Research Article

Statistically compared spectrophotometric analytical methods for the estimation of an anti-spasmodic drug and its application to pharmaceuticals

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ARTICLE INFO

Date of submission: 28-10-2020

Date of Revision: 22-11-2020

Date of acceptance: 03-12-2020

Key Words:

Tiemonium metylsulphate, First derivative, AUC−spectrophotometric, Statistical comparison

ABSTRACT

Statistically compared three analytical methods, like UV (Method I), first derivative (Method II) and AUC−spectrophotometric (Method III), were developed and validated by using 0.1N HCl as solvent for the assay of an anti-spasmodic drug i.e. Tiemonium metylsulphate (TMS) and also the methods were applied to its different pharmaceuticals for analysis. Response of the drug (extension/(dA/dλ) or AUC) was recorded at 235 nm, 248 nm and in the wavelength range of 230−240 nm against concentration for Method I, Method II and Method III, respectively and all the methods showed linearity in the concentration range of 2.5-80.0 µg/ml with correlation coefficient (r^2) value of 0.999. The methods were validated as per the parameters suggested by the International Conference on Harmonization and all the validation parameters were within the acceptable range. The proposed statistically verified analytical spectroscopic methods can be applied fruitfully for quantitative analysis of TMS in its different pharmaceutical dosages.

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1. INTRODUCTION

Tiemonium methylsulphate (TMS), chemically 4-[3-Hydroxy-3-phenyl-3-(2-thienyl) propyl]-4-methyl-morpholinium methylsulphate, is an achiral molecule having molecular formula $C_{19}H_{27}NO_6S_2$ and is existing in quaternary ammonium salt form [1]. It competitively inhibits muscarinic receptor (parasympatholytic / anticholinergic agent) and has weak competitive antagonistic effect on histamine receptor [2]. It also supports binding of calcium to membrane phospholipid which results in inhibition of intracellular contractile protein of visceral cell which eventually leads to inhibition of visceral spasm and pain [3].

Literature review suggests that a very few analytical techniques like spectrophotometric [4-6], HPTLC [7] and HPLC/UPLC [7-9] have been established so far for quantitation of TMS present in diverse biological samples as well as in pharmaceutical dosage forms. Islam et *al*. [4] have used distilled water as diluent in their study which is not advisable for intermediate precision study as well as dissolution studies because of chance of microbial growth in the medium. The methods described by Zaazaa et *al*. [5] and Ramadan et *al*. [6] are very complicated as they used multicomponent analysis techniques for detection of TMS in presence of its degradation product and used methanol and 2N H_2SO_4 as solvent, respectively, which are not economic and cannot recommended for dissolution studies. Some of the reported methods [7-9] necessitate highly sophisticated instruments and skilled personnel for their operation.

Keeping view on all mentioned troubles allied with various reported works and also unavailability of simple UV−spectroscopic method, authors felt that there is a need for development of uncomplicated and cost-efficient techniques for quantification of TMS either in raw material or in different pharmaceutical formulations without the interference of excipients and their degradation products using 0.1 N HCl as solvent; which can also be used in dissolution studies. The developed methods are statistically compared and found that there is no significant difference in the results among the different methods.

2. MATERIALS & METHODS

2.1. Chemicals and solvents

TMS reference standard (purity>98.34%) was kindly provided by Roland Institute of Pharmaceutical Sciences and further identified by obtaining its melting point and D^0 UV absorption spectra. Hydrochloric acid (35%w/w) of analytical reagent grade solution was purchased from Merck Life Science Pvt. Ltd., (Mumbai, India) and was used for preparation of 0.1N HCl in triple distilled water. A

commercial tablet formulation was purchased from the local market.

