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Validated Spectrophotometric Methods for the Determination of Memantine Hydrochloride in Tablets Using Eosin and 2,4-Dinitrofluorobenzene Reagents

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ABSTRACT

Memantine hydrochloride is a new member of the adamantane family and, likewise, it shares the non-chromophoric structural feature characterizing these compounds. It is approved for the treatment of moderate to severe Alzheimer's disease. In this work, we present two simple and sensitive spectrophotometric methods for its determination. Method I is based on forming a colored binary complex with eosin in acetate buffer (pH 3.6) which can be measured at 546 nm, while method II uses its reaction with 2,4-dinitrofluorobenzene in borate buffer (pH 8) to form a yellow colored product measured at 360 nm. Analytical performance of the proposed methods was validated and the values obtained met the validation acceptance criteria. Regression analysis showed good linearity over the concentration ranges of 1-10 and 5-30 µg/mL, for methods I and II, respectively with correlation coefficient values >0.9992. The applicability of the proposed methods was evaluated through the analysis of tablets and satisfactory recoveries were obtained. Moreover, the statistical comparison of the obtained results with those of the reference method revealed no significant differences. Thus, the simplicity and high sensitivity of the proposed methodologies can verify their suitability for the routine analysis of memantine hydrochloride in its tablet formulations.

Keywords: Memantine hydrochloride; Eosin; 2,4-Dinitrofluorobenzene; Spectrophotometric analysis; Tablet dosage forms.

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INTRODUCTION

Memantine hydrochloride (MEM), chemically known as 1-amino-3,5-dimethyladamantane hydrochloride, is an adamantane derivative with a unique non-planar tricyclic saturated ring structure. It exerts its action through the uncompetitive low affinity blockade of N-methyl-D-aspartate (NMDA) receptors. Therefore, it does not show the typical debilitating side effects normally associated with the other high affinity NMDA blocking analogues as ketamine and phencyclidine. In 2003, US-FDA approved its use in the treatment of moderate to severe Alzheimer's disease. In addition, it exhibits a broad therapeutic utility and it has shown efficacy in other neurological disorders as Parkinson's disease, central spasticity, brain injury and comatose states [1,2].

This aliphatic molecule lacks any significant UV absorption or fluorescence properties which hinder its determination through direct techniques such as conventional or derivative spectrophotometric or flourimetric methods. Consequently, derivatization of its primary amino group has been the basis of its high performance liquid chromatographic (HPLC) determination using UV or flourimetric detectors. Reagents as; 4-(4,5-diphenyl-1H-imidazol-2-yl)benzoyl chloride (DIB-Cl) [3], FMOC [4], flourescamine [5], OPA [6], anthraquinone-2-sulfonyl chloride [7], NBD-F [8], (2-Naphthoxy)acetyl chloride [9] and dansyl chloride [10] were used as pre-column derivatizing agents for its determination in various biological fluids. A micellar electrokinetic chromatography with laser-induced fluorescence detection method was also developed for the determination of its fluorescein derivative [11]. In addition, it was determined using other chromatographic techniques as; HPLC-MS [12], HPLC-MS/MS [13], UPLC-MS/MS [14], GC-MS [15], GC-FID [16] and capillary zone electrophoresis [17].

Despite the high sensitivity attained by the above mentioned techniques, the high cost of the used instruments represents a major obstacle to their wide availability in regular QC laboratories. Therefore, their use can be reserved to the analysis of MEM in complex biological fluids. However, spectrophotometric methods using various derivatization reagents appear as appealing simpler and affordable alternatives for the routine analysis of MEM in its bulk form and simple matrices as the case of pharmaceutical preparations. Indeed, methods utilizing reagents as NBD-Cl, OPA [18], Folin-Ciocalteau, 1,2-Napthaquinone-4-sulphonate [19], bromocresol green [20], bromothymol blue [20,21] and solochrome black T [21] were reported.

Eosin Y (ESN) is a tetrabromo derivative of fluorescein and it has been used for the spectrophotometric determination of many pharmaceutical compounds either through the formation of binary complexes [22-27] or ternary complexes in the presence of a metal ion [28,29]. On the other hand, 2,4-dinitrofluorobenzene (FDNB) or Sanger's reagent, the active aryl halide, is known to react with thiol, phenolic and amino group bearing compounds giving yellow colored products thus allowing their spectrophotometric determination [30]. In fact, literature contains several methods utilizing FDNB for the determination of drugs bearing primary [31] or secondary amino groups [32-34].

