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DEVELOPMENT AND VALIDATION OF SPECTROFLUOROMETRIC, SPECTROPHOTOMETRIC AND THIN LAYER CHROMATOGRAPHY STABILITY INDICATING METHODS FOR ANALYSIS OF TOPIRAMATE

Nahla Nour El-Din Ahmed Salama*, Afaf Osman Mohamed and Elham Anwer Taha
National Organization for Drug Control and Research, 6 Abou Hazem Street, Pyramids Ave, P.O.Box 29 Giza, `Egypt. (NODCAR)
Email: salama_nahla2004@hotmail.com

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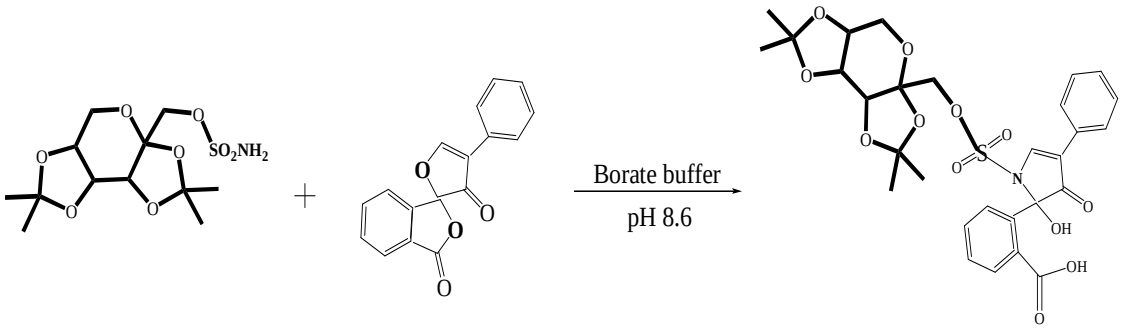
Abstract

Three selective, inexpensive and validated methods were developed and optimized for determination of antiepileptic drug, topiramate in drug substance and drug product as well as in the presence of its acid, base and thermal degradants. The first method is micelle-enhanced spectrofluorimetric assay based on the reaction between the studied drug and fluorescamine to give highly fluorescent derivative that was measured at 470 nm using an excitation wavelength 388 nm. The linearity range was found to be 0.01 – 0.10 µg mL^{-1} with mean recovery 98.5% ± 1.10. The second method is based on the reaction of the primary amino group of topiramate with ninhydrin reagent in ethanolic medium in the presence of 50 mM sodium bicarbonate. The colored product was measured at 570 nm. The linearity range was found to be 4 – 40 µg mL^{-1} with mean recovery 99.20%±1.08. All variables affecting the reaction conditions were thoroughly studied. The third method is based on separation of topiramate from its degradants (Acid, base, thermal). The results were found to agree statistically with those obtained from the official HPLC method (USP 2010). Furthermore, the methods were validated according to the ICH guidelines. The proposed methods are practical and valuable for their in quality control laboratories for analysis of topiramate.

***Keywords*:** Topiramate; Fluorescamine; ninhydrin; TLC; Drug product; Stability

1. Introduction:



Topiramate Fluorescamine Fluorophore

Scheme 1: Proposal reaction between fluorescamine and topiramate.

