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Research Article

GREEN VALIDATED METHOD FOR DETERMINATION OF TIEMONIUM METHYL SULFATE USING REVERSED-PHASE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY TECHNIQUE WITH STABILITY-INDICATING STUDIES

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ABSTRACT

Objective: The objective of this method was to develop a simple, sensitive, rapid reversed-phase high-performance liquid chromatography (RP-HPLC) and applied for determination of tiemonium methyl sulfate (TIM) in bulk, pharmaceutical formulations with stability-indicating studies.

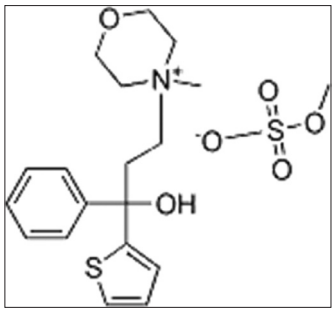
Methods: The stability-indicating capability of this method had been established by subjected TIM to several stress conditions of acidic, basic, oxidative, freezing, heating, photolytic, and catalytic degradation.

Results: Good separation between the target analyte and its degradation products without any interference referring to specificity and selectivity of this method had been reported. Reversed-phase C_{18} Kinetex® column (100 mm × 4.6 mm I.D., 2.6 µm), isocratic mobile phase composed of an aqueous solution adjusted to pH 2.3 by 0.1% orthophosphoric acid-acetonitrile (80:20, v/v) at 0.8 ml/minutes flow rate were used. The assay showed good linearity over the concentration range of 1-25 µg/ml with a correlation of coefficient >0.999 and with a detection limit of 0.249 µg/ml and a quantitation limit of 0.755 µg/ml. Results of the analysis were validated statistically by recovery studies.

Conclusion: Validated stability-indicating RP-HPLC has been developed for estimation of TIM in its pure and commercial forms in addition to good separation from its degradation products within reasonable time.

Keywords: Tiemonium methyl sulfate, Reversed-phase high-performance liquid chromatography, Stability indicating.

INTRODUCTION



Chemical structure of tiemonium methyl sulfate

Tiemonium methyl sulfate or tiemonium metil sulfate (TIM), 4-[3-hydroxy-3-phenyl-3-(2-thienyl)propyl]-4-methyl-morpholinium methyl sulfate with molecular formula $C_{19}H_{27}NO_6S_2$ = 429.6, is quaternary ammonium antimuscarinics which is used in the relief of visceral spasms [1]. The drug is not included in any official pharmacopeias. From the literature survey, there were few methods had been reported for TIM determination using particle-induced X-ray emission [2] and ultraviolet (UV) spectroscopic methods [3] for TIM, its dosage forms and in the presence of its degradation products [4,5]. Reported method about stability indicating using high-performance liquid chromatography (HPLC)/high-performance thin layer chromatography (TLC) techniques [6] had studied the acid degradation of TIM in its pure powder form, in pharmaceutical dosage form, and in laboratory prepared mixtures containing different percentages of the degradation product.

To our knowledge from the literature, till date, very few HPLC methods had been mentioned, so our study aimed to develop an easy, simple, and more sensitive method as quantitation was applied up to 1 µg/ml using a much simpler buffer free with relatively low organic composition of mobile phase that merits this green chromatographic method for determination of TIM in pure form and in pharmaceutical preparations with suitable retention time. Furthermore, the presented method investigated the stability of TIM under more stress testing than the previous published HPLC method according to International Conference on Harmonization (ICH)-recommended forced degradation conditions showing good selectivity for determination of TIM in the presence of its degradation product.

METHODS

Instrumentation

An agilent 1100 series chromatographic apparatus equipped with G1313A autosampler injector and 100 µg/L volume injection loop. The mobile phase was degassed using Agilent G1322A vacuum degasser with G1311A isocratic quaternary pump and solvent cabinets. UV lamp with short wavelength was obtained from Desaga (Waldbronn, Germany) and G1314A variable wavelength detector connected to a hp computer loaded with Agilent Chemstation Software were used. Chromatograms were recorded on Agilent integrator. Separation was carried out on Kinetex® 2.6 µ C18 100A (100 mm × 4.6 mm I.D., 2.6 µm particle size) supplied by Jones chromatography heater. Jenway 4330 conductivity and pH meter were used.

Chemicals and reagents

HPLC grade acetonitrile (DUKSAN Pure Chemicals), methanol (Lab-Scan Analytical Science), and water (TEDIA® high purity solvents) but all other solvents were of analytical grade such as orthophosphoric acid (Merck Co., Germany), hydrochloric acid, sodium hydroxide, and 30% H_2O_2 (El-NASR, Cairo, Egypt) were used in this study.

TIM raw material was generously supplied by Amoun Pharmaceutical Industries Co., (Cairo, Egypt). Its purity was found to be 100.217%

according to the comparison method and was used as received without further treatment.

Pharmaceutical preparations

The following available commercial formulations are subjected to analytical procedure:

1. Visceralgine® tablets, Batch No. 0515261 (Sedico Pharmaceutical Company, Giza, Egypt) labeled to contain 50 mg TIM per tablet
2. Viscera® ampoules, Batch No. 150846, 144766 (Amoun Pharmaceutical Industries Co., Cairo, Egypt) labeled to contain 5 mg TIM per 2 ml.