The aliphatic nature of MEM and the presence of a primary amino group have grabbed our attention towards the utilization of these two reagents in the attempt of forming stable colored chromogens of high sensitivity. To the best of our knowledge, no reports investigating the reaction of MEM with ESN or FDNB were found. Consequently, the objective of this work was to develop and validate two simple and sensitive derivatization reactions for the determination of MEM in bulk and dosage form.

EXPERIMENTAL

Apparatus:

Spectrophotometric analysis was carried on a T80 double beam UV/VIS spectrometer (PG instruments Ltd, London, UK) connected to a PC loaded with UV WIN 5 software (version 5.2.0) using a pair of 1 cm matched quartz cells. The data was recorded in the specified wavelength range at a 1 nm interval and a bandwidth fixed at 2 nm. A Mettler Toledo MP-230 pH meter (Switzerland) calibrated daily at room temperature with standard buffers pH 4 and 7 was used. A thermostatically-controlled water-bath accurate to ± 0.5 ºC (Köttermann, Germany) was utilized in the FDNB method.

Materials and Reagents:

MEM (99.8%) was kindly provided as a gift from Adwia Co. (Cairo, Egypt). All reagents and chemicals employed in the assays were of analytical grade. Distilled water was used throughout the work. ESN (BDH Laboratory Suppliers, Poole, England) was prepared as a 0.26 % aqueous solution. FDNB was purchased from Hopkin and Williams Ltd (Chadwell Health, Essex, England) and was freshly prepared as 0.15 % (v/v) in acetonitrile (Fisher Scientific, UK). Walpole's acetate buffer (0.2 M) of pH 3.6 was prepared by mixing 46.3 mL of 0.2 M acetic acid with 0.7 mL of 0.2 M sodium acetate and the volume was made up to 100 mL with water. Clark and Lub's borate buffer (0.3 M) of pH 8 was prepared by mixing 50 mL of 0.3 M boric acid in 0.3 M KCl solution with 3.9 mL of 0.3 M NaOH and completing the volume to 100 mL with water [35]. The pH values of the both buffers were further confirmed using the pH meter. Hydrochloric acid (32%) (BDH Laboratory Suppliers, Poole, England) was exploited for the acidification of the reaction mixture in the FDNB method. Pharmaceutical preparations involved in the study are Ebixa® tablets (Lundbeck Ltd., Copenhagen, Denmark) and Ravamantine® tablets (Eva Pharma, Giza, Egypt). Both are labeled to contain 10 mg MEM per tablet and were purchased from the local market.

Preparation of Standard Solutions:

MEM stock solution was prepared at a concentration of 0.5 mg/mL in water. This solution was further diluted with the same solvent to produce 0.1 and 0.25 mg/mL MEM standard working solutions for the ESN and FDNB methods, respectively.

General Procedure:

Method I (Reaction with Eosin):

Aliquots of 0.1 mg/mL MEM standard working solution were transferred into a set of 10 mL volumetric flasks to produce calibration solutions within the concentration range specified in Table 1. To each flask, water was added to complete the volume to 7 mL followed by 1.4 mL ESN reagent and the solutions were mixed well before adding 1.2 mL of 0.2 M acetate buffer pH 3.6. The volumes were finally completed with water and the colored solutions were scanned in the range of 500-600 nm against a similarly treated blank. The absorbance readings were recorded at 546 nm and the calibration curve was constructed.

Method II (Reaction with FDNB):

Serial volumes of 0.25 mg/mL MEM standard working solution covering the stated concentration range (Table 1) were transferred into a set of 10 mL volumetric flasks and the volumes were adjusted to 1.2 mL with water. To each flask, 0.3 mL of 0.3 M borate buffer pH 8 was added followed by 1.6 mL of FDNB reagent. The solutions were heated in a thermostatically-controlled water bath maintained at 80 ºC for 20 min then the flasks were cooled under tap water. Afterwards, the reaction mixtures were acidified through adding 0.1 mL 32% HCl and the volumes were completed to the mark with acetonitrile. The colored solutions were scanned from 300 to 500 nm against a similarly treated blank. The absorbance values at 360 nm were utilized for construction of the calibration curve.