Topiramate Scheme-1, a sulfamate-substituted monosaccharide (2,3:4,5-bis-O-(-1-methyl)-[beta]-D-fructopyranose sulfamate) is a new second generation antiepileptic agent[1-4]. The drug is structurally different from other anticonvulsants and has been proved in partial and generalized tonic-clonic seizure [5]. Topiramate has no ultraviolet, visible or fluorescence absorption and available methods for analysis of the drug in biological fluids and pharmaceutical products, consisted of gas chromatography (GC) coupled with flame ionization (FID) or nitrogen phosphorous detection (NPD) [6-8], fluorescence polarization immunoassay [9]. HPLC methods, including, ionic chromatography [10], or using refractive index (RI) or chemiluminescent nitrogen or MS detector are described [11-13]. Q NMR stability indicating method was also reported for its determination [14]. Analysis of the drug in human plasma following derivatization with 9-fluorenylmethyl chloroformate (FMOC-Cl) or 4-Chloro-7-nitrobenzofurazan (NBD-Cl) using fluorescence or UV detection have been reported [15-18]. The aim of this work is to follow the acid, base and thermal degradation of Top under stress conditions according to the ICH guidelines, and develop stability-indicating spectrofluorometric and spectrophotometric methods for determination of top in the presence of degradants. In addition, a TLC method is proposed for separation of Top from its degradants. The methods are alternatives and comparable in specificity and accuracy to chromatographic methods, which although highly specific and accurate, are more time consuming, performed in several steps, used environmentally hazardous solvents, and are rather expensive.

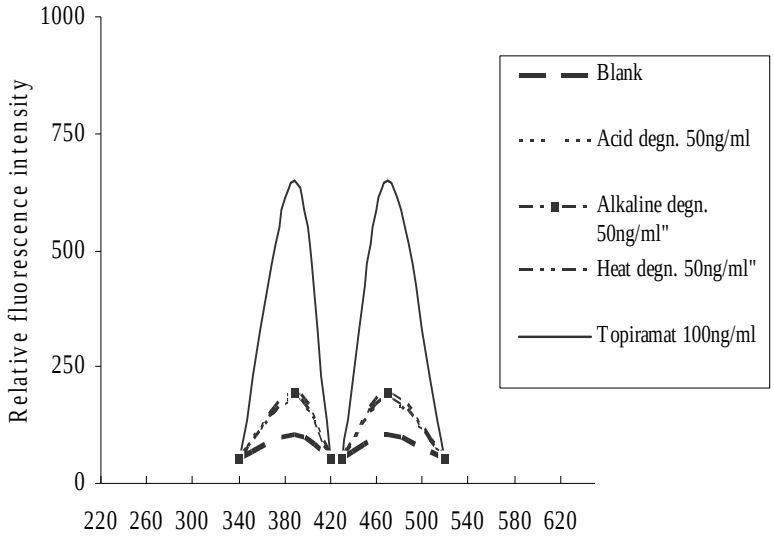


Fig-1: Excitation and emission spectra of the reaction products of topiramate (100 ng ml^{-1}) and its degradants (λ_{em} 470 nm, λ_{ex} 388nm).

2. Experimental:

2.1. Apparatus

Shimadzu Model RF-1501 spectrofluorometer equipped with xenon lamp and 1cm quartz cell was used for all measurements. Wavelength calibration was performed by measuring λ_{Ex} 388 nm and λ_{Em} 470 nm. Hanna Digital pH meter model 8417 was used for adjusting the pH. Shimadzu Model RF-1601, UV/VIS spectrophotometer was used. Pre-coated TLC plates (10 x 10 cm, aluminum plate coated with 0.25 mm silica gel F254 were purchased from Merck Co., Egypt. Samples were applied to the TLC plates with 25 µL Hamilton microsyringe. UV short wavelength lamp (Desaga Germany) was used**.**

2.2. Reagents and materials

- Topiramate certified to contain 99.40 %, according to USP 2010[19], was kindly supplied from Delta Pharma Co. Egypt.
- Delpiramate tablets, labeled to contain 100 mg topiramate per tablet was kindly supplied by Delta Pharma (Tenth of Ramadan City, Egypt).
- Fluorescamine(FC), Sigma Co., (1.0% w/v) in acetone aging for 24 hr.

- Borate buffer pH 8.6 (BP 2010).
- Sodium lauryl sulphate, Sigma Aldrich, St. Louis, USA.
- Tween 80, Tween 40, Merck, Munich, Germany.
- Triton® X 100, Loba-Chemie Indoaustranal, Co., India.
- Cetyl pyridinium bromide, Sigma Aldrich, St., Louis, USA.
- Ninhydrin BDH Co.,(2.0% w/v) in ethanol.
- Sodium bicarbonate, Adwic Co., Egypt, 0.05 M (BP 2010).
- Ethanol and acetone (Lab-Scan).