Chromatographic conditions

Samples were analyzed using Kinetex® C18 (100 mm × 4.6 mm I.D., 2.6 µm particle size) column. Elution pumps run isocratic mobile phase which consisted of acetonitrile and water adjusted pH 2.3 with 0.1% orthophosphoric acid (20:80, v/v). The autosampler utilized acetonitrile as a rinse solution; injection volume was 20 µl and flow rate of mobile phase was 0.8 ml/minutes with pressure 200 bar. The variable wavelength UV-visible detector was set at 225 nm. The column temperature was maintained at 50°C.

General procedures

Working standard solutions

A standard stock solution of TIM (100 µg/ml) was prepared by accurately weighted 10 mg of TIM powder into 100 ml volumetric flasks, 50 ml of HPLC water was added, shaken, and diluted to volume with the same solvent. The working standard solutions were prepared by transferring aliquots of stock solution to 10 ml volumetric flasks which were completed to final volume with the same solvent to obtain concentrations ranging from 1 to 25 µg/ml.

Construction of calibration curves

Aliquots (0.1, 0.15, 0.2, 0.5, 1, 2, 2.5 ml) of standard solutions were transferred into a series of 10 ml volumetric flasks then completed to the mark with the same solvent and mixed to give concentration equivalent to (1, 1.5, 2.5, 10, 15, 20, 25 µg/ml). 20 µl of the previously freshly prepared solutions were injected (triplicate) in the chromatographic system and eluted with the mobile phase under the optimum chromatographic conditions and detected at 225 nm. The standard calibration graph was constructed by plotting peak area versus the corresponding final concentration in µg/ml; then, the linear regression equation was computed.

Procedures for pharmaceutical preparations

Assay of Visceralgine® tablets

About 10 tablets of analyzed drugs were weighed, finely powdered then an amount equivalent to 10 mg of TIM was accurately transferred into a 100 ml volumetric flask, dissolved in 50 ml methanol and the flask was sonicated for 30 minutes, the volume was completed to the mark with methanol then followed by filtration [6]. The filtrate of TIM was evaporated, and residue of the drug was redissolved with 100 ml HPLC water. The general procedure was followed, and recovery experiments were performed by direct technique as an additional check on the accuracy of the proposed method.

Assay of Viscera® ampoules

About 4 ml of Viscera ampoules equivalent to 10 mg of TIM was transferred into 100 ml volumetric flask and completed to the mark with HPLC water. The assay was completed as under general procedure, and recovery experiments were performed as an additional check on the accuracy of the proposed method.

Procedures for forced degradation

To determine selectivity of the analytical method, the stability-indicating capability of the method was performed by exposure of the active ingredient to various stresses as acidic, basic, and oxidative conditions, adopting the reported degradation conditions [4] in addition to thermal, frozen, and photolytic stress (sunlight and UV light) as follows:

Degradation in solutions

Acidic hydrolysis

To 20 mg of TIM, 20 ml of 5 N HCl was added in round-bottomed flask.

The resultant solution was refluxed with stirring at 80°C for about 2 hrs to facilitate acid degradation of TIM and then tested for degradation occurrence by TLC using methanol:methylene chloride:glacial acetic acid (8:2:0.2, by volume) [4]. Degraded solution was allowed to cool to room temperature, and after neutralized with 5 N NaOH, the volume was completed to 50 ml using HPLC water in 50 ml volumetric flask. Aliquot 0.5 ml of degraded sample was transferred to 10 ml volumetric flask and diluted with water to yield concentration 20 µg/ml of TIM; then, it was finally injected into HPLC and compared with the control sample.

Alkaline hydrolysis

To 20 mg of TIM, 20 ml of 5 N NaOH was added in round-bottomed flask. The resultant solution was refluxed with stirring at 80°C for about 3 hrs to facilitate basic degradation of TIM. Degraded solution was allowed to cool to room temperature, and after neutralized with 5 N HCl, the procedure was completed as mentioned in acidic hydrolysis.

Oxidative hydrolysis

To 20 mg of TIM, 20 ml of 1 ml of 30% H_2O_2 diluted with water in round-bottomed flask. The resultant solution was refluxed with stirring at 80°C for about 3 hrs to facilitate oxidative degradation of TIM. Degraded yellowish solution was allowed to cool to room temperature and complete procedures of dilution using water to yield concentration 20 µg/ml of TIM; then, it was finally injected into HPLC and compared with the control sample.

Frozen stress

Solution of TIM (100 µg/ml) was prepared using water then frozen at −10°C for 14 hrs. Frozen solution was allowed to reach to room temperature complete procedures of dilution using water to yield concentration 20 µg/ml of TIM; then, it was finally injected into HPLC and compared with the control sample.

Degradation in solid form

Thermal stress studies

A thin layer of TIM bulk drug was spread on a petri dish and subjected to heat at 80°C in a dry heat oven for 8 hrs. 10 mg of TIM sample was accurately weighed and prepared for analysis as previously described.

Photo stability (sunlight and UV light)

Photolytic studies were conducted by exposing thin layer of TIM bulk spread on a petri dish to UV under UV cabinet at 25°C for 4 hrs and another portion of TIM bulk spread between two glass dishes as thin layer for 3 days to direct sunlight for determination the effect of light irradiation on the stability of TIM in the solid state. 10 mg degraded yellowish samples were accurately weighed and prepared for analysis as previously described.

