ISSN NO. 2320-5407

Journal homepage: http://www.journalijar.com

INTERNATIONAL JOURNAL OF ADVANCED RESEARCH

RESEARCH ARTICLE

Stability Indicating Chromatographic methods for the Determination of Tiemonium methylsulphate

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Manuscript Info

Manuscript History:

Received: 22 November 2013
Final Accepted: 25 December 2013
Published Online: January 2014

Key words:
Tiemonium methylsulphate**,** stability studies, Degradation**,** HPLC, TLC, H_2SO_4 .

Abstract

Two precise, accurate and sensitive high-performance liquid chromatographic and thin-layer chromatographic methods were developed and validated for the determination of Tiemonium methylsulphate in presence of its degradation product. Forced degradation study was performed using 2N H_2SO_4 on cold. Separation of the drug from its degradation product by HPLC was achieved using a X- Bridge C18 column and acetonitrile: methanol: 0.05M potassium dihydrogen phosphate in a ratio of (20: 5: 80 by volume) as a mobile phase, pH was adjusted to 3.0 ± 0.1 with ortho-phosphoric acid. The flow rate was 1.5 ml/min. Detection was performed at 235 nm. The linearity range was 2.0 to 20.0 µg/mL .The mean percentage recovery was 100.25 ± 0.660 %. The TLC method was used for separation of the drug from its degradation product using silica gel 60 F_{254} plates; the optimized mobile phase was water: methanol: glacial acetic acid (8: 4: 0.2 by volume). Quantitatively, the spots were scanned densitometrically at 235 nm. The linearity range was 0.5 to 10.0 µg/spot. The mean percentage recovery was 100.31 ± 0.655 %. Statistical comparison between the results obtained by these methods and those obtained by the manufacturer's method was done, and no significance difference was obtained.

Introduction

Tiemonium methylsulphate (Fig.1) 4-[3-Hydroxy-3-phenyl-3-(2-thienyl) propyl]-4-methyl-morpholinium methyl sulphate is used as antimuscarinic with peripheral effects similar to those of atropine and is used in the relief of visceral spasms. It has been used as an antispasmodic [1].

Determination of Tiemonium methylsulphate is described in manufacturer's method by UV spectrophotometry method; an aqueous solution of Tiemonium methylsulphate was scanned between (200 – 400 nm), measured at 235 nm.

Only one method was reported for the determination of Tiemonium methylsulphate, Swift Quantification of Fenofibrate and Tiemonium methylsulfate Active Ingredients in Solid Drugs Using Particle Induced X-Ray Emission [2].

This paper presented the study of the acid degradation of Tiemonium methylsulphate, followed by the development of two chromatographic stability-indicating methods for the determination of the drug in its pure powder form, in pharmaceutical dosage form and in laboratory prepared mixtures containing different percentages of the degradation product.

2. EXPERIMENTAL

2.1. Instruments

Agilent 1100 series, connected with an ultra violet detector set at 235 nm (Model G1316 A, Agilent 1100 series). The injector was a manual Rheodyne injector (Model 7725/7725I, Rohnert Park, CA., USA) equipped with 20 µl injector. The instrument was connected to an IBM compatible personal computer (PC), an HP disk jet 5652 printer and X- Bridge C18, (150×4.6mm.) column particle size 3.5µm (Waters). The mobile phase was filtered using a 0.45-mm Teflon membrane filter (Millipore, Milford, MA, USA) and degassed by ultrasonic vibrations J.P- Selecta, (Barcelona,Spain). For TLC method, the plates (Merck, Germany) used were 10 x 20 cm, precoated with 0.25mm silica gel 60 F_{254} (Merck, Germany). The sample was applied to the plates using Hamilton micro syringe (10µl). A TLC scanner , dual wavelength flying spot (Shimadzu CS-9301, Tokyo, Japan) was used for scanning. The experimental conditions were scan mode = Absorbance mode, and wavelength = 235 nm. The spots were visualized using UV lamp at 254 nm For identification of the degradation product, IR Bruker Vector 22 8201 PC spectrometer (Bruker Instruments Ltd, Rheinstetten/ Karlsruhe, Germany) and Mass Spectrophotometer, Hewlett Packard Model 5988A GC/MS (Agilent Technologies, Wilmington, DE) were used.