All other chemicals were of analytical grade.

2.3. Standard Solutions

An accurately weighted amount about 100 mg of Top was transferred in to 25 ml volumetric flask, dissolved in 20 ml ethanol, completed to the volume with the same solvent to obtain stock solution 4 mg ml^{-1}. This stock solution was further diluted with water and ethanol to obtain working solutions in the range of 0.1 - 1.0 μg ml^{-1} for FC, and 40 - 400 μg ml^{-1} for ninhydrin based methods respectively. All solutions were stored in refrigerator at 4 ^{o}C.

2.4. Preparation of degradants (acid, base, thermal)

The degradants were laboratory prepared as mentioned by heating 50 mg mL^{-1} of Top drug substance in 50 mL of 0.1 M HCl or 0.1 M NaOH on hot plate at 80 ^{o}C for 2 hrs [10, 14], while for thermal degradants the drug was kept in dry oven at 100 ^{o}C for 8 hrs[13]. The degradants were monitored by TLC, fluorescamine and ninhydrin based methods.

2.5. Analytical procedures

1- Fluorescamine based method

Aliquots of Top standard solution (0.1 mg mL^{-1}) containing in to transferred series of 10 mL volumetric flasks. To each flask 5 mL borate buffer pH 8.6 and, 0.1 mL surfactant 0.04% w/v (Tween 80) were added. The flasks were shaken and 1 mL of fluorescamine (1.0 % w/v in acetone) was added to each flask, made up to the volume

with water. The solution was shaked for five minutes and set a side for 15 min. The fluorescence intensity was measured at λ_{em}470 nm after excitation at λ_{em}388 nm against reagent blank prepared similarly omitting the drug.

2-Ninhydrin based method

Aliquots of Top standard solution (4 mg mL^{-1}) in ethanol containing 40 – 400 µg mL^{-1} were transferred into a series of stopper test tubes. To each tube 1 mL of 0.05 M $NaHCO_3$ and 2 mL of ninhydrin reagent (2.0% w/v in ethanol) were added. The tubes were heated for 15 min at 90 ^oC on water bath, cooled and transferred quantitatively in to 10 mL volumetric flasks. The volume was completed with ethanol/ 0.05 M $NaHCO_3$(50:50v/v). The absorbance was measured at 570 nm against reagent blank prepared similarly omitting the drug.

3-TLC based method

The TLC plates were developed in chloroform-dichloromethane-acetic acid, 4:4:2, (*v/v*). The chromatographic tank was saturated with the mobile phase for 15 minutes before development of the plates. For separation and detection 20 µL of Top (2 mg ml^{-1}) and 20 µL of each degradant(acid, base and heat)of Top were applied as separate compact spots 20 mm apart and 10 mm from the bottom of the TLC plates using a 25 µL Hamilton micro syringe. The chromatograms was developed up to 8 cm in the usual ascending way, air dried, and visualized by dipping in potassium permenganate solution. For acid degradants three spots were detected with $K_2M_nO_4$ with R_f values zero, 0.29 and 0.51. For base degradants two spots were appeared with R_f 0.51 and zero. While for dry heat one spot with R_f zero and another one has the R_f value similar to drug. The R_f value of drug was appear at 0.65.

2.6. Analysis of laboratory prepared mixtures

To assess the stability nature of the proposed methods, synthetic mixtures containing different ratios of Top and its degradants were analyzed by the proposed spectrofluorometric and spectrophotometric methods.

