regression (PCR) and partial least squares (PLS)] using the information contained in the absorption spectra. The proposed methods have been successfully applied to the analysis of PIM in pharmaceutical dosage forms without interference from other dosage form additives and the results were statistically compared with the official method.

1. Introduction

Pimozide; 1-[1-[4, 4-bis (4-fluorophenyl) butyl]-4-piperidinyl]-1, 3-dihydro-2H-benzimidazole-2-one [1] (Fig. 1). PIM is a neuroleptic of the diphenyl butyl piperidine series. It is a potent

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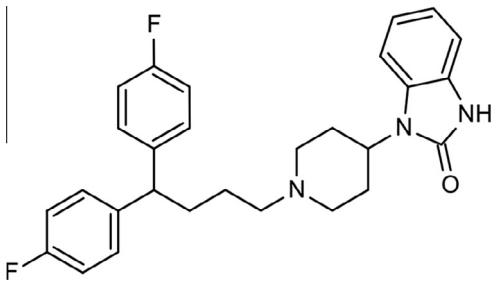

Figure 1 Chemical structure of intact Pimozide.

long-acting anti-psychotic, used mainly to control effectively hallucinations and delusions and to normalize incoherent thought processes and bizarre behavior patterns.[2] PIM appears to undergo significant first pass metabolism. The major route of elimination of its metabolites is via the kidney.[2] PIM is official in British Pharmacopoeia.[3] PIM was determined by several analytical techniques including; spectroscopic methods either colorimetric,[4–6] or fluorimetric[7] methods. PIM can also be determined by electrochemical methods,[8] and HPTLC.[9] High performance liquid chromatographic [HPLC] methods were widely used for analysis of PIM.[10–17]

To the best of our Knowledge, only one stability indicating HPTLC method[9] was reported for pimozide and no other spectrophotometric or chemometric stability indicating techniques was proposed for the determination of PIM in presence of its alkaline and acidic degradation products. So the aim of the present study is to develop and validate simple, accurate and specific stability indicating method for the quantification of pimozide in the presence of its degradation products.

2. Experimental

2.1. Instrumentation

A double beam UV–VIS spectrophotometer (UV-1800, Japan) connected to IBM compatible computer. The bundled software is UV probe software version 2.32 (Shimadzu) and the spectral bandwidth was 0.1 nm. The absorption spectra were carried out using 1 cm quartz cells. The chemometric calculations were performed in Matlab for Windows-version 7 Mathworks Inc. 2004. The PLS procedure was taken from PLS Toolbox 2.0, Eigenvector Research Inc. 2001 created by B.M. Wise, N.B. Gallagher for use with Matlab.

2.2. Materials and reagents

All chemicals were of analytical grade, the solvents were of spectroscopic grade.

PIM was purchased from Sigma Aldrich. Its purity was 100.09% ± 0.14 (n = 5) according to the official non aqueous titration.[3]

Orape forte®4 mg tablets, labeled to contain 4 mg of PIM per tablet were manufactured by Janssen Cilag, Batch No. 12CQ019 and purchased from the Egyptian market.

Sodium hydroxide, hydrochloric acid (Adwic-Cairo, Egypt), and methanol (Analar-Germany) were used.

2.3. Standard solutions of the intact Pimozide

2.3.1. Stock solution

A standard stock solution of PIM was prepared by transferring accurately 100 mg of pure drug into 100-mL volumetric flask, dissolving in 20 mL methanol with the aid of sonication and then the volume was completed to the mark with the same solvent to provide standard stock solution containing 1 mg mL^{-1}.

2.3.2. Working solution

PIM working solution was prepared by transferring 10 mL of the standard stock solution into 100-mL volumetric flask and then the volume was completed to the mark with methanol to obtain standard working solution containing 0.1 mg mL^{-1}.

2.4. Preparation of standard solution of acidic and alkaline degraded PIM

Methanolic PIM solutions containing accurately measured 50 mg were mixed with 25 mL of 2 M HCl and 2 M NaOH, separately then refluxed for 4 h. The solutions were cooled to room temperature, neutralized with 2 M NaOH and 2 M HCl, respectively till pH 7. Then the degradation products were extracted with multiple fractions of methanol (3 × 10 mL), then quantitatively transferred into 50 mL volumetric flasks and the volume was completed with methanol to reach concentration 1.00 mg mL^{-1}. Aliquot portions of these solutions were diluted with methanol to prepare working standard solutions of 0.1 mg mL^{-1}.

Complete acidic and alkaline degradation of the studied drug was confirmed by the TLC method using toluene: acetone: ammonia (5:5:0.1 by volume)[9] where no peaks corresponding to intact drug were detected in case of the degraded samples. The degradates were elucidated by IR spectrometry.

2.5. Procedures

2.5.1. Construction of calibration curves for D^1 spectrophotometric method

Accurately measured volumes of intact PIM working solution (0.1 mg mL^{-1}) were transferred into a series of 10-mL volumetric flasks and diluted to the mark with methanol to obtain concentrations from 10 to100 μg mL^{-1}. The D^1 spectra of each solution were recorded using $\Delta\lambda$ = 8 and scaling factor = 100. For determination of PIM in presence of its acidic and alkaline degradates, calibration curves were obtained by plotting the peak amplitudes of D^1 at 252 nm and 256.6 nm respectively (corresponding to zero-crossing of the degradation product) versus the corresponding drug concentrations, and regression equations were computed.

2.5.2. Construction of calibration curves of (DD^1) spectrophotometric method

Different aliquots of intact PIM working solution (0.1 mg mL^{-1}) were accurately transferred into a series of 10 mL volumetric flasks and diluted to the mark with methanol to obtain concentrations from 10 to100 μg mL^{-1}. The DD^1curves were recorded at $\Delta\lambda$ = 8 and scaling factor = 10.

