

Stability indicating spectrophotometric methods for determination of Tiemonium methylsulphate in the presence of its degradation products

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ABSTRACT

Simple, selective and sensitive stability indicating spectrophotometric methods have been developed and validated for Tiemonium methylsulphate (TIM) determination in pharmaceutical dosage form. Method A, isoabsorptive point comprised of measurement the total content of the mixture of TIM and its acid degradation product at 250 nm, while the content of acid degradation product was determined by measuring the peak amplitude of the ²D at 295.6nm, then TIM concentration can be determined by subtraction. Method B, the first derivative of ratio spectra was applied by measuring amplitudes of TIM at 224.4 nm and 247.2 nm using 20µg/mL of acid degradation product as a divisor. Method C is the ratio subtraction method. While method D is based on the measurement of first derivative amplitudes of TIM at the zero crossing point of its oxidative degradation product at 250 nm. The percentages mean accuracy for TIM was 100.39±0.33 for method A, 100.59±0.57 and 100.40±0.56 for B, 100.47±0.54% for C and 100.24±0.58% for D methods. The developed methods were validated as per International Conference of Harmonization guidelines. Furthermore, elucidation of the degradation pathway is described based on the use of the infrared spectroscopy and HPLC/MS techniques.

INTRODUCTION

Tiemonium methylsulphate (TIM), 4-[3- Hydroxy – 3-phenyl-3-(2-thienyl) propyl]-4 -methyl- morpholinium methylsulphate and tiemonium iodide are quaternary ammonium antimuscarinics with peripheral effects similar to those of atropine and are used in the relief of visceral spasms (Sweetman, 2011).

Few methods have been reported for TIM determination either alone by Ion Beam Analysis Elemental Technique (Bejjani *et al.*, 2011) or in combination with quaternary ammonium drugs (De Schutter and De Moerloose, 1988) and with other spasmolytics (De Schutter *et al.*, 1985) in the form of iodide salt. Human studies on the bioavailability of quaternary ammonium compounds, tiemonium iodide and tiemonium methosulphate, revealed no difference in the bioavailability of these two tiemonium salts (Scoular *et al.*, 1977). The International Conference on Harmonization (ICH) guideline entitled "Stability testing of new drug substances and products" requires that Stress testing of the drug substance can help identify the likely

degradation products, which can in turn help establish the degradation pathways and validate the stability indicating power of the analytical procedures used (ICH, 2003). No stability-indicating methods have been cited in the literature for determination of TIM and its degradation products; therefore the scientific novelty of the work presents a study of the degradation of TIM, identification of the obtained degradation using LC-MS, followed by the development of four simple stability-indicating methods for TIM determination either in raw material or in pharmaceutical dosage forms without the interference of excipients and their degradation products, which makes them suitable for quality control analysis.

EXPERIMENTAL

Instruments

A double beam UV-Visible spectrophotometer (SHIMADZU, Japan) model UV-1601 PC with quartz cell of 1 cm path length, connected to IBM compatible computer and HP680 inkjet printer. The bundled software was UVPC personal spectroscopy software version 3.7. The spectra bandwidth was 2 nm and wavelength scanning speed was 2800 nm/min.

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Structure elucidation of the degradation products was done using Jasco FT/IR-6100 spectrometer (Japan) and Agilent Technologies 6410 triple quadrupole mass spectrometer (U.S.A), fitted with ESI source where ESI-MS analyses were carried out on both positive and negative ion modes with scan range from m/z 50-500. The MS n data were acquired in the Auto MS n mode. The ESI source temperature was kept at 300°C and the capillary voltage was maintained at 4.0 Kv, while the fragmentor voltage was 135. Nitrogen was used as the nebulizing gas at a flow rate of 11 liter/min and the pressure was kept at 40 psi. The Agilent Mass Hunter software provided with the instrument for instrument control and data acquisition. Precoated TLC-plates, silica gel 60 F254 (20 cm×20 cm, 0.25 mm), E. Merck (Darmstadt-Germany). Ultrasonic, Bandelin electronic, Sonorex RK510S, HF-Frequency35KHz (10 liter capacity), Germany.

Materials

Chemicals and reagents

All chemicals and reagents used throughout this work were of analytical grade, and the solvents were of spectroscopic grade. Methanol AR (S.D Fine-Chem Limited, Mumbai, India), Hydrochloric acid (Riedel de Haen, Seelze, Germany).

Pure standard

Pharmaceutical grade Tiemonium methylsulphate was kindly supplied by ADWIA Pharmaceuticals Co., Cairo, Egypt, and certified to contain 100.6%.

Pharmaceutical formulation

The commercial Viscéralgine tablets Batch No. 710196, labeled to contain 50 mg of TIM / tablet manufactured by SEDICO Pharmaceutical Co., Egypt, under license of Riom laboratories – France. The dosage form was purchased from the Egyptian markets.

Degraded sample

Acid or Alkali-induced degradation product

Tiemonium methylsulphate (50 mg) was dissolved in the least amount of methanol, refluxed with 50 mL of 5 M HCl, or 5N NaOH solution in a 250 mL round-bottom flask for 2 hours, and tested for complete degradation by TLC using methanol: chloroform: glacial acetic acid (8:2:0.2, by volume) as the mobile phase. Only one spot was observed not corresponding to TIM. The degraded solution was then cooled, neutralized with an amount of acid or base equivalent to that of the previously added till pH was 7. The solution was nearly evaporated to dryness, cooled, transferred into a 50 mL volumetric flask with methanol. Then the volume was completed to the mark with the same solvent and filtered to obtain solution with final concentration equivalent to 1 mg /mL.

Hydrogen peroxide – induced degradation product

Two mL of 30% w/v of hydrogen peroxide was added to 50 mL of methanolic TIM solution (1mg/ mL), and then refluxed

at 90°C for 2 h. The solution was evaporated to dryness, and the residue was dissolved in methanol, transferred into a 50mL volumetric flask with methanol. Then the volume was completed to the mark with the same solvent to have a solution with final concentration equivalent to 1 mg /mL. The degraded solutions were used to scan the IR and LC-MS spectral scans, and also were used for testing the suggested methods.

Standard stock and working solutions

- Stock standard solutions of TIM, acid and oxidative degradation products 1 mg /mL (in 0.1M HCl for methods A and B) and (in methanol for C and D methods).
- Working standard solutions of TIM and acid degradation product (0.2 mg /mL in 0.1M HCl) of each for method A
- Working standard solutions of TIM, acid and oxidative degradation products (0.1 mg /mL in 0.1M HCl of each for method B and in methanol for method C and D)
- Solutions containing different ratios of TIM and its degradation products were prepared to contain 10–90% of degradation products of TIM.

Procedure

Linearity

Isoabsorptive method

The absorption spectra of 20 µg/mL of TIM and its acid degradation product prepared separately, and the spectrum of a binary mixture containing 10 µg/mL of TIM and its acid degradation product in a ratio of (1:1) using 0.1M HCl as a blank were recorded.

For TIM determination, working solutions containing 20-120 µg/mL TIM were prepared in 0.1M HCl using its corresponding working standard solution (0.2 mg/ mL in 0.1M HCl). The spectra were recorded from 200 – 400 nm and were stored in computer and their zero-order absorption spectra were measured at 250.0 nm (isoabsorptive point), the calibration curve relating the absorbance to the corresponding concentration of TIM in µg/mL was constructed and the regression equation was computed.

For the determination of acid degradation product, working solutions containing 20-120 µg/mL acid degradation product were prepared in 0.1M HCl from its working standard solution (0.2 mg/ mL in 0.1M HCl). The zero-order curves were recorded from 200 – 400 nm. The second derivative curves were manipulated at $\Delta\lambda = 4$ nm intervals and the scaling factor = 100. ²D values were measured at 295.6 nm. A calibration curve was constructed between the recorded peak amplitudes and the corresponding concentrations then the regression equation was computed.

