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Validated liquid chromatographic determination of a novel ACE inhibitor in the presence of its hydrolytic and oxidative degradation products as per ICH guidelines

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ABSTRACT

Imidapril hydrochloride (IMD) is a recently developed prodrug-type angiotensin-converting enzyme (ACE) inhibitor. Due to its instability under both hydrolytic and oxidative conditions, development of rapid, simple and sensitive methods for its determination in the presence of its possible degradation products is essential. We proposed two simple liquid chromatographic methods associated with ultraviolet detection. The first method is an HPTLC-densitometric one in which separation of IMD from its degradation products was achieved followed by densitometric scanning at 220 nm using silica gel F_{254} plates and chloroform:ethanol:acetic acid (3:0.5:0.1, v/v/v) as the developing system. The second method was based on RP-HPLC in which the separation was performed using C18 analytical column and isocratic elution system with acetonitrile: 0.15% triethylamine (pH=2.2) (40:60, v/v). The optimum flow rate was 1.5 mL min^{-1} and the detection was at 220 nm. Validation was conducted in compliance with the ICH guidelines and the methods were successfully applied for IMD determination in its commercial tablets. The obtained results were statistically compared to those obtained by applying reported HPLC method where no significant difference was found in accordance with accuracy and precision.

1. Introduction

Imidapril hydrochloride (IMD) or (4*S*)-3-[(2*S*)-2-[(1*S*)-*N*-(1-ethoxycarbonyl-3-phenylpropyl) amino]-propionyl]-1-methyl-2-oxo-imidazolidine-4-carboxylic acid mono-hydrochloride [1] is a recently developed prodrug-type angiotensin-converting enzyme (ACE) inhibitor, (Fig. 1). Clinically, IMD is used in the treatment of hypertension, chronic congestive heart failure and acute myocardial infarction. Unlike other ACE inhibitors, IMD has the advantage of being associated with lower incidence of dry cough. It is rapidly absorbed and metabolized in the liver to imidaprilat which is twice more potent than captopril (a well-known ACE inhibitor). Imidaprilat plasma level increases gradually and slowly declines [2].

IMD is a non-official drug which has only few reported methods for its determination such as UV spectrophotometry [3,4], HPLC and GC [4–10], an enantioselective chiral LC method [11] and TLC-densitometric method [12]. While, no stability studies or stability indicating analytical methods have been reported.

The quality of the active pharmaceutical ingredient (API) and its formulation can be improved through separation, identification, quantification and characterization of the most probable and possible degradation products produced under various conditions of forced degradation as per international conference on harmonization (ICH) guidelines [13]. An ideal stability-indicating method is the one that quantifies the drug as well as efficiently resolves its degradation products.

So, our aim in this work was to develop simple, accurate, specific and reproducible stability-indicating methods for the determination of IMD in the presence of its possible degradation products. The proposed methods were developed, validated [14] and compared to a reported HPLC method.

2. Materials and methods

2.1. Instruments

- Camag TLC scanner III S/N 130319 (Camag, Muttenz, Switzerland) operated with winCATS software version 3.15, Linomat IV

Abbreviations: IMD, imidapril hydrochloride; AKN, suggested alkaline induced degradation product of imidapril hydrochloride; OXI, suggested oxidative induced degradation product of imidapril hydrochloride; DKP, diketopiprazine; ES, external standard

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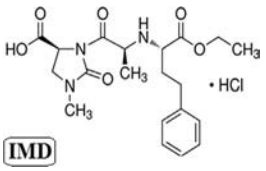

Fig. 1. Chemical structure of IMD.

auto-sampler (Camag, Muttenz, Switzerland), 100-μL Camag microsyringe (Hamilton, Bonaduz, Switzerland), Precoated silica gel aluminum Plates 60 F254 (20 cm × 10 cm) with 250 μm thickness (E. Merck, Darmstadt, Germany), Twin trough Automatic Developing Chamber ADC 2 chamber 20 cm × 10 cm (Camag, Muttenz, Switzerland).

- LaChrom Elite® HPLC chromatograph (VWR-Hitachi International GmbH, Darmstadt, Germany), which comprised L-2130 model pump, equipped with L-2400 UV detector and a 20-μL injection loop. EZChrom Elite Software Chromatography Data System, version 3.3.1 SP1. A Phenomenex C18 column (250 × 4.6 mm, 5 μm i.d.) was used. The mobile phase and samples were filtered using 0.45 μm membrane filter. Mobile phase was degassed by ultrasonic vibrations prior to use. The samples were injected with a 100-μL Hamilton analytical syringe.
- pH meter (Jenway 3310, UK) equipped with combined glass electrode was used for pH adjustment.
- Bandelin Sonorex RK 100 H DVE GS (gepüfte sicherheit) Sonicator.
- Perkin Elmer 1600 USA IR-Spectrometer; sampling was undertaken as potassium bromide discs.
- Electron impact MS spectra were recorded using Shimadzu QP-5050 Spectrometer.

