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Validated Stability Indicating HPTLC and UV-Spectrophotometric Techniques for the Determination of Avanafil

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Authors' contributions

This work was carried out in collaboration among all authors. Author MMS designed the study, performed the statistical analysis, wrote the protocol and wrote the first draft of the manuscript. Authors MKD and SAAR managed the analyses of the study. Author MMS managed the literature searches. All authors read and approved the final manuscript.

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ABSTRACT

Aims: To develop methods with complete validation according to ICH guidelines and to be applied for the determination of avanafil in pure form and in pharmaceutical formulation in the presence of its degradation products.
Study Design: High performance thin layer chromatography (HPTLC) and different spectrophotometric methods (dual wavelength, first derivative, first derivative of ratio spectra and ratio difference are developed for simultaneous determination of avanafil in laboratory-prepared mixtures of avanafil with its degradation products and in pharmaceutical formulation.
Place and Duration of Study: Sample: Department of Analytical Chemistry, Faculty of Pharmacy (Girls), Al-Azhar University, Cairo, Egypt, between May 2019 and September 2019.
Methodology: Two techniques have been developed for the determination of avanafil in the

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presence of its degradation products. The first was HPTLC where separation was performed on silica gel 60 F254 plates, with chloroform: toluene: methanol: conc. ammonia (6:5:3:0.1, by volume) as a developing system and UV detection at 230 nm. The second one was UV- spectrophotometry which included dual wavelength between 267 and 292 nm, first derivative determination of the drug at 261 nm, first derivative of ratio of peak amplitudes at 275.6, 305.4 and 329 nm and the ratio difference with the amplitude difference between (266 and 250 nm).

Results: HPTLC method was applied over the concentration range of 0.5-5. µg/spot, while spectrophotometric methods were linear over the concentration range 5-50 µg/mL for avanafil.

Conclusion: Novel, simple and accurate method for the determination of avanafil in laboratory-prepared mixtures of avanafil with its degradation products and in pharmaceutical formulation.

Keywords: Avanafil; HPTLC; dual wavelength; first derivative; first derivative of ratio spectra; ratio difference.

1. INTRODUCTION

Avanafil is a phosphodiesterase type 5 (PDE5) inhibitor approved for erectile dysfunction by the FDA. It acts by inhibiting a specific PDE5 enzyme which is found primarily in the corpus cavernosum penis, as well as the retina. The advantage of avanafil is that it has very fast onset of action compared with other PDE5 inhibitors [1]. It is chemically (S)-4-((3-chloro-4-methoxybenzyl)amino)-2-(2 (hydroxymethyl) pyrrolidin-1-yl)-N-(pyrimidin-2-ylmethyl) pyrimidine -5-carboxamide [2]. It is insoluble in water, sparingly soluble in methanol, slightly soluble in ethanol and soluble in 0.1 M hydrochloric acid [2]. Literature survey revealed that a number of HPLC [3-7], HPTLC [8,9], LC/MS [10], UV-Vis spectrophotometric [11-14] and fluorometric [15] methods were reported for the assay of avanafil alone and in combination with other drugs [16]. Screening of the literature revealed there are only one LC-MS/MS [10] that identified the degradation products of avanafil where avanafil undergo amide hydrolysis and replacement of the amide group with an acidic group after exposure to 1M HCL and 1M NaOH at 80°C for 24 h whose predicted m/z is 393 and opening of pyrolidine ring when exposed to 30% H_2O_2 at 80°C for 24 h whose predicted m/z is 367.1 was identified by MS analysis.

No densitometric or spectrophotometric methods concerning the determination of avanafil in presence of its degradation products. Thus the objective of the present study was to develop simple and accurate stability -indicating methods for selective determination of avanafil.

2. MATERIALS AND METHODS

2.1 Instrumentation

TLC plates used were 20 x 20 cm precoated with silicagel 60 F 254 (Flukachemie, Switzerland), a camag Linomate 5 sample applicator equipped with a 100 µL syringe (Hamilton,Germany). The plates were scanned with a camag TLC scanner 3 with WINCATS computer software (Switzerland) using UV lamp with short wavelength (254 nm) (Desega- Germany).

Shimadzu UV/Vis spectrophotometer (PC – 1601, Tokyo, Japan), using 1.0 cm quartz cells. Scans were carried out in the range from 200–400 nm at 0.5 nm intervals. Spectra were automatically obtained by Shimadzu UV-Probe 2.32 system software.

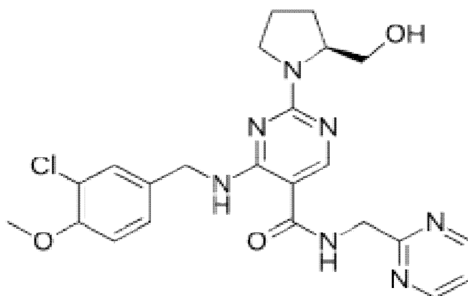


Fig. 1. Chemical structure of avanafil

2.2 Materials and Reagents

Pure samples: Avanafil was kindly supplied by Al – Andalous Pharmaceutical Company, 6th of October, Egypt. Its purity was 99.90% as stated by the supplier.

Market samples: Erovanafile® tablets; Batch no. 170236, labeled to contain 100 mg avanafil per tablet, the product of Al – Andalous Pharmaceutical Company, 6th of October, Egypt.

Sodium hydroxide, hydrochloric acid, tolune, conc. ammonia and Hydrogen peroxide (30%) were obtained from El-Nasr Co, Egypt. Methanol and chloroform were purchased from Sigma Aldrich (USA).

3N HCL was prepared in H_2O and 3N methanolic NaOH was prepared in methanol.