Table 1 The concentration of mixtures of PIM, and both its acidic and alkaline degradate in the training set.

Sample no.	PIM ($\mu g\ mL^{-1}$)	Alkaline degradate ($\mu g\ mL^{-1}$)	Acidic degradate ($\mu g\ mL^{-1}$)
1	40	40	40
2	40	20	20
3	20	60	30
4	60	30	60
5	60	40	30
6	40	30	30
7	30	50	60
8	50	60	50
9	50	40	60
10	40	60	60
11	60	20	50
12	20	50	20
13	20	40	50
14	40	50	50
15	50	30	20
16	30	20	30

The absorption spectra of these solutions were divided by the absorption spectrum of 80 $\mu g\ mL^{-1}$ of the acidic and 20ug $mL^{-1}$of the alkaline degradates separately (as divisors). The obtained ratio spectra were then differentiated with respect to wave length. The peak amplitudes at 292.5 nm were recorded for the determination of PIM in presence of either its acidic or alkaline degradates. The calibration curves representing the relationship between the measured amplitudes and the corresponding concentrations of the drug were constructed and the regression equations were computed.

2.5.3. Construction of calibration curves for Q-analysis (absorption ratio) method

Accurately measured volumes of intact PIM and its alkaline degradates working solutions were transferred separately into a series of 10-mL volumetric flasks and diluted to the mark with methanol to obtain concentrations from 10 to 100 $\mu g\ mL^{-1}$.The Zero-order spectrum of each dilution was recorded against methanol as a blank. The absorbance of PIM and its alkaline degradates was measured at 242.2 nm (isobestic point) and 281.7 nm (λ_{max} of PIM) for each dilution separately. Then the absorbances at the selected wavelengths were plotted against the corresponding concentrations and the regression equations were then computed. Absorptivity coefficients of PIM and its alkaline degradation product were determined at both selected wavelengths; the absorption equation was then formed. The concentration of the drug of interest is calculated from the equation.

2.5.4. Construction of calibration curve for dual wavelength method

Accurately measured volumes of intact PIM working solution (0.1 mg mL^{-1}) were transferred into a series of 10-mL volumetric flasks and diluted to the mark with methanol to obtain concentrations from 10 to 100 $\mu g\ mL^{-1}$. The Zero-order spectrum of each dilution was recorded against methanol as a blank. The absorbance of PIM was measured at 270.1 nm and 284 nm for each dilution separately. Then the difference between the absorbance at the selected wavelengths was calculated, plotted against the corresponding concentrations of the drug and the regression equation was then computed.

2.5.5. Construction of calibration curves for H-point standard addition analysis method

Synthetic samples containing different ratios of PIM and its alkaline degradates were prepared by transferring aliquots equivalent to 100 μg of PIM and its alkaline degradate into a set of 10-mL volumetric flasks from their respective working solutions, a standard addition of different aliquots of PIM in the range of 10–80 μg was added to the previously prepared synthetic samples and the volume was completed to the mark with methanol. The zero order absorption spectra of the prepared mixtures were recorded against methanol as a blank. The peak amplitudes were measured at 270.1 nm and 284 nm for each dilution separately, and then plotted against the corresponding added PIM concentrations .The regression equation was then computed.

2.5.6. Construction of calibration curve for mean centering spectrophotometric method

Accurately measured volumes of PIM working solution were transferred into a series of 10-mL volumetric flasks and diluted to the mark with methanol to obtain concentrations from 10 to 100 $\mu g\ mL^{-1}$. The Zero-order spectrum of each dilution was recorded against methanol as blank and stored. The stored spectra and a standard spectrum of the alkaline degradation product (20 $\mu g\ mL^{-1}$) were transferred to Matlab®, then each spectrum of the drug was divided by the standard spectrum of the degradation product, Mean centering of these vectors (spectra) with respect to wavelength was done and the mean centered curves were obtained .The values of the curves were recorded at 284 nm corresponding to a maximum wavelength, plotted each against its corresponding concentration and the regression parameters were computed.

2.5.7. Multivariate calibration technique (chemometric)

(a) Construction of training set: Different mixtures of PIM and its acidic and alkaline degradation product were prepared by diluting different volumes of their stock standard solutions into 10-mL volumetric flask. The volume was completed to the mark with methanol to reach the concentrations listed in Table 1. The absorbance of these mixtures was measured between 230 and 470 nm at 1 nm interval with respect to a blank of methanol. The composition of the samples was randomly designed according to five level calibration designs in order to obtain non correlated concentration profiles and this calibration design prepared to obey Beer's law.
(b) Construction of the models: To build the CLS model, feed the computer with absorbance and concentration matrices for training set. Carry out the calculations to obtain the "K" matrix. For the PCR and PLS models, use the training set absorbance and concentration matrices together with PLS-Toolbox 2.0 software for the calculations.
(c) Construction of the validation set: Prepare nine different mixtures of PIM and its acidic and alkaline degradates by transferring different volumes of their working standard solutions into a series of 10-mL volumetric flasks

and complete to volume with methanol, Table 2. Apply the developed models to predict the concentration of PIM in each mixture.

2.5.8. Laboratory prepared mixtures

Two solution sets containing different ratios of PIM and up to 60% of its acidic degradates in one set and up to 60% of its alkaline degradates in the other set were prepared. The solution mixtures were analyzed by the proposed methods for the determination of intact PIM in the presence of its acidic and alkaline degradation products.

2.5.9. Application to pharmaceutical preparation

Ten tablets were accurately weighed and finely powdered. A portion equivalent to 50 mg of PIM was weighed, sonicated in 20 mL methanol and filtered into 50-mL volumetric flask. The residue was washed three times each with 8 mL methanol and completed to the mark with the same solvent. Aliquots (according to linearity) were transferred to 10-mL volumetric flasks and diluted with methanol. The general procedures were followed and the concentration of PIM was calculated from its corresponding regression equation.