2.2. Chemicals and solvents

All chemicals used throughout the work were of analytical grade and solvents were of HPLC grade; acetonitrile (ACN) (Riedel-de Haen, Seelze, Germany), methanol (Riedel-de Haen, Seelze, Germany), *ortho*-phosphoric acid 85% (Merck, Darmstadt, Germany), ethanol 96% (Merck, Darmstadt, Germany), chloroform (Merck, Darmstadt, Germany), acetic acid (Riedel-de Haen, Seelze, Germany), triethylamine (TEA) (Fluka, Neu-Ulm, German), hydrochloric acid 35.4% (Merck, Darmstadt, Germany), sodium hydroxide (Merck, Darmstadt, Germany), hydrogen peroxide 30% (Fluka, Neu-Ulm, Germany).

2.3. Samples

- Imidapril hydrochloride (IM) reference standard material (with certified purity of 99.80%) was supplied by SMS Pharmaceuticals Limited, Hyderabad, India.
- Tanatril® tablets (Batch No. 8917) are labeled to contain 10 mg of IMD per tablet and manufactured by HIKMA Pharmaceuticals, Amman-Jordan. They were purchased from the local market.

2.4. Chromatographic conditions

2.4.1. HPTLC-densitometry

A Camag Linomat IV applicator along with Camag 100 μL microsyringe was used for application of pure and sample solutions onto precoated silica gel aluminum plate 60 F_{254}, (20 cm × 10 cm, 250 μm thickness) in the form of 6 mm band width. A mobile phase consisted of chloroform:ethanol:acetic acid (3:0.5:0.1, v/v/v) was used. Linear ascending development was carried out in (20 cm × 10 cm) twin trough automatic developing chamber and densitometric scanning was performed in the absorbance mode at 220 nm.

2.4.2. HPLC

A mobile phase consisting of acetonitrile and 0.15% triethylamine (pH 2.2; the pH was adjusted using ortho-phosphoric acid) in a ratio of 40:60 v/v was prepared. Then, the prepared mobile phase was subjected to filtration through a 0.45 μm pore size membrane filter and degassing. The optimum flow rate was 1.5 mL min^{-1} and the injection volume was 20 μL. Measurements were done at ambient temperature and detection was at 220 nm.

2.5. Standard IMD solutions

A stock standard solution of IMD (2.0 mg mL^{-1}) was prepared in methanol and used in HPTLC method. For HPLC, the prepared solution was further diluted by the mobile phase to get a working standard solution having the concentration of 1.0 mg mL^{-1}.

2.6. Construction of calibration curve

2.6.1. HPTLC-densitometry

A series of standard solutions were prepared by diluting IMD stock standard solution (2.0 mg mL^{-1}) with methanol to reach a concentration range of 0.1–1.6 mg mL^{-1}. Ten microliters from each standard solution were applied in triplicate onto HPTLC plates to obtain final concentration range of 1.0–16.0 μg $band^{-1}$. The plates were developed using the specified mobile phase. The separated components were scanned at 220 nm. The average peak area ratio was calculated for each concentration of IMD to that of external standard (ES) (IMD, 2.0 μg $band^{-1}$). The ratios were plotted versus their corresponding concentrations to obtain the calibration graph and the regression equation was then computed.

2.6.2. HPLC

A series of standard solutions were prepared by diluting IMD working standard solution (1.0 mg mL^{-1}) with the mobile phase to reach a concentration range of 0.01–0.4 mg mL^{-1}. Twenty microliters were injected for each concentration in triplicate and chromatographed using the HPLC conditions described above. The peak area ratio was calculated for each concentration of IMD to that of ES (IMD; 50.0 μg mL^{-1}). The ratios were plotted against the corresponding concentrations to obtain the calibration graph and the regression equation was computed.

2.7. Forced degradation conditions

For determination of the applicability of the developed analytical method as a stability-indicating one, a forced degradation study under different conditions was carried out in which IMD standard solution was subjected to the following conditions:

(a) Acidic conditions: IMD standard solution was treated with 20.0 mL of 2 mol L^{-1} HCl, Heated in thermostatic water bath at 80 °C for a period of 24 h and then, neutralized by adjusting the pH to 7.0 with 5 mol L^{-1} NaOH.
(b) Basic conditions: IMD standard solution was treated with 20.0 mL of 1 mol L^{-1} NaOH, heated in thermostatic water bath at 80 °C for a period of 12 h and then, neutralized by adjusting the pH to 7.0 with 5 mol L^{-1} HCL.
(c) Oxidation with H_2O_2: IMD standard solution was treated with 20.0 mL of hydrogen peroxide 3% (w/v) and heated in thermostatic water bath at 80 °C for a period of 48 h.

Complete degradation was ascertained by the disappearance of the intact peak. The obtained degradation products were subjected to IR and MS spectroscopic analyses for characterization and subsequent structural elucidation.