RESULTS AND DISCUSSION

Method development

Different conditions affecting the chromatographic performance were optimized to obtain an acceptable chromatographic resolution with symmetrical sharp peaks and suitable for good separation between the analyte and its main degradation products.

Type of column

Different columns were used for performance investigations, including Kinetex® C18 100A (100 mm × 4.6 mm I.D., 2.6 µm particle size), Thermo

Scientific BDS Hypersil® C18 (150 mm × 4.6 mm I.D., 5 µm particle size), Agilent HC C18 (150 mm × 4.6 mm I.D., 5 µm particle size), and Thermo Electron Corporation Hypersil Gold® (250 mm × 4.6 mm I.D., 5 µm particle size). The experimental studies revealed that the first column was the most suitable as TIM, and its degradation products were separated and eluted within reasonable analysis time showing good symmetrical peak shape and acceptable chromatographic resolution rather than other columns elongated retention time of TIM and gave broad peaks.

Mobile phase composition

Selection of mobile phase was based on peak parameters (symmetry, retention time, and resolution) and ease of preparation.

The optimum chromatographic performance was developed using acetonitrile and water adjusted pH 2.3 with 0.1% orthophosphoric acid (20:80, v/v) using the minimum quantity of organic solvent that merits this green chromatographic method. A buffer free aqueous phase was used due to buffer salts can precipitate and cause back pressure build-up inside the column in addition to the disadvantage of phosphate salts that have limited high organic solubility. For many liquid chromatography (LC)-UV and LC-mass spectrometry (MS) methods, a low pH is more important than the presence of a true buffer, so 0.1% phosphoric (UV) or formic (MS) acid can be used to satisfy this requirement as at pH 2.3 gave sharp peaks and high efficiency in our work. Acetonitrile was selected as organic solvent rather than methanol as giving good symmetrical peak. Various mobile phase compositions were tried in attempts to obtain good resolution of TIM and its degradants. Upon varying percentages of the organic phase (15-30%) revealed that increasing the percentage of acetonitrile (e.g., 30%) showed very early eluted analyte peak while decreasing the percentage (e.g., 15%) increase separation time with relatively broad peak.

Column oven temperature

Column oven temperature was also studied at room temperature 40 and 50°C. It was found that column temperature 50°C was optimum.

Choice of detection wavelength

TIM showed main absorption peaks at 225, 235, and 250 nm. 225 nm was found to be optimum for detection at which the highest detector response was obtained.

Choice of flow rate

The effect of flow rate was studied to optimize the chromatographic efficiency of the proposed method and improve the resolution of the eluted peaks.

The flow rate was changed over the range of 0.7-1 ml/minutes and a flow rate of 0.8 ml/minutes was optimum for good separation in a reasonable time.

Hence, the optimum chromatographic performances were achieved as summarized in Table 1 for quantification of the drug and separation from its degradation products without interference from each other within 8 minutes.

Satisfactory symmetrical peak shape (Fig. 1), number of theoretical plates (N), tailing factor (T), and capacity factor (k´) and their reference values [7,8] are shown in Table 2.

Stability studies

Results of degradation studies indicated that TIM underwent completely degradation in acid (heating for long time or at higher temperature under this acidic stress cause formation of small black particles), extensive oxidative degradation, moderate degradation occurred in alkaline medium and sunlight degradation while relatively stable in thermal, frozen, and UV light conditions as summarized in Tables 3 and 4.

Figs. 2-8 showed chromatograms obtained from TIM after forced degradation under several stress conditions.

Table 1: Analytical performance data and optimum chromatographic conditions for HPLC determination of TIM

Parameter	TIM
Beer's law limits µg/ml	1-25
Regression equation*	
Intercept (a)	6.439
Slope (b)	36.818
Correlation coefficient (r^2)	0.9999
Column	Kinetex® C18 100A (100 mm×4.6 mm I.D., 2.6 µm particle size)
Mobile phase	Acetonitrile - water adjusted pH 2.3 with 0.1% orthophosphoric acid (20:80%)
Flow rate	0.8 ml/minutes
Detector	UV-detection at 225 nm
Injection volume	20 µl
Temperature	50°C
Retention time	3.374 minutes

*A=a+bC, Where, C=Concentration of drug in µg/ml, A=Absorbance. HPLC: High-performance liquid chromatography, TIM: Tiemonium methyl sulfate, UV:Ultraviolet

Table 2: System suitability parameters of chromatogram for the determination of TIM by HPLC method

Parameters	TIM	Reference value
Retention time (t_R)	3.374	
Number of theoretical plates (N)	4581	>2000 increase with efficiency of separation
Tailing factor (T)	0.67	≤2 as T=1 for a typical symmetric
Capacity factor (K`)	1.48	1-10 acceptable
HETP	0.022 mm	The smaller the value, the higher the column efficacy

HETP: Height equivalent to one theoretical plate, TIM: Tiemonium methyl sulfate, HPLC: High-performance liquid chromatography

Method validation

The developed method was validated according to the ICH guidelines [9] by testing the followings:

Linearity

Calibration curve was constructed, for determination of TIM by the proposed method, by plotting peak area responses against concentrations of the drug in a range of 1-25 µg/ml to give a rectilinear graph. The linear regression equation was derived using the least-squares method, and statistical analysis of data for the drug was listed in Table 5 proving the good linearity over the working concentration range.