3. Results and discussion

The International Conference on Harmonization (ICH) guideline entitled "stability testing of new drugs substances and products" requires the stress testing of new substances and products, also requires the stress testing to be carried out to elucidate the inherent stability, characteristics of the active substance.[18] An ideal stability indicating method is one that quantifies the standard drug alone and also resolves its degradation products. PIM hydrolyzed under alkaline and acid conditions where complete degradation was observed using 2 M NaOH and 2 M HCl after refluxing for 4 h, respectively.

The structures of the intact drug, acidic and alkaline degradates were elucidated by IR (Fig. 2(a)–(c)). Considering the IR elucidation; it is suggested that complete hydrolysis of amide NH-group was achieved. Where IR spectrum of intact PIM showed a characteristic band at 3361.6 cm^{-1} indicating the presence of amide NH-group, and a band at 2940.1 cm^{-1}indicating the presence of alkane C–H bonds and another characteristic peak at 1643.0 cm^{-1} indicating the presence of C═O of the amide group. While IR spectrum of the acidic degradates showed a characteristic broad band at 3463.1 cm^{-1}, indicating the presence of OH group while the appearance of a band at 1639.2 cm^{-1} suggests the presence of C═O of the carboxylic acid. IR spectrum of the alkaline degradates showed a characteristic broad band at 3463.1 cm^{-1}, indicating the presence of OH group while the appearance of a band at 1639.2 cm^{-1} suggests the presence of C═O of the carboxylic acid.

The focus of the present work was to develop accurate, specific, reproducible and sensitive stability indicating methods for the determination of PIM in pure form, in pharmaceutical formulation, and in the presence of its acidic and alkaline degradates.

The Zero-order absorption spectrum of PIM and its acidic and alkaline degradates showed severe overlapping (Figs. 3 and 4) which interferes with the direct determination of PIM.

Table 2 The concentration of mixtures of PIM and both its acidic and alkaline degradates in the validation set.

Sample no.	PIM ($\mu g\ mL^{-1}$)	Alkaline degradate ($\mu g\ mL^{-1}$)	Acidic degradate ($\mu g\ mL^{-1}$)
1	20	20	60
2	30	60	40
3	30	30	50
4	60	50	40
5	60	60	20
6	50	20	40
7	50	50	30
8	20	30	40
9	30	40	20

3.1. Derivative spectrophotometry

The advantages of derivative spectrophotometry (D^n) are enhanced resolution of overlapping peaks for separation of superposed spectra particularly in multicomponent analysis and background absorption minimization or elimination.

A rapid, simple and low cost spectrophotometric method based on measuring the peak amplitude of D^1 at 252 nm (Fig. 5) and 256.6 nm (Fig. 6) was developed for determination of PIM in presence of its acidic and alkaline degradates, respectively (corresponding to zero crossing of the degradates) with good selectivity without interference of its acidic and alkaline degradates over concentration range of 10–100 $\mu g\ mL^{-1}$.

In order to optimize D^1 methods, different smoothing and scaling factors were tested, where a smoothing factor $\Delta\lambda = 8$ and scaling factor = 100 showed a suitable signal to noise ratio and the spectra showed good resolutions.

3.2. DR^1 method

In order to improve the selectivity of the analysis of PIM in presence of its acidic and alkaline degradates DR^1 method was established.

The main advantage of the method is that the whole spectrum of interfering substance is canceled [19]. Accordingly, the choice of the zero crossing wavelengths selected for calibration is not critical as in D^1 derivative method.

In order to optimize DR^1 method for determination of PIM in presence of its degradation products, it is necessary to test the influence of the variables: divisor concentration, smoothing and scaling factors. Several divisor concentrations of the acidic and alkaline degradates were tried, the best results was obtained when using 80 $\mu g\ mL^{-1}$ of its acidic degradates and 20 $\mu g\ mL^{-1}$ of its alkaline degradates as divisors. Different smoothing and scaling factors were also tested where a smoothing factor $\Delta\lambda = 8$ nm, and scaling factor = 10 were suitable to enlarge the signals of PIM to facilitate its measurement and to diminish error in reading the signal. DR^1 values showed good linearity and reproducibility at 292.5 nm without interference from its acidic or alkaline degradates, (Figs. 7 and 8).

Linearity of the peak amplitudes of the DR^1 curves at both wavelengths was obtained in range 10–100 $\mu g\ mL^{-1}$.