2.2 Materials and Reagents

Tiemonium methylsulphate-Pure sample was kindly supplied by Centaur Pharmaceuticals PVT. LTD. India, B.N. 20094607. Its purity was found to be 99.73%±0.504 according to the manufacturer's method. Methanol and acetonitrile for HPLC were purchased from s.d fine-chem limited (Mumbai, India). Sulphuric acid was purchased from Sigma Chemical Co. [St. Louis - USA]. While sodium hydroxide, potassium dihydrogen phosphate, methanol, glacial acetic acid were obtained from ADWIC (Cairo, Egypt). Spasmofree ampoule was supplied by Adwia Pharma, (Cairo, Egypt), B.N. 080904. Each ampoul is claimed to contain 5.0 mg of Tiemonium methylsulphate (5.0 mg/ampoul). Spasmofree tablet was supplied by Adwia Pharma, (Cairo, Egypt), B.N. 031013. Each tablet is claimed to contain 50.0 mg of Tiemonium methylsulphate.

2.3 Chromatographic Conditions

2.3.1. HPLC method. The mobile phase was prepared by mixing acetonitrile: methanol: 0.05M potassium dihydrogen phosphate in a ratio of (20: 5: 80 by volume) as a mobile phase, pH was adjusted to 3.0 ± 0.1 with ortho-phosphoric acid. The mobile phase was filtered using a 0.45 mm Teflon membrane filter (Millipore, Milford, MA, USA) and degassed by ultrasonic vibrations for 30 min prior to use. All determinations were performed at ambient temperature (25ºC) under the following chromatographic conditions:

- Column: X- Bridge C18, (Waters; 150×4.6mm, 3.5 µm).
- Flow rate: 1.5 ml/min
- Wavelength: 235 nm
- Injection volume: 20 µL

2.3.2. TLC method. The plates were first washed and developed with the mobile phase by mixing water: methanol: glacial acetic acid (8: 4: 0.2 by volume), then activated for 15 minutes by placing in an oven at 100ºC before use. Spots were applied as separate compact bands 20 mm apart and 20 mm from the bottom of the plates. The chromatographic tank was saturated with the mobile phase for one hour. The plates were developed in ascending manner to a distance of 7 cm from the spotting line at room temperature, air-dried, and the plates were scanned under the following conditions:

Source of radiation: deuterium lamp.
Photomode: Reflection.
Scan mode: Absorbance.
Result output: Chromatogram and area under the peak.
Swing width: 10 mm.
Wavelength: 235 nm.

2.4 Preparation of the degradation products

Tiemonium methylsulphate (100.0 mg) was accurately transferred into a 50-mL round bottom flask, 25.0 ml of 2N H_2SO_4 was added and left for 7 hours on cold then the volume was completed to the mark with 2N H_2SO_4. The solution was then tested for complete degradation by TLC using water: methanol: glacial acetic acid (8: 4: 0.2 by volume) as the mobile phase. One spot was visualized under UV lamp at 254 not corresponding to Tiemonium methylsulphate. The degraded solution was then neutralized with 2N NaOH solution respectively till pH was approximately 7. The solution was nearly evaporated to dryness, cooled and transferred quantitatively with methanol

to a volumetric flask 50-mL then the volume was completed to the mark to prepare solution of concentration (equivalent to 2.0 mg/mL of intact Tiemonium methylsulphate) in methanol and finally was filtered.

2.5 Standard solutions

2.5.1. For HPLC method, standard stock solutions of Tiemonium methylsulphate was prepared in a concentration of (0.1 mg/ml) by transferring 10.0 mg portion of Tiemonium methylsulphate powder to a 100-ml volumetric flask and dissolved in 20.0 ml mobile phase, and then the volume was completed with mobile phase.

2.5.2. For TLC method, standard stock solution of Tiemonium methylsulphate was prepared in a concentration of (2.0 mg/ml) by transferring 100.0 mg portion of Tiemonium methylsulphate powder to a 50-ml volumetric flask and dissolved in 20.0 ml methanol, and the volume was then completed with methanol.

2.6. Laboratory prepared mixtures containing different ratios of Tiemonium methylsulphate and its degradation product

2.6.1. HPLC method

Aliquots (1.8 – 0.2 mL) of Tiemonium methylsulphate were accurately transferred from its stock standard solution (0.1 mg/mL) equivalent to (180.0 – 20.0 µg) into a series of 10-mL volumetric flasks. Aliquots (0.2 –1.8 mL) of degradation product solution (0.1 mg/mL) equivalent to (20.0 – 180.0 µg) were added, the volume was completed with mobile phase to prepare mixtures containing 10 – 90 % of the degradation product.