Sensitivity

In accordance with ICH guideline Q2(R1) [9], several approaches to determine the detection and quantitation limits include visual evaluation, signal-to-noise ratio, and the use of standard deviation of the response and the slope of the calibration curve. In the present study, the limit of detection (LOD) and limit of quantitation (LOQ) were based on the third approach and were evaluated using the following equation:

$$LOD = 3.3\frac{\sigma}{s}$$

$$LOQ = 10\frac{\sigma}{s}$$

Where, σ = The standard deviation of replicate blank responses (under the same conditions as for sample analysis), and S = The slope of the calibration curve of the analyte.

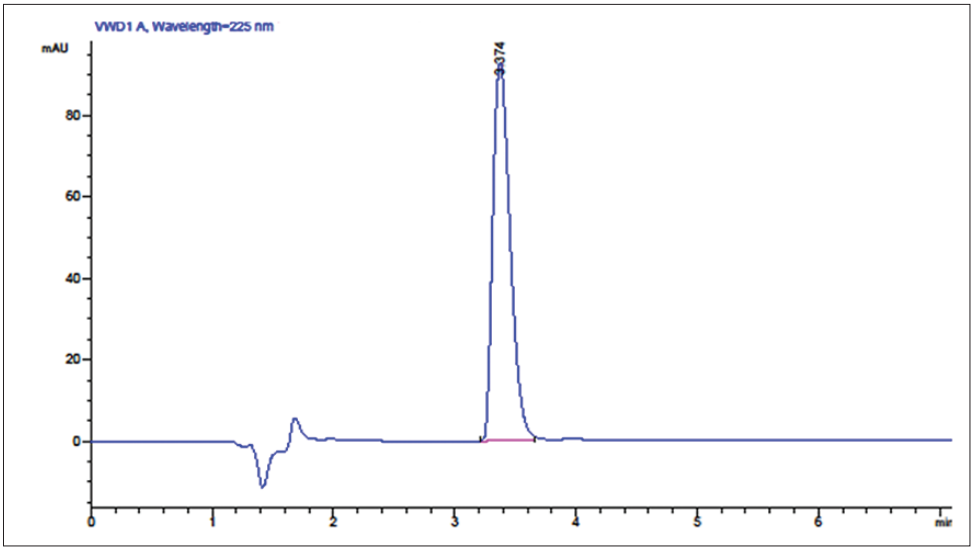


Fig. 1: Representative chromatogram of 25 µg/ml tiemonium methyl sulfate under the described conditions

Table 3: Results from analysis of samples after forced degradation study

Stress condition	Time and temperature	Retention time of degradants (minutes)	Recovery of intact drug (%)	Remarks
Acid degradation 5 N HCL	Reflux 2 hrs at 80°C	6.343 and 7.068	0	Completely degradation was observed at this two peaks
Alkaline degradation 5 N NaOH	Reflux 3 hrs at 80°C	6.540	84.1	One small peak
Oxidative degradation 30% H_2O_2	Reflux 3 hrs at 80°C	2.802, 4.800 and 5.963	28.94	Small three peaks
Frozen stress	14 hrs at 10°C		96	Relatively stable as no degradation product was observed
Thermal stress dry heat oven	8 hrs at 80°C		97.60	Relatively stable as no degradation product was observed
Sunlight degradation	3 days	3.987	87.82	Physical change in powder color (yellow) Small peak appears only when change wave length at 325 nm
UV light degradation	For 4 hrs at 25°C	4.057 and 4.781	96.51	Physical change in powder color (yellow) Two small peaks

UV: Ultraviolet

Table 4: System suitability parameters of degradation products of TIM

Degradation medium	Degradation product	Retention time (minutes)	Capacity factor (K´)	Selectivity (α)	Resolution (R_s)
Acidic	A1	6.343	3.879	1.84	9.00
	A2	7.068	4.437	1.11	1.68
Alkaline	B1	6.540	4.030	1.91	10.35
Oxidative	O1	2.802	1.155	-	-
	O2	4.800	2.692	1.39	5.53
	O3	5.963	3.587	1.24	3.89
Sun light	S1	3.987	1.492	1.33	2.311
UV light	U1	2.075	0.596	-	-
	U2	2.265	0.742	1.09	1.22
	U3	4.057	2.12	1.19	2.82
	U4	4.781	2.678	1.18	3.02

TIM: Tiemonium methyl sulfate, UV: Ultraviolet

Accuracy and precision

Accuracy

The accuracy of the proposed method was ascertained by determining pure samples of the cited drugs with reported method. Statistical analysis [10] of the results obtained by the proposed and comparison method [3] showed that the calculated values did not exceed the theoretical ones which indicated no significant differences found between the proposed method and comparison method. Statistical comparison of the results was performed using Student's t-test and variance ratio F-test at 95% confidence level (Table 6).