2.3 Standard Solutions

2.3.1 Preparation of standard solutions

Stock solution of avanafil (1 mg/mL) was prepared in methanol. Working solutions were freshly prepared by suitable dilution of stock solution with methanol to obtain a concentration of 0.1 mg/mL drug.

2.3.2 Preparation of degradation products

2.3.2.1 Acidic degradates

Degraded avanafil was laboratory prepared by refluxing 100 mg pure drug with 100 mL of 3M HCL for 2 hours. The solution was cooled, neutralized with 5M NaOH to pH about 7 and evaporated to dryness under vacuum. Residue was extracted three times, each with 25 mL methanol, then filtered into 100- mL volumetric flask and diluted to volume with the same solvent. The obtained solution were claimed to contain degradate derived from 1 mg mL^{-1} intact drug.

2.3.2.2 Oxidative degradates

Accurately weighed 100.0 mg of intact avanafil was dissolved in 25 mL 10% H_2O_2 and kept at room temperature for one day then allowed to dry at room temperature. Residue was extracted with methanol and diluted to 100 mL with the same solvent to obtain a solution claimed to contain degradates derived from 1 mg mL^{-1} intact avanafil.

2.4 Procedures

2.4.1 Linearity

HPTLC method: Different aliquots of stock standard solution (1 mg/mL) containing 0.5-5.0 mg avanafil were introduced into a series of 10-mL volumetric flasks and volume was completed with methanol. Ten µL from each solution was applied to pre-washed TLC activated plates of silica gel 60 F 254, as 6 mm apart, by means of a Camag Linomat IV automated spray-on band applicator equipped with a 100-µL syringe. The plates were developed with the mobile phase in a Camag twin-trough chamber previously saturated with mobile phase vapour chloroform: tolune: methanol: conc. ammonia (6:5:3:0.1, by volume) for 20 min. The plates were then removed and air dried. Densitometry was performed at 230 nm in reflectance mode with slit dimensions of 6.00 mm × 0.3 mm and scanning speed of 20 mm/s. Peak area of each concentration was then plotted against its corresponding drug concentration and regression equation was computed.

Spectrophotometric methods: Aliquots equivalent to 0.05-0.5 mg/mL avanafil were accurately transferred from its working standard solution (0.1 mg/mL) into a series of 10 -mL volumetric flasks and completed to volume with methanol. The spectra of the prepared solutions were scanned from 200 - 400 nm and stored in the computer.

- **Dual wavelength method**- The zero order spectra of above solutions were recorded in the range of 200 – 400 nm. The difference in absorbance between 267 nm and 292 nm were measured for each drug concentration.
- **First derivative (^{1}D) method** - 1D spectra of the drug above solutions were recorded against methanol as blank at 261 nm.
- **Derivative ratio (^{1}DR) method** - The stored zero-order spectra of avanafil were divided by the spectrum of 15 µg mL^{-1} acidic degradate. Then the first derivative of the ratio spectra (^{1}DR) with scaling factor 10 was obtained and smoothed at $\Delta\lambda$ = 8 nm. The amplitudes at 275.6 nm, 305.4nm and 329 nm of ^{1}DR spectra are then recorded.
- **Ratio difference (RD) method** - The above procedures detailed under derivative ratio (1DR) method were followed and after the division of the stored spectra by the spectrum of (15 µg mL^{-1}) of

the acidic drug degradate, the amplitude difference between 250 and 266 nm was measured.

In all the above spectrophotometric method the obtained response was plotted against the corresponding drug concentration and regression equation was computed.

2.4.2 Laboratory prepared mixtures

HPTLC method: Into a set of 10-mL volumetric flaks, different volumes (4.6 - 0.6 mL) of intact avanafil solution (1 mg mL^{-1}) were transferred and mixed with (0.2 – 2.2 mL) of each of acidic and oxidative degradates solutions. Volumes were completed to the mark with methanol. Ten µL were spotted onto a TLC plate following the above mentioned specific chromatographic conditions and scanned at 230 nm. Peak areas of the obtained chromatograms were measured and the concentration of the drug was calculated from its corresponding regression equation.

Spectrophotometric method: Aliquots of standard avanafil solution containing (0.45 - 0.1 mg) drug were introduced into a series of 10-mL volumetric flasks containing (0.05 - 0.40 mg) of the acidic degradate solution and then diluted to volume with methanol. The obtained solutions were analyzed by the proposed UV- methods as described under ‘’2.3.1. ii. Linearity’’. The intact drug concentrations were calculated from the corresponding regression equations.

2.4.3 Application to pharmaceutical formulations

Ten Erovanafile® tablets were accurately weighed and finely powdered. A weight equivalent to 100mg of avanafil was sonicated with about 30 mL of methanol in 100 ml volumetric flask for 20 minutes then the volume was completed with methanol to obtain a solution labeled to contain 1mg/mL avanafil. This solution was filtered and the filtrate used to analyze the drug by HPTLC as detailed under Linearity. The filtrate was diluted to10 folds with methanol to obtain a solution labeled to contain 0.1 mg/mL avanafil to be analyzed by UV-spectrophotometry as detailed under ‘’2.3.1. ii. Linearity’’.

3. RESULTS AND DISCUSSION

3.1 Forced Degradation

Stressed hydrolytic degradation of the studied drug was performed in acidic and alkaline media via refluxing the drug in different concentration of HCL and NaOH for different time intervals. The drug was completely degraded after about 2 or 3 hours using 3N HCL or 3N methanolic NaOH, respectively. Solutions were neutralized using 5M NaOH or 5M HCL, evaporated under vacuum and extracted with methanol.

Stressed oxidative degradation of the studied drug was performed by keeping the drug with different concentration of H_2O_2 for different time intervals. The drug was completely degraded after about one day at room temperature using 10% H_2O_2 then allowed to dry at room temperature then residue was extracted with methanol.

