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Selective determination of itraconazole in the presence of its oxidative degradation product

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ABSTRACT

Stability-indicative determination of itraconazole (in the presence of its oxidative degradation product, is investigated. The degradation product has been isolated, via oxidative-degradation, characterized and elucidated. Selective quantification of itraconazole, singly in bulk form, pharmaceutical formulations and/or in the presence of its oxidative degradation product is demonstrated. The indication of stability has been undertaken under conditions likely to be expected at normal storage conditions. Among the spectrophotometric methods adopted for quantification are second derivative (^{2}D), first derivative of ratio spectra (^{1}DD), Difference spectrophotometry and bivariate analysis.

KEYWORDS

Itraconazole;
Second derivative spectrophotometry;
Derivative-ratio;
Bivariate.

INTRODUCTION

Itraconazole, (4-[4-[4-[4-[[2-(2-(2, 4-Dichlorophenyl)-2-1H-1,2,4-triazol-1-ylmethyl)-1,3-dioxolan-4-yl] methoxy]phenyl]-1-piperazinyl]phenyl]-2, 4-dihydro-2-(1-methylpropyl)-3H-1,2,4-triazol-3-one[1] Figure 1.

Itraconazole is a triazole antifungal agent with a broad spectrum of activity. It acts primary by inhibiting the biosynthesis of ergosterol, an essential component of fungal cell membranes[2-5]. It is used in the treatment of a variety of fungal infections. The pharmacokinetics of orally administered itraconazole in humans are characterized by considerable inter-individual variation in drug absorption, extensive tissue distribution, with the concentrations in tissue being many times higher than those in plasma, and an elimination half-life of approximately 24 hours. Itraconazole is known to be extensively metabolized in humans, yielding over 30 metabolites, including the antifungal active metabolite hydroxy-

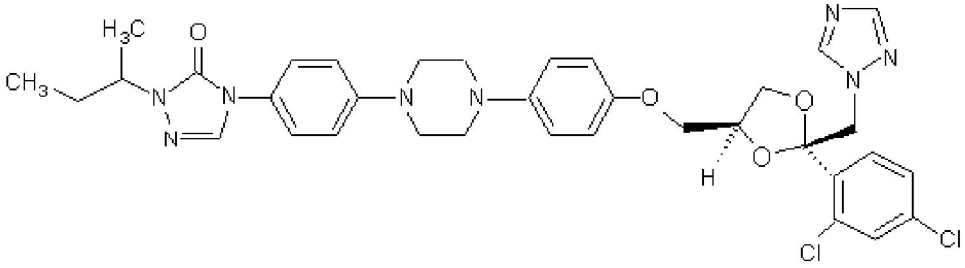

Figure 1

itraconazole.

Several methods have been reported to quantify itraconazole in biological fluids including HPLC and LC-MS-MS techniques. Methods with a sensitivity of 10 ng mL^{-1} having sample preparation using solid-phase extraction[6], a sensitivity of 20 ng mL^{-1} having sample preparation using three step liquid-liquid extraction[7] and a sensitivity of 25 ng mL^{-1} having sample preparation using protein precipitation are available[8]. More methods are also available for the determination of itraconazole with lower sensitivity using LCMS-MS as detection technique[9,10]. Spectrofluorimetry method has been published for assay of itraconazole in raw material and in dosage forms[11].

EXPERIMENTAL

Instruments

- Spectrophotometer: Shimadzu UV-1601 PC, dual-beam UV–visible spectrophotometer (Japan), with matched 1-cm quartz cells, connected to an IBM-compatible PC and an HP-600 inkjet printer. Bundled, UV-PC personal spectroscopy software Version 3.7 was used to process the absorption and the derivative spectra. The spectral bandwidth was 2nm with wavelength-scanning speed of 2800 $nmmin^{-1}$.
- IR Spectrophotometer: Mattson Genesis II FTIRTM (USA), sampling was undertaken as potassium bromide discs.
- UPLC MS-MS Acquity TQ-Waters (USA).