Precision

- Intra-day precision: To determine intra-day precision (repeatability) of the proposed methods, solutions containing three different concentrations (within working ranges) of each drug in its pure form were prepared and analyzed by proposed methods on three

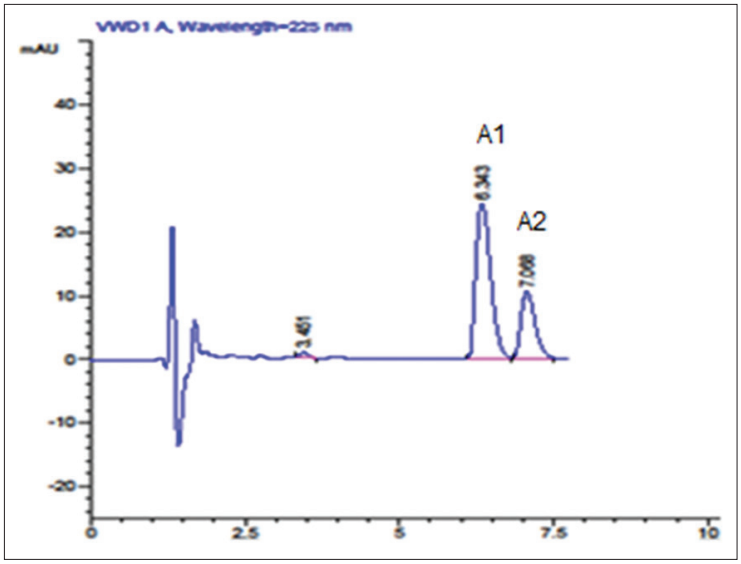


Fig. 2: Acid degradation chromatogram of 20 µg/ml tiemonium methyl sulfate

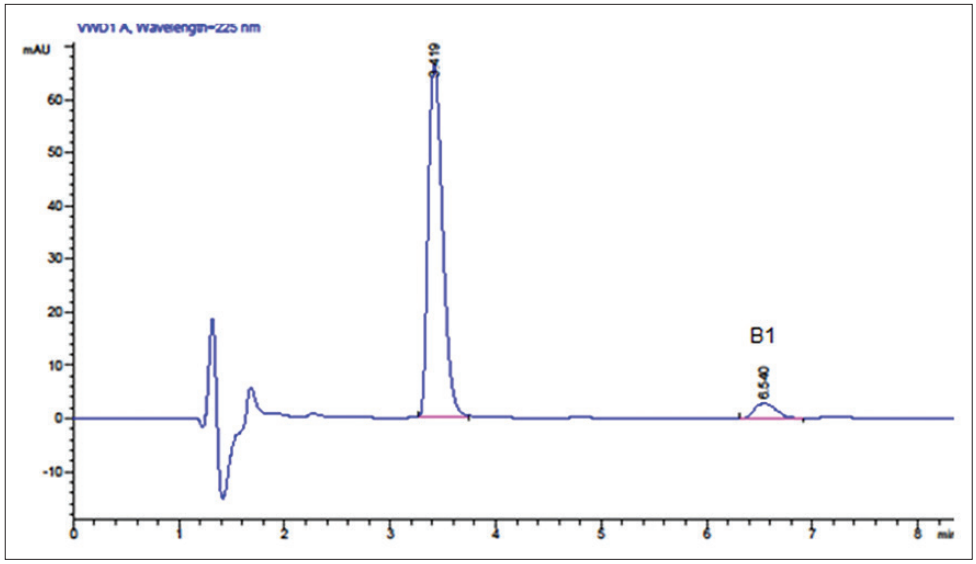


Fig. 3: Alkaline degradation chromatogram of 20 µg/ml tiemonium methyl sulfate