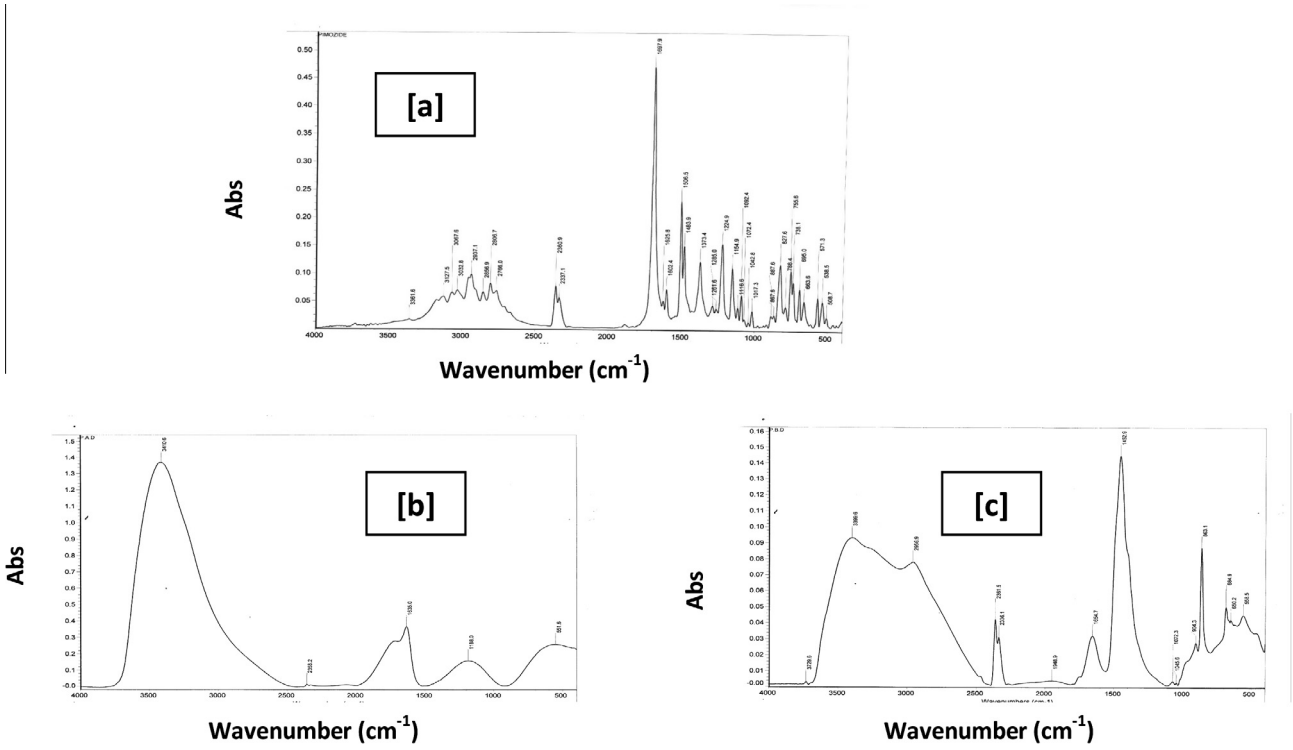

Figure 2 IR spectra of intact PIM [a], acidic degradates [b] and alkaline degradates [c].

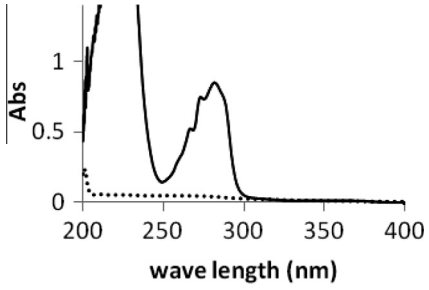

Figure 3 Zero order spectra of PIM (—) and acidic degradates (. . .), (60 μg mL^{-1} each) using methanol as a solvent.

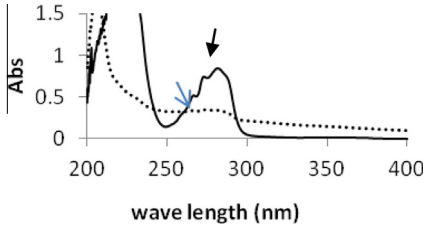

Figure 4 Zero order spectra of PIM (—) and alkaline degradates (. . .), (60 μg mL^{-1}) using methanol as a solvent.

3.3. Q-analysis (absorption ratio) method

In absorbance ratio method (Q-analysis), the primary requirement for developing a method for analysis is that the entire spectra should follow the Beer's law at all wavelengths, which was fulfilled in case of both these drugs. Absorbance ratio method uses the ratio of absorbances at two selected wavelengths,[20] one of which is an isoabsorptive point and other being the λ-max of PIM. From the overlain spectrum of the drug and its alkaline degradate, 242.2 nm (isoabsorptive point) and 281.7 nm (λ_{max} of PIM) were selected for the determination of PIM in presence of its alkaline degradation product.

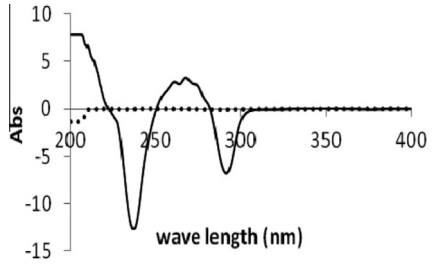

Figure 5 First derivative absorption spectra of PIM (—) and acidic degradates (·····), (60 μg mL^{-1} each) using methanol as a solvent.

Linear calibration curves were obtained in the range 10–100 μg mL^{-1} for both intact PIM and its alkaline degradation products relating the absorbance at the two selected wavelengths 242.2 nm and 281.7 nm to the corresponding concentrations of PIM and its alkaline degradate and the regression equations were computed.

The concentration of PIM was determined by substituting the absorbance and absorptivity coefficients in the following equation[21]

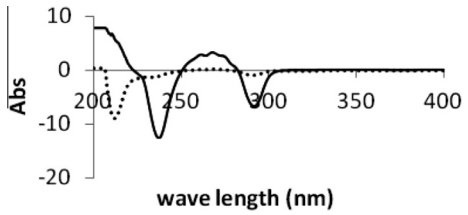

Figure 6 First derivative absorption spectra of PIM (—) and alkaline degradates (·····), (60 μg mL^{-1} each) using methanol as a solvent.

$$C_{PIM} = \frac{Q_m - Q_y}{Q_X - Q_y} \times \frac{A_1}{a_{x1}},$$

where A_1 and A_2 are the absorbances of mixture at 242.2 nm and 281.7 nm respectively. a_{x1} and a_{x2} are the absorptivity coefficients of PIM at 242.2 nm and 281.7 nm respectively. While a_{y1} and a_{y2} are the absorptivity coefficients of alkaline degradate at 242.2 nm and 281.7 nm respectively. $Q_m = A_2/A_1$, $Q_y = \frac{a_{y2}}{a_{y1}}$ and $Q_x = \frac{a_{x2}}{a_{x1}}$.