2.2. Instruments used

For development and validation of methods, a UV−Visible spectrophotometer of double beam (UV−1800, Shimadzu, Japan) linked to computer supported with UV Probe spectra manager software, with 1.0 cm duplicate cuvettes made up quartz was used. Different instrumental influencing parameters were maintained in the conditions such as; wavelength range: 200-400 nm; scanning speed: medium; sampling interval: 0.5 nm; derivative mode: ^{1}D (first order derivative); band width ($\Delta\lambda$): 5 nm; spectral slit width: 1 nm was used to obtain absorption spectra. All weight measurements were carried out on an electronic balance (Denver, Germany).

2.3. Preparation of Stock Solution and calibration standard solution

TMS stock solution (100 µg/mL) was prepared by dissolving 10 mg of standard drug in 0.1N HCl and made to 100 ml by using same solvent. For Method I, series of dilutions were made by pipetting out 0.25, 0.5, 1.0, 2.0, 3.0, 4.0, 5.0, 6.0 and 8.0 ml of stock solution into separate 10 ml volumetric flasks and diluting to volume with 0.1 N HCl to prepare the calibration standard solutions ranging from 2.5–80 µg/ml, scanned over the 400–200 nm range against 0.1N HCl solvent as blank and λ_{max} was found to be at 235.0 nm. For Method II, the obtained D^0 spectra were derivatized to get D^1 spectra and the response ($dA/d\lambda$) of the spectra were calculated at 248.0 nm. For Method III, AUC was calculated in the wavelength range between 230–240 nm. Linearity curves were constructed between concentration (2.5–80 µg/ml) and response [extension/ ($dA/d\lambda$) or AUC] of different methods [10, 11].

2.4. Estimation of TMS in marketed formulations

According to IP, to determine the TMS in the sample solutions, a total of 20 tablets (marketed pharmaceutical formulations) were weighed to get the average weight and then finely grounded with the help of mortar and pestle. Tablet powder equivalent to 10 mg was taken in a 100 mL volumetric flask, initially 50 ml of 0.1N HCl was added, sonicated for 15 min, diluted up to the mark with 0.1N HCl and filtered by using Whatman filter paper (No. 41). A proper dilution was carried out with 0.1N HCl from the filtrate sample solution in such a manner that the theoretical concentration of sample solution remains within the standard linearity range of TMS. Finally, the amount of TMS was determined by using the standard curves and percent labeled claim as well as Standard Deviation (SD) was determined.

The assay results obtained by using the different spectrophotometric methods were statistically compared with the assay results obtained by the **Method I** using t-test, F-test and one-way ANOVA at the 5% level of significance.

3. Validation of the Methods

The proposed methods were validated for its linearity, LOD, LOQ, precision, accuracy, specificity and ruggedness following USP and ICH Q2 (R1) guidelines [12-13].

For all the three methods Beer's range of the drug TMS was calculated over the concentration ranging from 2.5–80 µg/ml at six levels and each solution was prepared in triplicate on 3 different days. Linearity curves for TMS were obtained by linear least-squares regression analysis by plotting response on *y*-axis versus the concentration of standard on *x*-axis. Linearity range of the methods was indicated as correlation coefficient (r^2) and the value should be ≥0.9990. LOD and LOQ value for the methods were determined based on the SD of the response based on calibration curve and the slope.

Evaluation of precision i.e. both intraday and interday of the methods was performed by analysis of calibration standards which should be within the Beer's range and selected on the basis of smile curve (Conc. of standards on *x*-axis *vs.* % RSD of response on *y*-axis) [14] at three different concentrations (10, 20 and 40.0 µg/mL) and preparing each solution in triplicate. For repeatability precision study, three duplicates of the standard samples were analysed on the same day and for the intermediate precision, three duplicates of the standard samples were assessed on three different days. Results of the precision study are reported in terms of % RSD and also one-way ANOVA at the 5% level of significance were used to compare the intra and interday data.

Accuracy study of an analytical method measures the percentage of analyte recovered by assay procedure and was performed by adding three known quantities of reference material (16, 20 and 24 µg/mL; at three different levels 80, 100 and 120% of sample solution) into a predetermined sample solution of 20 µg/mL and each solution was prepared in triplicate. The percentage recovery of added reference drug was calculated by measuring the response and fitting these values to the regression equation of standard curves.