TLC separation of the intact and methanolic degraded solutions on silica gel GF254 plates using a mobile phase of chloroform- toluene - methanol - conc. ammonia (6: 5: 3: 0.1, by volume) revealed a spot for intact avanafil at R_f 0.61, two spots at R_f 0.0 and 0.51 corresponding to its acidic and alkaline degradates and another one at R_f 0.16 corresponding to its oxidative degradate.

Thus acidic and oxidative degradates were representatively used for the subsequent stability indicating analysis of the drug.

The obtained acid degradation product was separated on preparative TLC and only the spot at R_f 0.51 was identified by IR and mass spectroscopy. While oxidative degradate was confirmed by IR, mass and H^1NMR spectra.

The IR spectrum of the pure drug exhibits a sharp peak at 1639 cm^{-1} of –C=O of the amide group and two peaks at 3439 and 3255 cm^{-1} characteristic to the two –NH groups and aliphatic OH group; Fig. 2a. The appearance of a new broad band at 3417 cm^{-1} for –OH group with broad peak at 1695 cm^{-1} of –C=O group in the IR spectrum of the acid degradate suggested the hydrolysis of the amide group of the drug into a free carboxylic group; Fig. 2b. While IR spectrum of the oxidative degradate showed broad peak at 1690 cm^{-1} of –C=O group and appearance of new very broad band at 3210 cm^{-1} representing OH and NH groups; Fig. 2c [17].

EI (Electronic ionization) mass showed molecular ion peak at *m/z* = 483 for the intact drug; Fig. 3a. While the acidic degradate showed molecular ion peak at *m/z* = 393 indicating decreasing in molecular ion peak by about 90 unit. This means

loss of 2- methyl pyrimidine from the amine in the avanafile molecule and addition of OH of carboxylic group; Fig. 3b. While mass spectrum of oxidative degradate revealed molecular ion peak at *m/z* = 467 indicating the degradative effect of H_2O_2 by loss of 14 units from the molecular weight representing CH_2 from CH_2OH attached to pyrrolidine ring. In addition to the oxidative effect of CH_2NH to CH=N (imine group); Fig. 3c [17].

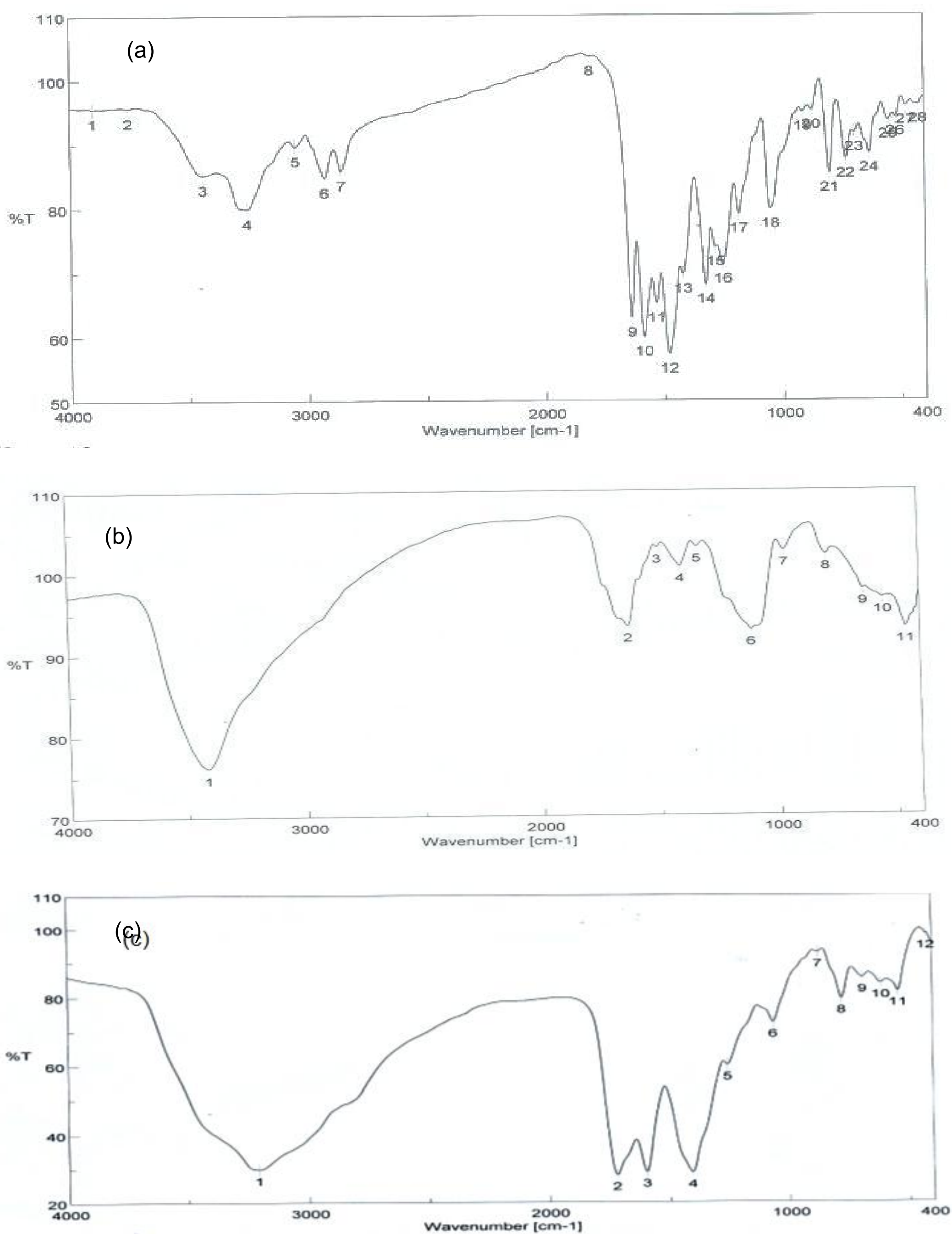


Fig. 2. IR Spectra of: (a) Intact avanafil, (b) avanafil acid degradate and (c) avanafil oxidative degradate on KBr disc