First-derivative of ratio spectra (¹DD) method

Working solutions containing 10-60 µg/mL TIM were prepared in 0.1M HCl using its corresponding working standard solution (0.1 mg/mL). Zero order absorption spectra of previous

solutions were divided by the absorption spectrum of 20 µg/mL of the acid degradation product (as a divisor), and then the obtained ratio spectra were differentiated with respect to wavelength using $\Delta\lambda = 4$ and scaling factor=10. The ¹DD peak amplitude at 224.4 nm and 247.2 nm were recorded. The calibration curves representing the relationship between the measured amplitudes and the corresponding concentrations of the drug were constructed, and regression equations were computed.

Ratio subtraction method

Working solutions containing 10-60 µg/mL TIM were prepared in methanol using its corresponding working standard solution (0.1 mg/ mL in methanol). A calibration curve was constructed relating the absorbance at 234.0 nm versus the corresponding concentrations of TIM and the regression equation was computed.

First derivative spectrophotometric method

Working solutions containing 10-60 µg/mL TIM were prepared in methanol using its corresponding working standard solution (0.1 mg/ mL in methanol). The zero order absorption spectra of the prepared solutions were recorded and stored in computer, then the first derivative spectra (¹D) were obtained at $\Delta\lambda = 2$ and scaling factor = 10. The calibration curve was obtained by plotting the peak amplitudes of ¹D spectra at 250.0 nm (corresponding to zero crossing of oxidative degradation product) versus the corresponding drug concentrations, and regression equation was computed.

Analysis of laboratory prepared mixtures

Mixtures containing different concentrations of TIM and its induced acid or oxidative degradation products were analyzed as mentioned under each method. The concentrations were calculated from the corresponding regression equations.

Assay of Pharmaceutical Formulations

The contents of ten tablets of Viscéralgine® tablets were powdered and mixed well; an accurately weighed amount of the powder equivalent to 0.1 g of TIM was transferred into 100mL volumetric flasks, 75 mL of the appropriate solvent added 0.1M HCl for (A, B methods) and methanol for (C, D methods) stirred well then cooled and completed to the volume to obtain 1mg/mL stock solutions, then filtered. The solutions were diluted to the same concentrations of the appropriate working solutions and preceded according to the procedure of each method mentioned above.

Liquid Chromatography- Mass Spectrometry

Aliquots of methanolic standard solution of pure TIM and its degradation products were diluted with mobile phase consisted of acetonitrile: 10 mM ammonium formate buffer pH 3.05 at a ratio of (70:30 v/v). An aliquot equivalent to 0.5 µL of each sample was separately injected at flow rate 0.7 mL /min,

using Inertsil ODS-3 C18 (50 x 4.6 mm, 5 µm particle size) as analytical column). Then samples were subjected to mass spectrometric analysis using positive and negative electrospray ionization modes.

RESULTS AND DISCUSSION

The stability-indicating assay is a method that is employed for the analysis of samples stability in pharmaceutical industry. With the advent of International Conference on Harmonization (ICH) guidelines, the requirement of establishment of stability-indicating assay method has become more clearly mandated (ICH, 2003). The guidelines explicitly require conduct of forced decomposition studies under a variety of conditions, like pH, light, oxidation, dry heat, etc. and separation of drug from degradation products (Bakshi and Singh, 2002). Reviewing the literature concerning determination of TIM revealed the lack of any stability- indicating methods for the determination of the intact drug in presence of its induced degradation products whose pharmacological effect was found to be less potent (Williams and Lemek, 2002). In this study, spectrophotometric methods were developed for determination of TIM after separation from degradation products resulted from the stress acid and oxidative conditions thus comprehensively making the entire study stability-indicating.

Identification of the degradation products

Different concentrations of HCl (1, 2, 4 and 5 M), NaOH (2 and 5 M) and (10% and 30%) of hydrogen peroxide were tried. Complete degradation has been achieved by reflux for 2 hrs with 5 M HCl for acid degradation, and for 2 hrs with 30% hydrogen peroxide in case of oxidative one, meanwhile the drug showed stability against alkaline degradation. Degradation process was followed by TLC method for complete disappearance of the intact drug's spot.

Since impurities and degradation products are usually present in relatively small quantities compared to the drug, an analytical technique capable of separating a mixture containing highly varied concentrations of analytes with sensitive and specific detection is required. LC-MS is therefore widely used for this purpose (Lim and Lord, 2002; Volk *et al.*, 1996; Wu, 2000; Eckers *et al.*, 1997). The acid and oxidative degradation products were subjected to LC-MS for their identification. The positive ion ESI-MS-scan of the solution of TIM in the mobile phase showed a peak at m/z 429 which refers to the protonated molecular ion of the drug, Figure 1a. Scanning of acid degradation product solution showed a product at m/z 413, Figure 1b, which suggests that the drug undergoes dehydration during acid hydrolysis to produce 3-(4-methylmorpholin-4-ium-4-yl)-1-phenyl-1-thiophen-2-ylprop-1-ene. The positive ion ESI-MS-scan of oxidative degradation product solution showed a peak at m/z 445, Figure 1c. This result led to the suggestion of oxidative reaction with an increment increase of mass of +16, which corresponds to the reactive [O] atom with the production of 2-[1-hydroxy-1-phenyl-3-(4-

methylmorpholin-4-ium-4-yl) prop-1-yl] thiophene -1-oxide. The IR spectrum on KBr discs of oxidative degradation product showed a band at 1046 cm^{-1} corresponds to S=O, Fig. 2, which gave an additional proof of the suggested structure. The drug showed stability under alkaline induced degradation. The predicted structural formula of TIM degradation products are shown in the scheme, Figure 3.

Isoabsorptive method

In the isoabsorptive method, the spectra of two components are crossed at certain points, these points are so-called "isoabsorptive points" at which the two components have the same absorptivity, so the mixture of the two components act as single component that condemns the absorbance at isoabsorptive points as good measurements of the total concentration of both in mixture, if the concentration of either two components could be determined, separately, the concentration of the second one could be calculated by subtraction (Abdel-Kawy *et al.*, 2006)

In the isoabsorptive method, total concentration of TIM and its acid degradation product concentration in the mixture (T) were determined at the previously chosen isoabsorptive point. This point is determined experimentally by recording the absorbance spectra of $20\text{ }\mu\text{g/mL}$ of each TIM and its acid degradation product separately, and that a mixture containing same total concentration ($10\text{ }\mu\text{g/mL}$ of each) as shown in Figure 4.

Acid degradation product can be determined by measuring the second order derivative of its spectra at 295.6 nm with no interference from TIM, and the concentration of TIM could be calculated by subtraction (Total – acid degradation product). The linearity between the zero order absorption of its spectra of TIM at 250.0 nm (isoabsorptive point) and the corresponding concentrations of the drug was studied. Also the linearity of second-order absorption spectra of acid degradation product at 295.6 nm and its corresponding concentration was studied. The regression equations were computed for TIM and its acid degradation product, respectively;

$$A_{\text{iso}} = 0.0091 C_T + 0.0111 \quad r_T = 0.9997$$

$${}^2D_{295.6} = -0.0033 C_{\text{ac}} + 0.0005 \quad r_{\text{ac}} = 0.999$$

Where A_{iso} is the absorbance of TIM at 250.0 nm , C_T is the total concentration of the mixture and r_T is the correlation coefficient of equation. ${}^2D_{295.6}$ is the peak amplitude of the second derivative absorption spectra of acid degradation product at 295.6 , C_{ac} is its concentration and r_{ac} is the correlation coefficient of equation.

First-derivative of ratio spectra (${}^1\text{DD}$) method

Figure 5 shows the UV absorption spectral overlap of TIM and its acid degradation product at their nominal concentrations. In order to improve the selectivity of the analysis of TIM in the presence of its acid degradation product, ${}^1\text{DD}$ spectrophotometric method was established. The main advantage of the method is that the whole spectrum of the interfering substance is cancelled. Accordingly, the choice of the wavelength selected for calibration is not critical as in the ${}^1\text{DD}$ method.

In order to optimize ${}^1\text{DD}$ method, several divisor concentrations 10 , 20 , and $50\text{ }\mu\text{g/mL}$ of the acid degradation product were tried, the best result was obtained when using $20\text{ }\mu\text{g/mL}$ of the acid degradation product as a divisor. Different smoothing and scaling factors were tested, where a smoothing factor $\Delta\lambda = 4$ and a scaling factor = 10 were suitable to enlarge the signal of TIM to facilitate its measurement and to diminish error in reading the signal.

