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ORIGINAL ARTICLE

Determination of a novel ACE inhibitor in the presence of alkaline and oxidative degradation products using smart spectrophotometric and chemometric methods

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Abstract Simple, accurate, sensitive and validated UV spectrophotometric and chemometric methods were developed for the determination of imidapril hydrochloride (IMD) in the presence of both its alkaline (AKN) and oxidative (OXI) degradation products and in its pharmaceutical formulation. Method A is the fourth derivative spectra (D4) which allows the determination of IMD in the presence of both AKN and OXD, in pure form and in tablets by measuring the peak amplitude at 243.0 nm. Methods B, C and D, manipulating ratio spectra, were also developed. Method B is the double divisor–ratio difference spectrophotometric one (DD–RD) by computing the difference between the amplitudes of IMD ratio spectra at 232 and 256.3 nm. Method C is the double divisor-first derivative of ratio spectra method (DD–DR1) at 243.2 nm, while method D is the mean centering of ratio spectra (MCR) at 288.0 nm. Methods A, B, C and D could successfully determine IMD in a concentration range of 4.0–32.0 μg/mL. Methods E and F are principal component regression (PCR) and partial least-squares (PLS), respectively, for the simultaneous determination of IMD in the presence of both AKN and OXI, in pure form and in its tablets. The developed methods have the advantage of simultaneous determination of the cited components without any pre-treatment. The accuracy, precision and linearity ranges of the developed

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methods were determined. The results obtained were statistically compared with those of a reported HPLC method, and there was no significant difference between the proposed methods and the reported method regarding both accuracy and precision.

1. Introduction

Arterial hypertension is one of the most common cardiovascular disorders. It is considered to be a social disease due to its high incidence, contribution in the development of arteriosclerosis and its clinical forms, coronary disease, cardiac infarction, and cerebral stroke. The principal action of hypotensive medicines is based on controlling blood pressure in various pressure points. Renin–angiotensin–aldosterone system plays an important role in controlling blood pressure and volume of body fluids. This necessitates searching for medicines inhibiting the renin–angiotensin activity, including angiotensin converting enzyme (ACE) inhibitors. These inhibitors are dipeptides or compounds which are structurally very similar. ACE inhibitors differ in terms of pharmacokinetic properties and affinity for ACE. This is related to the strength and time of their activity. At present, ACE inhibitors are the first-choice medicines used in the treatment of hypertension. They may also be used in the treatment of ischemia and heart failure. Their active metabolites created as a result of hydrolysis cause the fall in ACE activity, a decrease in releasing aldosterone, an increase in the concentration of vaso-dialytic kinins and prostaglandins and indirectly reducing the synthesis of catecholamines and general sympathetic activity. This results in the diastole of vessels and reduction of circulating blood volume, which leads to the drop in blood pressure and the reduction of peripheral resistance [1].

IMD is a recently developed prodrug-type angiotensin-converting enzyme (ACE) inhibitor. IMD, with an ethyl ester structure, is converted into a dicarboxy-type compound (imidaprilat) in the body. Imidaprilat shows about a 500 times higher activity than the ester form, IMD [2]. From the literature survey on the subject, many methods have already been tested including HPLC and GC methods with the use of MS detector [3–7], chiral LC method [2], other HPLC methods [8,9], densitometry [1] and spectrophotometric determinations [8,10].

With the intent of improving the quality of the active pharmaceutical ingredient (API) and its formulation, there is an increasing need of separation, identification, quantification, and characterization of the most possible degradation products generated under the various ICH guidelines for forced degradation [11]. These guidelines require the stress testing to be conducted to elucidate the inherent stability characteristics of the active substance. An ideal stability-indicating method is one that quantifies the drug and also resolves its degradation products.

IMD is a novel ACE inhibitor and a non-official pharmacopeial raw material and therefore, there is no officially approved method neither for its determination in the pharmaceutical dosage forms nor for its stability or purity assessment: in solid state formulations, as a raw material or in body fluids. Most of the reported methods for the determination of IMD are based on chromatographic techniques, presenting a relatively high cost and time consumption. The aim of the present work is to develop accurate, specific, reproducible, rapid and less expensive stability-indicating methods for the determination of IMD in the presence of both its alkaline and oxidative degradation products for assessment of the drug stability in its dosage form, which can be performed easily with adequate software support and thus providing a clear example of the high resolving power of those techniques. Therefore, in this study, the proposed methods were firstly developed for the determination of IMD in the presence of its possible degradation products, which was not accomplished by the previously reported methods. This important feature of the adopted methods can resolve the intact drug in the presence of more than one degradation product in the same sample. Other important features of the developed methods are the higher sensitivity and the wider range of quantitation of IMD offering high flexibility in the determination of the intact drug at very low and high concentrations. Also, the application of newly developed spectrophotometric methods, double divisor–derivative ratio, double divisor–ratio difference methods and mean centering of ratio spectra, and multivariate calibration methods: principal component regression (PCR) and partial least-squares (PLS), was considered and adopted as stability-indicating methods. The developed methods were validated as per ICH guidelines [12]. The results obtained by means of the adopted methods were subsequently statistically compared. The analytical procedure for both methods comprised the following stages: (1) validation; (2) evaluation of the key parameters (i.e., linearity, accuracy and precision); and (3) assay of IMD content in the pharmaceutical formulation. The chemical structure of IMD is illustrated in Fig. 1.

2. Experimental

2.1. Samples

2.1.1. Pure sample

Imidapril hydrochloride (with certified purity of 99.80%) was kindly supplied by SMS Pharmaceuticals Limited, Hyderabad, India.

2.1.2. Pharmaceutical formulation

Tanatril® tablets (Batch no. 8917) labeled to contain 10 mg of IMD per tablet and manufactured by HIKMA Pharmaceuticals, Amman–Jordan were purchased from the local market.

2.2. Chemicals and reagents

All chemicals used throughout the work were of spectroscopic grade. Methanol was purchased from Riedel-de Haen (Seelze,

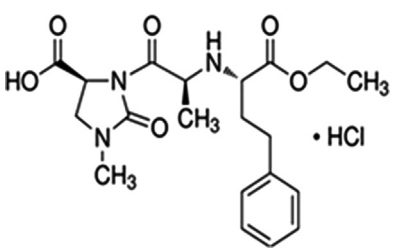

Fig. 1 Chemical structure of IMD.