To know the specificity of the developed analytical methods, absorption spectra for sample solution within the linearity range was recorded and compared with the same strength of standard solution. This study provides information about the

interference of excipients during sample analysis.

Ruggedness study was performed by examining aliquots from homogenous slot (40 µg/mL) in various laboratories by two different analysts and by using two different instruments under identical operational and environmental conditions. The outcomes were presented in terms of % RSD and t-test at the 5% level of significance was used to compare the data.

4. RESULTS AND DISCUSSION

Purity of Standard: Before starting the experiment, the purity of the standard drug was evaluated by recording D^0 UV absorption spectrum and melting point. The shape of the spectrum matches with standard reported spectrum (**Figure. 1**) and the experimental melting point value (143.24 ºC) is very close with the reported standard value (144.32 ºC); which proves the absence of impurities in the reference standards, hence the drug was used without further purification.

Selection of suitable solvent: For selection of suitable solvent, initially solubility of the drug in different solvents such as distilled water, 0.1N NaOH, 0.1N HCl, methanol, phosphate buffer (pH 6.8) etc. was carried out. The drug was soluble in water but 0.1N HCl was selected as suitable solvent on the basis of stability of the drug in the solvent and usefulness in dissolution studies.

Optimization of wavelength and selection of method: To fix the detection wavelength for Method I, the drug was prepared in the 0.1N HCl and scanned in the wavelength range between 200−400 nm (**Figure. 1)**. Then a particular wavelength was selected as detection wavelength where the drug showed the maximum absorbance (λ_{max}), followed the Beer−Lambert's law properly and showed wide linearity range.

Derivative spectrophotometry is an analytical technique which enhances the sensitivity and specificity of method for qualitative and quantitative analysis of various compounds including pharmaceuticals. Hence Method II was carried out. To fix the detection wavelength for D^1technique, the spectra obtained in D^0 method were derivatised to get first order derivative spectra. Then 248 nm was selected as detection wavelength because at this wavelength there is no shift of maximum wavelength and zero crossing point of the maxima/minima of derivative spectra with increase in concentration, there is absence of distortion in shape of maxima/minima, drug followed the Beer-Lambert's law and showed wide linearity range as compared to detection at other wavelength (**Figure. 2)**.

The AUC spectroscopic method is applied when D^0 spectra is not showing sharp peak or when broad spectra were obtained or

spectra was split. It involves the calculation of integrated value of absorbance with respect to the selected wavelength range, which is selected on the basis of repeated observation so as to get the linearity between AUC and concentration. As D^0spectra of the drug is broad, hence Method III was performed and AUC was calculated in the wavelength range 230-240 nm and plotted against concentration to get the linearity curve (**Figure. 3)**.

The evaluation of the linearity was performed with a six-point calibration curve over the 2.5-80.0 µg/mL specified concentration range with a % RSD of less than 2 based on three successive readings. The slope and intercept of the calibration graph was calculated by using linear regression analysis and it was observed that with the increase in drug concentration, the response is increased proportionately (**Figure. 1, Figure. 2 and Figure. 3**).