3.4. Dual wave length method

The privilege for utility of dual wavelength data processing program is to calculate the unknown concentration of a component of interest independent of the extensively or even completely interfering components. The only pre-requisite for dual wavelength method is the selection of two such wavelengths at which the difference in the analyte signals had to be linear while the difference in the interferent signal is remaining zero. The overlain spectrum of PIM and its alkaline degradates suggested that dual wavelength method is a suitable method for the simultaneous determination of PIM in presence of its alkaline degradates.[22]

From the overlain spectra (Fig. 4) 270.1 nm and 284 nm were selected for the determination of PIM, where the alkaline degradates shows the same absorbance.

A linear Calibration curve was obtained in the range 10–100 μg mL^{-1} relating the difference between the absorbances at the two selected wavelengths 270.1 nm and 284 nm to the corresponding drug concentrations in presence of alkaline degradates.

3.5. H-point standard addition analysis method

PIM was also determined and resolved from its alkaline degradates by using H-point standard addition analysis method.

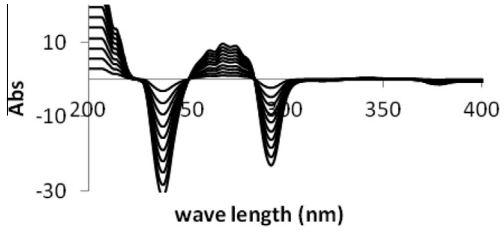

Figure 7 First derivative ratio spectra of different concentrations of PIM (10–100 μg mL^{-1}) using 80 μg mL^{-1} acidic degradates as a divisor.

The H-point standard addition method (HPSAM) permits both proportional and constant errors produced by the matrix of the sample to be corrected. This method is based on the principles of dual wavelength spectrophotometry and the standard addition method. The method has been applied to binary mixtures of drugs with overlapped absorption spectra.[23,24]. The greatest advantage of HPSAM is that it can remove the errors resulting from other components in the system.

HPSAM uses the analytical signal at two accurately selected wavelengths corresponding to constant absorbance of interfering substance to be plotted versus the added analyte concentration. The following principles have to be followed for selection of appropriate wavelengths for applying HPSAM:

(i) At two selected wavelengths, the signal of interfering substance must remain the same, even if the analyte is changed.
(ii) The analytical signals of the mixture composed of the analyte and interfering substance should be equal to the sum of individual signals of the two species.
(iii) The slope difference of the two straight lines obtained at two selected wavelengths must be as large as possible in order to get good accuracy.

By plotting the analytical signal against the added analyte concentration, two straight lines are obtained that have a common point with coordinates H ($-C_H$, A_H), where $-C_H$ is the unknown analyte concentration and A_H the analytical signal due to the interfering species.[25]

For determination of PIM in presence of its alkaline degradate by HPSAM, two wavelengths λ_1 (270.1 nm) and λ_2 (284 nm) were selected at which the spectrum of its degradation products show the same value of absorbance as presented in Fig. 4.

Different calibrations were plotted for various laboratory prepared mixtures to assist the accuracy and the specificity of the selected pair of wavelengths, for accuracy, different concentrations of PIM (10, 20 and 30 μg mL^{-1}) were separately added each to a constant concentration degradation products (10 μg mL^{-1}) to prepare three mixtures containing different ratios of PIM and its degradation products. To the previously prepared mixtures, different concentrations of PIM were

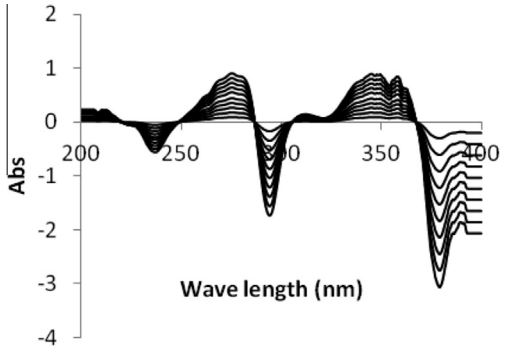

Figure 8 First derivative ratio spectra of different concentrations of PIM (10–100 μg mL^{-1}) using 20 μg mL^{-1} alkaline degradates as a divisor.

added and the absorbances at the two selected wavelengths were plotted against the added PIM concentrations.

Another set of mixtures were prepared in which PIM concentration is constant (10 μg mL^{-1}) while the concentration of the degradation products is variable (10, 20, 40 μg mL^{-1}) to assist the specificity of the method. Different concentrations of the previously prepared mixtures and the absorbances at the two selected wavelengths were plotted against the added PIM concentration.

Table 3, shows the regression parameters calculated for each calibration, it is evident from the results that the slopes at each wavelength were almost equal, and the calculated recoveries of PIM were satisfactory to assist the accuracy and specificity of the selected pair wavelengths.

Samples containing 10 μg mL^{-1} PIM and 10 μg mL^{-1} alkaline degradates were prepared, then known amounts of PIM (10, 20, 30, 40, 50, 60, 70, 80 μg) were successively added to the mixtures and the resulting absorbance at the two selected wavelengths; λ_1 (270.1 nm) and λ_2 (284 nm) at which the degradation products have the same absorbances values.

By plotting the absorbance at selected pair of wavelengths against added concentration of PIM, two straight lines are obtained that intersect at so-called H-point (its x axis value refer to original PIM concentration in the prepared mixture which is constant while its y axis value corresponds to the absorbance of the degradation products) as presented in Fig. 9.