Germany). 35.4% of hydrochloric acid (0.1, 3 and 5 M aqueous solutions) and sodium hydroxide (1 and 5 M aqueous solutions) were purchased from Merck (Darmstadt, Germany). Thirty percent of hydrogen peroxide (3% aqueous solution) was purchased from Fluka (Neu-Ulm, Germany). Two degraded solutions "alkaline (AKN) and oxidative (OXI)" of IMD, having concentrations of 2.0 mg/mL, were prepared by dissolving 100 mg of the drug in 10 mL of methanol and then mixed with 1 M NaOH and 3% H_2O_2, respectively; heating in thermostatic water bath at 80 °C for 12 h and 48 h, respectively. After cooling and neutralizing with 5 M HCl for AKN the volume was obtained by adding methanol for all the degraded solutions. Complete degradation was ascertained by the disappearance of the intact drug peak at 220 nm in HPLC using the mobile phase consisting of ACN:0.15% TEA, pH = 2.2 (40:60, v/v), at a flow rate of 1.5 mL/min. The obtained degradation products were subjected to infrared spectroscopy (IR) and mass spectrometry (MS) analyses for subsequent structure elucidation.

2.3. Apparatus

UV–vis spectra were recorded using a Double Beam UV–vis spectrophotometer, model-T60 (PG Instruments Ltd., UK) with spectral bandwidth of 0.2 nm connected to an IBM compatible computer and analyzed using accompanying UVWin Spectrophotometer software version 5.0.5. pH-meter (Jenway 3310, UK), equipped with combined glass electrode was used for pH adjustment.

High-performance thin layer chromatography (HPTLC) system used to ensure complete degradation of IMD drug molecule consisted of a Camag TLC scanner III S/N 130319 (Camag, Muttenz, Switzerland) operated with winCATS software version 3.15, Linomat IV autosampler (Camag, Muttenz, Switzerland), Camag microsyringe 100 μL (Hamilton, Bonaduz, Switzerland), precoated silica gel aluminum plates 60 F254 (20 cm × 10 cm) with 250 μm thickness (E. Merck, Darmstadt, Germany), and twin trough Automatic Developing Chamber ADC 2 chamber 20 cm × 10 cm (Camag, Muttenz, Switzerland).

Statistical comparison of the proposed methods with a reported HPLC method was carried out using LaChrom Elite® HPLC system (VWR-Hitachi International GmbH, Darmstadt, Germany), which comprised L-2130 model pump, equipped with a L-2400 model UV detector and a 20-μL volume injection loop. LaChrom Elite® HPLC system was controlled by EZChrom Elite Software Chromatography Data System, version 3.3.1 SP1. A Phenomenex C18 column (250 mm × 4.6 mm i.d., 5 μm) was used as the stationary phase.

2.4. Software

All computations were performed in Matlab® version 7.10 [13]. The PLS procedure was computed using PLS Toolbox version 5.8 [14].

2.5. Spectral characteristic

The zero-order (D0) absorption spectra of IMD and its degradates, AKN and OXI in 0.1 M HCl, were recorded separately over the range of 200–350 nm using 0.1 M HCl as blank.

2.6. Procedure

2.6.1. UV spectrophotometric methods

2.6.1.1. Stock and working solutions. Stock standard solution of IMD (2.0 mg/mL) was prepared by accurately weighing 100 mg of pure powder into a 50 mL volumetric flask, dissolved in methanol and diluted to the required volume with methanol. Stock solutions of AKN and OXI (2.0 mg/mL) were prepared as previously mentioned in Section 2.2.

Working standard solution of IMD and working solutions of AKN and OXI (100 μg/mL; each) were prepared by transferring 5 mL from the stock standard solution of IMD and the stock solutions of AKN and OXI (2.0 mg/mL; each) into three separate 100-mL volumetric flasks, dissolved in methanol and diluted to the volume with methanol.

The binary mixture solution of AKN and OXI (3 μg/mL; each), was used as double divisor (DD).

Aliquots equivalent to 40–320 μg IMD were accurately transferred from its working standard solution (100 μg/mL) into a series of 10-mL volumetric flasks. The volume was brought to the mark with 0.1 M HCl to obtain a final concentration range of 4–32 μg/mL. The zero order absorption spectra of the resulting solutions were scanned from 200 to 350 nm and stored in the computer.

2.6.1.2. Derivative specrophotometry (D4). Construction of the calibration curve. The fourth derivative (D4) spectra were computed using scaling factor = 10^4 and $\Delta\lambda = 4$ nm. The peak amplitude at 243.0 nm was measured for IMD, then plotted each against its corresponding concentration and the regression parameters were computed.

Assay of laboratory-prepared mixtures. The absorption spectrum was recorded for each laboratory-prepared mixture, containing different ratios of IMD, AKN and OXI against 0.1 M HCl as a blank and stored in the computer. The peak amplitudes of (D4) spectra of those laboratory-prepared mixtures were measured at 243.0 nm for IMD determination. The concentrations of IMD were then calculated from its corresponding regression equation.

2.6.1.3. Double divisor–ratio difference spectrophotometric (DD–RD) method. Construction of the calibration curve. The stored spectra of IMD were divided by the spectrum of DD and stored in the computer as ratio spectra. Calibration curve for IMD was constructed by plotting the difference between the amplitudes of ratio spectra at 232.0 nm and 256.3 nm, versus the corresponding concentration and the regression equation was computed.

Assay of laboratory-prepared mixtures. The scanned spectra of the laboratory-prepared mixtures were divided by the absorption spectrum of DD and stored in the computer, and then the difference between the amplitudes of ratio spectra at 232.0 nm and 256.3 nm was computed. The concentration of IMD in the mixtures was calculated from the corresponding regression equation.

2.6.1.4. Double divisor–derivative ratio spectrophotometric (DD–DR1) method. Construction of the calibration curve. The peak amplitude of the first derivative of the stored ratio spectra of IMD at 243.2 nm was differentiated using scaling factor = 10 and $\Delta\lambda = 4$ nm, then plotted versus the corresponding concentration of IMD, and the regression equation was computed.

Assay of laboratory-prepared mixtures. The first derivative of the stored ratio spectra of the laboratory-prepared mixtures was computed. The concentration of IMD in the mixtures was calculated from the corresponding regression equation at 243.2 nm.

2.6.1.5. Mean centering of ratio spectra (MCR) method. Construction of the calibration curve. The scanned (D0) spectra of IMD, AKN and OXI were exported to Matlab for subsequent calculation, then the spectra of IMD were divided by the normalized spectrum of AKN, the obtained ratio spectrum was mean centered and then the mean centered ratio spectra were divided by the mean centered normalized vector of AKN/OXI and then mean centered. The calibration curve for IMD was constructed by plotting the mean centered values at 288.0 nm, versus the corresponding concentration of IMD and the regression equation was computed.

Assay of laboratory-prepared mixtures. The general procedure of the proposed method mentioned under "*Construction of the calibration curve*" was followed. The concentration of IMD was calculated using the mean centered values at 288.0 nm and the specified regression equation.

2.6.2. Chemometric-assisted spectrophotometric methods

2.6.2.1. Stock and working solutions. Stock standard solution of IMD (2.0 mg/mL) was prepared as mentioned previously in Section 2.6.1. Stock solutions of AKN and OXI (2.0 mg/mL; each) were prepared as mentioned previously in Section 2.2.