2.7. Analysis of topiramate in drug product

Ten tablets (claimed to contain 100 mg / tablet) were finally powdered**;** an accurately weighed amount of powdered tablets equivalent to 400 mg of Top was transferred into 150 mL beaker and extracted into sufficient volume of ethanol (80 mL). The content was stirred with magnetic stirrer for 15 min and transferred

quantitatively to 100 mL volumetric flask. The volume was completed with the same solvent, mixed well and filtered. The first portion of the filtrate was rejected. The prepared solution was diluted quantitatively with distilled water and ethanol/0.05 M $NaHCO_3$(50:50v/v) to obtain a suitable concentration for FC and ninhydrin methods respectively.

3. Results and discussion:

3.1. Strategy for Assays Development, Involved Reaction, and Spectral Characteristics.

Because of the absence of any chromophoric group in the top molecule, it has no absorption in the ultraviolet-visible region, and it has no native fluorescence as well. Therefore, direct spectrofluorometric and spectrophotometric determination of Top were not possible. Therefore, derivatization of Top was attempted in the present study for the development of both spectrofluorometric and spectrophotometric methods for its determination. Fluorescamine has been used as fluorogenic reagent for primary amines while ninhydrin was used as chromogenic reagent for primary and secondary amines [20 - 23], however, its reaction with Top has not been investigated yet. Therefore, the present study was devoted to explore FC and ninhydrin as a derivatizing reagent in the development of spectrofluorometric and spectrophotometric methods for the determination of Top in tablets and in the presence of its acid, base, and thermal degradants. Our preliminary experiments in investigating the reaction between Top and each of FC and ninhydrin revealed that FC-Top derivative exhibiting a highest fluorescence intensity at 470 nm after excitation at 388 nm. While, ninhydrin-derivative was found to has maximum absorption at 570 nm. Scheme 1 shows the reaction pathway between Top and FC, and Figure 1 shows the excitation, and emission spectra of the reaction products.

While Figure.2 shows the absorption spectra of ninhydrin-Top reaction products. The following sections describe the optimization of the assay variables and validation for the performance of both spectrofluorometric and spectrophotometric methods. Moreover, TLC method was used to monitor and detect Top degradants (acid, base and thermal).

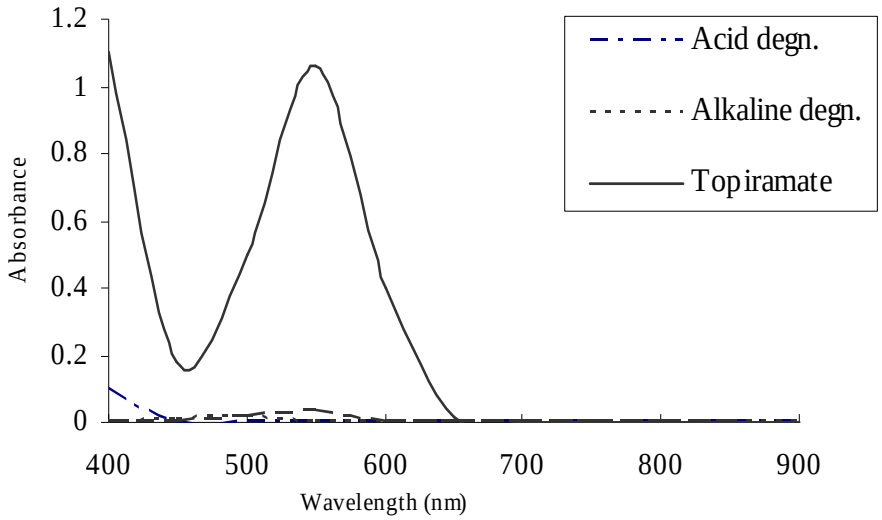


Fig-2: Zero order absorption spectra of topiramate--ninhydin reaction products 40 µg ml^{-1}, and its degradants 20 µg ml^{-1}.