A linear correlation was obtained between the absorbance against added PIM concentration at the two selected wavelengths 270.1 nm and 284 nm. The regression equations were computed and found to be:

$$A_{270.1} = 0.0093C + 0.145 \quad r = 0.9998$$

$$A_{284} = 0.0138C + 0.1902 \quad r = 0.9999$$

where, A is the absorbance value at the two selected wavelengths 270.1 nm and 284 nm, C is the concentration of PIM in μg mL^{-1} in the mixture and r is the correlation coefficient.

The equation used for the determination of PIM concentration using the parameters in the last two equations is:

$$C = (A_{270.1} - A_{284})/(M_{\lambda 1} - M_{\lambda 2})$$

To assess the efficiency of the proposed method as stability indicating, it was applied to laboratory prepared mixtures containing different ratios of intact PIM and its alkaline degradate.

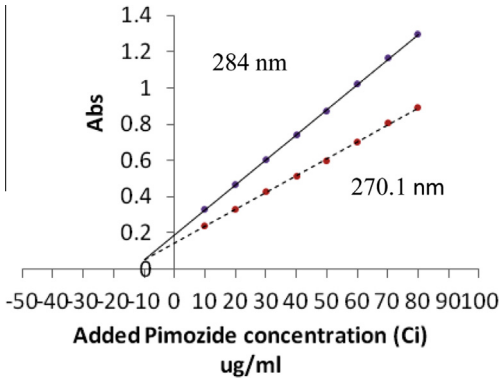

Figure 9 Plots of H-point standard method between the absorbance at 270.1 nm and 284 nm and the added concentration of PIM to mixture of PIM and alkaline degradation products (10 μg mL^{-1}each).

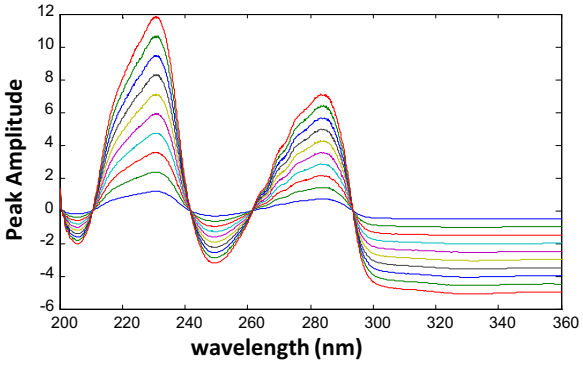

Figure 10 Mean centered ratio spectra of PIM 10–100 μg mL^{-1} in methanol using 20 μg $mL^{-1}$of degradation products as a divisor.

3.6. Mean centering method

Mean centering is a recently established spectrophotometric method that was developed for the simultaneous determination of binary and ternary mixtures without preliminary separation. This method differs from the ratio derivative method in

Table 3 Results of several experiments for the analysis of Pimozide and its alkaline degradation products in synthetic samples by HPSAM.

A–C equation	R	Taken (μg mL^{-1})		Recovery %
		PIM	Alkaline deg.	PIM
$A_{270.1} = 0.0093C_i + 0.145$	0.9998	10	10	102
$A_{284} = 0.0138C_i + 0.1902$	0.9999			
$A_{270.1} = 0.0093C_i + 0.1988$	0.9998	10	20	100
$A_{284} = 0.0138C_i + 0.2439$	0.9998			
$A_{270.1} = 0.0092C_i + 0.32$	0.9998	10	40	102.2
$A_{284} = 0.0137C_i + 0.3655$	0.9998			
$A_{270.1} = 0.0092C_i + 0.2413$	0.9997	20	10	98.90
$A_{284} = 0.0138C_i + 0.3309$	0.9999			
$A_{270.1} = 0.0093C_i + 0.3299$	0.9996	30	10	98.53
$A_{284} = 0.0139C_i + 0.4694$	0.9998			

Table 4 Validation parameters for the proposed stability-indicating spectrophotometric methods.

Validation parameters	D[1]		DR[1]		Q-analysis method		Dual wave length	HPSAM		MC
	252 nm	256.3 nm	292.5 nm	292.6 nm	242.1 nm	281.7 nm		270.1 nm	284 nm	284 nm
Linearity (μg mL^{-1})	10–100	10–100	10–100	10–100	10–100	10–100		10–100		10–100
Slope	0.0115	0.031	0.231	1.007	0.0058	0.0142	0.0045	0.0093	0.0138	0.0704
Intercept	−0.002	0.011	0.0205	−0.24	0.0027	0.0044	0.0002	0.145	0.1902	−0.00806
Correlation coefficient (*r*)	0.9999	0.9999	1	1	0.9998	0.9999	1	0.9998	0.9999	0.9999
LOD[a] (μg mL^{-1})	0.85	1.08	0.72	0.85	1.35	1.16	1.05	1.91	1.91	0.53
LOQ[a] (μg mL^{-1})	2.59	3.3	2.19	2.57	4.11	3.52	0.34	5.8	4.11	1.63
Precision *Intra-day*[b]	0.36	0.36	0.13	0.52	0.12		0.25	0.28		0.15
Inter-day[c]	0.46	0.24	0.12	0.92	0.13		0.28	0.31		0.44

[a] Limits of detection and quantitation are determined via calculations: LOD = (SD of the response/slope) × 3.3. LOQ = (SD of the response/slope) × 10.
[b] The intraday (n = 3) average of three concentrations (30, 50, 70) repeated three times within day.
[c] The interday (n = 9) average of three concentrations repeated three times in three successive days.

that it is based on mean centering of the ratio spectra instead of calculating their derivative and therefore the signal-to-noise ratio is enhanced.[26] It was used in many applications as kinetic analysis of binary mixtures[27] and determination of reactions' rate constant.[28]

As shown in Fig. 4, the absorption spectra of PIM and its alkaline degradation product in methanol are severely overlapped. So, the absorption spectra of the standard solutions of the PIM with different concentrations (10–100 μg mL^{-1}) were recorded and divided by the spectrum of 20 μg mL^{-1} of its alkaline degradate to obtain the ratio spectra. The obtained ratio spectra were mean centered and the calculated mean centered curves are shown in (Fig. 10).The concentration of PIM was determined by measuring the amplitude at 284 nm corresponding to a maximum wavelength (Fig. 10). For determination of the concentration of PIM in laboratory-prepared mixtures and samples of a pharmaceutical preparation, the same procedure was used, except that the spectra of the mixture were used instead of those of a standard solution of PIM.