Working standard solution of IMD (100 μg/mL) was prepared as mentioned previously in Section 2.6.1. Working solutions of AKN and OXI (10 μg/mL; each) were prepared by transferring 0.5 mL from the stock solutions of AKN and OXI (2.0 mg/mL; each) into two separate 100-mL volumetric flasks, dissolved in and diluted to the volume with methanol.

Multilevel multifactor design was used for the construction of the calibration and validation sets [15]. A five-level, five-factor calibration design was used. The concentrations details are given in Table 1.

Table 1 Concentrations of IMD, AKN and OXI in the calibration and validation sets for PCR and PLS.

Sample number	IMD (μg/mL)	AKN (μg/mL)	OXI (μg/mL)
1	15.0	3.0	3.0
2	15.0	2.4	2.4
3	12.0	2.4	3.6
4	12.0	3.6	2.7
5	18.0	2.7	3.6
6	**13.5**	**3.6**	**3.0**
7	18.0	3.0	2.7
8	**15.0**	**2.7**	**2.7**
9	**13.5**	**2.7**	**3.3**
10	**13.5**	**3.3**	**3.6**
11	**16.5**	**3.6**	**3.3**
12	18.0	3.3	3.0
13	**16.5**	**3.0**	**3.6**
14	15.0	3.6	3.6
15	18.0	3.6	2.4
16	18.0	2.4	3.3
17	12.0	3.3	2.4
18	**16.5**	**2.4**	**3.0**
19	12.0	3.0	3.3
20	**15.0**	**3.3**	**3.3**
21	16.5	3.3	2.7
22	16.5	2.7	2.4
23	13.5	2.4	2.7
24	12.0	2.7	3.0
25	13.5	3.0	2.4

The boldfaced samples are those of the validation set.

2.6.2.2. Chemometric PCR and PLS methods. Building the calibration models. A calibration set of seventeen different laboratory-prepared mixtures of IMD, AKN and OXI in different ratios was prepared by transferring different aliquots from the working standard solution of IMD (100 μg/mL) and the working solutions of AKN (10 μg/mL) and OXI (10 μg/mL) into 10-mL volumetric flasks and the volumes were completed with 0.1 M HCl. The absorption spectra of the prepared mixtures were recorded in the range of 205–305 nm against 0.1 M HCl as blank. The recorded spectra were then transferred to MATLAB® 7.10 for subsequent data analysis and the calibration models (PCR, PLS) were constructed.

Assay of external validation set. The absorption spectra of the validation set that consisted of eight laboratory-prepared mixtures containing different ratios of IMD, AKN and OXI were recorded in the range of 205–305 nm. The concentrations of IMD, AKN and OXI were calculated using the optimized PCR and PLS calibration models.

2.6.3. Determination of IMD in its pharmaceutical formulation (Tanatril® tablets)

Ten tablets of Tanatril® were finely powdered. A portion of the powdered tablets equivalent to 20 mg of IMD was transferred into a 25-mL volumetric flask and sonicated for 20 min with 20 mL methanol, then the volume was completed with the same solvent and filtered to prepare stock solution, having a concentration of 0.8 mg/mL. Aliquot of 1.5 mL was transferred to 25-mL volumetric flasks and the volume was completed with methanol; then from this solution, second dilutions were made by transferring aliquots of 2.5, 3.0 and 3.5 mL into three separate 10-mL volumetric flasks and the volume was completed with 0.1 M HCl. The general procedures described above for each of the proposed methods were followed to determine the concentration of IMD in its pharmaceutical preparation.

2.6.4. Application of standard addition technique

To check the validity of the proposed methods, the standard addition technique was applied. Three portions of the previously powdered tablets, each claimed to contain 10 mg of IMD were accurately weighed and mixed with 8, 10 and 12 mg of the pure standard IMD, separately. Each spiked sample was transferred to a 25 mL volumetric flask and sonicated for 20 min with 20 mL methanol then the volume was adjusted with the same solvent and filtered to obtain three solutions having the concentrations of 0.72, 0.8 and 0.88 mg/mL. Aliquot of 2.5 mL from each spiked solution was transferred into three separate 25-mL volumetric flasks and the volume was obtained with methanol. Then from these solutions, second dilutions were made by transferring aliquot of 2.0 mL from each spiked sample into three separate 10-mL volumetric flasks and diluting with 0.1 M HCl. The general procedures described in Sections 2.6.1 and 2.6.2 were then proceeded.

3. Results and discussion

The main task of this work was to establish simple, sensitive and accurate analytical methods for the simultaneous determination of IMD in the presence of both its alkaline and oxidative degradation

products and in its pharmaceutical formulation with satisfactory precision for good analytical practice (GAP) and to develop methods of comparable sensitivity and selectivity as chromatographic methods with lower cost and higher speed. Also, to construct a comparison between the efficiency of the proposed methods and a reported HPLC method.

Upon stress testing of IMD with alkali, the drug underwent degradation giving a diacid derivative of IMD through hydrolysis of ester linkage on the basis of the studies of Nishi et al. [2]. Degradation of IMD was also examined upon stress testing using peroxide. It was noticed that diketopiperazine (DKP) derivative was produced via oxidation and internal cyclization of IMD as suggested by Stofik et al. [16]. IMD was exposed to different time periods (12 h and 48 h) at 80 °C as an accelerated stress test to carry out complete drug degradation under alkaline and oxidative conditions, respectively. Complete drug degradation under previously mentioned conditions was ascertained using previously developed HPTLC method as presented in Fig. 2.

The assignment of IMD degradation was based on comparison of IR and MS spectral data for the degradation products with that of the intact drug as shown in Figs. 3 and 4. IR spectrum of IMD shows