The absorption spectra of TIM in the range of 10 - $60\text{ }\mu\text{g/mL}$ were divided by the absorption spectrum of $20\text{ }\mu\text{g/mL}$ of the acid degradation product (as a divisor). The obtained spectra were differentiated with respect to wavelength (Figure 6). ${}^1\text{DD}$ values showed good linearity and reproducibility at 224.4 nm and 247.2 nm , linear regression equations were computed and found to be:

$${}^1\text{DD}_{224.4} = 0.0332C_{224.4} + 0.0147; r = 0.9998 \text{ at } 224.4\text{ nm}$$

$${}^1\text{DD}_{247.2} = -0.0538C_{247.2} - 0.0267; r = 0.9999 \text{ at } 247.2\text{ nm}$$

Where ${}^1\text{DD}$ is the first derivative of the ratio peak amplitudes at the specified wavelength 224.4 nm and 247.2 nm for TIM, C is the concentration of TIM ($\mu\text{g/mL}$) and r is the correlation coefficient.

Ratio subtraction method

Based on the theory of ratio subtraction spectrophotometric method (El-Bardicy, 2008); TIM could be selectively determined in the presence of its oxidative degradation product.

Practically, the ratio subtraction method starts by scanning zero - order spectra of the prepared working solutions of TIM in methanol, then the linearity is checked between absorbance at the selected wavelength at 234.0 nm and the corresponding concentration of TIM. The method depends on that, when a mixture of TIM (X) and oxidative degradation product (Y); where the spectrum of (Y) is more extended (Figure 7), the determination of (X) could be done by scanning the zero order absorption spectra of mixtures containing different concentration of TIM and oxidative degradation product in methanol which were then divided by the spectrum of the oxidative degradation product as a divisor (\hat{Y}) producing new ratio spectra that represent $(X/Y) + \text{constant } (Y/\hat{Y})$ as shown in Figure 8. Different divisor concentrations (20 , 40 and $60\text{ }\mu\text{g/mL}$) were tried and the divisor of concentration $60\text{ }\mu\text{g/mL}$ of degradation product was found to be the best regarding accuracy and precision. The value of the absorbance in the plateau region at λ above 278 nm (Y/\hat{Y}) was subtracted from the spectrum of the divided mixtures (Figure 9); the obtained spectra were then multiplied by the spectrum of the divisor (\hat{Y}) as shown in Figure 10. Finally, the original spectra of TIM (X) could be obtained which are used for direct determination of TIM at 234.0 nm and calculation of the concentration from the corresponding regression equation. A linear correlation is obtained between the absorbance and the corresponding concentration of TIM at 234.0 nm . The regression equation is:

$$A = 0.0219 C - 0.0209 \quad r = 0.9997$$

Where A is the absorbance, C is concentration of TIM in $\mu\text{g/mL}$ and r is the correlation coefficient.

First derivative spectrophotometric method

The zero-order absorption spectra of TIM and its oxidative degradation product in methanol are shown in Figure 7. The spectra display overlapping in the region of 200- 300 nm. This makes the determination of TIM in the presence of its oxidative degradation product by conventional UV spectrophotometry difficult.

The derivative spectrophotometry technique was, therefore, chosen for the determination of the drug since it could remove broadband contributions from excipients and might also overcome the interference from peak overlapping. The experiments showed that the first derivative spectra of TIM was simple and give results with suitable precision at $\Delta\lambda=2$ nm, scaling factor= 10. In this first derivative spectrum the signals at 250.0 nm corresponding to zero crossing of the oxidative degradation product, are proportional to TIM concentration, Figure 11, and the regression equation was then computed.

$$A = - 0.0124 C - 0.0047 \quad r = 0.9999$$

Where A is the amplitude of the first-derivative curve at the corresponding wavelength, C is concentration of TIM in $\mu\text{g/mL}$ and r is the correlation coefficient.

Methods Validation

The suggested procedures were subjected to the validation scheme according to ICH guidelines (ICH, 2005) and showed good specificity and reproducibility, Table 1.

Range and Linearity

The linearity of the isoabsorptive, ^1DD , ratio subtraction and ^1D methods for determination of TIM was evaluated by analyzing a series of different concentrations. In this study six concentrations ranging between 20-120 $\mu\text{g/mL}$ for the isoabsorptive and 10-60 $\mu\text{g/mL}$ for the other spectrophotometric methods. Each concentration was repeated three times; in order to provide information on the variation peak area and spectrophotometric values between samples of same concentration. The linearity of the calculated graphs was validated by the high value of the correlation coefficient and the intercept value (Table 1).

Characteristic parameters for regression equations of the studied methods obtained by least squares treatment of the results are given in Table 1.

Precision

The precision of the methods was evaluated by calculating the relative standard deviation of the assay results. The mean relative standard deviations are presented in tables 1 and can be considered to be satisfactory.

Limit of Detection and Limit of Quantitation

According to the International Conference on Harmonization (ICH) recommendations, the approach based on the standard deviation (SD) of the response and the slope was used for

determining the detection and quantitation limits. The theoretical values for the proposed methods were assessed practically and given in Table 1.

Accuracy

The accuracy of the investigated methods was validated by analyzing pure sample of TIM in triplicate. The concentrations of the active drug were calculated from the calculated regression equations. Good results are shown in Table 1. When results obtained by applying the proposed methods for analysis of pure TIM compared to those obtained by applying the manufacturer method, they showed no significant difference regarding accuracy and precision, and results were given in Table 2.

Selectivity

Methods selectivity was achieved by preparing several laboratory-prepared mixtures of the studied compounds at various concentrations within the linearity range. The laboratory-prepared mixtures were analyzed according to the previous procedures described under the proposed methods. Satisfactory results were obtained. Table 3 indicates the high selectivity of the proposed methods for TIM and its acid and oxidative degradation products.

Application to commercial tablets

The suggested methods were successfully applied for determination of TIM in its pharmaceutical formulations (Viscéralgine tablets). The results were satisfactory and with good agreement with the labeled amounts. Also applying the standard addition technique, no interference due to excipients was observed as shown from the results in Table 4

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 TIM in its pharmaceutical formulation without interference due to the excipients or the degradation products.

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. They could be applied for routine analysis of pure drug or in its pharmaceutical formulation. It is one of the useful studies where forced decomposition was undertaken under different suggested conditions. As the methods separate the drug from its acid and oxidative degradation products, they can be employed as stability indicating techniques.

Table 1: Regression Equation Parameters and Determination of Pure Sample of TIM by the Proposed Methods.

Parameters	Isosbestic	¹ DD		Ratio Subtraction	¹ D
		At 224.4	At 247.2		
Linearity					
range	20-120 µg/mL		10-60 µg/mL	10-60 µg/mL	10-60 µg/mL
Intercept	0.0111	0.0147	-0.0267	- 0.0209	- 0.0047
Slope	0.0091	0.0332	0.0538	0.0219	-0.0124
Correlation coefficient (r)	0.9997	0.9998	0.9999	0.9997	0.9999
Accuracy					
Mean±%RSD	100.39±0.33	100.59±0.57	100.40±0.56	100.47±0.54	100.24±0.58
Precision (%RSD)					
Repeatability ^a	± 0.66	± 0.63	± 0.72	± 0.59	± 0.63
Intermediate Precision ^b	± 0.98	± 0.97	± 0.76	± 0.91	± 0.92
LOD*	2.68 µg/mL	1.21 µg/mL	0.96 µg/mL	1.57 µg/mL	0.52 µg/mL
LOQ*	8.12 µg/mL	3.67 µg/mL	2.91 µg/mL	4.76 µg/mL	1.57 µg/mL

^aIntraday precision (average of 3 different concentrations of / 3 replicate each (n=9) within the same day)

^bInterday precision (average of 3 different concentrations of / 3 replicate each (n=9) repeated on 3 successive days).

* LOD = 3.3 (S.D / S), LOQ = 10 (S. D / S); where S.D is the residual standard deviation of the slope and S is the slope for TLC-densitometric and HPLC methods.