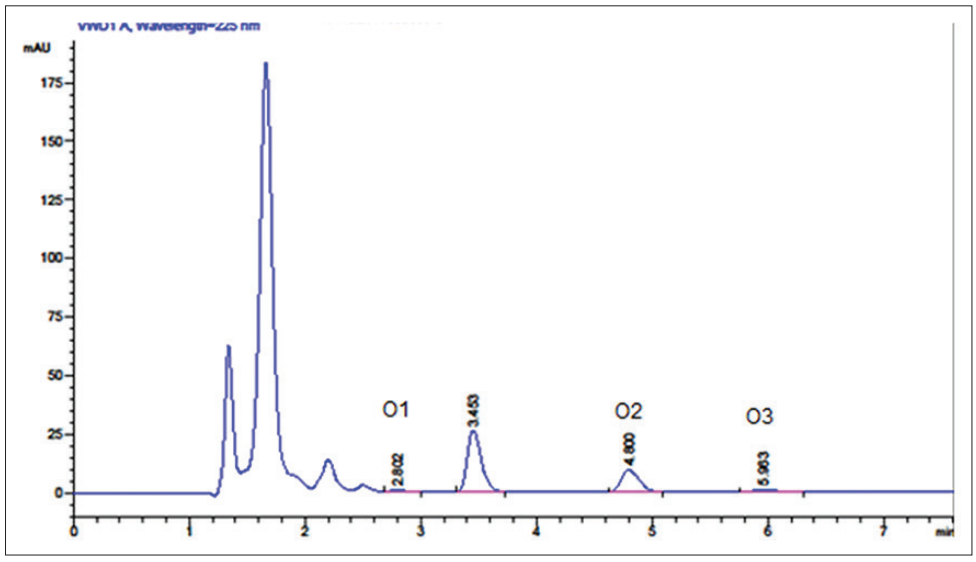


Fig. 4: Oxidative degradation chromatogram of 20 µg/ml tiemonium methyl sulfate

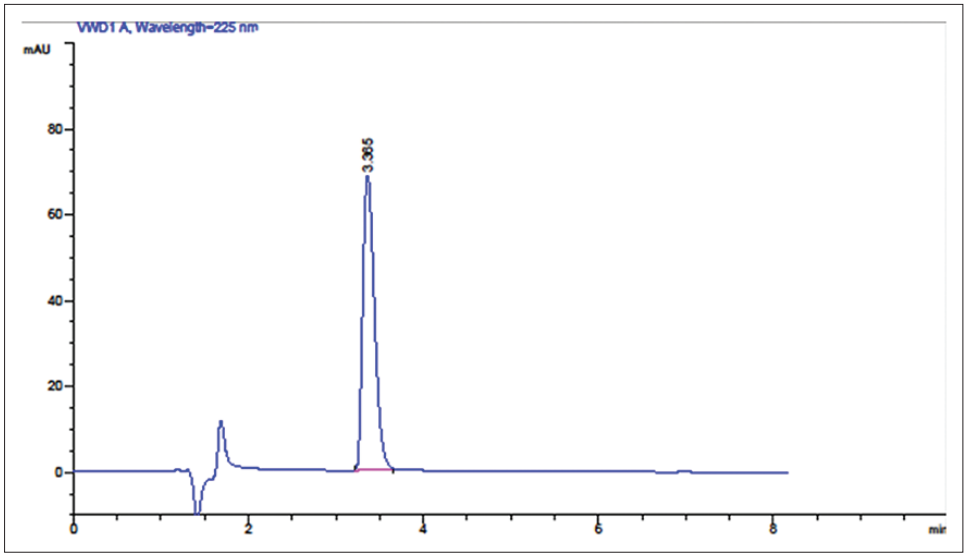


Fig. 5: Chromatogram of 20 µg/ml tiemonium methyl sulfate under frozen stress

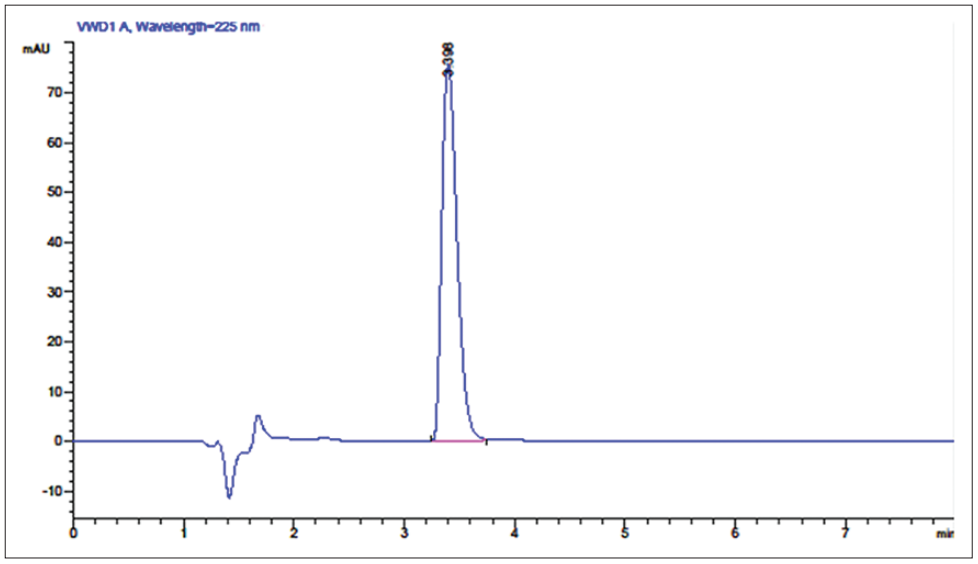


Fig. 6: Chromatogram of 20 µg/ml tiemonium methyl sulfate under thermal stress

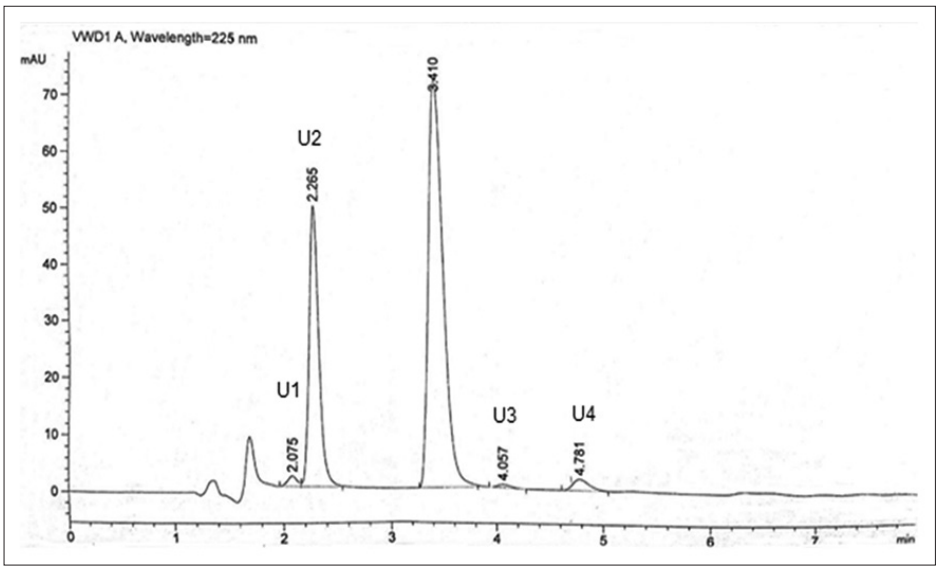


Fig. 7: Photodegradation chromatogram of 20 µg/ml tiemonium methyl sulfate using ultraviolet light