The effect of divisor concentration on analytical parameters, such as slope, intercept, and correlation coefficient of the calibration graphs was also tested. Different concentrations of divisor were used, but it was observed that changing the concentration had no significant effect on the linear calibration range and calculated analytical parameters.

Results of assay validation obtained by applying the proposed spectrophotometric methods for determination of the intact PIM in presence of its degradation products are presented in Table 4.

3.7. Multivariate method

In this section, different chemometric approaches were applied for the determination of PIM in presence of its acidic and alkaline degradates, including CLS, PCR, PLS. these multivariate calibrations were useful in spectral analysis because the simultaneous inclusion of many spectral wavelengths instead of single wavelength greatly improved the precision and predictive ability.[29]

The first step in the simultaneous determination of ternary components by multivariate calibration methods involves constructing the calibration matrix for ternary mixture. The

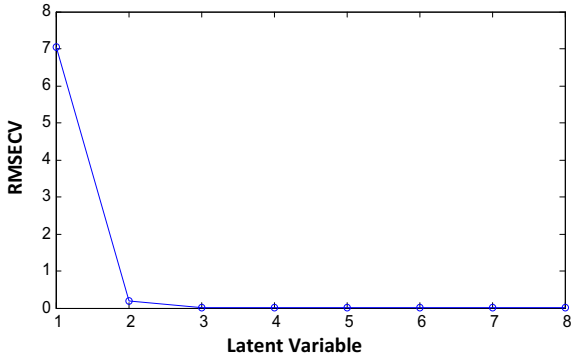

Figure 11 RMSECV plot of a training set prediction using cross validation (principal component regression model).

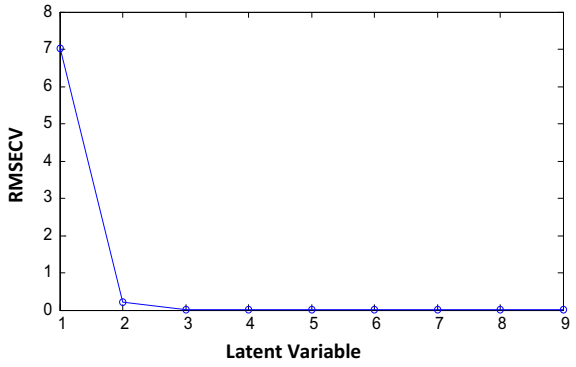

Figure 12 RMSECV plot of a training set prediction using cross validation (partial least squares model).

calibration set was obtained by using the absorption spectra of a set of 16 mixtures of PIM and both of its degradation products with different ratios of each component and their concentrations as given in Table 1. Different concentrations of PIM and both its acidic and alkaline degradates were used in the validation set, Table 2.

The UV spectra of the prepared solutions were recorded over the range 240–370 nm. Wavelengths (200–239 nm)

Table 5 Results of assay validation obtained by applying the proposed chemometric methods for the determination of Pimozide in presence of both its acidic and alkaline degradates.

Validation parameters	CLS	PCR	PLS
Predicted versus actual concentration plot			
Slope	1.000	1.001	0.9999
Intercept	0.00028	0.0011	0.0081
Correlation coefficient (r)	1.000	1.000	1.000
RMSEP	0.0030	0.0028	0.0072

dominated by noise and non informative spectral region after 370 nm are not included. Spectra were digitized each at 0.1 nm interval, and the experimental data points were exposed to MATLAB_version 7.0 for calculations.

To build the CLS model, feed the computer with absorbance and concentration matrices for training set. Carry out the calculations to obtain the "K" matrix. For the PCR and PLS models, use the training set absorbance and concentration matrices together with PLS-Toolbox 2.0 software for the calculations.

The selection of the optimum number of factors for the PLS technique was a very important step before constructing the models because if the number of factors retained was more than the required, more noise will be added to the data. On the other hand, if the number retained was too small meaningful data that could be necessary for the calibration might be discarded. In this study the leave one out cross validation method was used.[30,31] Two factors were found to be suitable for both PLS and PCR methods (Figs. 11 and 12).

The root mean square error of prediction (RMSEP) was calculated as diagnostic tool for examining the errors in the predicted concentrations. It indicated both the precision and accuracy of predictions as it played the same role of standard deviation in indicating the spread of the concentration errors.

Results of assay validation parameters obtained by applying the proposed chemometric methods for the determination of PIM in presence of its acidic and alkaline degradation products were calculated and listed in Table 5.

The proposed spectrophotometric and chemometric methods were applied successfully for the determination of PIM in presence of its acidic and alkaline degradates in different laboratory prepared mixtures and in pharmaceutical formulation. The results obtained are listed in Table 6.

Table 6 Determination of the studied drug in the laboratory prepared (L.P.) mixtures with its degradation products and in tablets by the proposed methods.