Method validation

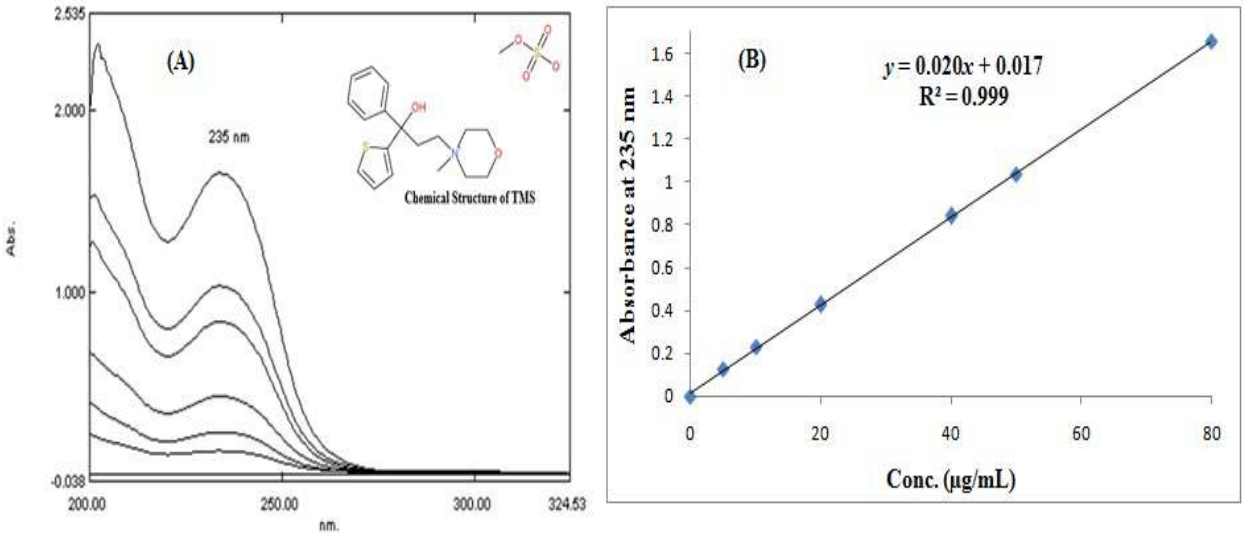

Figure. 1: (A): Overlaid UV absorption spectrum of TMS in 0.1N HCl solvent (2.5-80.0 µg/mL); (B): Calibration curve of TMS in 0.1N HCl by Method I (2.5-80.0 µg/ml)

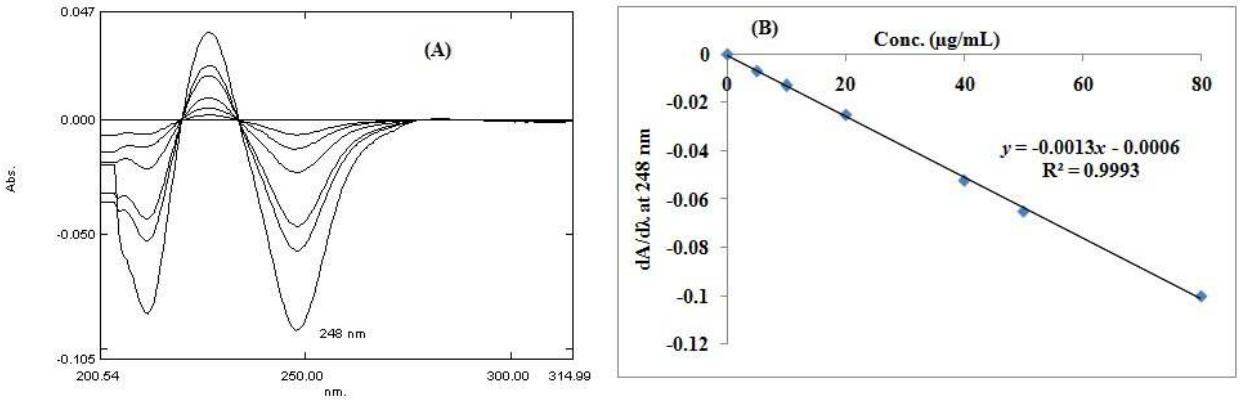

Figure. 2: (A): Overlaid D^1 UV absorption spectrum of TMS in 0.1N HCl solvent (2.5-80.0 µg/mL); (B): Calibration curve of TMS in 0.1N HCl by Method II (2.5-80.0 µg/ml)