Table 2: Comparison between the Results of Analysis of TIM Using the Proposed Methods and the Manufacturer's Method in Pure Form.

Parameter	Isosbestic	¹ DD		Ratio Subtraction	¹ D	Manufacturer's Method ^a
		At 224.4	At 247.2			
Mean±S.D.	100.39±0.33	100.59±0.57	100.40±0.56	100.47±0.54	100.24±0.58	100.57±0.60
n	6	6	6	6	6	5
Variance	0.11	0.33	0.32	0.29	0.34	0.36
t-test (2.262) ^b	0.63	0.06	0.49	0.30	0.91	---
F ratio(5.19) ^b	3.27	1.09	1.13	1.24	1.06	---

^aChromatographic conditions: column, microbond pack C18; mobile phase, methanol : aqueous phase pH 3 with 10% ammonium acetate (45:55 v/v); flow rate 1.2 mL/min; temperature ambient; detection, UV at 235 nm

^bFigures in parentheses represent the corresponding tabulated values for F and t at P=0.05

Table 3: Results of laboratory prepared mixtures of TIM in presence of its acid and oxidative degradation products by the proposed methods.

Degradates %	Isosbestic	Recovery%		Ratio Subtraction	¹ D
		¹ DD			
		At 224.4	At 247.2		
10	100.36	100.39	101.37	100.82	101.21
30	100.68	100.78	100.62	100.11	100.61
50	99.35	99.36	100.83	101.41	99.12
70	101.36	99.73	100.46	99.78	100.1
80	100.57	---	--	--	--
90	103.98*	100.52	99.56	100.28	100.28
Mean±S.D.	100.46±0.73	100.16±0.59	100.57±0.66	100.48±0.64	100.26±0.76

Each result is an average of three experiments.

* Rejected value

Table 4: Determination of TIM in Its Pharmaceutical dosage form using the Proposed Methods and application of Standard Addition Technique.

Method	Viscéralgine @ 50 mg TIM /tablet		Standard Addition Technique	
	B.N. 710196		Pure added (µg/mL)	Recovery%
	Recovery% ±S.D.			
Isosbestic	100.77 ± 0.79		20	101.76
			40	100.63
			60	99.89
			Mean ±S.D	100.76 ± 0.94
At 247.2	100.45 ± 0.83		10	100.12
			20	99.71
			40	100.38
			Mean ±S.D	100.07 ± 0.34
¹ DD	100.47 ± 0.89		10	101.01
			20	100.20
			40	100.63
			Mean ±S.D	100.61 ± 0.41
Ratio Subtraction	100.29 ± 0.96		10	100.52
			20	100.45
			40	99.10
			Mean ±S.D	100.02 ± 0.80
¹ D	100.36 ± 1.00		10	99.61
			20	100.25
			40	101.22
			Mean ±S.D	100.36 ± 0.82

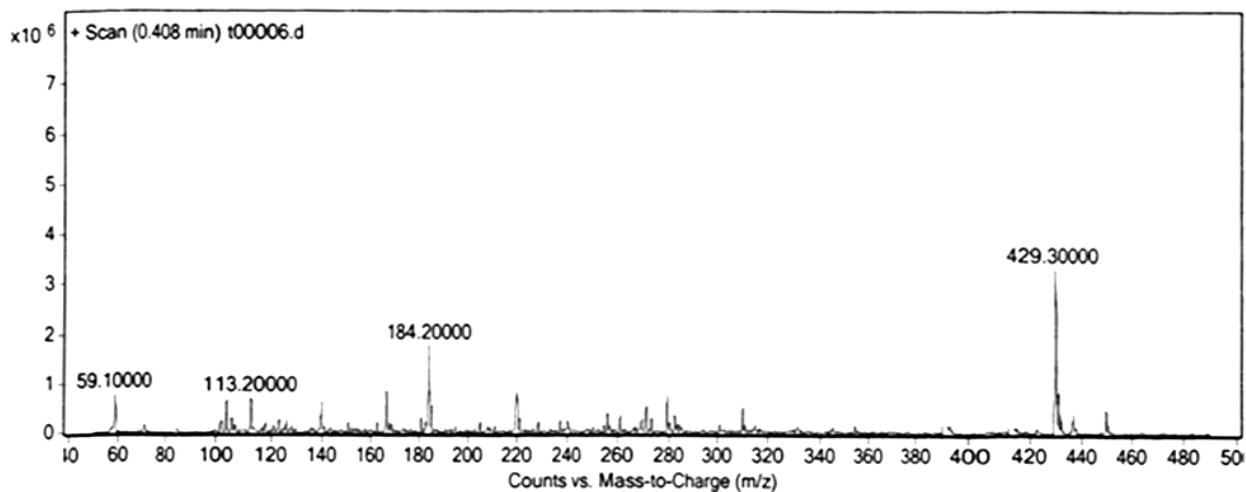


Fig. 1a: Positive ESI-MS of tiemonium methyl sulphate ($[M+H]^+$, m/z 429.3).

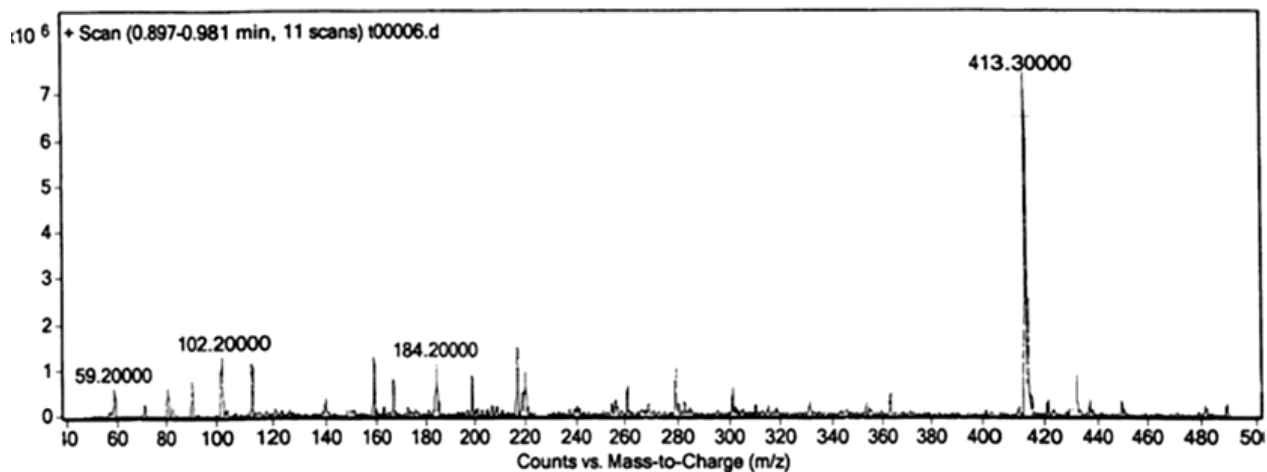


Fig. 1b: Positive ESI- MS scan of tiemonium methyl sulphate acid degradation product ($[M+H]^+$, m/z 413.3).