Materials and reagents

All chemicals and reagents were of analytical grade and the solvents were of spectroscopic grade. Pure sample was kindly supplied by the by Adwia pharmaceutical industry, Egypt; it was assayed for its purity according to a pharmacopoeial method[1] and found to contain 99.85 ±0.886. Itranox capsules: Manufactured by Adwia Parmaceutical industry, Batch No. 060175, labelled to contain 100 mg itraconazole/capsule.

Methanol (E. Merck, Darmstadt, Germany), Methylene chloride (Sigma), 30% hydrogen peroxide (Sigma), ethyl acetate, methylene chloride, concentrated ammonia (specific gravity 0.91) were obtained from Adwic. Co, Egypt.

Preparation of standard solutions

Itraconazole standard solution (0.1 mg mL^{-1}) and drug degradation product standard solution (0.1mg mL^{-1}) were prepared in methylene chloride.

Procedures

Degradation of itraconazole

The drug (300 mg) was weighed in a conical flask, dissolved in 30 ml methylene chloride, 5 ml hydrogen peroxide 30% (v/v) was added and the solution was subjected to reflux at 100 °C for ten hours. Where complete degradation was achieved, as investigated by thin layer chromatography using ethyl acetate: methanol: ammonium hydroxide (9: 1: 0.01, v/v/v) as a developer solvent. The solution was concentrated to a small volume and extracted with methylene chloride. The methylene chlorideic extract was evaporated under vacuum. The structure of the isolated degradation product was elucidated using IR, and MS spectrometry.

Second derivative (^2D) method

a. Spectral characteristics of itraconazole and its degradation product

Two aliquots equivalent to 10 µg of itraconazole and 5 µg of its degradation product standard stock solutions (each, 0.1mg mL^{-1}) were transferred separately into two 10-mL volumetric flasks. Then the volumes were completed with methylene chloride. The zero order (^0D) and the second derivative (^2D) spectra of the prepared solutions were recorded.

b. Linearity

Portions equivalent to (50–250 µg) of itraconazole standard solution (0.1 mg mL^{-1}) were separately transferred to a series of 10-mL volumetric flasks. Each flask was completed to the volume with methylene chloride to reach the concentration range of 5–25 µg mL^{-1}. The amplitudes of the second derivative peaks were measured at 261 nm with $\Delta\lambda = 4$ nm and a scaling factor = 10. Calibration graph was constructed by plotting peak amplitude *versus* concentration. The regression equation was then computed at the specified wavelength and used for determination of unknown samples containing itraconazole.

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First derivative of ratio spectra (^{1}DD) method

Linearity

Standard serial concentrations in the range of 5–25 μg mL^{-1} solutions of itraconazole were prepared as under Section 2.3.2.2. Accurately 2.5 mL of the degradation product standard solution (0.1 mg mL^{-1}) was transferred into a 10-mL volumetric flask and the volume was completed with methylene chloride to get a final concentration of 25 μg mL^{-1} to be used as a divisor. The spectra of the prepared standard solutions were scanned (200–400 nm) and stored into the computer. The stored spectra of itraconazole were divided (amplitude at each wavelength) by the spectrum of 25 μg mL^{-1} of the degradation product. The first derivative of the ratio spectra (^{1}DD) with $\Delta\lambda$= 4 nm and a scaling factor = 10 was obtained. The amplitudes of the first derivative peaks of itraconazole were measured at 252 nm. Calibration graphs were constructed relating the peak amplitudes of (^{1}DD) to the corresponding concentrations. The regression equations were then computed at the specified wavelength and used for determination of unknown samples containing itraconazole.

Difference spectrophotometric method

Linearity

Aliquots 2.5–15 mL^{-1} of itraconazole standard solution (0.1 mg mL^{-1}) were transferred into a series of 50-ml volumetric flasks then completed to volume with methylene chloride; For blank, aliquots (2.5 – 15 ml) of the itraconazole standard solution were transferred into a series of conical flasks; two ml of H_2O_2 were added to each solution then heated by refluxing in a water bath till complete degradation. Excess H_2O_2 was removed by boiling. The solutions were accurately transferred into a series of 50-ml volumetric flasks; then the volumes were completed to the mark with methylene chloride. Each of itraconazole solution in methylene chloride was measured against the corresponding blank. The (ΔA) spectra were obtained and the absorbance difference at 265 nm was measured. A linear calibration curve was constructed relating the absorbance difference at 265 nm to the corresponding concentrations of itraconazole and the corresponding regression equation was computed.