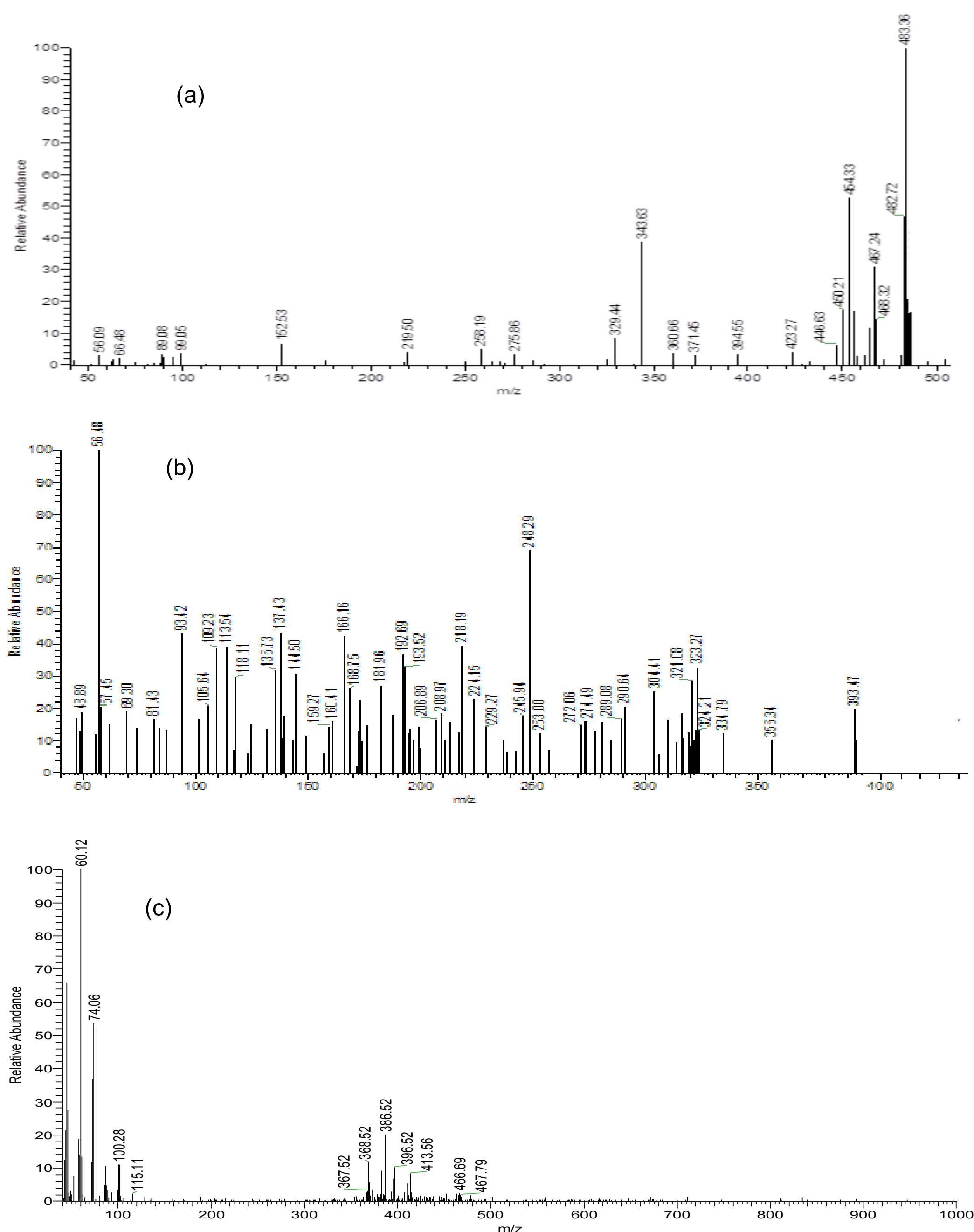


Fig. 3. Mass spectra of: (a) intact avanafil , (b) its acid degradate and (c) its oxidative degradate

H^1NMR spectrum of intact avanafil showed some multiplet signals expressing different proton types. Noticeably, pyrrolidine and methoxy protons exhibited multiplets ranged from 1.8-3.8 ppm, while CH_2NH, NH CH_2 and OH protons showed some signals in range 4.4-5 ppm. On the other hand, multiplets of phenyl, two pyrimidine and NH of amide appearing in the range of 7.0-8.7 ppm; Fig. 4a. Combatively, the spectrum of the oxidative degradate records the presence of all signals except signal of CH_2 at 3.6 ppm of (CH_2OH), signals of $NHCH_2$ at 4.6 ppm and appearance of new deshielded peak for CH=N at the aromatic region 8.65 ppm; Fig. 4b [17]. The suggested acidic and oxidative degradation pathway of avanafil were illustrated in the following scheme.