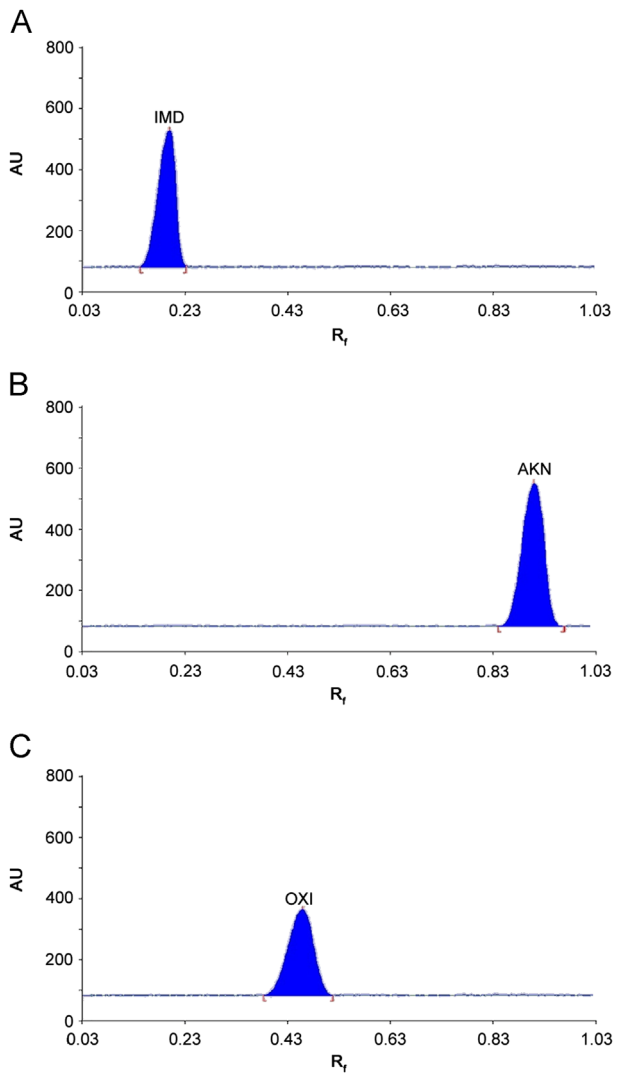

Fig. 2 HPTLC chromatograms of IMD (A), AKN (B) and OXI (C), using chloroform:ethanol:acetic acid (3:0.5:0.1, by volume) at 220 nm.

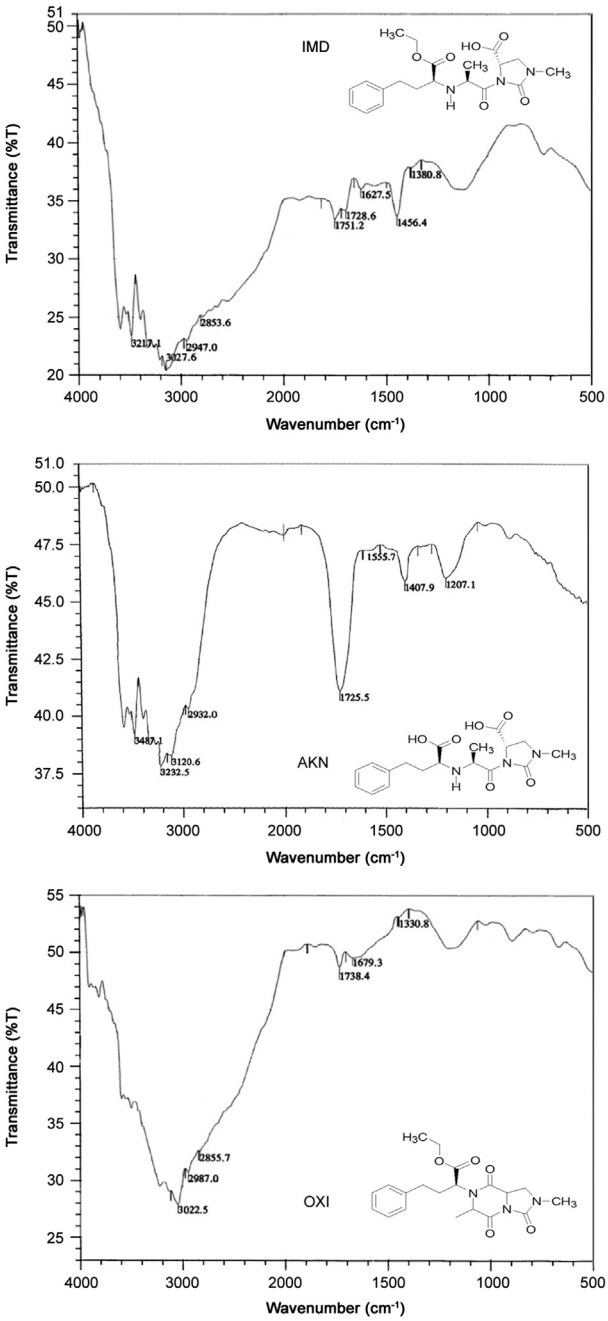

Fig. 3 IR spectra of IMD, AKN and OXI.

peaks at 3217 and 3027 cm^{-1}, which are assigned to the stretching vibrations of N–H (the secondary amine) and C–H bands of aromatic ring. The peaks at 2947 and 2853 cm^{-1} are due to the asymmetric CH_3 and CH_2 stretching vibrations, respectively. The peaks at 1751 and 1728 cm^{-1} are attributed to the carbonyl stretching of ester and carboxylic acid, respectively; while the peak at 1627 cm^{-1} corresponds to the carbonyl stretching of tertiary amide; the peak at 1456 cm^{-1} corresponds to CH_2 scissoring; the peak at 1380 cm^{-1} is due to C–H bending. The IR spectrum of AKN shows the absence of the peak at 1751 cm^{-1}, which corresponds to the carbonyl stretching of ester. But the presence of intense and sharp peak at 1725 cm^{-1} is attributed to the carbonyl stretching of the two carboxylic acid groups of the diacid derivative of IMD produced through hydrolysis of ester linkage. The disappearance of IR peaks in OXI spectrum

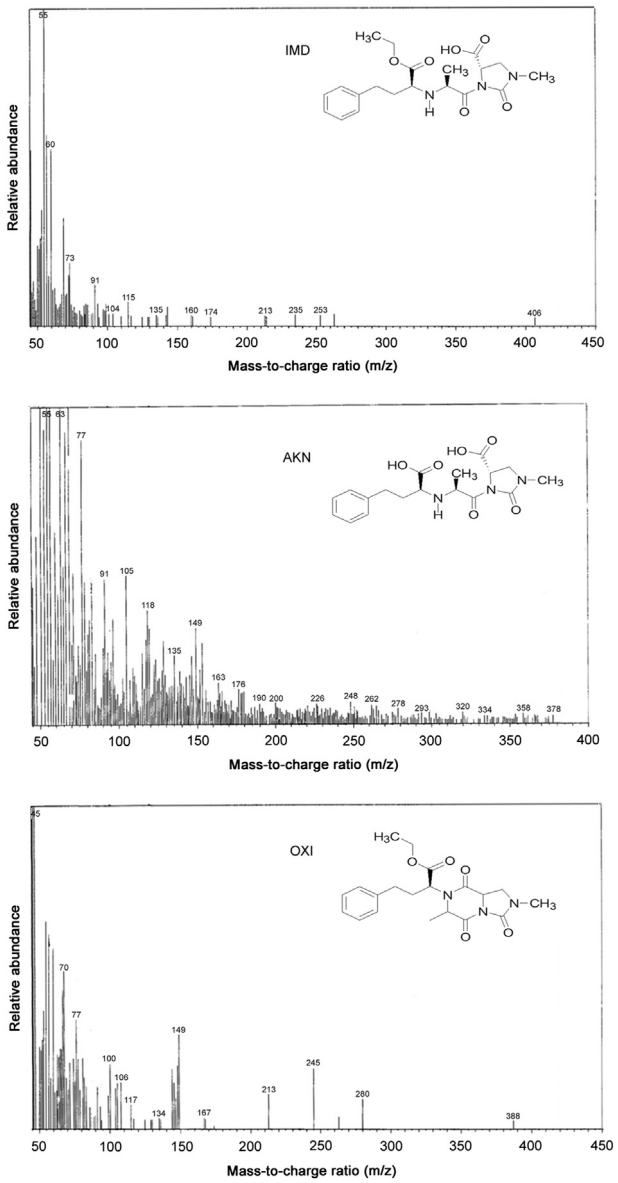

Fig. 4 MS spectra of IMD, AKN and OXI.