Bivariate method

Two series of standard solutions containing aliquots equivalent to (5–25 μg mL^{-1}) of itraconazole and (5–25 μg mL^{-1}) of its degradation product were prepared from the stock solution (0.1mg mL^{-1}, each) for the bivariate calibration. Spectra of the obtained solutions were recorded and stored into the computer. The regression equations were computed at λ= 230 and 260 nm. The concentrations of itraconazole and its degradation product were calculated using the parameters of the linear regression functions evaluated individually for each component at the same wavelength and substituting in the following equations:

$$C_{degradate} = \frac{m_{A2}(A_{AB1} - e_{AB1}) + m_{A1}(e_{AB2} - A_{AB2})}{m_{A2}m_{B1} - m_{A1}m_{B2}}$$

$$C_{BH} = \frac{A_{AB1} - e_{AB1} - m_{B1}C_{degradate}}{m_{A1}}$$

where A_{AB1} and A_{AB2} are the absorbances of *A* and *B* at λ_1 and λ_2, respectively, e_{AB1} and e_{AB2} are the sum of the intercepts of the linear calibration at two, wavelengths λ_1 and λ_2 ($e_{AB1} = e_{A1} + e_{B1}$), m_A and m_B are the slopes of linear regression and *C* is the concentrations (μg mL^{-1}) of itraconazole and its degradate.

The accuracy of the results was checked by applying the proposed bivariate calibration method for determination of different blind samples of pure itraconazole and its degradate. The concentrations were obtained from the corresponding regression equations from which percentage recoveries were calculated.

Analysis of laboratory prepared mixtures

Aliquots of intact drug and its degradation product were mixed to prepare different mixtures and proceed as mentioned under each method. The concentration of itraconazole was calculated from the corresponding regression equations.

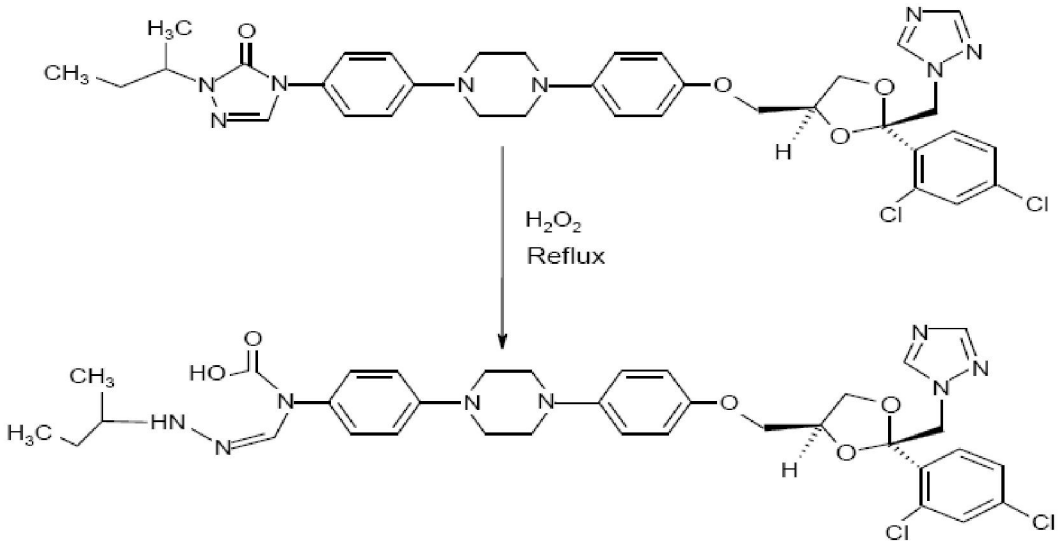
Assay of pharmaceutical formulations

The contents of ten capsules were emptied then grinded. A portion of the powder equivalent to 10 mg itraconazole was accurately weighed into a 100-mL beaker, dissolved in methylene chloride and filtered into a 100-mL measuring flask. The volume was completed by the same solvent to reach a final drug concentration of 0.1 mg mL^{-1} for the proposed methods and proceed as mentioned under each method.