The prepared solutions under acidic, alkaline and oxidative conditions were completed with methanol to obtain stock solutions of IMD degradation products equivalent to 2.0 mg mL^{-1} IMD. These stock solutions were used directly in HPTLC. While, for HPLC they were diluted using the mobile phase [ACN: 0.15% TEA; pH=2.2 (40:60, v/v)] to get solutions having a concentration of 1.0 mg mL^{-1}.

2.8. Assay of laboratory prepared mixtures

In HPTLC-densitometric method, solutions containing different ratios of IMD and its possible degradation products in different ratios were prepared from their respective stock solutions and diluted with methanol. On the other hand, in HPLC method, the solutions were prepared from the working solutions and diluted with the mobile phase. The peak areas ratios of the laboratory-prepared mixtures were calculated and processed as described above for the two proposed methods. The concentration of IMD was calculated using the computed regression equations.

2.9. Application to pharmaceutical preparation (Tanatril® tablets)

Ten tablets of Tanatril® were finely powdered. A portion of the powdered tablets equivalent to 20 mg of IMD was transferred into 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 a stock solution having the concentration 0.8 mg mL^{-1}.

In HPTLC-densitometric method, Aliquots of 4.0, 6.0 and 8.0 mL were transferred from the prepared solution to 10-mL volumetric flasks and diluted with methanol. Then, 10 μL from each solution were applied onto HPTLC plates in triplicate. While, in HPLC method, aliquots of 1.0, 2.0 and 4.0 mL were transferred to 10-mL volumetric flasks and the volume was completed with the mobile phase. Then, 20 μL from each final dilution were injected in triplicate.

The general procedure described above for each method was followed. Then, the concentration of IMD in its pharmaceutical preparation was calculated.

2.10. Application of standard addition technique

To check the validity of the proposed chromatographic methods, 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 5, 10 and 15 mg of the pure standard IMD, separately. Each spiked sample was transferred to 25-mL volumetric flask, sonicated for 20 min with 20 mL methanol. Then, the volume was completed with the same solvent and filtered to obtain three spiked solutions of concentrations 0.6, 0.8 and 1.0 mg mL^{-1}.

In HPTLC-densitometric method, 6.0 mL from each spiked sample were separately transferred to 10-mL volumetric flask and diluted with methanol. Then, 10 μL from each dilution were applied onto HPTLC plates in triplicate. While, in HPLC-UV method, 2.0 mL from each spiked sample were separately transferred to 10-mL volumetric flasks and the volume was completed with the mobile phase. Then, 20 μL from each final dilution were injected in triplicate.

These general procedures described above for each method was followed and the concentration of the added pure IMD standard was calculated from the specified regression equation.

3. Results and discussion

Development of analytical methods for the determination of pharmaceuticals in the presence of their degradation products without previous chemical separation is always a matter of interest. The main task of this work was to establish simple, sensitive and accurate analytical methods for the determination of IMD in the presence of hydrolytic and oxidative induced degradation products, in its bulk powder and commercial tablets with satisfactory precision for good analytical practice (GAP).

3.1. Degradation behavior of IMD

Under acidic and alkaline stress conditions, IMD undergoes degradation with the production of a diacid derivative of IMD through hydrolysis of ester linkage on the basis of the studies of Nishi et al. [11]. Although acid and alkaline conditions lead to the formation of the same degradation product, NaOH was used as the hydrolyzing agent according to ICH guidelines[13]. IMD was also subjected to oxidative degradation using hydrogen peroxide. It was noticed that diketopiperazine derivative (DKP) was produced via oxidation through internal cyclization of IMD as suggested by Stofik et al. [15]. In order to achieve complete degradation, IMD was exposed to different time periods (24 h, 12 h and 48 h) at 80 °C as an accelearted stress testing under acidic, alkaline and oxidative conditions, respectively.

The assignment of IMD degradation products was based on the comparison of their IR and MS spectral data with those of IMD. IR spectrum of IMD (Fig. 2) shows 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 band 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 the alkaline degradation product (Fig. 3) showed the absence of the peak at 1751 cm^{-1}, which corresponds to the carbonyl stretching of ester. However, 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 oxidative degradation product spectrum (Fig. 4) at 3217 cm^{-1} (the secondary amine), 1728 cm^{-1} (the carbonyl group of carboxylic acid) and

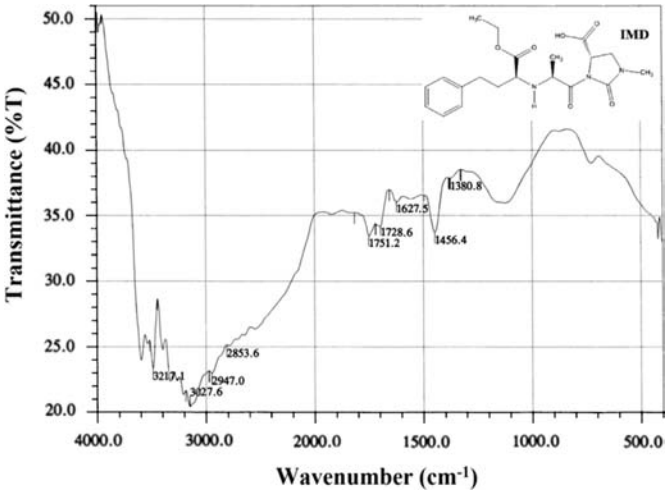

Fig. 2. IR spectrum of IMD.