at 3217 cm^{-1} (the secondary amine), 1728 cm^{-1} (the carbonyl group of carboxylic acid), and 1627 cm^{-1} (the carbonyl stretching of tertiary amide) confirmed the DKP formation. But several new peaks at 2987, 1738 and 1679 cm^{-1} were observed. The peak at 2987 cm^{-1} was due to the asymmetric CH_3 stretching vibration, and the peaks at 1738 and 1679 cm^{-1} were assigned to the carbonyl bands of ester and DKP groups, respectively[17].

The mass ion peak of IMD was identified at *m/z* 407, while that of AKN and OXI was at *m/z* 378 and *m/z* 387, respectively. Therefore, one can conclude that carrying out the alkaline hydrolysis and oxidative degradation of IMD may proceed as shown in Fig. 5.

IMD spectrum suffers a strong overlap with those of its degradation products, AKN and OXI as shown in Fig. 6, which prevents direct determination of IMD. Derivative spectrophotometry (D4), DD–RD, DD–DR1 and MCR were applied to allow the resolution of IMD. Also, multivariate calibration for the resolution of these components had been successfully performed using chemometric methods, PCR and PLS.

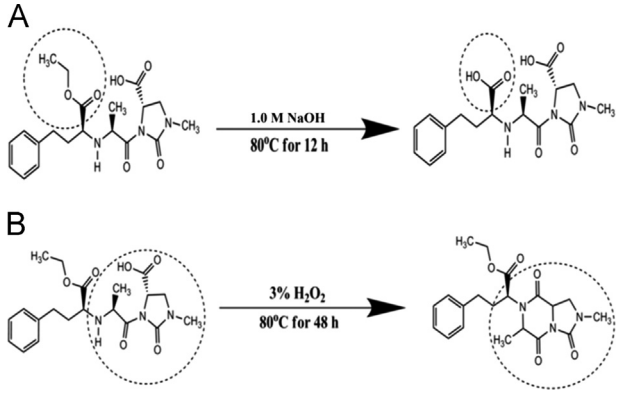

Fig. 5 Schemes of IMD degradation under alkaline (A) and oxidative (B) conditions.

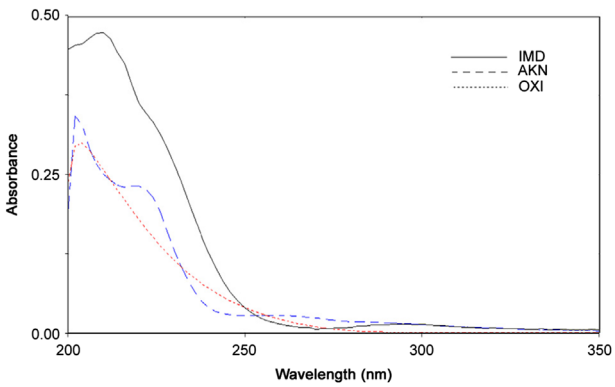

Fig. 6 Zero order absorption spectra of IMD (12 μg/mL) and of its degradates, AKN and OXI (3 μg/mL each) using 0.1 M HCl as a solvent.

3.1. UV spectrophotometric methods

3.1.1. D4

Derivative spectrophotometry was first suggested during the last decade and soon became a well established technique for the assay of drug in mixtures and in pharmaceutical dosage forms [18]. It is a good technique and capable of enhancing the resolution of overlapped bands [19]. It can be applied for the determination of a drug in the presence of another by selecting a wavelength where contribution of one compound is zero or almost zero while the compound to be determined has a reasonable value.

The fourth derivative (D4) showed that IMD could be determined by measuring the peak amplitude at 243.0 nm, without any interference in its measurement from either AKN or OXI; whose contributions are almost zero, as shown in Fig. 7.

To optimize this method, it was necessary to test the influence of variables:

(a) Different $\Delta\lambda$ values were tried (2–8) where $\Delta\lambda = 4$ showed a suitable signal to noise ratio and the spectra showed good resolution.
(b) Different scaling factor values were tried (10–10^4) where scaling factor 10^4 was suitable to enlarge the signal of IMD to facilitate its measurement and to diminish error in reading signal without any interference from AKN or OXI.

A linear correlation was obtained between the peak amplitude values and the corresponding concentrations for IMD at 243.0 nm.

The characteristic parameters of the regression equation of the (D4) method for the determination of IMD are given in Table 2.

3.1.2. DD–RD

This is a newly developed method having the ability for solving severely overlapped spectra without prior separation mean while it does not require any sophisticated apparatus or computer programs [20]. The most striking feature of the DD–RD method is its simplicity, rapidity and accuracy. The utility of the DD–RD method is to calculate the unknown concentration of a component present in a ternary mixture containing the component of interest and other two unwanted interfering components. For the determination of concentration of component of interest by the DD–RD method, the only requirement is the contribution of the three components at the two selected wavelengths $\lambda 1$ and $\lambda 2$ where the ratio spectrum of the interfering components shows the same amplitudes (constant) whereas the component of interest shows significant difference in these two amplitude values at these two selected wavelengths with respect to concentration. The overlapped spectra of the cited components suggested that the DD–RD method was a suitable method for the determination of IMD in the presence of both its degradates, AKN and OXI.

Ratio difference method began with scanning the zero order absorption spectra of their laboratory-prepared mixtures (IMD, AKI and OXI). For determination of IMD, each spectrum of the previous mixtures was divided by the absorption spectrum of a solution of binary mixture of AKN and OXI (3 μg/mL; each) as a double divisor (DD) producing new ratio spectra which represent IMD/(AKN+OXI)′+constant, as shown in Fig. 8. The amplitudes at 232.0 nm and 256.3 nm were selected. The amplitudes at these two wavelengths were subtracted, so the constant (AKN+OXI)/(AKN+OXI)′ will be canceled.

To optimize this method, it was necessary to test the influence of the divisor and its concentration. Therefore, different concentrations of AKN and OXI were tried as double divisor. It was found that changing the concentration had no significant effect on the linear calibration range and the calculated analytical parameters.