RESULTS AND DISCUSSION

Degradation of itraconazole

In this study, itraconazole was degraded by refluxing in 30% H_2O_2. (Scheme 1)



Scheme 1 : Suggested scheme for the degradation of itraconazole

The IR spectrum of intact itraconazole revealed carbonyl stretching band at 1699.64 cm^{-1}

Once complete degradation was reached, The IR spectrum of itraconazole degradation product showed one stretching band characteristic of secondary amine at 3500 cm^{-1}, a broad band of carboxylic OH stretching vibration at 2600 cm^{-1} and carbonyl stretching band at 1723.81 cm^{-1} indicating the presence of carboxylic group. This confirmed the hydrolysis at the cyclic – carbonyl linkage.

In the GC/MS-chart, the parent peak was identified at *m/z 723* (mol. w. of degradate).

TLC monitoring of the drug degradation was done on thin layer plates of silica gel F254 using ethyl acetate: methanol: ammonium hydroxide (9:1: 0.01, v/v/v) as a developing solvent. The developed plates were visualized under short UV lamp. The degradate (R_f value = 0.82) could be separated elegantly from the intact drug (R_f value = 0.35).

Second derivative (^{2}D) method

Derivative spectrophotometry is a useful tool in quantification of mixture of drugs. It could be even used as a stability-indicating technique for the analysis of drugs in presence of their degradation products, by solving the problem of the overlapping absorption bands.

A simple, rapid and selective spectrophotometric procedure was proposed and applied for the determination of itraconazole in the presence of its degradation product, either as raw material or in pharmaceutical formulations. This was done by applying the second derivative (^{2}D) ultraviolet spectrophotometry. The method can solve the problem of spectral bands overlapping between itraconazole and its degradate without sample pretreatment or extra separation steps. The absorption spectra of itraconazole and its degradation product (Figure 2) show overlapping, little interference and error probability that make the use of direct measurement of itraconazole in the presence of its degradate inaccurate, especially at higher level of degradation.

When the second-derivative spectra (Figure 3) were examined, it was found that itraconazole could be determined at 261 nm, where its degradate has no contri-

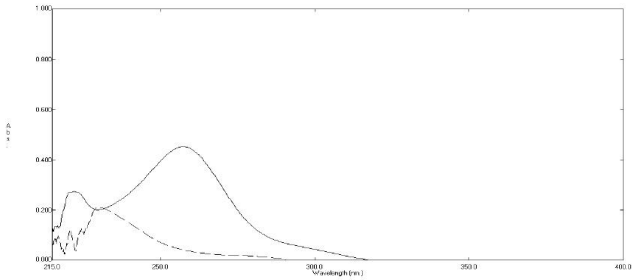


Figure 2 : Absorption spectra of itraconazole 10 µg ml^{-1} (___) and its degradation product 5 µg ml^{-1} (---) using methylene chloride as a blank

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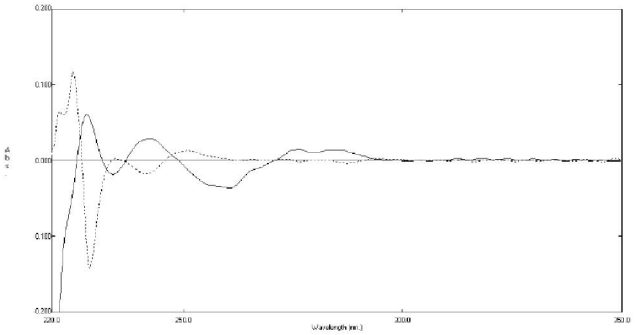

Figure 3 : Second-derivative absorption spectra of itraconazole 15 µg ml^{-1} (__) and degradation product 5 µg ml^{-1} (---) using methylene chloride as a solvent