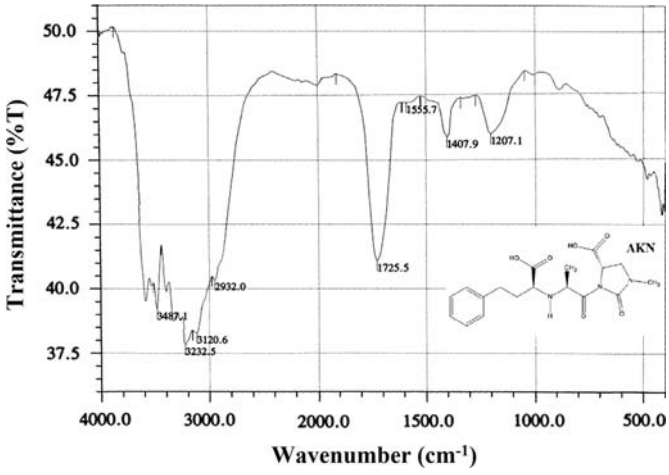

Fig. 3. IR spectrum of AKN.

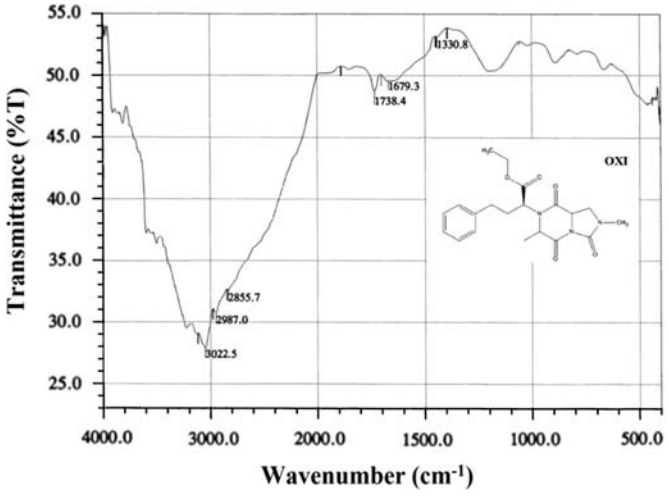

Fig. 4. IR spectrum of OXI.

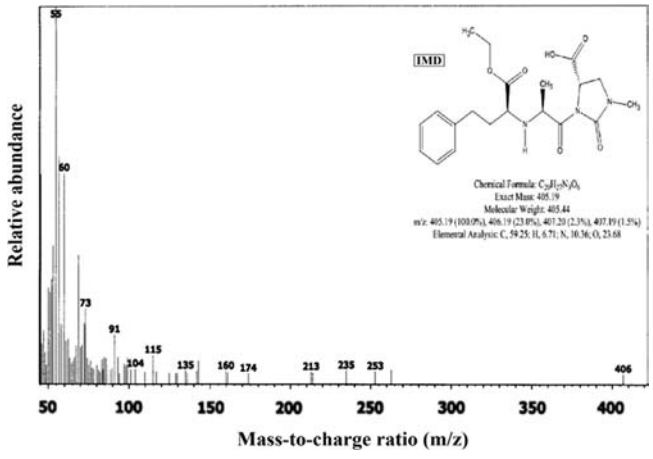

Fig. 5. MS spectrum of IMD.

1627 cm^{-1} (the carbonyl stretching of tertiary amide) confirmed the DKP formation. While, several new peaks at 2987, 1738 and 1679 cm^{-1} were observed. The peak at 2987 cm^{-1} was due to the asymmetric CH3 stretching vibration, and the peaks at 1738 and 1679 cm^{-1} were assigned to the carbonyl bands of ester and DKP groups, respectively [16].

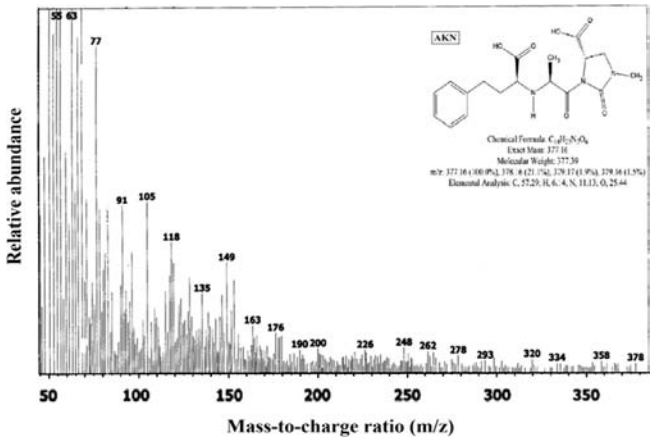

Fig. 6. MS spectrum of AKN.