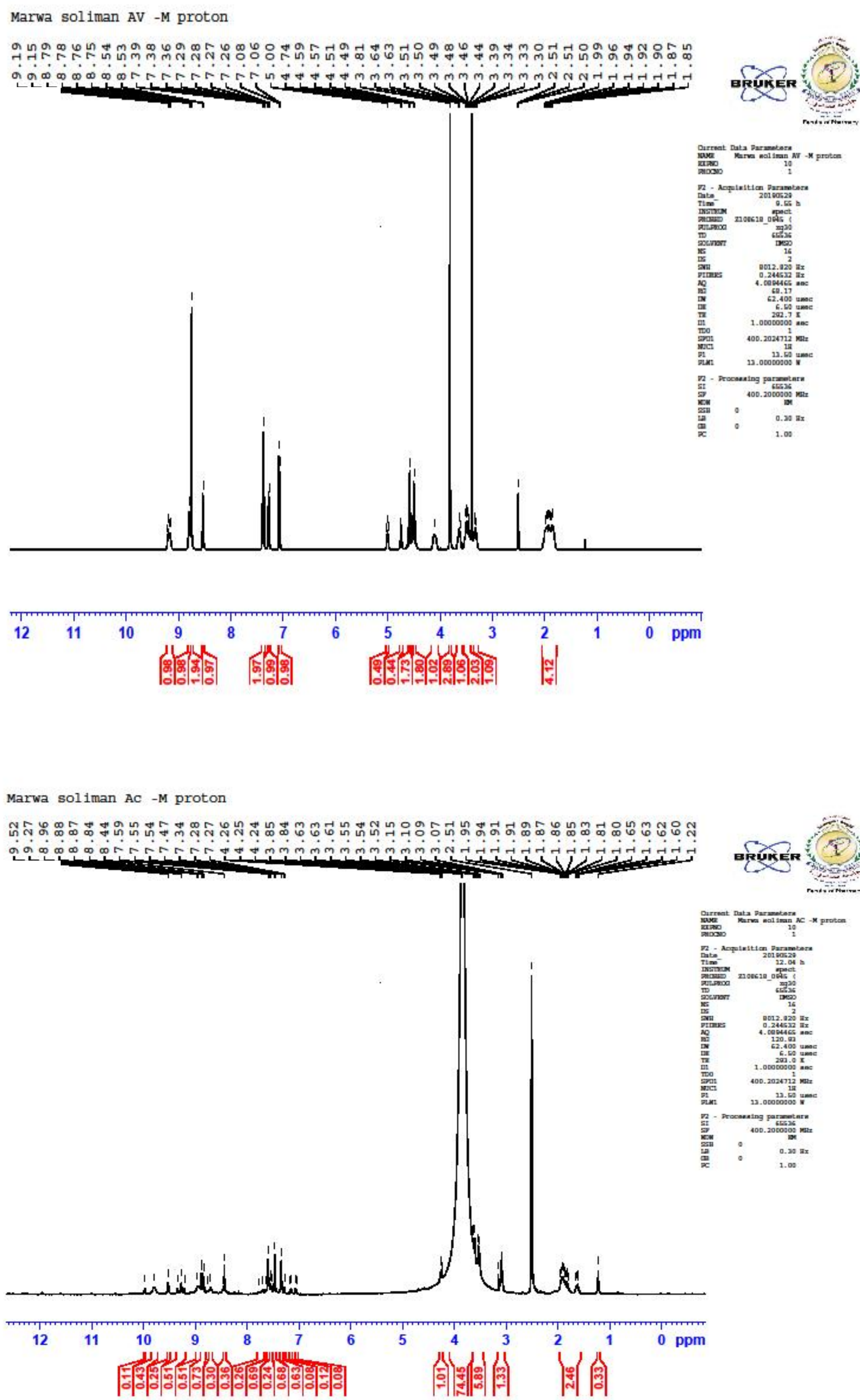
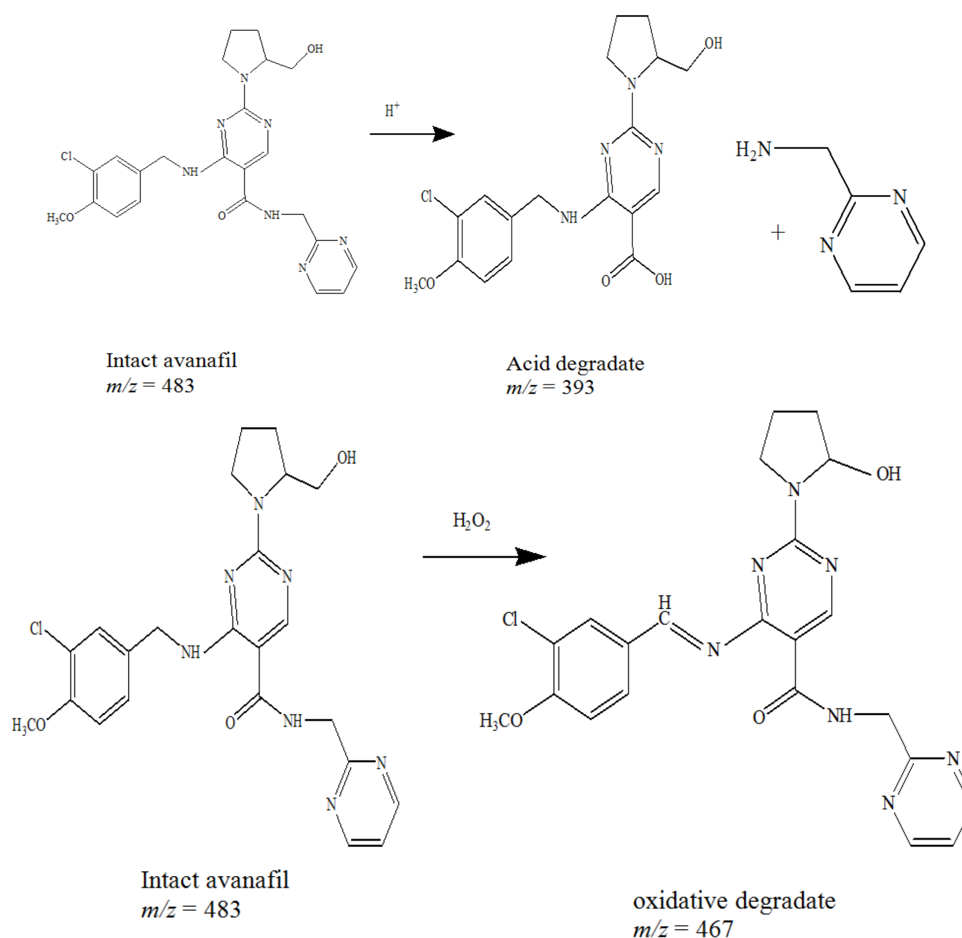