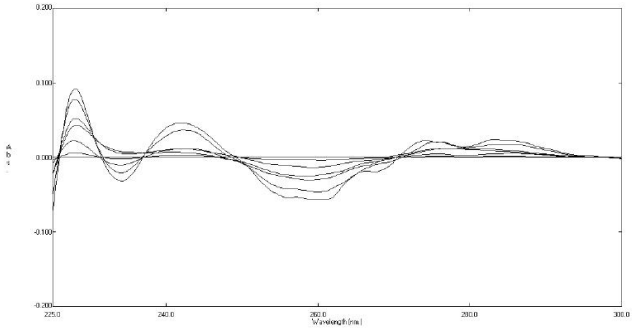

Figure 4 : Second-derivative absorption spectra of 5–25 µgmL^{-1} itraconazole

bution allowing accurate determination of itraconazole in presence of its degradate.

A linear relationship was obtained in the range of 5–25 µgmL^{-1} itraconazole (Figure 4).

The regression equation was computed and found to be:

^{2}D = - 0.0022 C + 0.0003 (r = 0.9991), at 261 nm

where ^{2}D is the peak amplitude of the second derivative curve at the corresponding wavelength, *C* the concentration of itraconazole (µgmL^{-1}) and *r* is the correlation coefficient..

Derivative-ratio spectrophotometric method

The derivative-ratio spectroscopy is a useful tool in quantification of drugs. It could be applied as a stability-indicating method for the determination of itraconazole in presence of its degradate. The first order of the ratio spectra of itraconazole is presented in Figure 5. It was found that upon dividing by 25 µg mL^{-1} of the degradation product, best results were obtained in terms of sensitivity, repeatability and signal to noise ratio.

Linear calibration graphs were obtained for itraconazole in concentration range of 5–25µg mL^{-1} by

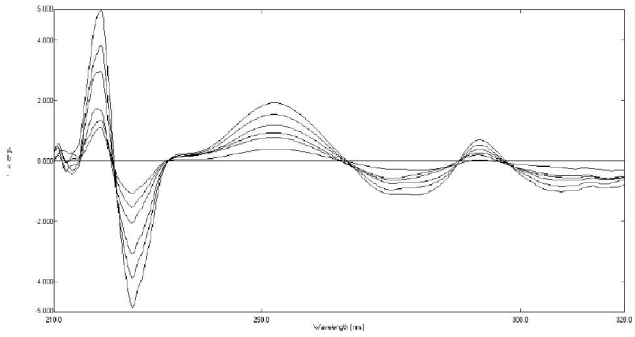

Figure 5: First derivative of ratio spectra of itraconazole (5-25 ¼g mL-1) using the spectrum of 25 ¼g mL^{-1} of degradation product as a divisor

recording the peak amplitudes at 252 nm using 25 µg mL^{-1} of the degradate as a divisor.

The regression equations were computed and found to be:

^{1}DD = 0.0757 C + 0.0266 (r = 0.9995), at 252 nm

where ^{1}DD is the peak amplitude of the first derivative curve for (itraconazole /its degradate), *C* the concentration of itraconazole (µg mL^{-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.

Difference spectrophotometric method

The method depends on measuring the difference between the spectrum of the degradation product and the spectrum of the same concentration of the intact drug, in methylene chloride. The absorbance difference of the intact drug versus its degradation product (Figure 6). The absorbance difference at the selected wavelength is proportional to the drug concentration and independent on the degradation product, (Figure 7).

Linear calibration graphs were obtained for

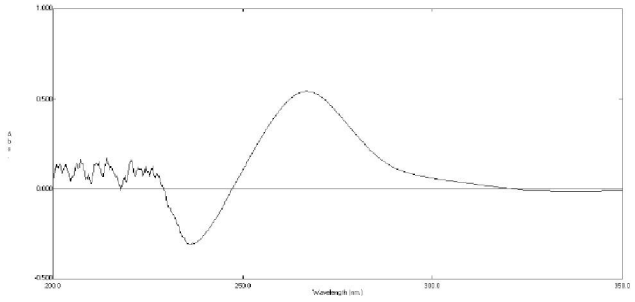

Figure 6 : Difference absorption (ΔA) spectrum of itraconazole versus its degradation product in methylene chloride, concentration of each was 15¼g mL-1.