Sample	D^1-method[a]		DR^1-method[a]		Q-analysis method[a]	Dual wave length method[a]	HPSAM[a]	MC[a]	CLS[a]	PCR[a]	PLS[a]
	252 nm	256.3 nm	292.5 nm	292.6 nm							
L.P.-mixtures (n = 5)[b]	98.84 ± 0.72	99.88 ± 0.93	100.23 ± 0.51	101.22 ± 0.44	100.64 ± 0.68	100.61 ± 0.30	100.38 ± 0.34	100.78 ± 0.56	99.99 ± 0.0078	100.00 ± 0.0070	99.99 ± 0.0183
Standard error of mean	0.322	0.416	0.228	0.197	0.304	0.134	0.152	0.250	0.0035	0.0031	0.0082
Variance	0.518	0.865	0.260	0.194	0.462	0.09	0.116	0.314	0.00006	0.00005	0.0003
Orape-forte tablets 4 mg, B.N. 12Q019	101.41 ± 0.20	101.09 ± 0.34	101.22 ± 0.28	101.23 ± 0.14	100.02 ± 0.16	99.65 ± 0.13	99.77 ± 0.30	99.88 ± 0.26	100.20 ± 0.19	100.25 ± 0.15	100.27 ± 0.33
Standard error of mean	0.089	0.152	0.125	0.063	0.072	0.058	0.134	0.116	0.085	0.067	0.148
Variance	0.0004	0.116	0.078	0.0196	0.026	0.017	0.09	0.068	0.008	0.023	0.109

[a] Recovery ± SD.
[b] Sets each of 3 replicates.

Table 7 Application of the standard addition technique to the analysis of the studied drug by the proposed methods.

Pharmaceutical preparation	Authentic added (μg mL^{-1})	Standard addition										
		Recovery%*										
		D^1		DR^1		Q-analysis	Dual wave length	HPSAM	MC	CLS	PCR	PLS
		252 nm	256.3 nm	292.5 nm	292.6 nm							
Orape-forte ®tablets 4 mg B.N. 12Q019	20	102.56	98.54	99.68	101.40	99.88	102.77	101.61	98.92	99.20	99.50	99.75
	30	102.57	99.89	101.27	101.75	99.48	101.11	100.33	100.80	100.06	99.80	99.76
	40	100.63	99.83	100.12	101.20	99.62	101.38	10.80	100.28	100.30	100.42	100.42
	50	101.20	100.58	102.82	101.64	99.77	101.55	101.08	101.77	99.76	99.68	99.84
	60	100.42	99.83	100.08	100.72	99.80	101.29	100.90	101.09			
	Mean	101.47	99.73	100.97	101.34	99.71	101.62	100.94	100.57	99.83	99.85	99.94
	SD	1.03	0.73	1.27	0.41	1.15	0.66	0.46	1.06	0.47	0.40	0.32

* Average of at least 3 separate determinations.

Table 8 Statistical comparison of the results obtained by the proposed methods and the manufacturer method for the determination of Pimozide in pharmaceutical preparation.

Items	D[1]		DR[1]		Q-analyss	Dual wave length	HPSAM	MC	CLS	PCR	PLS	Reported method[*]
	252 nm	256.3 nm	292.5 nm	292.6 nm								
Mean	101.41	100.09	101.22	101.23	100.02	99.65	99.77	99.88	100.20	100.25	100.27	100.09
SD	0.20	0.34	0.28	0.14	0.16	0.13	0.30	0.16	0.19	0.13	0.13	0.14
RSD%	0.19	0.33	0.27	0.14	0.15	0.13	0.30	0.16	0.18	0.12	0.12	0.13
n	5	5	5	5	5	5	5	5	5	5	5	5
Variance	0.046	0.122	0.08	0.021	0.030	0.017	0.09	0.02	0.03	0.02	0.018	0.02
Student's *t*-test (2.306)	0.85	2.14	1.83	2.03	0.66	1.80	2.08	2.14	1.03	1.80	2.05	
F-test (6.388)	2.03	4.89	4.11	1.07	1.39	1.14	4.62	1.33	1.81	1.03	1.08	

The values between parenthesis are the theoretical values of *t*-test and *F*-test at $P = 0.05$.

[*] HPLC system using C_{18}, a mobile phase consisting methanol: phosphate buffer (8.1) (85:15) and UV detection at 254 nm.[12]

Table 9 One-way ANOVA testing for the different proposed methods used for the determination of Pimozide.

Source of variation	Sum of squares (SS)	Degree of freedom[a] (df)	Mean squares (MS)	*F*-value[b]	*F*-critical
Between groups	8.527028	10	0.852703	1.614337	2.070965
Within groups	21.65646	41	0.528206		
Total	30.18348	51			

[a] At the 0.05 level.

[b] The population means are not significantly different.

To assess the accuracy of the all proposed methods developed in this work, standard addition technique was carried out and satisfactory results were obtained, Table 7. A statistical comparison of the results obtained by the suggested methods and the official non aqueous titration[3] for the determination of pure drug was done. The values of calculated *t* and *F*-values are less than the tabulated ones,[32], which reveals that there is no significant difference with respect to accuracy and precision between the proposed methods and the reported one, Table 8. In order to compare the ability of the proposed methods for the determination of PIM, the results obtained by applying the proposed methods were subjected to statistical analysis using a one-way analysis of variance (ANOVA) test; there was no significant difference among all of the proposed methods as shown in Table 9.

4. Conclusion

The proposed methods are simple, very sensitive, precise, and can be easily applied in QC laboratories for determination of PIM in presence of its acidic and alkaline degradates. The presented work has advantages over the published stability study HPTLC work[9] of being simple, needs no such drastic organic solvent system consisting of toluene: acetone: ammonia; using simple spectrophotometer which is available in all quality control labs; and our work suggested many alternative methods to facilitate the quantitative determination of PIM.

The proposed methods could be also successfully applied for routine analysis of PIM either in its bulk powder or in dosage form in QC laboratories, without any preliminary separation step.

5. Conflict of interest

None declared.

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