The concentration of IMD was calculated using the corresponding regression equation obtained by plotting the difference in the amplitudes at 232.0 nm and 256.3 nm of the ratio spectra of IMD/(AKN+OXI)′ against its corresponding concentrations. The characteristic parameters of the regression equation of the DD–RD method for the determination of IMD are given in Table 2.

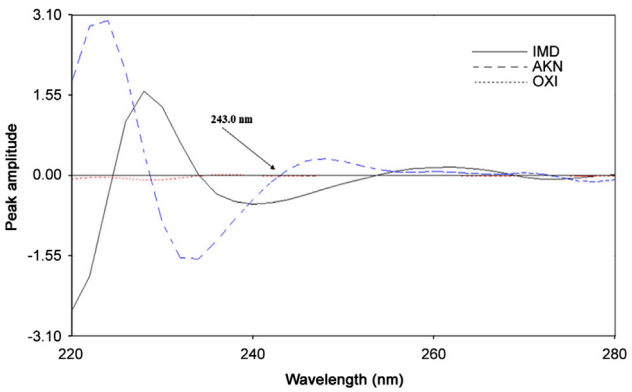

Fig. 7 The fourth derivative (D4) spectra of IMD (12 μg/mL) and of its degradates, AKN and OXI (3 μg/mL, each) using 0.1 M HCl as a solvent.

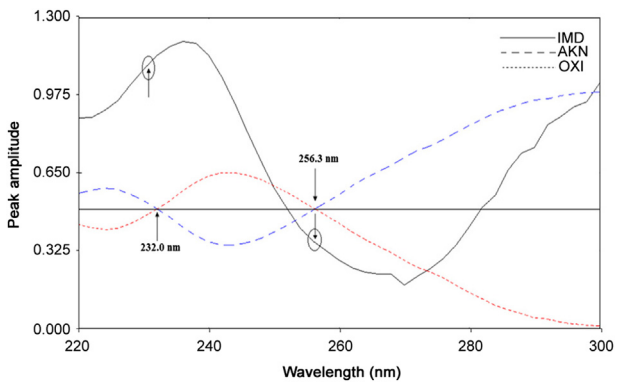

Fig. 8 Ratio spectra of IMD (12 μg/mL) and of its degradates, AKN and OXI (3μg/mL each) using DD as double divisor of AKN and OXI (3 μg/mL each) and 0.1 M HCl as a blank.

Table 2 Results of regression and assay validation parameters of the developed spectrophotometric methods for determination of IMD in the presence of both AKN and OXI.

Validation parameters	D4	DD–RD	DD–DR1	MCR
Linearity range (μg/mL)	4–32	4–32	4–32	4–32
Slope	0.0413	0.0645	0.0444	2.5018
SE of slope	0.0003	0.0004	0.0004	0.0194
Intercept	−0.0025	0.0144	−0.0116	0.1989
SE of intercept	0.0048	0.0074	0.0075	0.3612
Correlation coefficient (r)	0.9998	0.9998	0.9996	0.9997
SE of regression	0.0060	0.0093	0.0095	0.4525
SE of residuals	0.0054	0.0083	0.0085	0.4047
LOD (μg/mL)	0.43	0.43	0.63	0.53
LOQ (μg/mL)	1.30	1.29	1.90	1.62
Accuracy (Mean ± SD)	100.14 ± 0.52	99.78 ± 1.01	100.91 ± 0.79	99.87 ± 0.96
Precision (RSD, %)				
Intra-day	0.250	0.254	0.294	0.307
Inter-day	0.891	0.962	0.900	0.958

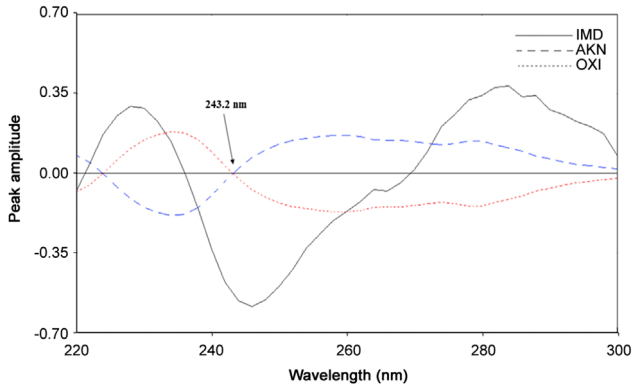

Fig. 9 The first derivative of the ratio spectra of IMD (12 µg/mL) and of its degradates, AKN and OXI (3 µg/mL each) using DD as double divisor of AKN and OXI (3 µg/mL each) and 0.1 M HCl as a blank.

3.1.3. *DD–DR1*

Another method for resolving ternary mixtures without previous separation is the DD–DR1 method, which was developed by Dinc et al. [21]. In this method the absorption spectrum of the mixture is obtained and divided by the absorption spectrum of a solution of binary mixture of AKN and OXI (3 µg/mL; each) as a double divisor (DD) and then the first derivative of the ratio spectrum is obtained. This method permits the determination of IMD in its mixture at 243.2 nm, as presented in Fig. 9.

To optimize this method, it was necessary to test the influence of variables:

(a) The divisor and its concentration: different concentrations of AKN and OXI were tried as double divisor. It was found that changing the concentration had no significant effect on the linear calibration range and the calculated analytical parameters.
(b) Different $\Delta\lambda$ values were tried where $\Delta\lambda=4$ showed a suitable signal to noise ratio and the spectra showed good resolution.
(c) Different scaling factor values were tried where scaling factor 10 was suitable to enlarge the signal of IMD to facilitate its measurement and to diminish errors in reading signal.

Calibration curve was constructed at 243.2 nm, representing the relationship between the peak amplitudes of DD–DR1 and the corresponding concentrations of IMD. The characteristic parameters of the regression equation of the DD-DR1 method for the determination of IMD are given in Table 2.

3.1.4. *MCR*

It is a new spectrophotometric method that was developed for the simultaneous determination of binary and ternary mixtures without preliminary separation. This method is based on the mean centering of ratio spectra instead of calculating the derivative and therefore signal-to-noise ratio is enhanced [22].

As shown in Fig. 6, the absorption spectra of IMD, AKI and OXI are severely overlapped. So, the absorption spectra of the standard solutions of IMD with different concentrations were recorded in the wavelength range of 200–350 nm and divided by the normalized spectrum of AKN (3 µg/mL) and the obtained ratio spectra were then divided by the mean centered vector of AKN/OXI (OXI, 3 µg/mL), then the obtained second ratio spectra were mean centered as presented in Fig. 10.

The effect of divisor concentration on the 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 the calculated analytical parameters. A calibration curve could be constructed by plotting the amplitude at 288.0 nm against the corresponding concentrations of IMD. The characteristic parameters of the regression equation of the MCR method for the determination of IMD are given in Table 2.