2.6.2. TLC method

Aliquots (4.5 – 0.5 mL) of Tiemonium methylsulphate were accurately transferred from its stock standard solution (2.0 mg/mL) equivalent to (9.0 – 1.0 mg) into a series of 10-mL volumetric flasks. Aliquots (0.5 –4.5 mL) of degradation product solution (2.0 mg/mL) equivalent to (1.0– 9.0 mg) were added, the volume was completed with the methanol to prepare mixtures containing 10 – 90 % of the degradation product.

2.7. Construction of Calibration Curves

2.7.1. For HPLC method

Aliquots (0.2, 0.4, 0.8, 1.2, 1.6 and 2.0 mL) of Tiemonium methylsulphate were accurately transferred from its stock standard solution (0.1mg/mL) equivalent to (20.0, 40.0, 80.0, 120.0, 160.0 and 200.0 µg) into a series of 10-mL volumetric flasks. The volume was completed to the mark with mobile phase. Aliquots equivalent to 20 µl of the previously prepared solutions were injected in triplicate into the liquid chromatograph at ambient temperature (25ºC) under the previously mentioned chromatographic conditions.

The chromatogram was obtained, the average peak area ratios obtained for each concentration of Tiemonium methylsulphate to that of external standard 20.0 µg/ml were plotted versus concentrations, and the regression equation was computed.

2.7.2 For TLC method

Aliquots (0.25, 1.0, 2.0, 3.0, 4.0 and 5.0 mL) of Tiemonium methylsulphate were accurately transferred from its stock standard solution (2.0mg/mL) equivalent to (0.5, 2.0, 4.0, 6.0, 8.0, and 10.0 mg) into a series of 10-mL volumetric flasks. The volume was completed to the mark with methanol. Aliquots equivalent to ten µl of the prepared solutions, using 10 µl Hamilton syringe, were applied as separate compact bands 20 mm apart and 20 mm from the bottom of the plates. The chromatographic tank was saturated with the mobile phase for one hour in an ascending manner to a distance of 7 cm from the spotting line at room temperature, air-dried, and the plates were scanned under the previously mentioned chromatographic conditions .The scanning profile for Tiemonium methylsulphate was obtained. The calibration curve relating the integrated peak area to the corresponding concentration was constructed and the regression equation was computed.

2.8 Application of the proposed methods for the analysis of laboratory prepared mixtures of Tiemonium methylsulphate and its degradation products.

2.8.1 HPLC method

Aliquots equivalent to twenty µl from the prepared mixture were injected into the liquid chromatograph. Then the procedure was completed as described in subsection of **2.7.**1. The concentration of Tiemonium methylsulphate was calculated by substitution in the corresponding regression equation.

2.8.2 TLC Method

Aliquots equivalent to ten µl from the prepared mixture were spotted on TLC plates and the procedure was completed as described in subsection of **2.7.2.** The concentration of Tiemonium methylsulphate was calculated by substitution in the corresponding regression equation.

2.9 Application of the proposed methods for the analysis of Tiemonium methylsulphate in pharmaceutical preparation.

2.9.1 HPLC method

2.9.1.1. Spasmofree ampoule

Five ampoules of Spasmofree were mixed carefully. A volume of solution equivalent to 10.0 mg Tiemonium methylsulphate was accurately transferred into a 100-mL volumetric flask, the volume was then completed to the mark with methanol HPLC grade to prepare solution of concentration (0.1 mg/mL). Further Dilution with the mobile phase was done to obtain solution of final concentrations of (10.0 µg/mL). Then the procedure was completed as described in subsection of 2.7.1. The concentrations of Tiemonium methylsulphate were calculated by substitution in the corresponding regression equation.