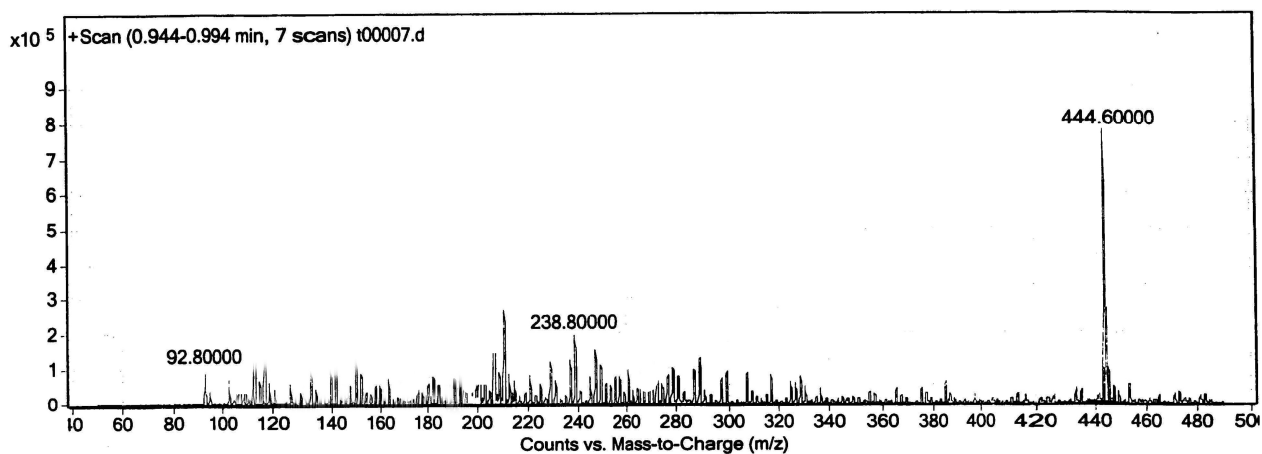


Fig. 1c: Positive ESI-MS scan of tiemonium methyl sulphate oxidative degradation product ($[M+H]^+$, m/z 444.6)

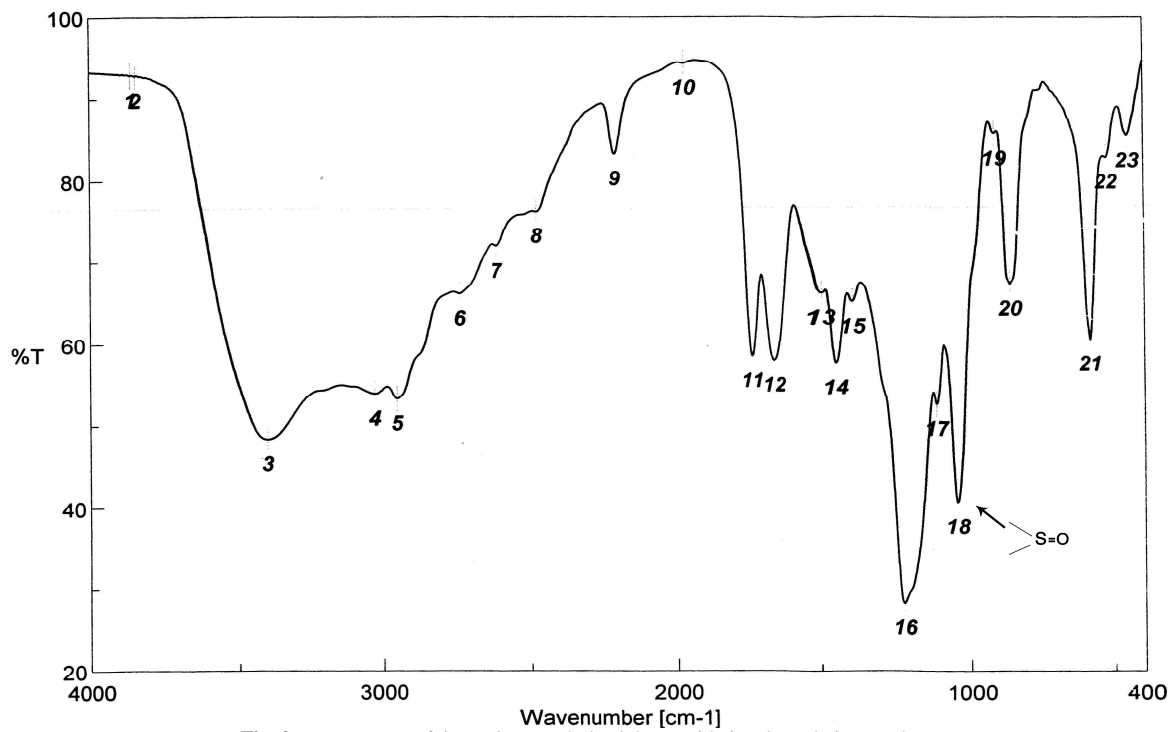


Fig. 2: IR spectrum of tiemonium methyl sulphate oxidative degradation product.

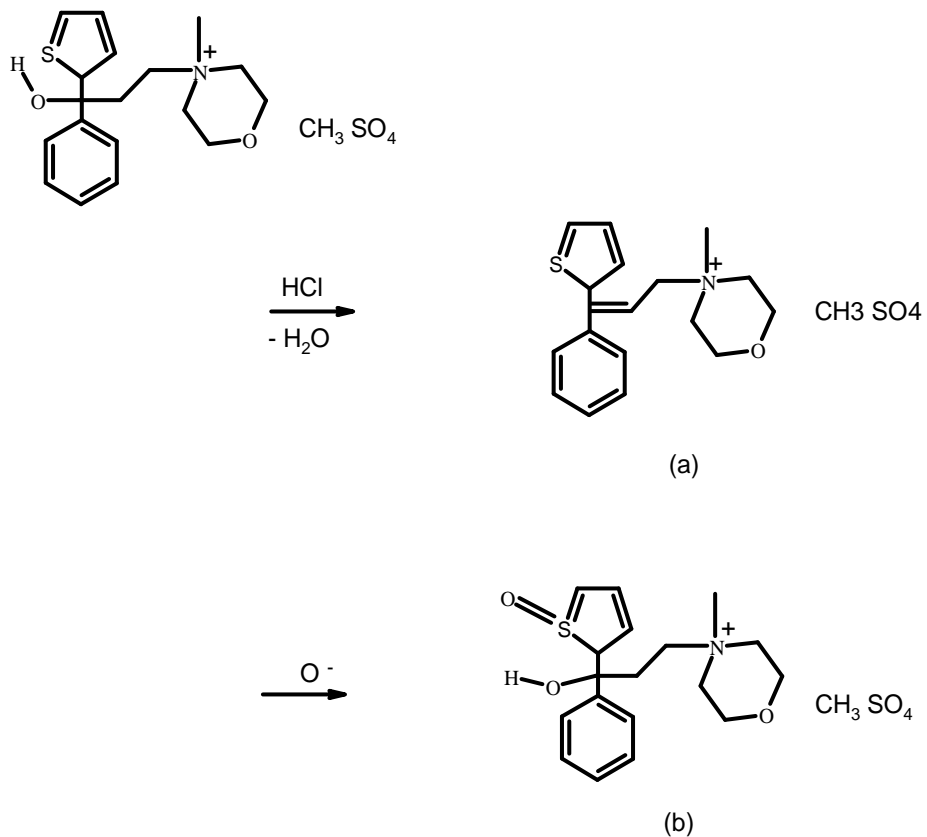


Fig. 3: Scheme of the suggested structures of (a) acid degradation product and (b) oxidative degradation product of tiemonium methyl sulphate.