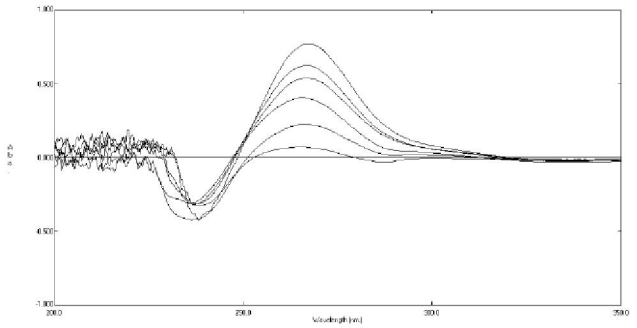

Figure 7 : Difference absorption (ΔA) spectra of itraconazole and its degradation product in methylene chloride (5 –30 μg mL^{-1})

itraconazole in concentration range of 5–30μg mL^{-1} by recording the peak amplitudes at 265 nm. The regression equations were computed and found to be

ΔA = 0.0355C + 0.0239 (r = 0.9995), at 265 nm

where ΔA is the absorbance difference for itraconazole, *C* the concentration of itraconazole (μg mL^{-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.

Bivariate method

The bivariate calibration method may be competitive and in some cases even superior to commonly use derivative spectrophotometric methods as applied for the resolution of binary mixtures. The advantage of bivariate calibration method is its simplicity and the fact that derivatization procedures are not necessary. Unlike other chemometric techniques, there is no need for full spectrum information and no data processing is required. Calibration function was calculated ($r > 0.9990$), m_i- and e_i-values were taken for the bivariate algorithm. In order to apply the bivariate method to the resolution of binary mixture of itraconazole and its degradate, we first select the signals of the two components located at six wavelengths: 230, 240, 250,260, 270 and 275 nm. The calibration curve equations and their respective linear regression coefficients are obtained with the aim of ensuring that there is a linear relationship between the absorbance values and the concentrations. All the calibration curves at the selected wavelengths showed satisfactory linear regression coefficients ($r >$ 0.9990). The slope values of the linear regression were estimated for both components at the selected wavelengths and used for determination of the sensitivity matrices *K*, proposed by Kaiser's method[12].

TABLE 1 : Application of Kaiser's method in the selection of wavelength pair for the mixture of itraconazole and its degradate: the absolute values of determinants of sensitivity matrices (K×10^{-5})

λ/λ	230	240	250	260	270	275
230	0	15.58	98.38	140.07	100.04	69.13
240		0	76.83	115.40	82.59	56.58
250			0	38.11	28.28	16.27
260				0	1.53	-3.60
270					0	-3.33
275						0

The determinants of these matrices were calculated as shown in TABLE 1.

The wavelength set was selected for which the highest matrix determinant value was obtained. For the bivariate determination of itraconazole and its degradate the wavelengths 230 and 260 nm were used. At these selected wavelengths, the one-component calibration curves were obtained in the range of 5–25μg mL^{-1} for both components. The linear regression calibration formulae used for the bivariate algorithm are presented in TABLE 2.

TABLE 2 : Linear regression calibration formulae used for the bivariate algorithm for itraconazole

Component	Calibration Equation	
	λ= 230 nm	λ= 260 nm
Itraconazole	A =0.033x-0.014 (r =0.9991)	A=0.045x+0.003 (r =0.9991)
Degradate	A =0.040x +0.036 (r =0.9995)	A =0.012x-0.012 (r =0.9991)

The advantage of this method over the other spectrophotometric methods is the ability for simultaneous determination of the intact drug and its degradate in mixtures.