2.9.1.2. Spasmoofree tablet

Ten tablets of Spasmofree were weighed accurately and finely powdered in a small dish. An amount of powder equivalent to 10.0 mg Tiemonium methylsulphate was accurately transferred into a 100-mL volumetric flask, 50.0 mL of the methanol HPLC grade was added. The flask was sonicated for 30 minutes, the volume was then completed to the mark with the same solvent and finally was filtered to prepare solution of concentration equivalent to (2.0 mg/mL). Further Dilution with the mobile phase was done to obtain solution of final concentrations of (10.0 µg/mL). Then the procedure was completed as described in subsection of 2.7.1. The concentrations of Tiemonium methylsulphate were calculated by substitution in the corresponding regression equation.

2.9.2 TLC Method

2.9.2.1. Spasmofree ampoule

Twenty ampoules of Spasmofree were mixed carefully. A volume of solution equivalent to 50.0 mg Tiemonium methylsulphate was accurately transferred into a 25-mL volumetric flask, the volume was then completed to the mark with methanol to prepare solution of concentration (2.0 mg/mL). Further Dilution with methanol was done to obtain solution of final concentrations of (0.2 mg/mL). Then the procedure was completed as described in subsection of 2.7.2**.** The concentrations of Tiemonium methylsulphate were calculated by substitution in the corresponding regression equation.

2.9.2.2. Spasmoofree tablet

Ten tablets of Spasmofree were weighed accurately and finely powdered in a small dish. An amount of powder equivalent to 200.0 mg Tiemonium methylsulphate was accurately transferred into a 100-mL volumetric flask, 50.0 mL of the methanol was added. The flask was sonicated for 30 minutes, the volume was then completed to the mark with the same solvent and finally was filtered to prepare solution of concentration equivalent to (2.0 mg/mL). Further Dilution with methanol was done to obtain solution of final concentrations of (0.2 mg/mL). Then the procedure was completed as described in subsection of 2.7.2**.** The concentrations of Tiemonium methylsulphate were calculated by substitution in the corresponding regression equation.

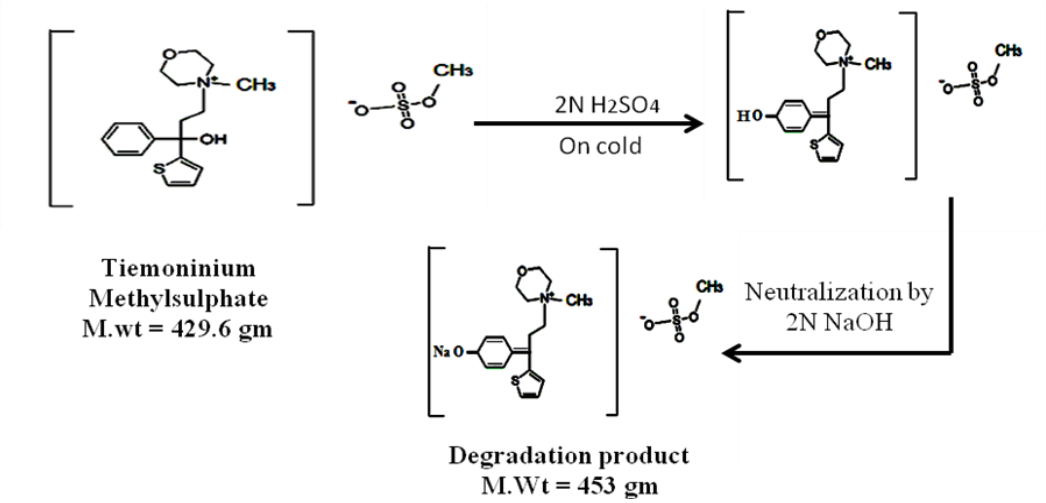
3. Result and discussion

3.1 Separation and identification of degradation products

The stability of the drug was studied according to ICH guidelines Q2 (R1) [3] for:

(a) Stress Acid and Alkaline: 7M HCl/7M NaOH for 5 hours, 6M HCl/6M NaOH for 7 hours and 2N H2SO4/2N NaOH 7hours.
(b) Oxidative Condition: 3% H_2O_2 for 2, 4, 6 and for 10 hours.
(c) Thermal Degradation: at 100°C in an oven for 2, 4 and for 6 hours.

The degradation process under the previously mentioned conditions was followed using TLC and the compound was found to be liable to acid degradations by using 2N H_2SO_4 on cold. There is one component which was confirmed by TLC.
The structure of the acidic-induced degradation product was confirmed using IR and mass spectroscopy.



Scheme 1: The degradation pathway of Tiemonium methylsulphate

In the present study, two stability-indicating methods for the simultaneous determination of Tiemonium methylsulphate, in presence of its acidic-induced degradation product were suggested.