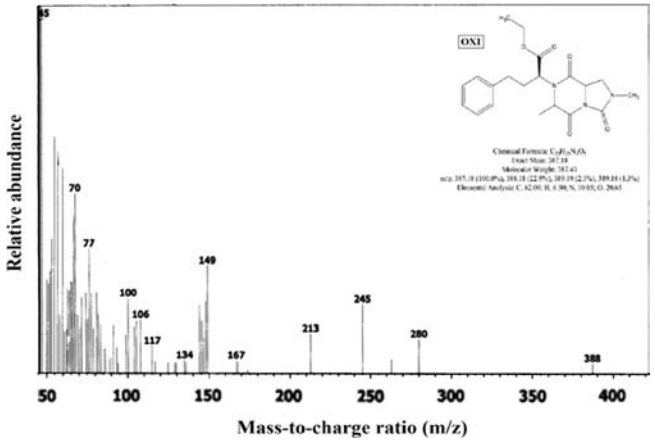

Fig. 7. MS spectrum of OXI.

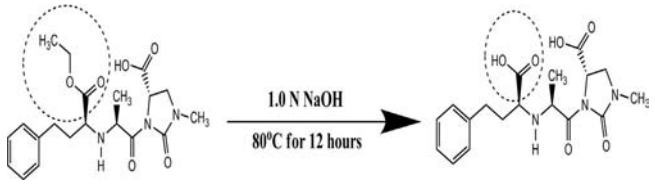

Fig. 8. Scheme of alkaline hydrolysis of IMD.

The mass ion peak of IMD was identified at *m/z* 407 (Fig. 5), while those of alkaline (AKN) and oxidative (OXI) degradation products were at *m/z* 378 (Fig. 6) and at *m/z* 387 (Fig. 7), respectively. Therefore one can conclude that carrying out the alkaline (or acidic) hydrolysis of IMD may proceed as shown in Fig. 8, while oxidative degradation of IMD may proceed as shown in Fig. 9.

3.2. Method optimization

3.2.1. HPTLC-densitometric method

A sensitive stability-indicating HPTLC-densitometric method is described for the determination of IMD in the presence of its possible degradation products either through hydrolysis or oxidation. Different solvent systems were tried for the separation of IMD and its degradation products. Satisfactory results were obtained by using a mobile phase composed of chloroform: ethanol:acetic acid (3:0.5:0.1, v/v/v) which gave good resolution and sharp symmetrical peaks. In order to minimize band diffusion,

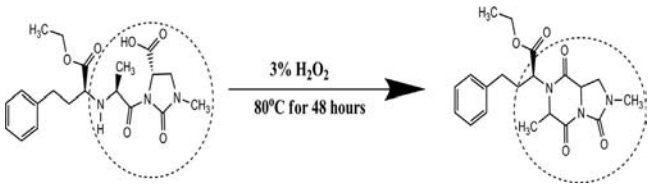

Fig. 9. Scheme of oxidative degradation of IMD.

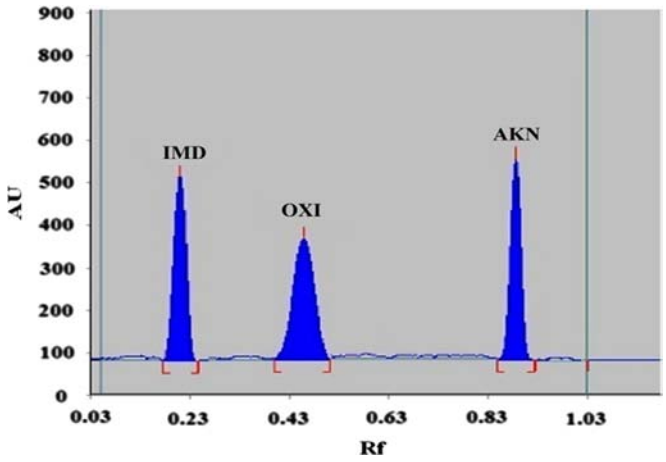

Fig. 10. HPTLC chromatogram of IMD, OXI and AKN mixture, using chloroform: ethanol:acetic acid (3:0.5:0.1, by volume) at 220 nm.

the optimum bandwidth chosen was 6 mm. Different scanning wavelengths were tried; on using 220 nm the separated peaks were more sharp and symmetrical with minimum noise. The R_f values were 0.19, 0.45 and 0.89 for IMD, OXI and AKN, respectively. A typical chromatogram of the three components is shown in Fig. 10, in which the separation allows the determination of IMD without any interference from its degradation products.

The shape of the calibration curves in densitometry is generally inherently non-linear due to scattering of light. Calibration curves generally comprise a pseudo-linear region at low sample concentrations where Beer's law is obeyed. The departure from linearity begins at higher sample concentrations. So linearity may cause problems when wide concentration range is required. The relationship between the integrated peak area ratio and the concentration was evaluated with linear and polynomial regression functions. Fitting with polynomial function gave better correlation and lower values of standard deviation and was therefore used for quantitative analysis.