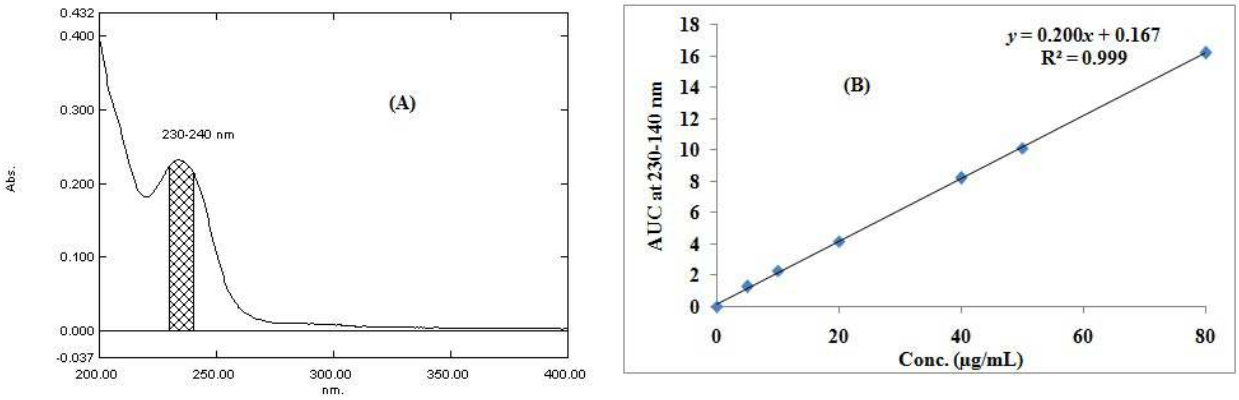

Figure. 3: (A) Absorption spectrum of TMS (10 µg/mL) in 0.1N HCl [230-240 nm range was selected for AUC measurement]; (B) Calibration curve of TMS in 0.1N HCl by Method III (2.5-80.0 µg/ml)

The linearity range and regression equation of the calibration curve for the proposed spectrophotometric methods are presented in the **Table 1**. A correlation coefficient of 0.999 suggests that the developed spectrophotometric methods had an excellent linearity over the investigated range.

The LOD and LOQ value for the proposed spectrophotometric methods were determined and the values were very low which indicates that the developed Spectrophotometric methods are sensitive (**Table 1**). LOD and LOQ value determined by the Method II was less as compared to Method I and II which indicates that the derivative spectroscopic method is more sensitive as compared to the D^0 spectroscopic and AUC spectroscopic method.

Molar absorptivity is an intrinsic property of the species and it was calculated to know, how strongly a chemical species absorbs light at a given wavelength and the

value varies from 10^0-10^4. The value in the order of 10^4 indicates strong absorption and the value in the order of 10^3 indicates weak absorption. The molar absorptivity (ε) for the Method I was calculated and showed in the Table 1.

Sandell's sensitivity is the amount/cm^2 producing an absorbance 0.001 and has dimension μg cm^{-2}. The Sandell's sensitivity of the developed D^0 method was calculated and tabulated in the Table 1.

The intra and interday precision studies for the three methods were satisfactory with %RSD value less than 2 (Table 1), which indicate that the developed methods were reproducible. Furthermore, one-way ANOVA was applied to contrast the intra and interday data and the obtained *p*-value was 0.999 (for three methods) which was more than 0.05 and $F_{Calculated}$ values were less than $F_{Tabulated}$ value (5.143), this indicates that there was no difference in the results obtained in different days by different methods.

The accuracy of the methods was carried out and the results were found to be in the acceptable range which indicates that the developed methods were accurate (Table 1). The developed methods were specific because the absorption spectrum for sample was overlapping with the standard which showed that there was lack of interference from excipients.

Ruggedness of the methods was determined as explained in the experimental section and the results of the study are shown in Table 1. The results obtained by two analysts proved the ruggedness of the spectrophotometric methods, since the %RSD values were less than 2. Moreover t-test was applied to compare the data obtained by two analysts and *p*-value obtained was more than 0.05 and $t_{Calculated}$ value was less than $t_{Tabulated}$ value (2.776), which indicates that there was no difference in the results obtained by two analysts.