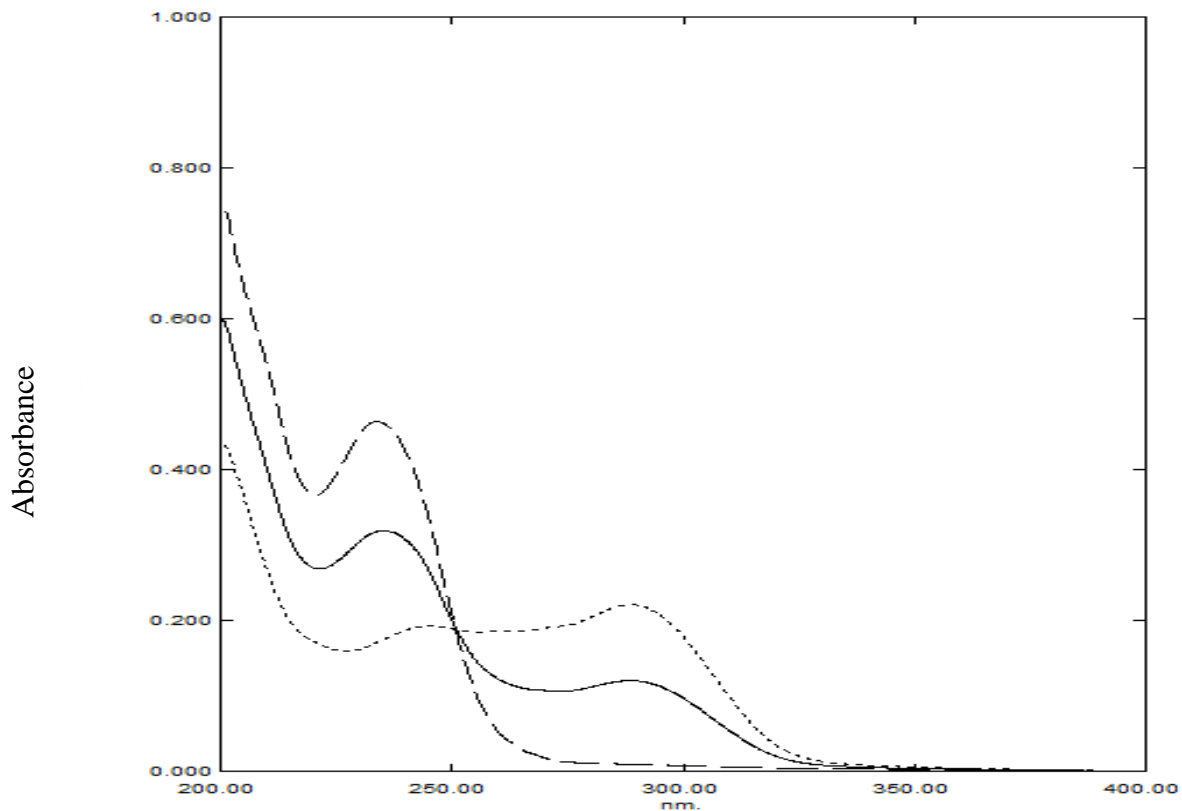


Fig. 4: Zero-order absorption spectra of 20 µg/mL of tiemonium methyl sulphate (— —), 20 µg/mL of acid degradation product (.....) and a (1: 1) mixture contain 10 µg/mL of each (—) in 0.1M HCl.

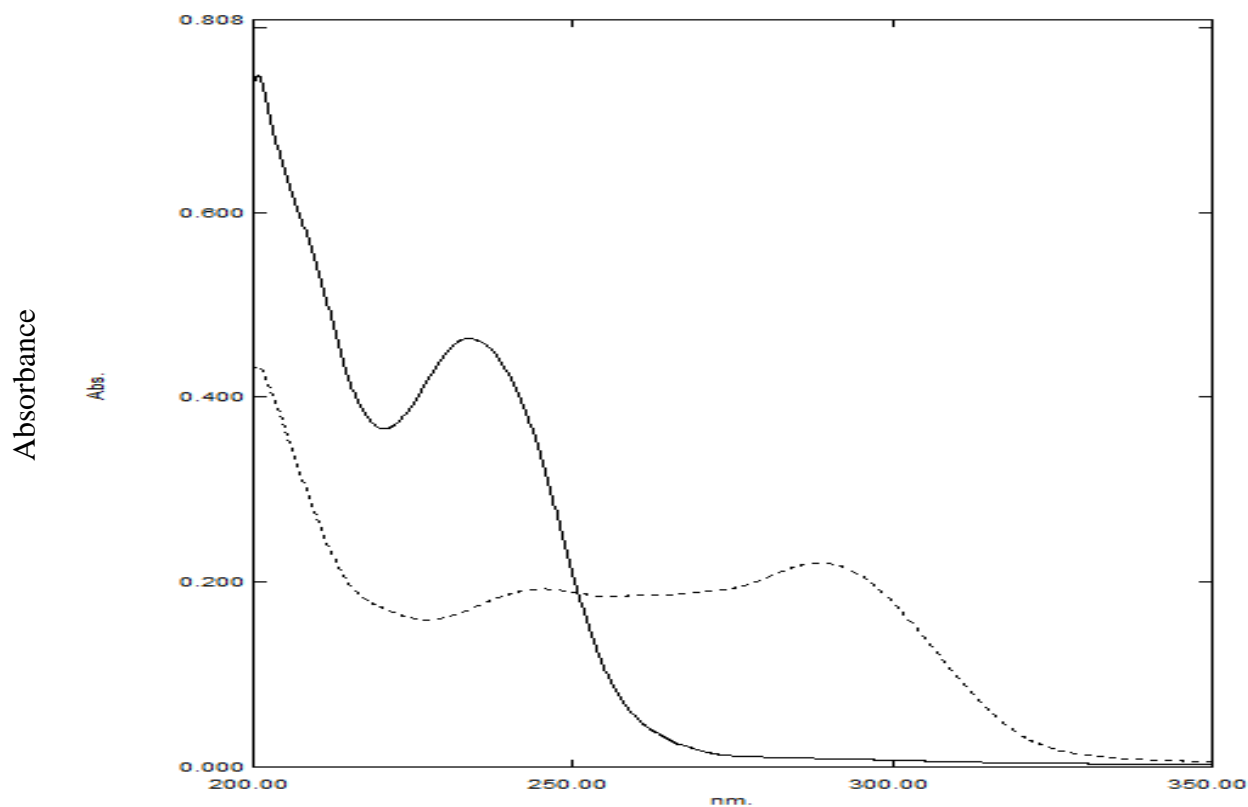


Fig. 5: Zero-order absorption spectra of tiemonium methyl sulphate 20 µg/mL (—) and its acid degradation product 20 µg/mL (.....) using 0.1M HCl as a blank.

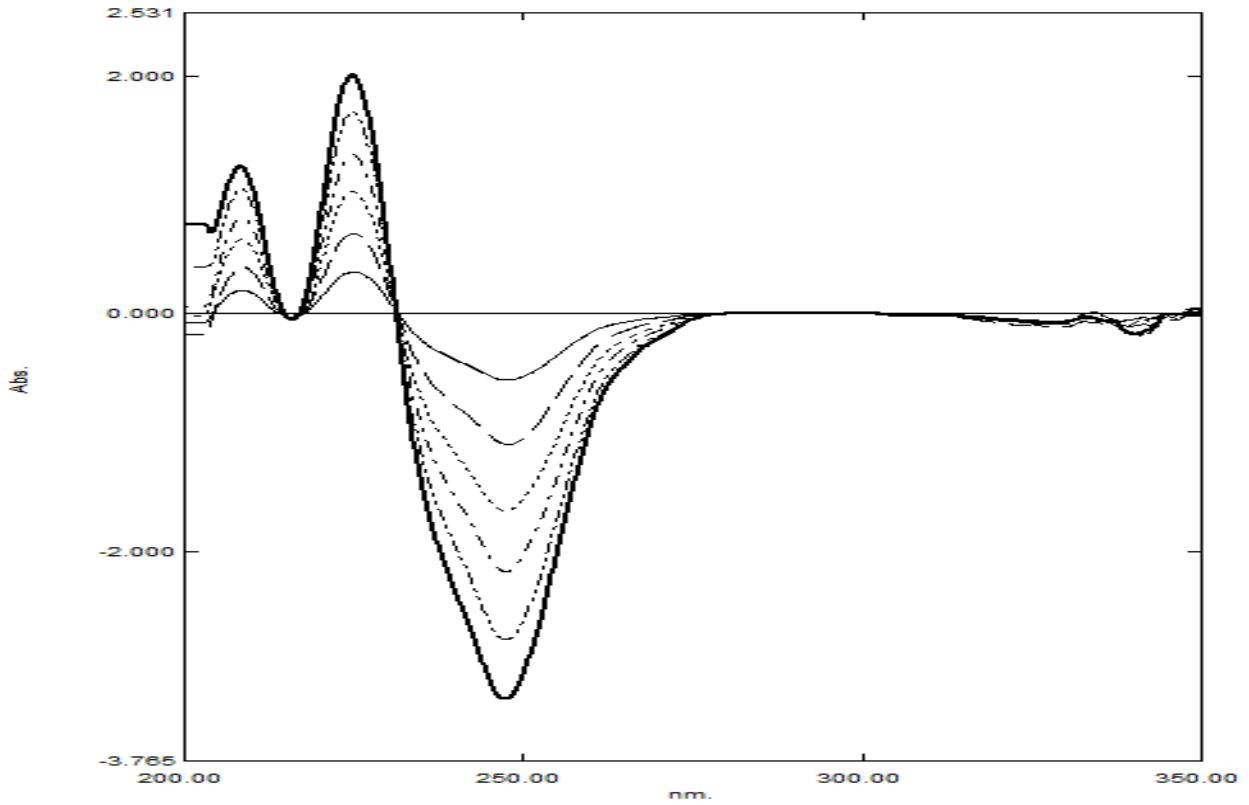


Fig. 6: First derivative of ratio spectra of tiemonium methyl sulphate (10-60 $\mu\text{g/mL}$) using the spectrum of 20 $\mu\text{g/mL}$ of the acid degradation product as a divisor.