The second-order polynomial regression was used and the regression equation was computed and found to be:

$$A = -0.0040C^2 + 0.2762C + 0.4492 \; r = 0.9998$$

where A is the integrated peak area ratio, C is the concentration in μg band^{-1} and r is the correlation coefficient.

The precision of the proposed method was checked by the analysis of different concentrations of authentic samples in triplicates. The mean percentage recovery was found to be 99.72 ± 0.716.

3.2.2. HPLC method

A simple isocratic HPLC method was developed for the determination of IMD in its pure powder, in the presence of its possible degradation products and in pharmaceutical preparation using a Phenomenex C18 column (250 × 4.6 mm, 5 μm i.d.). The mobile phase was consisted of [acetonitrile: 0.15% triethylamine (40:60, v/v)] and the pH of 0.15% triethylamine was adjusted to 2.2 using *o*-phosphoric acid. The mobile phase was chosen after several trials to reach the optimum stationary/mobile-phase matching. Reasonable separation with good resolution and suitable analysis time was obtained upon using flow rate of 1.5 mL min^{-1}. System suitability parameters were tested by calculating the capacity factor, tailing factor, the asymmetry factor, selectivity factor, no. of theoretical plates and resolution. Under the optimum chromatographic conditions, AKN, OXI and IMD were eluted at 2.40, 2.67, and 4.88 min, respectively, as shown in Fig. 11. The chromatographic system described in this work allows complete separation of IMD from its possible degradation products. Calibration graph was obtained by plotting the peak area ratios against concentration of IMD (μg mL^{-1}). Linearity range was found to be 10.0–400.0 μg mL^{-1} using the following regression equation:

$$A = 0.0173C + 0.0873 \; r = 0.9999$$

where A is the peak area ratio, C is the concentration in μg mL^{-1} and r is the correlation coefficient.

The mean percentage recovery of pure samples was found to be 99.94 ± 0.765. The precision of the proposed method was checked by the analysis of different concentrations of authentic samples in triplicates.

3.3. Analysis of pharmaceutical preparation

The proposed methods were applied to the determination of IMD in Tanatril® tablets. The results shown in Table 1 are satisfactory and with good agreement with the labeled amount.

3.4. Method validation

ICH guidelines[14] for method validation were followed for validation of the suggested methods.

3.4.1. Linearity

The linearity of the proposed chromatographic methods for determination of IMD was evaluated by analyzing a series of different concentrations of the drug. In this study six concentrations were chosen, ranging between 1.0 and 16.0 μg band^{-1} for HPTLC-densitometric method and 10.0 and 400.0 μg mL^{-1} for HPLC-UV method. Each concentration was repeated three times, in order to provide information on the variation in peak area values among samples of the same concentration. Linear relationships were obtained by plotting the drug concentrations against the average peak area ratios obtained for each concentration of IMD to that of external standard (2.0 μg band^{-1} for TLC method and 50.0 μg mL^{-1} for HPLC method). The linearity of the calibration

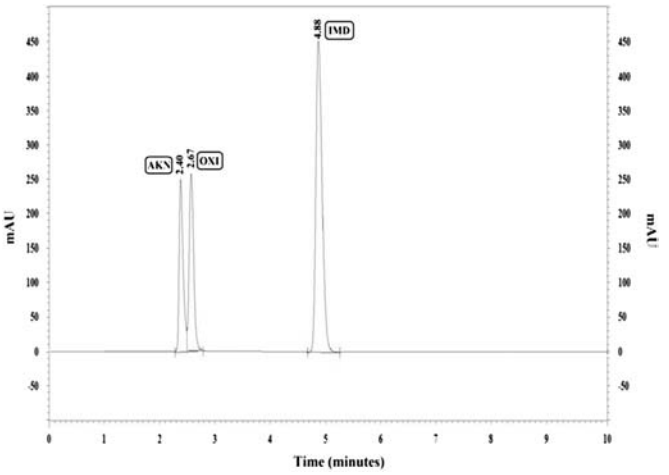

Fig. 11. HPLC chromatogram of IMD, AKN and OXI mixture, using 'ACN – 0.15% TEA; pH=2.2' (40:60, v/v) at 220 nm.

Table 1
Determination of IMD in Tanatril® tablets using the proposed chromatographic methods.

HPTLC method				
Pharmaceutical preparation	Sample No.	Taken amount IMD (μg band^{-1})	Found[a] amount IMD (μg band^{-1})	% Recovery
Tanatril® tablets labeled to contain 10 mg IMD per tablet B.N.: 8917	1	3.20	3.18	99.38
	2	4.80	4.83	100.63
	3	6.40	6.42	100.31
	Mean ± SD			100.11 ± 0.649
HPLC method				
Pharmaceutical preparation	Sample No.	Taken amount IMD (μg mL^{-1})	Found[a] amount IMD (μg mL^{-1})	% Recovery
Tanatril® tablets labeled to contain 10 mg IMD per tablet B.N.: 8917	1	80.00	80.16	100.20
	2	160.00	160.86	100.54
	3	320.00	318.54	99.54
	Mean ± SD			100.09 ± 0.505

[a] Mean of three determinations.