Analysis of Tablets:

Ten tablets of either Ebixa® or Ravamantine® tablets were accurately weighed, powdered and thoroughly mixed. An accurate weight of the finely powdered sample equivalent to 25 mg of MEM was extracted into 25 mL water with the aid of vortex mixing for 15 min then filtered into a 50 mL-volumetric flask. The residue was washed with 2 × 10 mL portions of water and washings were added to the filtrate. The filtrate was diluted to volume with the same solvent to reach a final concentration 0.5 mg/mL for MEM. Portions of this stock tablet extract were diluted with water to produce 0.1 and 0.25 mg/mL tablet extract solutions. For each method, aliquots from the appropriate solution were treated as described under *general procedure* and the contents of the tablets were calculated from the corresponding regression equation.

RESULTS AND DISCUSSION

Spectral Characteristics and Optimization of the Proposed Methods

Method I: Reaction with Eosin (ESN)

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MEM reacts instantly with ESN in acidic medium forming an orange-red ion-pair complex with a maximum absorption at 547 nm (Fig. 1). In general, limited aqueous solubility of the produced drug-eosin complexes is of major concern and such problem is usually observed in the spectrophotometric measurements as the reagent is of high concentration. Previous reports solved that problem by either extracting the complex with an organic solvent [22,23] or adding a non-ionic surfactant as methyl cellulose [24]. Alternatively, El-Brashy *et al*. [25] reported a simpler solution for such a problem, which is based on keeping the sample concentration at maximum dilution before adding the dye solution and mixing the solution well before the addition of the acetate buffer. In fact, this technique was later used for the determination of several drugs bearing basic centers [26,27]. Accordingly, by adopting the above procedure, the stability and solubility of the complex were achieved without the need of lengthy extraction steps or the use of non-ionic surfactants. In order to optimize the investigated reaction, different parameters were extensively studied in order to yield the highest and most reproducible absorbance readings. The acidic pH is a fundamental factor affecting the ionization of ESN thus allowing its interaction with MEM. For this reason, the use of different buffers (acetate, phosphate and citrate) was studied among which the acetate buffer gave the best results. Accordingly, this buffer was further tried in different ranges of molarity (0.1-0.5 M), volumes (0.2-1.6 mL) and pH values (3.2-4.4) and 1.2 mL of 0.2 M acetate buffer pH 3.6 gave the highest absorbance readings. Fig. 2 shows the effect of acetate buffer pH on the absorbance of the colored complex. Investigation of the effect of the reagent volume revealed that 1.4 mL of ESN reagent was sufficient to develop the color to its maximum intensity (Fig. 3). Finally, the colored complex is formed instantaneously; therefore, the absorbance readings were taken at zero time which offers the fast processing of large number of samples.

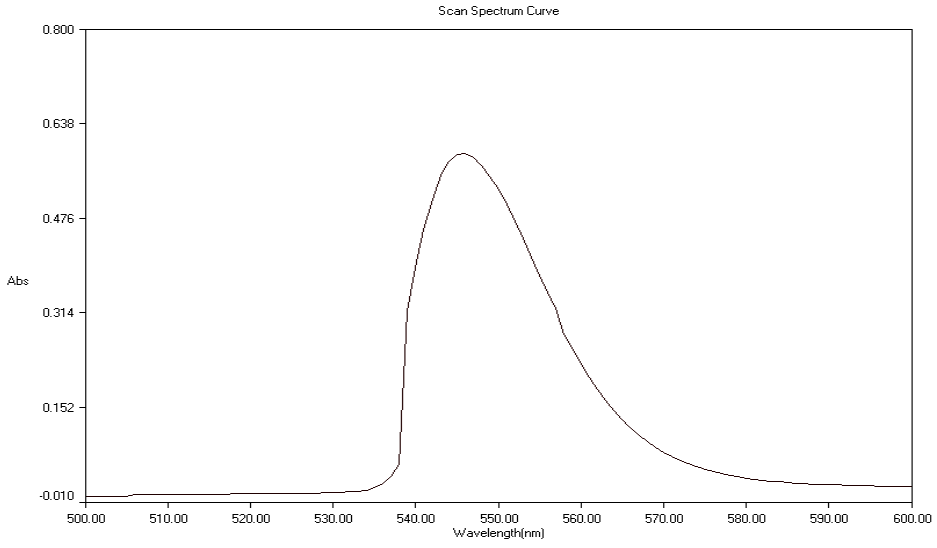

Figure 1: The absorption spectrum of the binary complex of 6 µg/mL MEM with ESN.