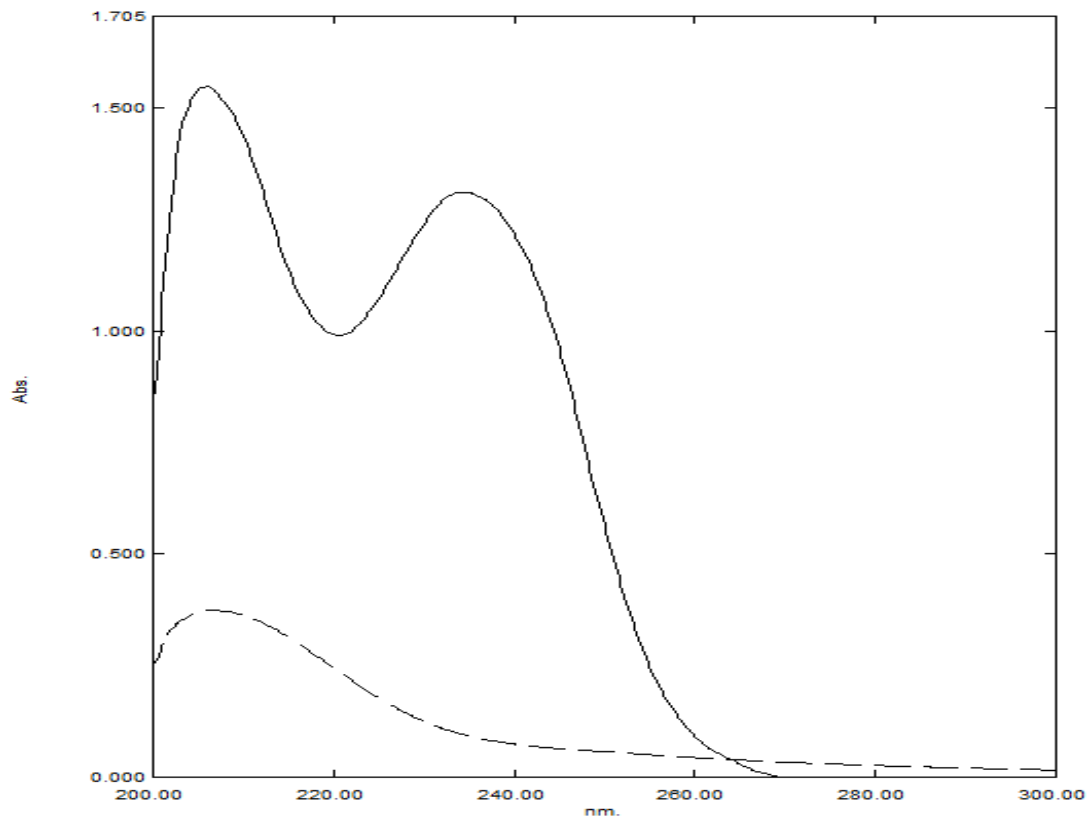


Fig.7: Zero-order absorption spectra of 60 $\mu\text{g/mL}$ of both tiemonium methyl sulphate (—) and its oxidative degradation product (---) using methanol as a blank.

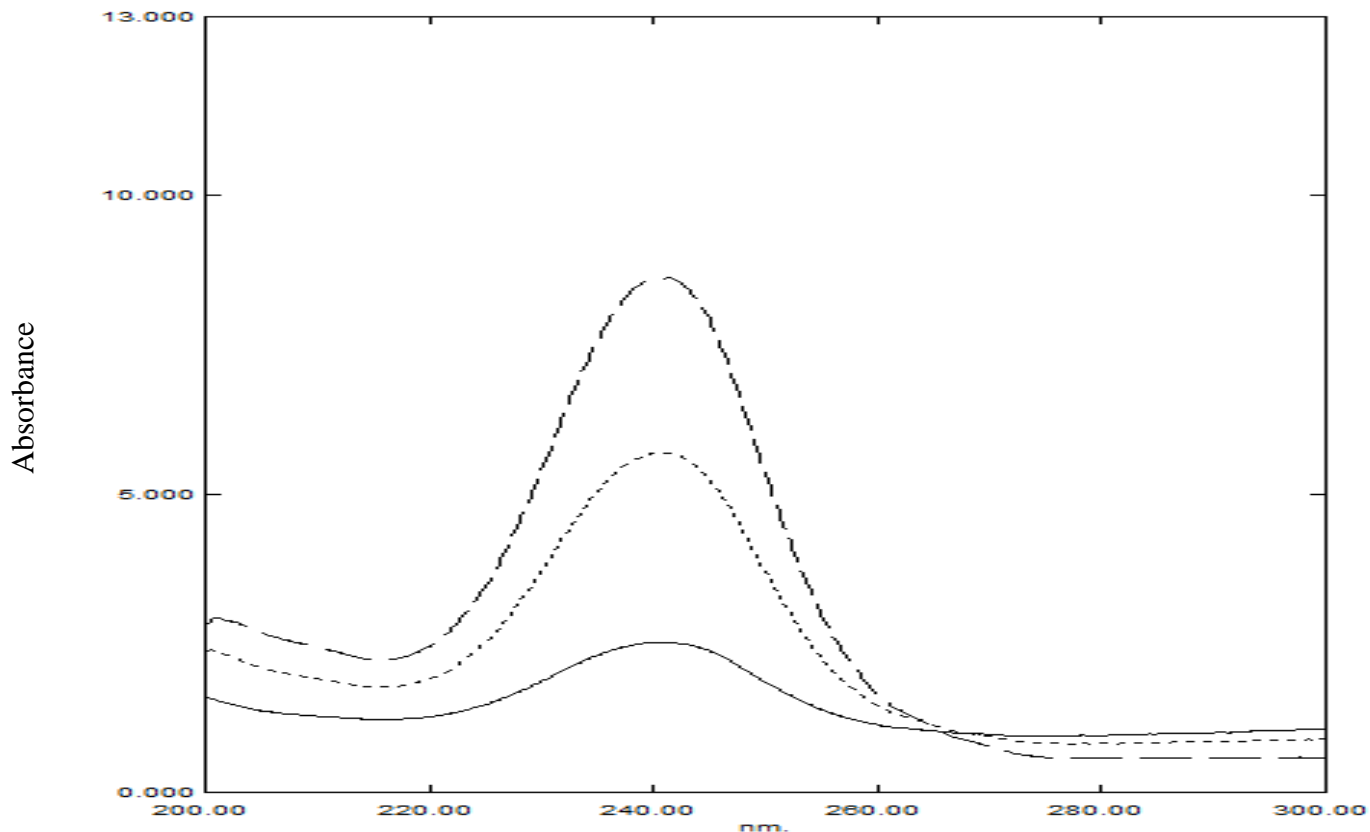


Fig. 8: Division spectra of laboratory prepared mixtures of tiemonium methyl sulphate and oxidative degradation product in the ratio of 10:90 (—), 30:70 (...) and 50:50 (---) using 60µg/mL of its oxidative degradation product as a divisor and methanol as a blank.