Table 2
Characteristic parameters for the regression equations of the proposed methods for determination of IMD.

Validation parameters	**HPTLC Method**	**HPLC Method**
Linearity range	1.0–16.0 (μg band^{-1})	10.0–400.0 (μg mL^{-1})
Slope (X^2 coefficient)	−0.0040	–
Slope (X coefficient)	0.2762	0.0173
Intercept	0.4492	0.0873
SE of slope (X^2 coefficient)	0.0016	–
SE of slope (X coefficient)	0.0069	0.0001
SE of intercept	0.0211	0.0146
Correlation coefficient (r)	0.9998	0.9999
LOD	0.25 (μg band^{-1})	2.78(μg mL^{-1})
LOQ	0.76 (μg band^{-1})	8.42 (μg mL^{-1})
Precision (RSD%, n=9)		
Intra-day	0.407	0.627
Inter-day	0.885	0.947

Table 3
Determination of IMD in presence of its degradation products in laboratory prepared mixtures by the proposed stability-indicating chromatographic methods.

IMD (μg band^{-1})	AKN (μg band^{-1})	OXI (μg band^{-1})	% Recovery[a] of IMD
HPTLC method			
9.0	0.5	0.5	100.93
7.0	1.5	1.5	99.44
5.0	2.5	2.5	100.17
3.0	3.5	3.5	98.54
1.0	4.5	4.5	99.56
Mean% ± SD			99.73 ± 0.889
HPLC method			
180.0	10.0	10.0	99.18
140.0	30.0	30.0	99.06
100.0	50.0	50.0	101.06
60.0	70.0	70.0	100.94
20.0	90.0	90.0	100.84
Mean% ± SD			100.22 ± 1.003

[a] Average of 3 experiments.

graphs was validated by the high value of the correlation coefficient and the intercept value, which was not statistically different from zero (P=0.05). Characteristic parameters for regression equations of the adopted chromatographic methods are given in Table 2.

3.4.2. Range

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

3.4.3. Precision

In order to judge the quality of the elaborated methods, precision was determined. For evaluation of the precision estimates, intra-assay and inter-assay were performed by repeating the assay of three different concentrations of IMD; in triplicate, three times in the same day and assaying the same selected concentrations on three successive days using the developed chromatographic methods and calculating the RSD%. Results in Table 2 indicate satisfactory precision of the proposed methods.

3.4.4. Detection and quantitation limits

According to the International Conference on Harmonization (ICH) recommendations [14], the approach based on both the standard deviation (SD) and the slope of the response, was used for calculating the detection and quantitation limits as presented in Table 2.

3.4.5. Specificity

The specificity of a method is the extent to which it can be used for analysis of a particular analyte in a mixture or matrix without interference from other components. The specificity of the proposed methods was tested by the analysis of five laboratory prepared mixtures containing different percentages of IMD at various concentrations within the linearity range with its degradants produced in forced degradation studies. The laboratory-prepared mixtures were analyzed according to the previous procedures described under each of the proposed methods. The specificity was demonstrated by the chromatograms recorded for mixtures of IMD and its degradants, indicating that the methods enabled highly specific analysis of the drug. Well-resolved peaks for IMD, AKN and OXI were observed (Figs. 10 and 11). Satisfactory results were obtained (Table 3) indicating the high specificity of the proposed methods for determination of IMD in presence of up to 90% of its degradation products.

3.4.6. System suitability

System suitability test parameters must be checked to ensure that the system is working correctly during the analysis. Method

Table 4
(a) The system suitability test results of the developed HPTLC method for determination of IMD.

Parameters	IMD	OXI	AKN	Reference values
Retardation factor (R_f)	0.19	0.45	0.89	
Resolution (R_s)	–	3.42[a]	4.73[b]	$R_s \geq 2$
Tailing factor (T)	0.90	0.67	0.75	$T \leq 2$
Capacity factor (K')	2.36	6.14	10.91	$1 < K' < 10$
Selectivity (α)	–	2.60[a]	1.78[b]	$\alpha > 1$

(b) The system suitability test results of the developed HPLC method for determination of IMD.