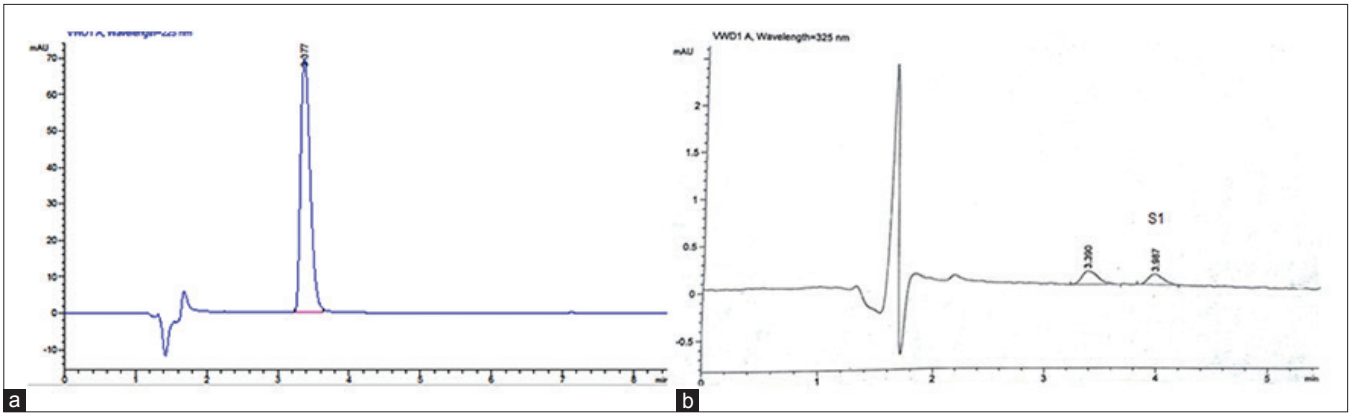


Fig. 8: (a) Photodegradation chromatogram of 20 µg/ml tiemonium methyl sulfate (TIM) under sunlight at 225 nm, (b) photodegradation chromatogram of 20 µg/ml TIM under sunlight at 325 nm

Table 5: Assay results for the determination of TIM in pure form by the proposed method

Parameters	TIM		
	Taken µg/ml	Found µg/ml	Recovery*%
	1	0.985	98.49
	1.5	1.482	98.79
	2	1.971	98.54
	5	4.986	99.71
	10	10.149	101.49
	15	14.989	99.93
	20	19.924	99.62
	25	25.015	100.06
Mean±SD	99.58±0.994		
N	8		
RSD	0.998		
SE	0.351		
Variance	0.988		
Slope	36.818		
LOD (µg/ml)	0.249		
LOQ (µg/ml)	0.755		
ε (×10⁴)** L.mol⁻¹.cm⁻¹	1649.46		

*Average of three different determinations, **Apparent molar absorptivity was calculated on the basis of the molecular weight of the drug. TIM: Tiemonium methyl sulfate, SD: Standard deviation, RSD: Relative standard deviation, SE: Standard error, LOD: Limit of detection, LOQ: Limit of quantitation

Table 6: Statistical analysis of results obtained by the proposed method compared with reported method

Drug	Proposed methods	Comparison method
TIM		
Mean±SD	99.58±0.994	100.22±0.964
Variance	0.988	0.930
N	8	6
Student's t-test	1.207 (2.179)*	
F-test	1.062 (3.97)*	

*The corresponding theoretical values for t- and F-tests at p=0.05. SD: Standard deviation, TIM: Tiemonium methyl sulfate

successive times in the same day. The values of relative standard deviation (RSD) were calculated, and percentages relative error (Er%) of the suggested method were also calculated using the following equation:

Er% = [(found − added)/added] × 100

- Inter-day precision: To establish inter-day precision (intermediate), three experimental replicates including three different concentrations (within working ranges) of the cited drugs were carried out using proposed method over period of 3-day.

Intra- and inter-day precisions and accuracy results were summarized in Table 7 indicating the validity, applicability of the proposed methods, and the reproducibility of the results.

Specificity

Specificity of the proposed method allowed separation and determination of TIM in its dosage forms as tablet, ampoule and in the presence of its degradation products without any interference.

Robustness

Robustness is the measure of the capacity of proposed methods to remain unaffected by small variations of the method variables. It was evaluated by making small incremental changes in one parameter while the others were kept unchanged as flow rate (0.77, 0.8 and 0.83 ml/minutes) and injected volume (19.5, 20, and 20.5 µl). The effect of the changes was studied on the peak area and retention time by calculating (recovery ± % RSD) as shown in Table 8, and this changes had negligible influence on the shape of the peak and tailing factor.

System suitability

System suitability test parameters: Column efficiency number of theoretical plates (N), capacity factor (K´), tailing factor (T), resolution (R_s), and selectivity (α) are used to verify that the resolution and the reproducibility of the chromatographic system are adequate for the analysis to be done according to *United States Pharmacopeia* [11] as shown in Tables 2 and 4.