Fig. 4. H^1NMR spectra of: (a) intact avanafil and (b) its oxidative degradate



Scheme 1. Suggested acidic and oxidative degradation pathway of avanafil

3.2 Method Development

3.2.1 HPTLC method

Different mobile phases in different ratios and at different wavelengths for detection were tried. It was found that chloroform: toluene: methanol: conc. ammonia (6:5:3:0.1, by volume) as a developing system followed by densitometric determination at 230 nm offered best separation, resolution and detection. Where spots appeared at Rf 0.61 for intact avanafil, at Rf 0.51 corresponding to its acidic degradate and another one at Rf 0.16 corresponding to its oxidative degradate, Fig. 5.

3.2.2 Spectrophotometric method

The zero-order absorption spectra of avanafil and its acidic degradate showed severe overlapping over the entire spectrum of the intact drug; Fig. 6. Therefore, the mathematical methods were used for assaying avanafil in the presence of its acid degradation product was not possible. It is noteworthy to mention that oxidative degradate showed also sever overlapping in zero order spectra and upon applying these UV-methods the presence of this degradate hindering the determination of the drug.

Dual wavelength method: From the overlain spectra shown in Fig. 6, the difference between 267 nm and 292 was selected for the estimation of avanafil in presence of its acid degradation product which showed zero difference at these wavelengths.

First-derivative method: It was found the overlapping in zero order spectra was somewhat resolved in ^{1}D spectra and avanafil can be determined at 261 nm, at which its acid degradate has no contribution (zero crossing); Fig. 7.

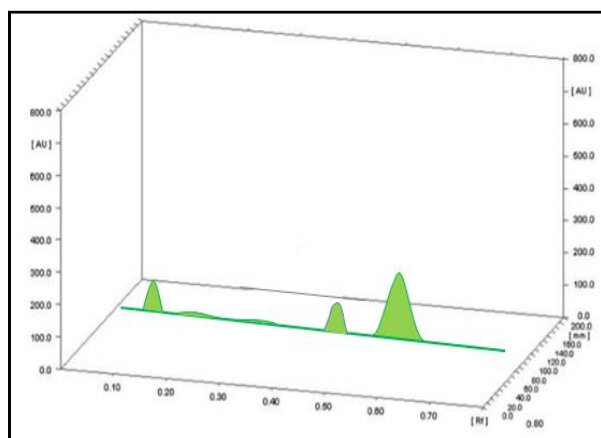


Fig. 5. Densitometric chromatogram of intact avanafil (2 µg/ spot) and of each of acidic and oxidative degradates (1.5 µg/ spot)

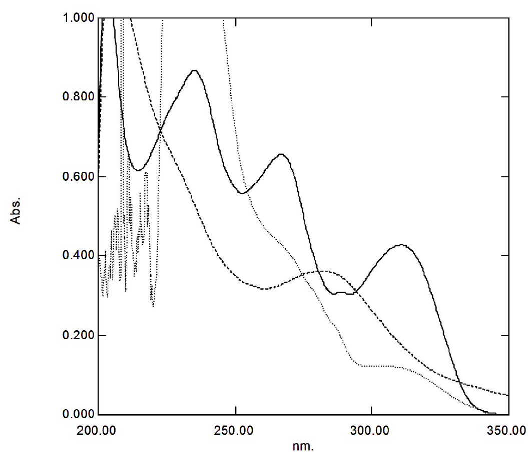


Fig. 6. Zero-order spectra of intact avanafil (20 µg mL^{-1}) (—) and its acidic degradate (15 µg mL^{-1}) (- - -) and its oxidative degradation product (20 µg mL^{-1}) (……) in methanol

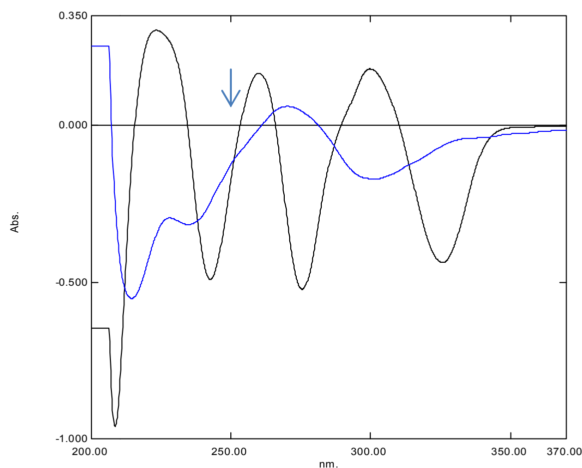


Fig. 7. 1D spectra of intact avanafil (20 µg mL^{-1}) (—) and its acid degradation product (15 µg mL^{-1}) (- - -) in methanol

Another wavelength at 281.4 nm at which the acid degradate showed zero absorbance in ^{1}D was studied but it gave non reliable results.

- **Derivative ratio (^{1}DR) method:** In this method the absorption spectrum of avanafil is divided by the absorption spectrum of its acid degradate and the first derivative of the ratio spectrum is obtained. Different concentrations of its acid degradate were tried as a divisor (10-50 µg/ml) where acidic degradate derived from 15 µg/ml avanafil was the best regarding average % recovery. A good linearity and recovery percent were observed at 275.6 nm, 305.4 nm and 329 nm using Δλ = 8 nm and scaling factor 10; Fig. 8b.
- **Ratio difference (RD) method [18]:** Linear correlation was obtained between the differences in amplitudes between 250 and 266 nm against the corresponding concentration of avanafil using 15 µg/ml of its acid degradate as divisor; Fig. 8a.