Parameters	AKN	OXI	IMD	Reference values
Retention time (R_t; min)	2.40	2.67	4.88	
Resolution (R_s)	–	1.10[c]	9.34[d]	$R_s \geq 2$
Tailing factor (T)	–	1.36	1.02	$T \leq 2$
Asymmetry factor (A_f)	–	1.03	1.00	$0.9 < A_f < 1.1$
Capacity factor (K')	3.71	4.23	8.72	$1 < K' < 10$
Selectivity (α)	-	1.14[c]	2.06[d]	$\alpha > 1$
Injection repeatability[e]	0.986	0.897	0.949	*RSD ≤ 1% for n ≥ 5*
Theoretical plates (N)	1195	2593	4999	$N > 2000$
Height equivalent to theoretical plate (HETP; cm plate^{-1})	0.0209	0.0096	0.0050	*The smaller the value, the higher the column efficiency*

[a] To IMD.
[b] To OXI.
[c] To AKN.
[d] To OXI.
[e] RSD% for five injections.

Table 5
Results[a] of robustness testing of the proposed chromatographic methods for determination of IMD.

HPTLC method	R_f		T	
Mobile phase ratio (chloroform:ethanol:acetic acid)	1.015		1.075	
HPLC method	R_t	N	T	R_s[b]
Mobile phase composition (ACN: 0.15% TEA; pH=2.2)	1.539	0.924	1.493	1.451
pH of 0.15% TEA	0.615	0.563	1.125	0.587

[a] RSD% (n=3).
[b] Resolution to the nearest degradation product (OXI).

performance data including capacity factor, selectivity, resolution, and tailing factor are listed in Table 4a and b. All data was satisfactory and indicative of the good specificity of the method for the determination of IMD in presence of AKN and OXI.

3.4.7. Robustness

The robustness of the chromatographic methods was investigated by the analysis of samples under a variety of experimental conditions such as small changes in TLC mobile phase ratio; chloroform:ethanol:acetic acid (3.2:0.3:0.1 and 2.8:0.7:0.1, v/v) and deliberate variations in HPLC mobile phase ratio; ACN: 0.15% TEA; pH= 2.2 (38:62 and 42:58, v/v) and pH value of TEA (at 2.1 and 2.3). Results presented in Table 5 indicate that the capacity of the utilized methods remain unaffected by these small deliberate variations, providing an indication for the reliability of the proposed chromatographic methods during routine work.

Table 6
Application of standard addition technique on Tanatril® tablets to the analysis of IMD using the proposed chromatographic methods.

Sample No.	Claimed conc. (μg band^{-1})	Pure added conc. (μg band^{-1})	Pure found conc. (μg band^{-1})	% Recovery[a] of the added conc.
HPTLC method				
1	2.40	1.20	1.20	100.00
2	2.40	2.40	2.37	98.75
3	2.40	3.60	3.64	101.11
Mean ± SD%				99.95 ± 1.181
HPLC method				
1	80.00	40.00	40.71	101.77
2	80.00	80.00	79.44	99.30
3	80.00	120.00	121.48	101.23
Mean ± SD%				100.77 ± 1.299

[a] Mean of three determinations.

3.4.8. Accuracy

The interference of excipients in the pharmaceutical formulations was studied using the proposed methods. For this reason, standard addition method was applied to the commercial pharmaceutical formulation containing IMD. In application of standard addition method the mean percentage recoveries and their standard deviation for the proposed methods were calculated (Table 6). According to the obtained results a good precision and accuracy was observed for this method. Consequently, the excipients in pharmaceutical formulations do not interfere in the analysis of IMD in its pharmaceutical formulation.

The results obtained by applying the proposed chromatographic methods were statistically compared to those of the reported HPLC method by Stanisz et al. [4] used for IMD analysis. It is concluded that; with 95% confidence, there is no significant difference between them since the calculated t and F values are less than the theoretical values; as presented in Table 7.

Table 7
Statistical analysis of the proposed methods and the reported method for determination of IMD in its pure powdered form.

Parameters	HPTLC method	HPLC method	Reported method
Mean	99.72	99.94	100.75
SD	0.716	0.765	0.955
Variance	0.513	0.586	0.912
***t*-test**[a]	1.920	1.472	–
***F*-test**[a]	1.778	1.556	–

[a] The theoretical values of t and F at $P=$ 0.05 are (2.306) and (6.388), respectively, where $n=5$.

4. Conclusion

The quality of pharmaceutical products is of vital importance for patients' safety. The presence of degradation products may affect the efficacy and safety of pharmaceuticals. Degradation could change the chemical, pharmacological and toxicological properties of drugs and have a significant effect on product quality and safety. Drug stability is regarded as a secure way of ensuring delivery of therapeutic doses to patients. In this work simple, sensitive, accurate, precise, reproducible, repeatable, specific, and robust stability-indicating HPTLC-densitometric and HPLC-UV methods were established for the determination of IMD in the presence of its degradation products. The behavior of IMD under different stress conditions was studied. The developed HPTLC-densitometric method is highly sensitive and has the advantages of short run time, large sample capacity, and use of minimal volume of solvents. While, the HPLC-UV method offers high specificity and good resolution between the three proposed components within suitable analysis time. The developed methods are quite sensitive for quantitative detection of the IMD in its pharmaceutical preparation and can thus be used for routine analysis, quality control and for quality check during stability studies of its pharmaceutical preparations.

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