3.2. *Chemometric two-way PCR and PLS methods*

The spectral data acquisition was taken with 0.1 nm intervals, thus producing 1001 data points per spectrum. In order to decrease the initial number of wavelengths, every 10th wavelength was selected, thus the produced spectral data matrix has 17 rows representing different samples and 101 columns representing wavelengths (17×101). In order to build the PCR and PLS models, the raw data of the calibration samples were mean centered [23] as a pre-processing step and the 'random subsets' cross-validation method was used [24]; each subset consisted of six and iterated four times. The appropriate selection of the number of factors to be used for building the model is crucial for achieving correct quantitation in PCR and PLS calibrations.

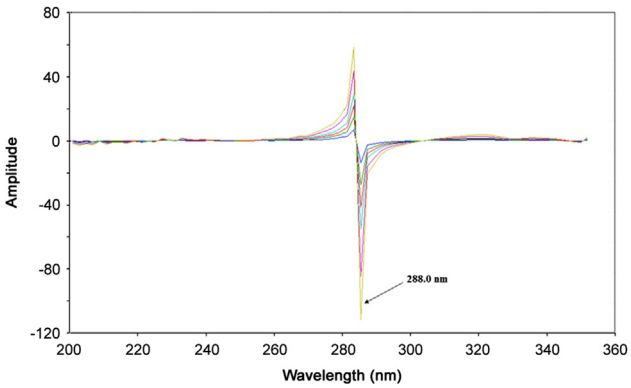

Fig. 10 Mean centering of ratio spectra of IMD, 4–32 µg/mL in 0.1 M HCl using the spectra of its degradation products as divisor of AKN and OXI (3 µg/mL each).

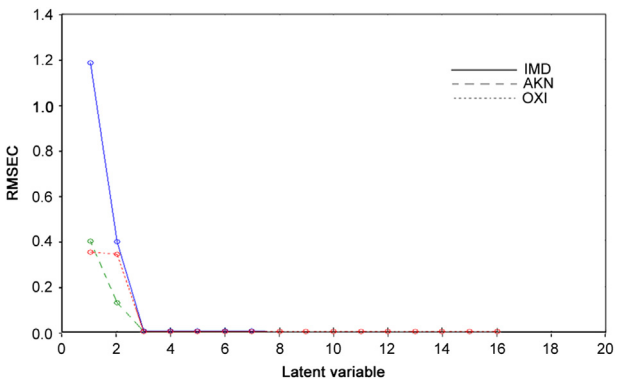

Fig. 11 Root mean square error of calibration (RMSEC) plot of the cross validation results of the training set as a function of the number of principal components used to construct the PCR calibration.

The optimum number of latent variables was selected according to Haaland and Thomas criteria [25]. The selected model was that with the smallest number of factors such that root mean square error of calibration for that model was not significantly greater than root mean square error of calibration from model with an additional factor. The optimum number of latent variables described by the constructed models was found to be four factors for both PCR and PLS as shown in Figs. 11 and 12.

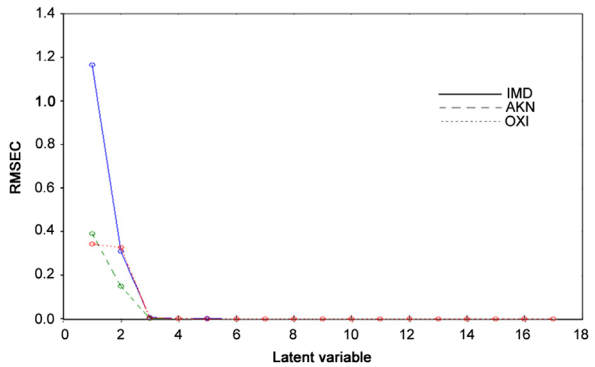

Fig. 12 Root mean square error of calibration (RMSEC) plot of the cross validation results of the training set as a function of the number of principal components used to construct the PLS calibration.

In order to assess the predictive ability of each of the developed models, it was applied on an external validation set for determination of the three components. The recoveries, mean recoveries and RMSEP are summarized in Table 3.

The validation of the developed PCR and PLS models was assessed using several diagnostic tools. The predicted concentrations of the validation samples were plotted against the true concentration values. This was used to determine whether the model accounted for the concentration variation in the validation set. All plots had a slope of nearly one and an intercept close to zero. The results in Table 4 indicate the high predictive abilities of the two models.

3.3. Determination of IMD in its pharmaceutical formulation (Tanatril® tablets)

The suggested methods were successfully applied for determination of IMD in Tanatril® tablets. The results shown in Table 5 are satisfactory and with good agreement with the labeled amount.

ICH guidelines [12] for method validation were followed for validation of the suggested methods.

3.3.1. Linearity

The linearity of the proposed spectrophotometric methods was evaluated by analyzing six concentrations of IMD ranging from 4 to 32 μg/mL for all of the proposed UV spectrophotometric methods. Each concentration was repeated three times. The assay

Table 3 Percentage recoveries of IMD, AKN and OXI in the validation set using PCR and PLS models.

Sample no.	IMD			AKN			OXI		
	Conc. (μg/mL)	Recovery (%)		Conc. (μg/mL)	Recovery (%)		Conc. (μg/mL)	Recovery (%)	
		PCR	PLS		PCR	PLS		PCR	PLS
1	13.5	99.90	99.94	3.6	100.00	100.00	3.0	100.20	100.11
2	15.0	99.91	99.89	2.7	99.97	99.96	2.7	100.30	100.36
3	13.5	100.04	100.02	2.7	100.02	100.00	3.3	99.91	99.97
4	13.5	99.98	99.99	3.3	99.87	99.88	3.6	100.16	100.11
5	16.5	99.95	99.95	3.6	100.02	100.04	3.3	100.10	100.07
6	16.5	99.85	99.86	3.0	99.85	99.86	3.6	100.48	100.44
7	16.5	100.01	100.07	2.4	99.96	99.95	3.0	99.98	99.86
8	15.0	100.07	100.07	3.3	100.23	100.24	3.3	99.61	99.58
Mean		99.96	99.97		99.99	99.99		100.09	100.06
RMSEP		0.012	0.012		0.004	0.004		0.009	0.009

Table 4 Summary of results obtained by applying the diagnostic tools for model validation of PCR and PLS chemometric methods.