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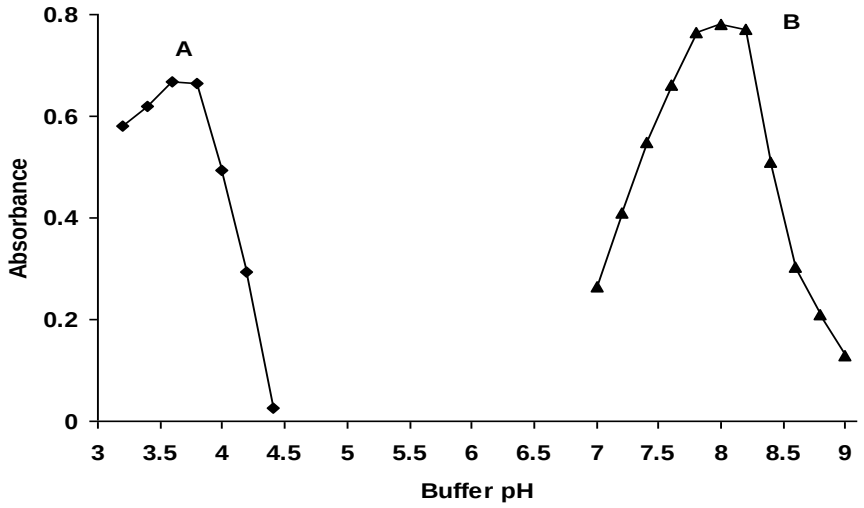

Figure 2: The effect of acetate buffer pH (A) and borate buffer pH (B) on the absorbance of the colored products of MEM with ESN and FDNB respectively.

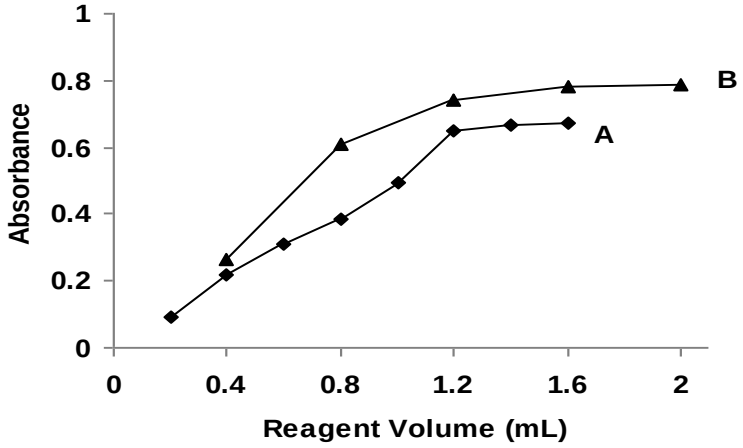

Figure 3: The effect of reagent volume (A = ESN , B = FDNB) on the absorbance of the colored products with MEM.

Method II: Reaction with 2,4-Dinitrofluorobenzene (FDNB)

FDNB is an active aryl halide that reacts with primary and secondary amines in alkaline medium through nucleophilic aromatic substitution reaction [30]. MEM was found to react with FDNB giving a yellow colored adduct which absorbs maximally at 360 nm (Fig. 4). Different experimental parameters affecting the reaction were carefully studied and optimized. As the reaction proceeds in alkaline medium, serial volumes (0.1-0.6 mL) of (0.2-0.5 M) borate buffer were tried in the pH range (7-9) and highest absorbance readings were obtained using 0.3 mL of 0.3 M, pH 8 buffer solution. Fig. 2 shows the effect of borate buffer pH on the absorbance of the colored product. The effect of the reagent volume was also investigated and 1.6 mL was