Stability-indication

To assess the stability-indicating efficiency of the proposed methods, the degradation product of itraconazole was mixed with its pure sample at different ratios and the mixtures were analyzed by the proposed methods. TABLE 3 illustrates good selectivity in the determination of itraconazole in the presence of up to 80% of its degradate in the first-derivative, derivative-ratio and bivariate spectrophotometric meth-

TABLE 3 : Determination of itraconazole in laboratory prepared mixtures by the proposed spectrophotometric methods

Methods	^{2}D at 261 nm	^{1}DD at 252 nm	Difference spectrophotometry at 265 nm	Bivariate method
Mean± S.D.	99.99 ±1.101	100.13±0.0.952	100.78±0.0.661	100.29±0.760

ods, but up to 83% by difference spectrophotometry method.

Application of the proposed methods to the pharmaceutical formulations

The suggested methods were successfully applied for the determination of itraconazole in itranox capsules showing good percentage recoveries. The validity of the suggested methods was further assessed by applying the standard addition technique (TABLE 4), and the precision was also expressed in terms of relative standard deviation of the inter-day and intra-day analysis results (TABLE 5).

Statistical analysis

Results of the suggested methods for determination of itraconazole were statistically compared with those obtained by applying pharmacopoeial non aqueous titration method[1]. The calculated *t*- and *F*-values[13] were found to be less than the corresponding

TABLE 4 : Quantitative determination of itraconazole in itranox capsules by the proposed spectrophotometric method

Itranox capsules	^{2}D at 261 nm	^{1}DD at 252 nm	Difference Spectrophotometry at 265 nm	Bivariate method
Batch No. 0401109 Mean±S.D.	100.36±0.996	100.04±1.149	99.67±1.421	99.79±0.425

TABLE 5 : Assay validation parameters of the proposed spectrophotometric methods for the determination of pure samples of itraconazole

Parameter	^{2}D at λ= 261 nm	DD_1 method at 252 nm	Difference Spectrophotometry at 265 nm	Bivariate method
Accuracy (mean ± S.D.)	100.34±1.372	99.81±1.241	100.06±0.984	99.99±1.542
Specificity	100.36±0.996	100.04±1.149	99.67±1.421	99.79±0.425
Precision				
Repeatability*	99.78±0.981	100.48±0.781	99.21±0.971	99.61±0.981
Intermediate precision**	98.85±0.756	100.81±1.016	99.64±0.736	99.88±0..954
Linear range (µg/ml)	5-25	5-25	5-30	5-25
Slope	-0.0022	0.0757	0.0355	0.0449
Standard error of the Slope	3.27×10^{-5}	0.000824	0.000394	0.000663
Intercept	0.0003	0.0266	0.0239	0.0026
Standard error of the intercept	0.000521	0.013103	0.007664	0.010543
Correlation coefficient (r)	0.9991	0.9995	0.9995	0.9991

***the intraday and **the inter-day mean values ± standard deviations of samples of concentration of 10, 15, 20 µg/ml of itraconazole**

TABLE 6 : Statistical analysis of the results obtained by the proposed spectrophotometric methods and the compendial method for the determination of itraconazole in pure powder form

Item	^{2}D method	^{1}DD method	Difference Spectrophotometry	Bivariate method	Pharmacopoeial method*[3]
Mean	100.34	99.81	100.06	99.99	99.85
S.D.	1.372	1.241	0.984	0.760	0.886
Variance	1.882	1.54	0.968	0.578	0.785
n	5	6	5	4	5
Student's t test	0.729 (2.306)**	0.063 (2.306)**	0.344 (2.306)**	0.719 (2.365)**	
F value	2.397 (6.390)**	1.962 (6.260)**	1.230 (6.390)**	1.358 (9.120)**	

***non aqueous titration method**

****the values in parenthesis are the corresponding tabulated t and f values at p=0.05**

theoretical ones, confirming good accuracy and excellent precision (TABLE 6).

CONCLUSION

Unlike the mostly recommended HPLC-procedures, the proposed spectrophotometric methods are simple and not expensive. The reagents used in the proposed methods are cheap and readily available. The procedures applied in each method do not involve any critical reactions or tedious sample preparations. This aspect of spectrophotometric analysis is of major interest in analytical pharmacy since it offers distinct possibility of assaying itraconazole in itranox capsules without interference due to the excipient or the degradation product.

The suggested methods are found to be simple, accurate, selective and equally sensitive with no significant difference of the precision compared with the reference method[1].

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