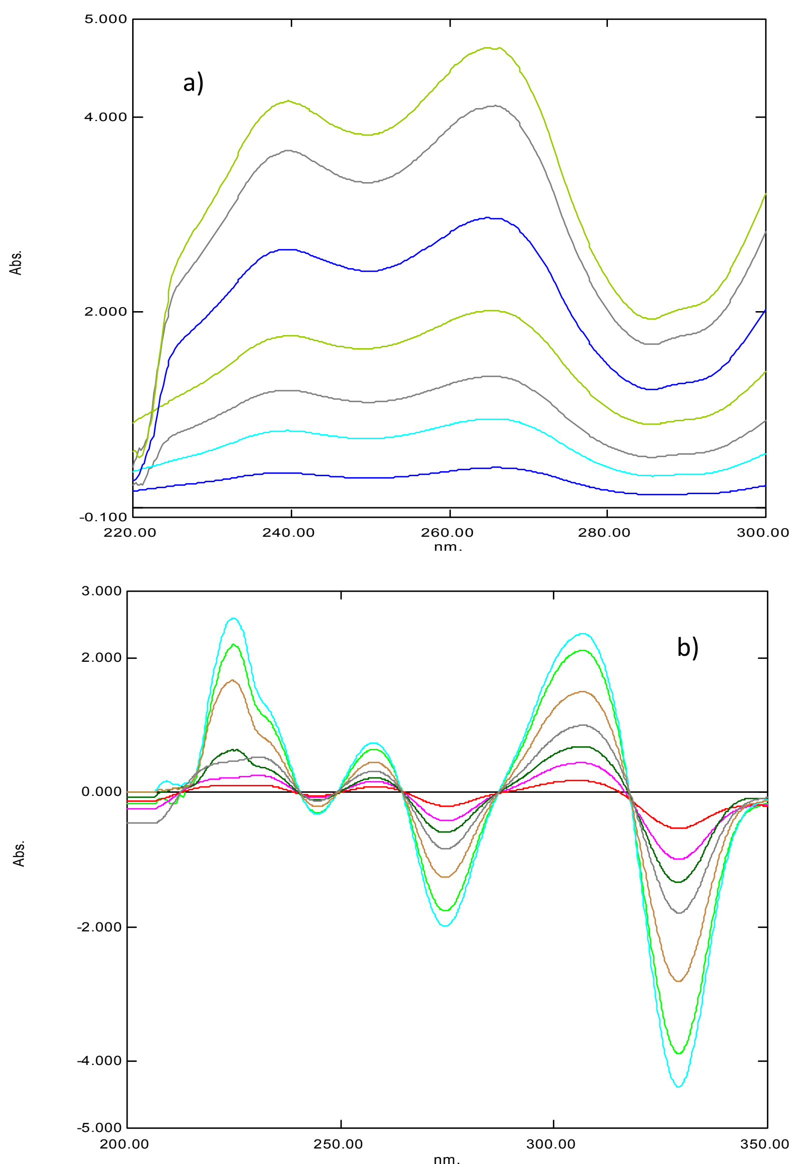


Fig. 8. (a) Ratio spectra and (b) First derivative of smoothed ratio spectra of avanafil (5-50 µg/mL) using 15 µg/mL its acid degradate as divisor

Table 1. Regression and validation parameters for the determination of avanafil by the proposed methods

	HPTLC	Dual wavelength	(1D) method	1DR method			RD method
λ $_{max}$ (nm)	230	Between 267 nm & 292 nm	261 nm	275.6	305.4	329	Between 250 nm & 266 nm
Linearity range	**0.5-5 µg/ spot**	**5-50 µg/ mL**					
Regression parameters							
Slope (b)± SD	1316.01±9.35	0.0166± 0.0002	0.0073± 9.82E-05	0.0385± 0.0005	0.0477± 0.0006	0.0850± 0.0012	0.0169± 0.0002
Intercept (a)± SD	934.76± 29.95	0.0122± 0.0064	0.0094± 0.0029	0.0271± 0.0161	-0.0309± 0.0185	0.1162± 0.00359	0.0325± 0.0064
Coefficient of determination (r^2)	0.9998	0.9992	0.9991	0.9990	0.9991	0.9990	0.9992
Accuracy (R%) (n=9)	99.34	99.01	99.83	98.91	98.49	100.07	98.39
Precision (RSD %)							
Intraday	0.99-1.58	0.31-0.67	0.64-1.27	0.29-0.62	0.51-1.26	0.21-1.06	0.31-0.67
Interday (n=9)	1.16-1.99	0.44-1.00	0.73-1.69	0.52-0.95	0.99-1.85	0.42-0.84	0.44-1.00

Table 2. Determination of avanafil in mixtures with its acid and oxidative degradate by the proposed densitometric method

Intact µg/spot	Acid degradate µg/spot	Oxidative degradate	% degradate	Recovery % of Intact
4.6	0.2	0.2	8	101.84
4.4	0.4	0.4	16	99.16
4	0.5	0.5	20	100.37
3	1	1	40	100.12
2	1.5	1.5	60	101.39
1	2	2	80	102.77
0.6	2.2	2.2	88	106.41*
Mean% ±SD			100.94±1.31	

** rejected*

Table 3. Determination of avanafil in mixtures with its acid degradate by the proposed UV- methods