sufficient to develop full color and larger volumes provided no significant increase in the intensity of the measurements (Fig. 3). As for the effect of the temperature, it was found that the increase in temperature results in a corresponding increase in the absorbance intensity of the colored product, however, heating the solution mixture at 80 ºC gave the most reproducible results and was consequently set as the optimum temperature. Furthermore, the reaction mixtures were heated at the pre-studied temperature for different time periods (5-40 min) and the reaction was found to proceed to completion after 15 min of heating, consequently, a heating time of 20 min was chosen (Fig. 5). In addition, a volume of 0.1 mL 32% HCl was sufficient to remove the interference caused by the excess colored reagent through its hydrolysis into the corresponding colorless 2,4-dinitrophenol [31,32]. Finally, among all factors; the choice of the diluting solvent had the most profound effect on the sensitivity of the measurements. Methanol, ethanol, acetone and water were significantly less effective than acetonitrile, and therefore, acetonitrile was chosen as the optimum diluting solvent (Fig. 6).

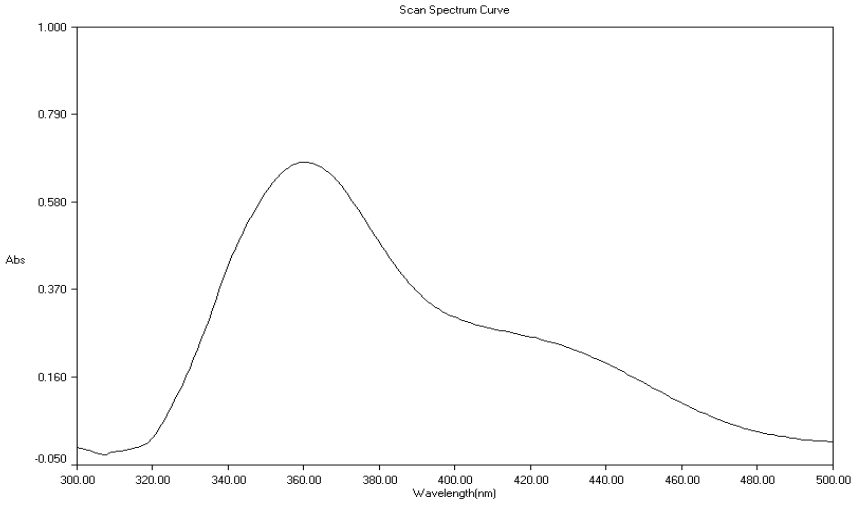

Figure 4: The absorption spectrum of the reaction product of 20 µg/mL MEM with FDNB.

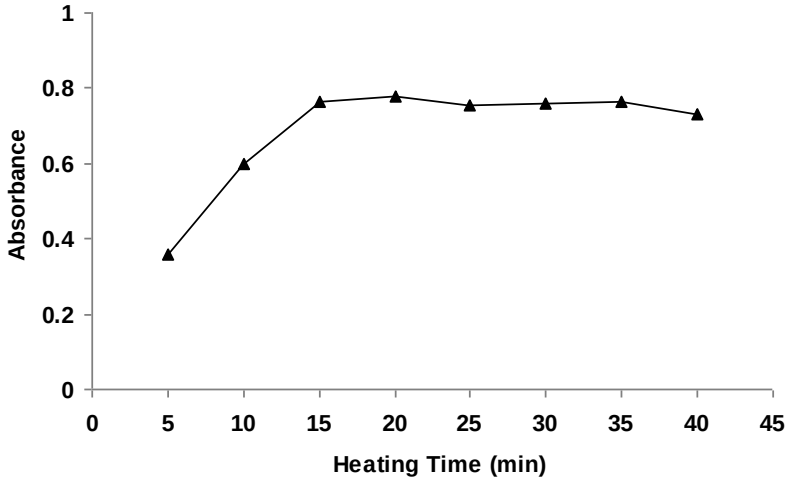

Figure 5: The effect of heating time on the reaction of MEM with FDNB.