3.2. Optimization of Chromatographic Conditions

3.2.1. HPLC method

HPLC has become the most versatile and wide spread technique used by the pharmaceutical industry for quality control. It has many applications in the field of pharmaceuticals including the quantitative determination of drugs present either alone or in presence of their degradation [4, 5]. The proposed method is based on the difference in the retention time between the intact drug and its degradation product. The suitable mobile phase has been selected to achieve the best separation the drug from its degradation product.
Different solvent systems with different ratios were tried; best separation was achieved upon using acetonitrile: methanol: 0.05M potassium dihydrogen phosphate in a ratio of (20: 5: 80 by volume) as a mobile phase, pH was adjusted to 3.0 ± 0.1 with ortho-phosphoric acid. The flow rate was 1.5 ml/min and the detector wavelength was 235 nm. Tiemonium methylsulphate was completely resolved from its degradation product and its R_t value was 2.74±0.03 min, on the other hand the R_t value of the acidic-induced degradation product was 7.31±0.03. This would permit quantitative determination of Tiemonium methylsulphate in presence of its acidic-induced degradation product (Fig. 2).
System suitability test according to the United States Pharmacopoeia was used to verify that the resolution and reproducibility of the chromatographic system were adequate for the analysis to be done [6]. Accordingly, system suitability was checked by calculating the column efficiency (N), resolution (R), selectivity (α) and tailing factor (T), where the system was found to be suitable, (Table 2).

3.2.2 TLC Method

Thin layer chromatography has become a well established technique for the assay of drugs either in binary or in multi-component mixtures [7].
The proposed method is based on the difference in the R_f between the intact drug and its degradation product. The suitable mobile phase has been selected to achieve the best separation the drug from its degradation product; other necessary conditions have been established. Different solvent systems with different ratios were tried; best separation was achieved upon using water: methanol: glacial acetic acid (8: 4: 0.2 by volume). The instrumental conditions for densitometric measurement such as scan mode and wavelength detection were optimized. The scan mode chosen was absorbance mode, and the wavelength was 235 nm. Tiemonium methylsulphate was completely resolved from its degradation product and its R_f value was 0.33. On the other hand the R_f value of the degradation product was 0.24. This would permit quantitative determination of the drug in presence of its degradation products (Fig. 3).

3.3. Method Validation

Validation of the proposed methods was made by measuring range, accuracy, precision, repeatabilities, interday precision, linearity and specificity. Results obtained are depicted in Table (1). This data render the applicability of the proposed methods for the quality control of the drug formulation.

3.3.1. Linearity

3.3.1.1. HPLC. The linear regression data for the calibration curves showed a good linear relationship over a concentration range of 2.0 – 20.0 µg/ml and the regression equation was computed and found to be:

$A = 0.0491C + 0.0284$ $r = 0.9997$

Where A is the peak area ratio, C is the concentration of the drug in µg/ml and r is the correlation coefficient.

3.3.1.2. TLC. A linear relationship between the concentration of Tiemonium methylsulphate and the integrated peak area was existing. The proposed method was found to be valid in the range of 0.5 – 10.0 µg/ spot and the regression equation was computed and found to be:

$A = 0.0316C + 0.0233$ $r = 0.9996$

Where A is the integrated peak area, C is the concentration of the drug in µg/ spot and r is the correlation coefficient.

3.3.2. Accuracy. The accuracy of two methods were assessed by the determination of pure Tiemonium methylsulphate samples within the linearity ranges, the mean accuracies are given in Table (1).The recovery percentages (recovery %) and relative standard deviations (RSD) revealed excellent accuracy.

3.3.3. Repeatability and intermediate precision. The repeatability and interday precision were evaluated by assaying three freshly prepared solutions of the drug in triplicate on the same day and on three successive days respectively at concentrations within the linearity range for the two methods. RSD% shows the precision of the methods (Table 1).

3.3.4. The specificity: The specificity of the methods was proved by the analysis of laboratory prepared mixtures containing different percentages of the degradation products. The specificity of the two methods for Tiemonium methylsulphate was achieved in presence of its degradation up to 90% (Table 3).