Table 1: Method validation parameters of the proposed spectrophotometric methods

Method Validation Parameters	Method I	Method II	Method III
Linearity range (µg/mL)	2.5−80.0	2.5−80.0	2.5−80.0
λ_{max}(nm)/ wave length range (nm)	235.0	248.0	230.0-240.0
(a) Slope of Regression equation	0.020	-0.0013	0.200
(b) Intercept of Regression equation	0.017	0.0006	0.167
Correlation coefficient (r)	0.999 (Figure. 1)	0.999 (Figure. 2)	0.999 (Figure 3)
LOD (µg/mL)	0.484	0.417	0.479
LOQ (µg/mL)	1.466	1.263	1.454
Molar absorptivity (L/ mol cm) ± SD	$0.07\times 10^{4}\pm$ 0.0064	---	----
Sandell's sensitivity (µg cm^{-2}/0.001 A) ± SD	0.045 ± 0.004	----	-----
Precision (RSD, %) **Intraday (n=3)** **Interday (n=3)** **F Calculated**	0.745−1.542 0.452−1.687 3.3×10^{-5}	1.071−1.178 1.012−1.634 0.65×10^{-5}	0.371−0.912 0.205−1.514 0.13×10^{-5}
Accuracy (% recovery)	99.11−99.93	98.85−99.99	98.31−99.32
Ruggedness **(40µg/ ml; RSD, %)** **Analyst1 /Instrument1** **Analyst 2/Instrument 2** **t Calculated** **P value**	 1.320 1.505 0.744 0.498	 1.643 1.670 1.177 0.304	 0.278 0.881 1.821 0.143
Assay results(50mg/tab) **% Label Claimed ±SD(n=5)** **%RSD**	98.99±0.56 0.56	99.56±0.32 0.32	98.42±0.67 0.68

Application of the proposed spectrophotometric methods for assay of the drugs in marketed formulations

Commercially available different pharmaceutical formulations for the drug was obtained and assayed as described in section 2.4 and the results obtained are presented in Table 1. The assay results for pharmaceutical dosage form were comparable with the label value claimed. The results indicate the recovery of drugs from the pharmaceutical preparation was quantitative and there was no interference from the excipients present in the dosage form when compared to the control.

Statistical Comparison among spectrophotometric methods

The assay results obtained by using the three different spectrophotometric methods were statistically compared with the assay results obtained by the Method I using t-test at the 5% level of significance. One-way ANOVA at the 5% level of significance was used to compare the assay results obtained by using the three different spectrophotometric methods. *p*-value obtained was more than 0.05, $F_{Calculated}$ value was less than $F_{Tabulated}$ value and $t_{Calculated}$ value was less than $t_{Tabulated}$ value (2.776), which indicates that there was no significant difference in the content of drug determined by the three different spectrophotometric methods (**Table 2**). Hence all the developed methods can be used equally to quantify the drug from their marketed formulations.

Table 2: Statistical Comparison of the developed methods

Comparison among methods	**One-way ANOVA test**		
	P Calculated	***F*** Calculated	***F*** tabulated
Method I, Method II and Method III	0.952	0.050	9.552
Comparison between methods	**t-test**		
	$P_{Calculated}$	$t_{Calculated}$	
Method I and Method II	0.992	0.010	
Method I and Method III	0.917	0.111	

CONCLUSION

Three methods that were developed for the determination of TMS are based on different analytical spectroscopic techniques i.e., zero-derivative, first-derivative spectrophotometry and AUC method. All the analytical methods were validated and statistically compared and found to be simple, sensitive, accurate, and precise. Hence, all the methods can be used successfully for routine analysis of pharmaceutical dosage forms of TMS.

Acknowledgments

The authors are grateful to Roland Institute of Pharmaceutical Sciences, Brahampur-10, Odisha, India for providing the financial and research facilities.

DISCLOSURE

The authors declared that there is no conflict of interest in this work.

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