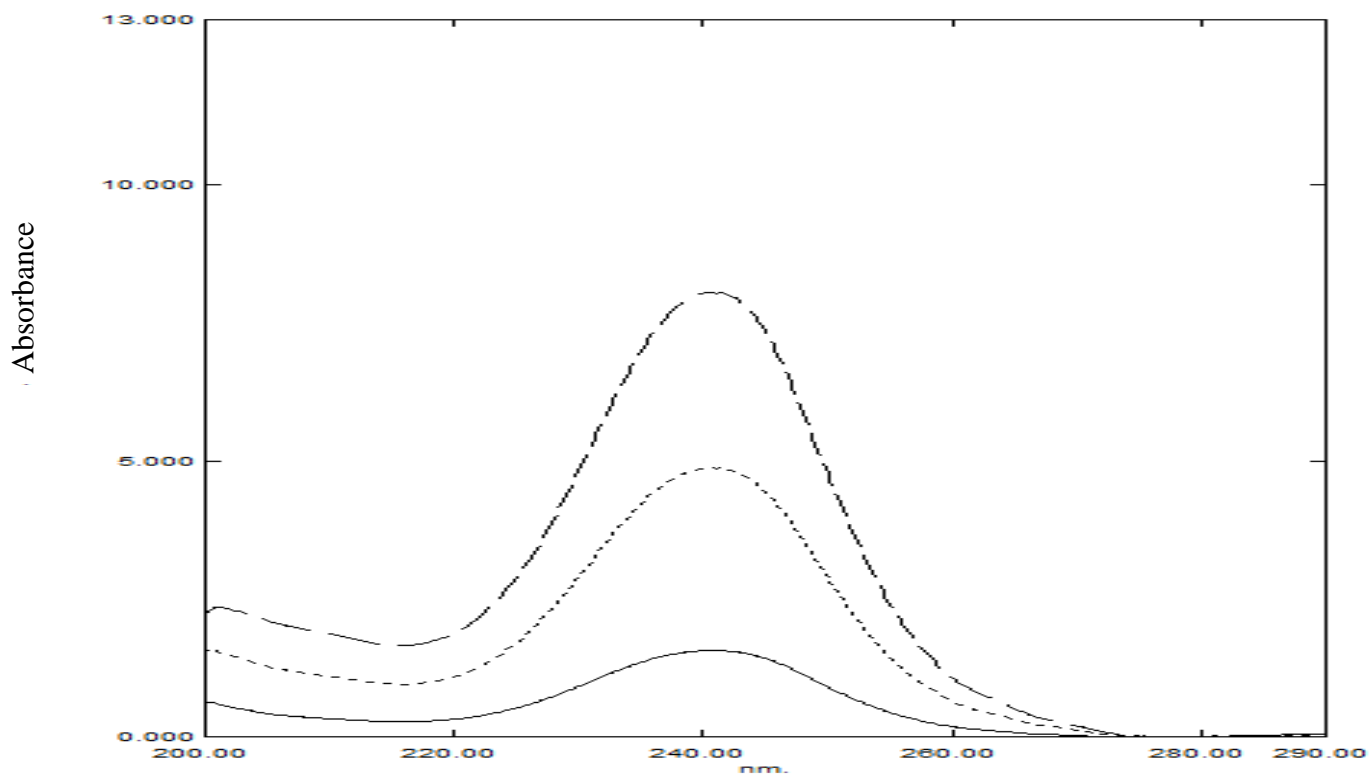


Fig. 9: Division spectra of laboratory prepared mixtures of tiemonium methyl sulphate and its oxidative degradation product in the ratio of 10:90 (—), 30:70 (...) and 50:50 (---) using 60 µg/mL of oxidative degradation product as a divisor and methanol as a blank after subtraction of the constant.

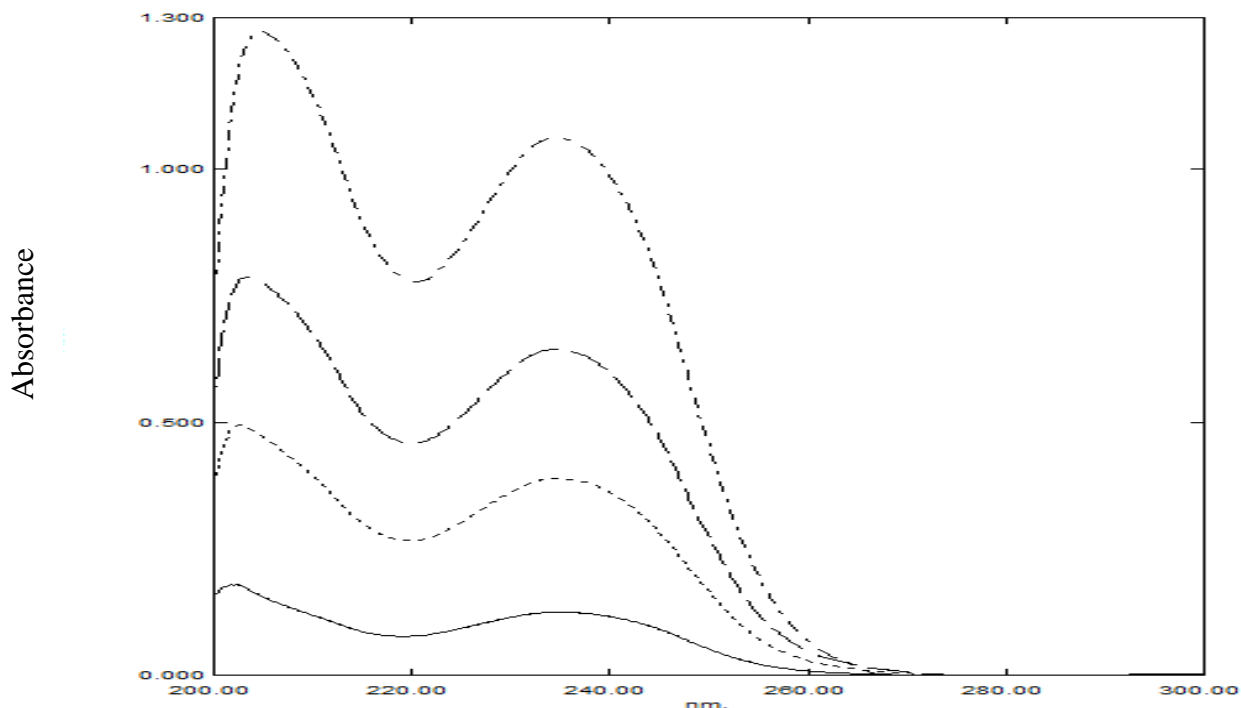


Fig. 10: Zero order absorption spectra of tiemonium methyl sulphate obtained by the proposed ratio subtraction method for the analysis of laboratory prepared mixtures after multiplication by the divisor and the original spectrum of 50 µg/mL of (pure) tiemonium methyl sulphate (---).

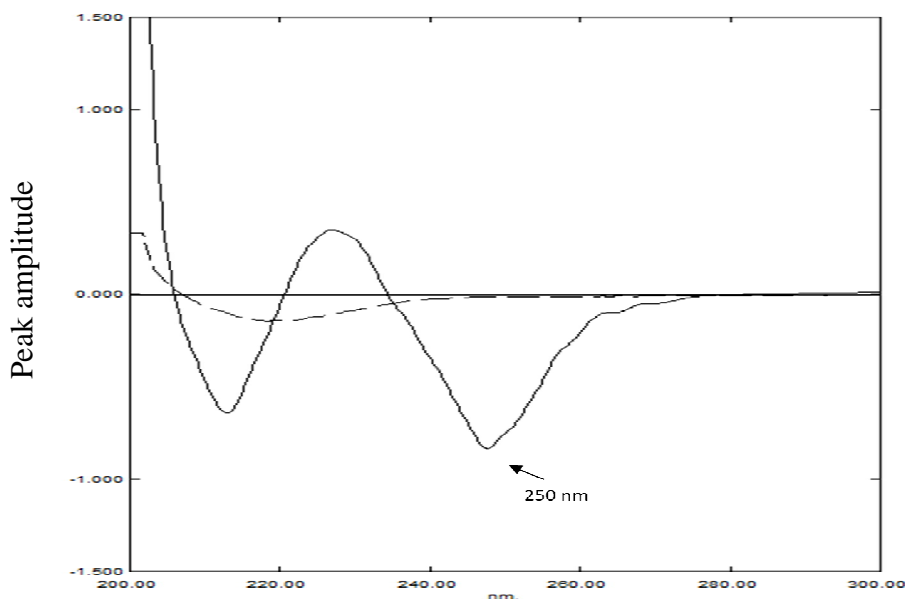


Fig. 11: First-derivative absorption spectra of 60 µg/mL of tiemonium methyl sulphate (—) and oxidative degradation product (---) using methanol as a solvent.

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