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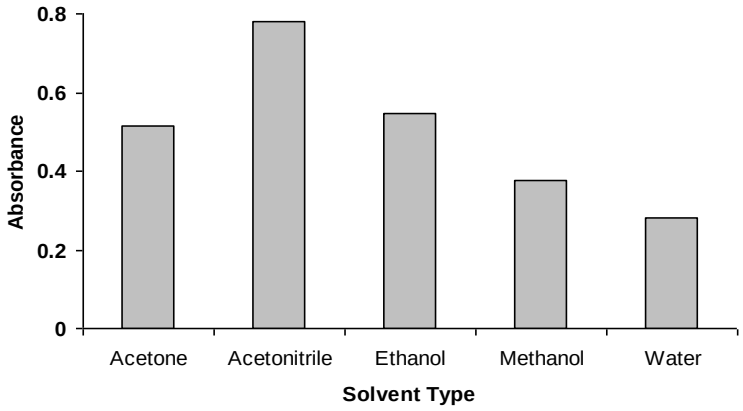

Figure 6: The effect of the solvent type on the reaction of MEM with FDNB

Pathways of the Reactions

MEM is an aliphatic compound bearing only a primary amino group which has always represented the target for derivatization in the attempts of increasing the sensitivity of its determination. ESN bears a carboxylic group and a phenolic group; the latter is highly affected by the presence of the 4-electron withdrawing bromine atoms. As a result, the pK_a(OH) becomes less than pK_a(COOH) (2.02 and 3.8, respectively) [36]. Therefore, at pH 3.6 the OH group becomes most likely fully ionized and thus electrostatically interacts with the primary amino group of MEM forming the colored complex. On the other hand, the yellow product formed upon the reaction of MEM and FDNB is due to the nucleophilic attack of MEM primary amino group on FDNB (nucleophilic substitution reaction). The suggested reaction pathways are outlined in the schemes illustrated in Fig. 7.

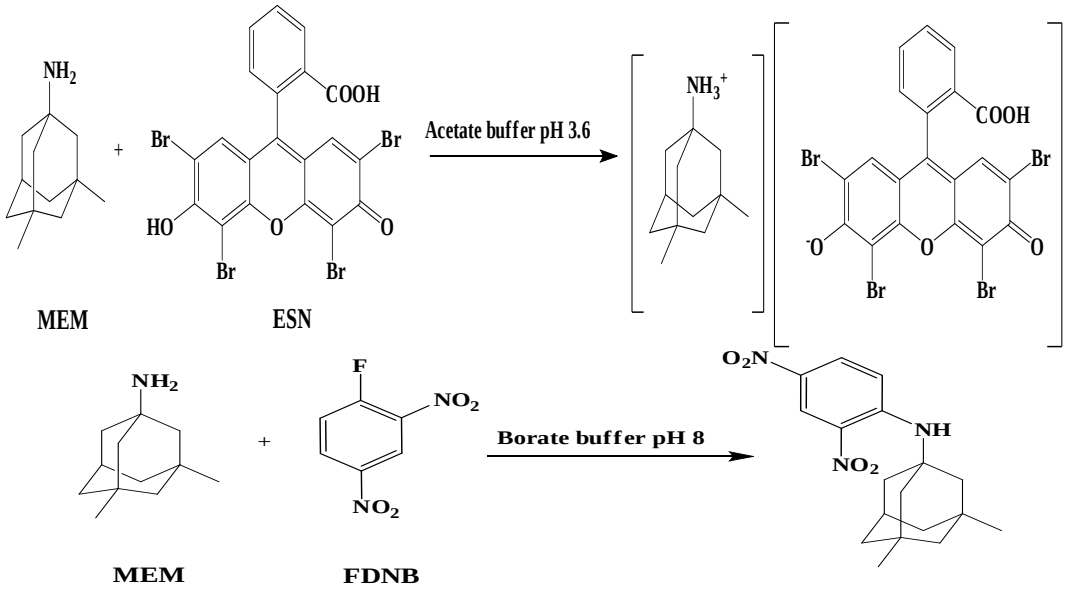

Figure 7: Proposed pathways for the reactions of MEM with ESN (Method I) and FDNB (Method II).

ISSN: 0975-8585

Validation of the Proposed Methods

Linearity and Concentration Ranges

In order to assess the linearity of each method, calibration curve was constructed by plotting the produced response versus the appropriate concentration and the regression parameters were calculated by the use of the least square regression model. As seen from Table 1; the good linearity of either methods can be verified by the high value of the correlation coefficient in conjunction with the low values of the intercept, standard deviation of the intercept (S_a), standard deviation of the slope (S_b) and RSD% of the slope (S_b%) which was in both methods less than 2%. The low values of the standard deviation of residuals ($S_{y/x}$) also confirm the insignificant differences between the found and the calculated y-values and hence the negligible scatter of the practical points around the best fitted line. Additionally, the regression lines showed high F values indicating an increase in the mean of squares due to regression (steeper regression lines) and low significance F values indicating a decrease in the mean of squares due to residuals.