Validation parameters	IMD		AKN		OXI	
	PCR	PLS	PCR	PLS	PCR	PLS
Predicted vs. known concentration plot						
1—Slope	0.9979	0.9985	1.0016	1.0022	1.0034	1.0024
2—SE of slope	0.003	0.004	0.003	0.003	0.011	0.012
3—Intercept	0.0260	0.0180	−0.0050	−0.0069	−0.0079	−0.0056
4—SE of intercept	0.050	0.054	0.011	0.010	0.037	0.038
6—Correlation coefficient	0.9999	0.9999	0.9999	0.9999	0.9991	0.9991
7—SE of regression	0.012	0.013	0.004	0.004	0.009	0.010
Residual vs. actual concentration plot ± error in prediction	0.011	0.012	0.004	0.004	0.009	0.009

Table 5 Quantitative determination of IMD in its pharmaceutical preparation by the proposed methods and application of standard addition technique.

Proposed methods	Pharmaceutical preparation Tanatril® tablets (10 mg IMD per tablet)	Recovery of IMD(%)[a]			
	(Mean (%) ± SD)	Authentic added (μg/mL)			Mean (%)±SD
		6.4	8.0	9.6	
D4	99.93±0.87	99.91	101.11	99.89	100.30±0.70
DD–RD	100.12±0.91	100.02	101.36	100.25	100.54±0.72
DD–DR1	100.08±0.81	100.72	101.48	100.31	100.83±0.59
MCR	100.05±0.69	99.12	101.10	100.28	100.17±1.00
PCR	99.86±0.70	99.18	100.11	100.03	99.77±0.52
PLS	99.96±0.70	100.21	100.10	99.05	99.79±0.64

[a]Mean of three determinations; standard addition technique; pharmaceutical preparation taken was 8 μg/mL.

Table 6 Determination of IMD in the presence of different proportions of its degradates, AKN and OXI, in laboratory prepared mixtures by the proposed spectrophotometric methods.

Laboratory prepared mixture				Recovery* of IMD (%)			
% Degradation	IMD (μg/mL)	AKN (μg/mL)	OXI (μg/mL)	D4	DD–RD	DD–DR1	MCR
20	16.0	3.0	1.0	98.24	100.10	100.49	98.24
	16.0	1.0	3.0	99.45	100.19	100.49	99.29
	16.0	2.0	2.0	99.29	100.19	100.49	99.78
	16.0	4.0	0.0	100.50	100.29	100.49	98.84
	16.0	0.0	4.0	100.35	100.29	100.49	98.48
50	8.0	6.0	2.0	99.07	98.41	99.45	98.75
	8.0	2.0	6.0	99.67	98.61	99.45	100.52
	8.0	4.0	4.0	100.28	98.80	99.45	98.21
	8.0	8.0	0.0	99.37	99.00	99.45	98.46
	8.0	0.0	8.0	101.49	99.19	99.45	99.16
80	4.0	12.0	4.0	101.34	99.51	99.88	99.11
	4.0	4.0	12.0	103.16	99.12	99.88	98.15
	4.0	8.0	8.0	100.13	99.90	99.88	100.49
	4.0	16.0	0.0	102.55	98.73	99.88	100.68
	4.0	0.0	16.0	100.74	98.35	99.88	98.91
Mean (%)±SD				100.38 ±1.33	99.38 ±0.73	99.94±0.44	99.14 ±0.86

*Mean of three determinations.

was performed according to the experimental conditions previously mentioned (Table 2).

3.3.2. *Accuracy*

The accuracy of the results was checked by applying the proposed spectrophotometric methods for the determination of different blind samples of IMD. The concentrations were obtained from the corresponding regression equations, from which the percentage recoveries were calculated with mean percentage recovery (Table 2).

Accuracy of the method was further assured by the use of the standard addition technique, it was performed by the addition of known amounts of pure IMD to known concentrations of the pharmaceutical formulation. The resulting mixtures were assayed, and the results obtained were compared with the expected results (Table 5). The good recoveries of the standard addition technique suggested good accuracy of the proposed methods.

3.3.3. *Precision*

3.3.3.1. Repeatability. Three concentrations of IMD (6, 14, and 28 μg/mL) were analyzed three times intra- daily using the proposed spectrophotometric methods. The percentage recoveries and relative standard deviation were calculated (Table 2).

3.3.3.2. Intermediate precision. The previous procedures were repeated inter-daily three times on three different days for the analysis of the three chosen concentrations. The percentage recoveries and relative standard deviation were calculated (Table 2).

Table 7 Statistical comparison between the reported HPLC method and the adopted methods for the determination of IMD.

Method	Mean (%)	S.D.	Variance	*t*-Test [a]	*F*-test [a]
Reported method[b]	100.75	0.96	0.912	–	–
D4	99.93	0.87	0.751	1.55	1.22
DD–RD	100.12	0.91	0.826	1.16	1.10
DD–DR1	100.08	0.81	0.650	1.32	1.40
MCR	100.05	0.69	0.474	1.45	1.92
PCR	99.86	0.70	0.485	1.85	1.88
PLS	99.96	0.70	0.491	1.63	1.86

[a]The theoretical values of t and F at $P=0.05$ are 2.23 and 5.05, respectively ($n=6$).

[b]HPLC method using C18 column, flow rate of 1.0 mL/min, mobile phase composed of acetonitrile–methanol–phosphate buffer, pH 2.0 (60:10:30, v/v/v) and UV detection at 254 nm.

3.3.4. *Range*

The calibration range was established through considerations of the practical range necessary according to adherence to Beer's law and the concentration of IMD present in the pharmaceutical preparations to give accurate, precise and linear results (Table 2).

3.3.5. *Detection and quantitation limits*

According to the ICH recommendations [12], the approach based on the SD of the response and the slope was used for determining the limits of detection (LOD) and quantitation (LOQ) (Table 2).

$$\text{LOD} = 3.3 \times \text{SD of the response/slope}$$

$$\text{LOQ} = 10 \times \text{SD of the response/slope}$$

3.3.6. *Specificity*

Specificity of the proposed spectrophotometric methods was achieved by the analysis of different laboratory prepared mixtures of IMD and its degradation products, AKN and OXI, within the linearity range. Satisfactory results are shown in Table 6.

Table 7 shows statistical comparison of the results obtained by the proposed methods and the reported HPLC method by Stanisz et al. [8]. The calculated t and F values are less than the theoretical ones indicating that there is no significant difference between the proposed methods and the reported HPLC method with respect to accuracy and precision.

4. Conclusion

The present work is concerned with the determination of IMD in the presence of both its alkaline and oxidative degradation products, in pure form or in its pharmaceutical formulation. The proposed spectrophotometric and chemometric methods are considered to be simple, convenient, less time consuming and economic stability-indicating methods compared to other published LC methods. The suggested methods showed high sensitivity, accuracy, reproducibility and specificity. These methods can be used as stability-indicating methods and can determine IMD in its tablets without interference from excipients. Moreover, the adopted methods are inexpensive and do not require sophisticated techniques or instruments. They can be considered useful and promising for developing routine quality control analysis of pharmaceuticals without any preliminary separation step. They are valid for application in laboratories lacking liquid chromatographic instruments.

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