Analytical applications

Dosage form analysis

The proposed method was successfully applied to the assay of the studied drugs in their pharmaceutical formulations Visceralgine® tablets and Viscera® ampoules. Satisfactory results obtained for the recoveries of the drugs were in good agreement with the label claim and proved the suitability of the proposed methods. The percentage recoveries of the pure drugs using the proposed methods shown in Table 9 are in good agreement with those obtained with the comparison methods [3]. Fig. 9 show representative chromatograms for determination of TIM in its dosage forms.

CONCLUSION

Validated stability-indicating reversed-phase HPLC has been developed for estimation of TIM in its pure and commercial forms in addition to good separation from its degradation products within a reasonable time. It has the advantage of being much simpler, and friendly environmental

Table 7: Precision data for the determination of TIM by the proposed method

TIM							
Intra-day				**Inter-day**			
Added (µg/ml)	**Found±SE (µg/ml)**	**RSD%**	**Er%**	**Added (µg/ml)**	**Found±SE (µg/ml)**	**RSD%**	**Er%**
5	5.14±0.526	0.886	2.74	5	5.01±0.608	1.052	0.14
15	15.08±0.299	0.516	0.51	15	14.97±0.573	0.994	−0.18
25	24.86±0.313	0.545	−0.57	20	20.03±0.612	1.058	0.16

TIM: Tiemonium methyl sulfate, SE: Standard error, RSD: Relative standard deviation

Table 8: Robustness of the proposed method

Concentration	Variation	Retention time	Peak area	Tailing factor	Theoretical plates
5 µg	Flow (0.77 ml/minutes)	3.543	183.102	0.77	4723
	Flow (0.83 ml/minutes)	3.266	182.641	0.81	5184
	Without variations (0.8 ml/minutes)	3.376	186.018	0.7	4655
	RSD of affected parameters	4.108	1.032		
10 µg	Injected volume (20.5 µl)	3.392	392.105	0.68	4438
	Injected volume (19.5 µl)	3.369	368.671	0.68	4675
	Without variations (20 µl)	3.354	379.277	0.69	4614
	RSD of affected parameters	0.568	3.141		

RSD: Relative standard deviation

Table 9: Application of the proposed method for determination of TIM in its dosage forms

Parameters	TIM					
	Visceralgine® tablets			**Viscera® ampoules**		
	Taken µg/ml	**Added µg/ml**	**Recovery* %**	**Taken µg/ml**	**Added µg/ml**	**Recovery* %**
	3	2.921	97.38	6	6.118	101.97
	4	3.954	98.84	7	7.105	101.50
	5	4.847	96.94	8	8.157	101.96
	6	5.823	97.05	10	10.126	101.26
Mean±SD	97.55±0.878			101.67±0.350		
N	4			4		
SE	0.439			0.175		
Variance	0.771			0.122		

*Mean of three different experiments. SE: Standard error, SD: Standard deviation, TIM: Tiemonium methyl sulfate

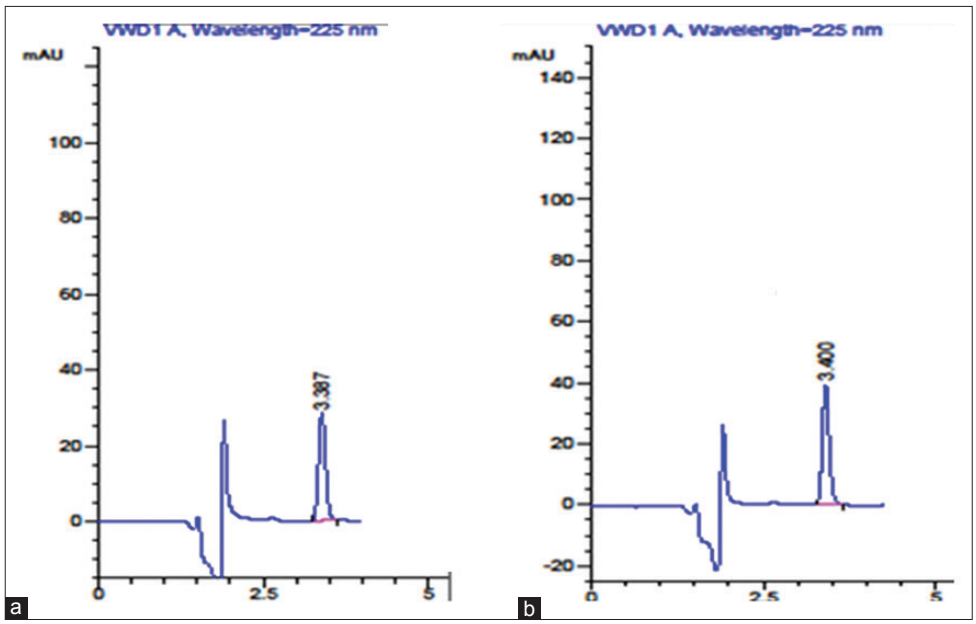


Fig. 9: Chromatogram of tiemonium methyl sulfate sample solutions from (a) The tablets (6 µg/ml), (b) the ampoules (7 µg/ml)

method as buffer free mobile phase and a low proportion of organic phase was used. Besides, it is more sensitive than the previously published HPLC method as it applied up to 1 µg/ml and studied its stability under many stress testing.

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