Limits of Detection and Quantification

The limit of detection (LOD) and the limit of quantification (LOQ) were calculated in accordance to the equations provided by the ICH guidelines [37]. They are defined as 3.3 S_a/b and 10 S_a/b, respectively, where S_a is the standard deviation of the intercept and b is the slope of the calibration curve. The low values obtained indicate the high sensitivity of the proposed methods and the slightly higher sensitivity of the ESN method in comparison with the FDNB method (Table 1).

Table 1 Regression and Statistical Parameters for the Determination of MEM Using ESN and FDNB Methods

Parameter	Method I ESN	Method II FDNB
Wavelength (nm)	546	360
Concentration range (µg/mL)	1-10	5-30
Molar absorptivity (L mol^{-1} cm^{-1})	19249	6301
Intercept (a)	0.0447	0.0558
S_a [a]	0.0088	0.0112
Slope (b)	0.0892	0.0292
S_b [b]	1.46×10^{-3}	5.70×10^{-4}
RSD% of the slope (S_b%)	1.64	1.95
Correlation coefficient (r)	0.99946	0.99923
$S_{y/x}$ [c]	0.0114	0.0120
F [d]	3711	2584
Significance F	4.35×10^{-7}	8.97×10^{-7}
LOD [e] (µg/mL)	0.33	1.27
LOQ [f] (µg/mL)	0.99	3.84

[a] Standard deviation of the intercept
[b] Standard deviation of the slope
[c] Standard deviation of residuals

[d] Variance ratio, equals the mean of squares due to regression divided by the mean of squares about regression (due to residuals)
[e] Limit of detection
[f] Limit of quantification

Accuracy and Precision

For each method, the within-day (intra-day) precision and accuracy were examined by analyzing three concentrations using three replicate determinations for each concentration within one day. Similarly, the between-day (inter-day) precision and accuracy were tested by analyzing the same three concentrations using three replicate determinations repeated on three days [37]. The adequate recovered concentrations calculated from the corresponding regression equations confirm the accuracy of the proposed methods (Table 2). The percentage relative standard deviation (RSD %) and percentage relative error (E_r %) did not exceed 2.0 % proving the high repeatability and accuracy of the developed methods for the estimation of MEM in its bulk form (Table 2).

Robustness

The robustness of the proposed methods was examined by evaluating the influence of small deliberate variations of the reaction conditions as buffer pH, buffer volume, reagent volume, heating temperature and time. These variables did not have any significant effect on the measured responses as seen from the absorbance values gathered in Table 3. In addition, the RSD % in all cases did not exceed 3% indicating the reliability of the proposed methods during routine work.

Table 2 Precision and Accuracy for the Determination of MEM Using ESN and FDNB Methods

Method	Nominal value (µg/mL)	Within-day			Between-day		
		Found ± SD[a] (µg/mL)	RSD(%)[b]	E_r(%)[c]	Found ± SD[a] (µg/mL)	RSD(%)[b]	E_r(%)[c]
Method I ESN	3	2.97 ± 0.03	1.01	-1.00	3.05 ± 0.05	1.64	1.67
	6	6.02 ± 0.08	1.33	0.33	5.96 ± 0.10	1.68	-0.67
	9	8.91 ± 0.05	0.56	-1.00	8.95 ± 0.12	1.34	-0.56
Method II FDNB	10	9.86 ± 0.10	1.01	-1.40	10.04 ± 0.12	1.20	0.40
	20	20.18 ± 0.27	1.34	0.90	20.08 ± 0.35	1.74	0.40
	30	29.86 ± 0.29	0.97	-0.47	29.50 ± 0.43	1.46	-1.67

[a] Mean ± standard deviation for three determinations.
[b] % Relative standard deviation.
[c] % Relative error.