Table 1: Results of validation parameters of the responses and the regression equations obtained by the proposed methods

Parameter	HPLC method	Densitometric method
Validation of regression equation:		
Slope [a] S.E. of slope	0.0491 0.000578	0.0316 0.000427
Intercept [a] S.E. of intercept	0.0284 0.007013	0.0233 0.002588
Correlation coefficient	0.9997	0.9996
Validation of response:		
Concentration range	2.0 – 20.0 (µg/mL)	0.5 – 10.0 (µg/Spot)
Average accuracy % S.D. R.S.D. %	100.25 0.662 0.660	100.31 0.657 0.655
Specificity± R.S.D. %	100.28±1.077	100.59±0.871
Repeatability[*b] %	100.77±0.783	100.43±0.176
Intermediate precision[*c] %	100.26±0.555	100.29±0.469

[a] Results of six determinations
[*b] =3×3
[*c] =3×3

Table 2: Parameters of system suitability test of HPLC Method

Parameter	Obtained value		Reference value
	Tiemonium methylsulphate	Degradation product	
Relative retention time (α)	3.24		>1
Resolution (R)	9.58		>1.5
Capacity factor (K')	2.91	9.44	0.5-10
Tailing factor (T)	0.85	0.89	T=1 for a typical symmetric peak
Column efficiency (N)	2212	1625	Increase with the efficiency of the separation
HETP	0.006781 cm/plate	0.009231 cm/plate	The smaller the value the higher the column efficiency

Table 3: Results of analysis of Tiemonium methylsulphate in laboratory prepared mixtures containing different ratios of Tiemonium methylsulphate and its degradation product in pure powder form by the proposed methods

Degradation %	HPLC Method			Densitometric Method		
	Tiemonium methylsulphate (µg/mL)	Degradation (µg/mL)	Recovery %*	Tiemonium methylsulphate (µg/mL)	Degradation product (µg/mL)	Recovery %*
10	18.0	2.0	98.71	9.0	1.0	99.68
20	16.0	4.0	99.54	8.0	2.0	99.76
30	14.0	6.0	100.80	7.0	3.0	99.82
50	10.0	10.0	101.68	5.0	5.0	100.45
70	6.0	14.0	100.42	3.0	7.0	101.41
80	4.0	16.0	101.32	2.0	8.0	101.76
90	2.0	18.0	99.47	1.0	9.0	101.25
Mean			100.28			100.59
S.D.			1.080			0.876
R.S.D. %			1.077			0.871

* Average of three determinations

Table 4: Quantitative determination of Tiemonium methylsulphate in pharmaceutical formulation by the proposed methods and results of application of standard addition technique

Pharmaceutical formulation *Spasmofree ampoule (5mg/ampoul) B.N. 080904*	**HPLC Method**	**TLC Method**
Found %[a]	100.51±0.870%	100.68 ± 0.344%
Recovery of standard added %[b]	100.72 ± 1.333%	100.25 ± 0.277%
Spasmofree tablet (50mg/tablet) B.N. 031013	**HPLC Method**	**TLC Method**
Found %[a]	99.78±0.651%	99.36±0.978%
Recovery of standard added %[b]	99.51±0.668%	99.20±0.434%

[a] Average of six determinations
[b] Average of six determinations

Table 5: Statistical analysis between the results obtained for the determination of Tiemonium methylsulphate in pure samples by the proposed methods and those obtained by the reported method

Parameters	**HPLC Method**	**TLC Method**	**Manufacturer's method****
Mean	100.25	100.31	99.73
S.D	0.662	0.657	0.503
R.S.D%	0.660	0.655	0.504
Variance	0.438	0.432	0.253
n	6	6	6
Student's t	1.532 (2.228)*	1.717 (2.228)*	
F test	1.731 (5.05)*	1.708 (5.05)*	

*The values between parenthesis are the theoretical values of t and F at (p = 0.05).
** UV spectrophotometry method; an aqueous solution of Tiemonium methylsulphate was scanned between (200 – 400 nm), measured at 235 nm.