Intact µg/mL	Degradate µg/mL	% degradate	DW method	1D method		1DR method		RD method
			R % of Intact between 267 and 292 nm	R % of Intact at 261 nm	R % of Intact at 275.6 nm	R % of Intact at 305.4 nm	R % of Intact at 329 nm	R % of Intact between 250 and 266 nm
45	5	10	99.93	98.68	101.13	100.93	99.78	100.11
40	10	20	100.46	99.42	101.47	101.19	100.24	100.53
35	15	30	100.81	99.95	101.35	101.53	100.68	100.95
30	20	40	101.06	101.59	101.81	101.89	101.46	101.31
25	25	50	101.72	102.06	102.07	102.43	101.89	101.84
20	30	60	102.56	102.85	102.49	102.95	102.13	102.67
15	35	70	104.97*	106.26*	105.19*	106.23*	105.39*	105.23*
10	40	80	107.32*	109.15*	108.66*	109.66*	107.87*	108.34*
Mean% ±SD			101.09±0.94	100.76±1.64	101.72±0.50	101.82±0.77	100.94±1.03	101.24±0.92

** rejected*

Table 4. Application of standard addition technique for the determination of avanafil Erovanafile® tablets by the proposed methods

Method		Pharmaceutical* formulations	Taken (ug mL^{-1})	Pure added (ug mL^{-1})	% recovery of added	Mean%±SD
HPTLC		99.26%±1.33	2	0.5	100.46	100.80±1.27
				1	101.59	
				2	99.16	
				3	101.98	
Dual wavelength at 267 and 292 nm		100.05±1.35	20	5	101.88	100.90±0.86
				10	101.15	
				20	99.82	
				30	100.75	
(1D) method at 261nm		99.31±1.06	20	5	101.23	100.33±1.17
				10	101.42	
				20	99.56	
				30	99.11	
1DR method	At 275.6 nm	100.55±1.21	20	5	98.52	99.77±0.91
				10	100.17	
				20	100.65	
				30	99.73	
	At 305.4 nm	100.11±1.50	20	5	98.88	99.86±0.68
				10	100.37	
				20	100.28	
				30	99.89	
	At 329 nm	99.73±1.17	20	5	99.20	100.25±0.75
				10	100.53	
				20	100.32	
				30	100.96	
RD method at 250 and 266 nm		99.25±0.79	20	5	98.37	99.45±1.08
				10	100.89	
				20	98.95	
				30	99.57	

**Average of 5 determinations*

Table 5. Statistical analysis of the proposed analytical and reported methods [11] for the determination of avanafil in Erovanafile® tablets

Parameter	HPTLC	Dual wavelength	(1D) method	1DR method			RD method	Reported method [11]
				At 233.4 nm	At 250 nm	At 275.6 nm		
Linearity	0.5-5 µg/ spot	5-50 µg/ mL						5-35 µg/ mL
N	5	5	5	5	5	5	5	5
Mean%±SD	99.26±1.33	100.05±1.35	99.31±1.06	100.55±1.21	100.11±1.50	99.73±1.17	99.25±0.79	99.16±1.25
Variance	1.77	1.82	1.12	1.46	2.25	1.37	0.62	1.56
t-	0.12	1.09	0.21	1.79	1.09	0.73	0.14	-
F-	1.13	1.17	1.39	1.07	1.44	1.14	2.52	-

-The theortical t- and f- values at p= 0.05 are 2.31 and 6.39, respectively.
-Reported method [11] involved UV- spectrophotometric method of avanafil in methanol at 307 nm

3.3 Method Validation

The proposed method was validated according to the ICH guidelines [19].

- **Linearity:** Under the described experimental conditions, linear calibration curves between responses to respective drug concentrations were obtained along the ranges of 0.5 -5 µg/ spot and 5-50 µg/mL of avanafil using HPTLC and spectrophotometric methods, respectively. Regression parameters were computed, where values of r^2 ranged between 0.9990 and 0.9998 indicating good linearity, Tables 1.
- **Accuracy and precision:** Accuracy ranged from 98.39 to 100.07% for avanafil. Intraday precision RSD % ranged from 0.21 to 1.58%, while inter day one ranged from 0.42 to 1.99%; indicating good repeatability and reproducibility of the proposed methods, Tables 1.
- **Selectivity:** The selectivity of the proposed methods was assured by application to laboratory prepared mixtures of the intact drug together with its acidic and oxidative degradation products. They were successfully applied for the determination of avanafil in the presence of up to 80% of both degradates for HPTLC method and up to 60% of its acid degradate for spectrophotometric methods with mean % recovery of 100.94±1.31 for HPTLC method, Table 2 and 101.09±0.94, 100.76±1.64, 101.72±0.50, 101.82±0.77, 100.94±1.03 and 101.24±0.92 for DW, ^{1}D, ^{1}DR at 275.6 nm, 305.4 nm and 329 nm and RD methods, respectively, Table 3. It is noteworthy to mention that oxidative degradate gave non-reliable results upon applying the UV- methods for determination of the drug in presence of it.

3.4 Application to Pharmaceutical Formulations

The proposed techniques were successfully applied for analysis of avanafil in the Erovanafile® Tablets with mean % recovery of 99.25±0.79 to 100.55±1.50. The validity of the proposed methods was further assessed by applying the standard addition technique. The results obtained were reproducible with acceptable SD, Table 4. Statistical analysis of the results obtained by the proposed methods compared with a reported UV method [11] showed that the calculated t and F values were less than the tabulated ones indicating no significant difference between them at 95% confidence limit, Table 5. The chromatographic method was more selective and sensitive than the reported one, while UV-spectrophotometric methods are more selective; determining the intact drug in presence of its acidic degradate.

4. CONCLUSION

The proposed two techniques are rapid, accurate and precise and can be used for the analysis of avanafil in pure form and in pharmaceutical formulations in the presence of its degradation products. The sample recovery for all the proposed methods was in good agreement with their respective label claims which suggested no interference from additives and excipients.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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