Table 3 Robustness of the Proposed Spectrophotometric Methods for the Determination of MEM

Reagent	Parameters	Absorbance ± SD[a]	RSD(%)
Method I ESN	Buffer pH 3.6 ± 0.1	0.411 ± 0.006	1.46
	Buffer Volume (mL) 1.2 ± 0.2	0.410 ± 0.007	1.71
	Reagent Volume (mL) 1.4 ± 0.2	0.395 ± 0.011	2.78
Method II FDNB	Buffer pH 8 ± 0.1	0.777 ± 0.016	2.06
	Buffer Volume (mL) 0.3 ± 0.05	0.770 ± 0.018	2.34
	Reagent Volume (mL) 1.6 ± 0.2	0.775 ± 0.005	0.65
	Heating Temperature (ºC) 70 ± 2	0.780 ± 0.017	2.18
	Heating Time (min) 20 ± 3	0.771 ± 0.014	1.82

[a] Average ± standard deviation for three absorbance values.

Stability of Standard Solutions and Reaction Products

The standard solutions of MEM were found stable for 2 weeks when stored at 4 ºC. Furthermore, the reaction products of both methods were stable for at least 2 hours.

Application to Tablet Dosage Forms

In order to test the applicability of the proposed methods, commercial preparations were analyzed as previously described and MEM recoveries were calculated from similarly treated external standards. The recovered concentrations were in good agreement with the label claim and the assay revealed satisfactory precision as seen from the values of RSD % gathered in Table 4. A reference spectrophotometric method was applied for the estimation of MEM in its commercial products [18]. For each pharmaceutical preparation, the results of the proposed methods were statistically compared with those of the reference method using the one-way analysis of variance test (Single factor ANOVA) [38]. The ANOVA test is a useful statistical tool for comparing recovery data obtained from more than two methods of analysis. For each preparation, the results obtained from the proposed methods were statistically compared with one another as well as with the reference method and the calculated F values were always less than the critical one (Table 4). In addition, possible interference from excipients was tested through the standard addition technique, where the tablet extracts were spiked with known amounts of the appropriate MEM standard solution. The acceptable recovered values and RSD % confirm that inactive ingredients do not interfere with the proposed methods. In conclusion, it could be inferred from the data gathered in Table 4 that both methods are applicable for the routine analysis of MEM with comparable analytical performance, and are suitable for the quality control of MEM in tablet dosage forms.

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Table 4 Analysis of MEM in tablets by the proposed spectrophotometric methods and the reference method

Using external standard analysis						
Method / Results	**Method I ESN**		**Method II FDNB**		**Reference Method**	
Ebixa® tablets						
%Recovery ± SD[a]	98.84 ± 0.64		99.12 ± 0.78		98.56 ± 0.95	
RSD%[b]	0.65		0.79		0.96	
ANOVA (single factor)						
Source of Variation	SS	df	MS	F	P-value	F critical
Between Groups	0.79570	2	0.39785	**0.62691**	0.55086	**3.88529**
Within Groups	7.61549	12	0.63462			
Total	8.41119	14				
Ravamantine® tablets						
%Recovery ± SD[a]	99.15 ± 0.76		100.13 ± 1.04		99.60 ± 0.87	
RSD%[b]	0.77		1.04		0.87	
ANOVA (single factor)						
Source of Variation	SS	df	MS	F	P-value	F critical
Between Groups	2.43165	2	1.21583	**1.50593**	0.26090	**3.88529**
Within Groups	9.68831	12	0.80736			
Total	12.11996	14				

Using standard addition analysis		
Method / Results	**Method I ESN**	**Method II FDNB**
Ebixa® tablets		
%Recovery ± SD[a]	100.08 ± 0.73	99.53 ± 0.99
RSD%[b]	0.73	1.00
Ravamantine ® tablets		
%Recovery ± SD[a]	99.86 ± 0.92	99.78 ± 1.21
RSD%[b]	0.92	1.21

[a] Mean ± standard deviation for five determinations.
[b] % Relative standard deviation.

CONCLUSION

This work describes the development and validation of two colorimetric methods for the determination of memantine hydrochloride in its pure and dosage forms. The proposed methods offer high level of sensitivity without compromising the simplicity and low cost of the analysis. Therefore, both methods can be regarded as cost-effective methods adequate for the routine quality control purposes. The proposed methods are superior or comparable to the already available spectrophotometric methods [18-21] regarding sensitivity of measurement and/or simplicity (no extraction with harmful organic solvents such as chloroform). In addition, the reliability and robustness of the developed methodologies were confirmed through different validation parameters. Statistical comparison of the results obtained from the analysis

of commercially available tablets by the proposed methods did not significantly differ from those obtained from the reference method.

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