Fig. 1. Tiemonium methylsulphate
Molecular formula = $C_{19}H_{27}NO_6S_2$ Molecular weight = 429.6

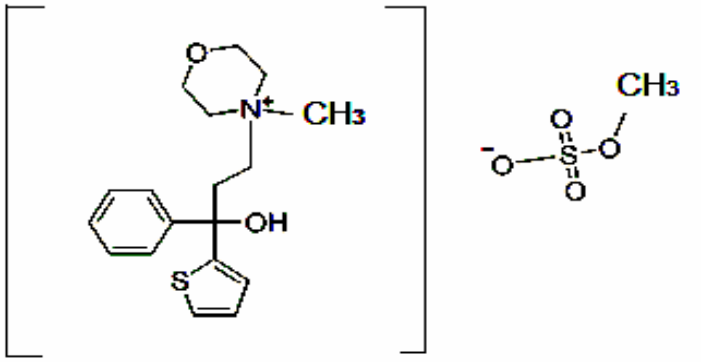


Fig. 2: HPLC chromatogram of a resolved mixture of Tiemonium methylsulphate (10.0 µg/ml) and its acidic-induced degradation product (5.0 µg/ml) using the specified chromatographic conditions.

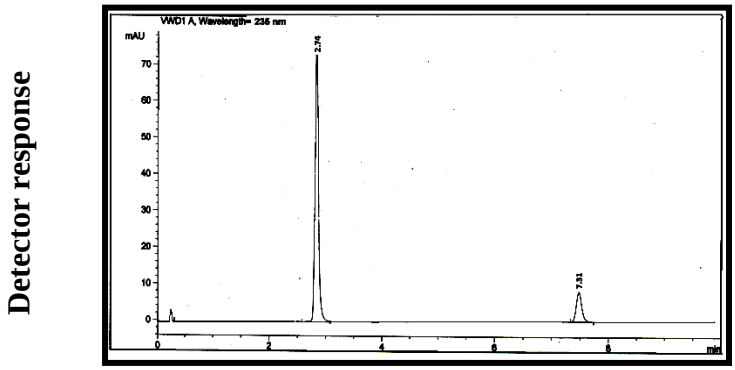
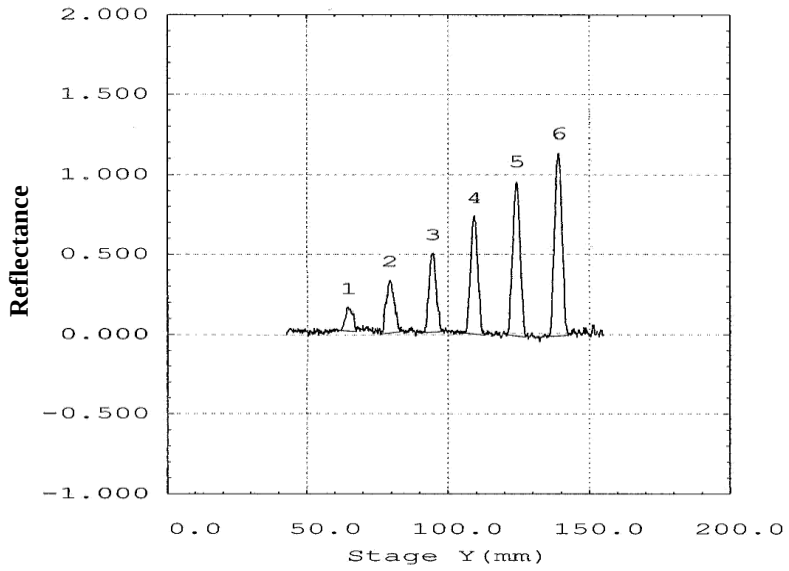


Fig.3: Scanning profile of TLC chromatogram of Tiemonium methylsulphate (0.5 – 10.0 µg/spot) at 235nm.



Spot position (mm)

3.3.5. Assay of pharmaceutical formulation:

The usefulness of the proposed methods for the analysis of Tiemonium methylsulphate was studied by assaying Spasmofree ampoule and Spasmofree tablet (Table 4). Standard addition technique was also applied to assess the validity of the proposed method (Table 4).

3.3.6. Comparison with the manufacturer's method:

Results obtained by the proposed method for the determination of pure samples of the drug were statistically [8] compared to those obtained by manufacturer's method and no significant differences were observed (Table 5).

4. Conclusion

The TLC and HPLC methods proposed are accurate, precise and reproducible. They are stability-indicating methods, so can be used for stability studies to predict the expiry dates of pharmaceuticals. Both methods complied with the validation guidelines of the International Conference on Harmonization and could be used for purity testing, stability studies, quality control, and routine analysis of Tiemonium methylsulphate.

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