3.2. Method Development:

3.2.1. Optimization of Derivatization Reaction and Spectrofluorometric Procedure: The factors affecting the derivatization reaction (the concentrations of FC and pH, surfactants, reaction time, and the diluting solvents) were investigated by altering each variable in a turn while keeping the others constant. The studying of FC concentrations revealed that the reaction was dependent on FC reagent (Figure 3). The highest fluorescence intensity was attained when the concentration of FC was 0.3 ml of 1.0 % (w/v) in the final solution. The results of investigating the effect of pH on the reaction revealed that the optimum pH was 8.6 (Figure 4). The effect of surfactants on the fluorescence intensity was studied by carrying out the reaction using different surfactants and the highest fluorescence was obtained with Tween 80 (Figure 5). Different solvents were tested for dilution, the highest fluorescence intensity was obtained when water was used for dilution.

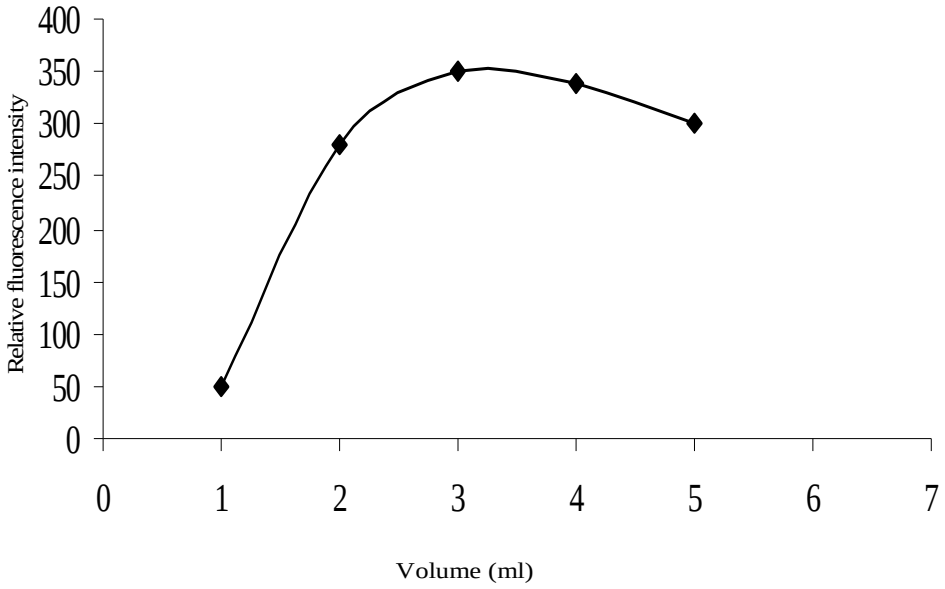


Fig-3: Effect of fluorescamine (1.0% w/v) on the formation of topiramate fluorescamine reaction product (100ng ml^{-1})

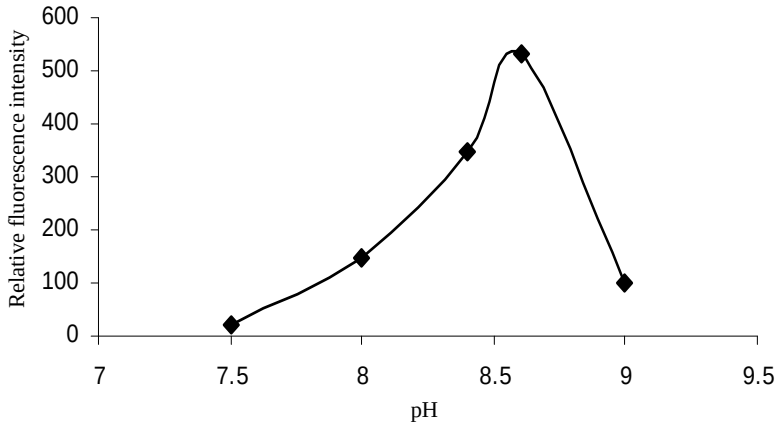


Fig-4: Effect of pH on the formation of colored reaction product of topiramate (100 ng ml^{-1}) with fluorescamine.

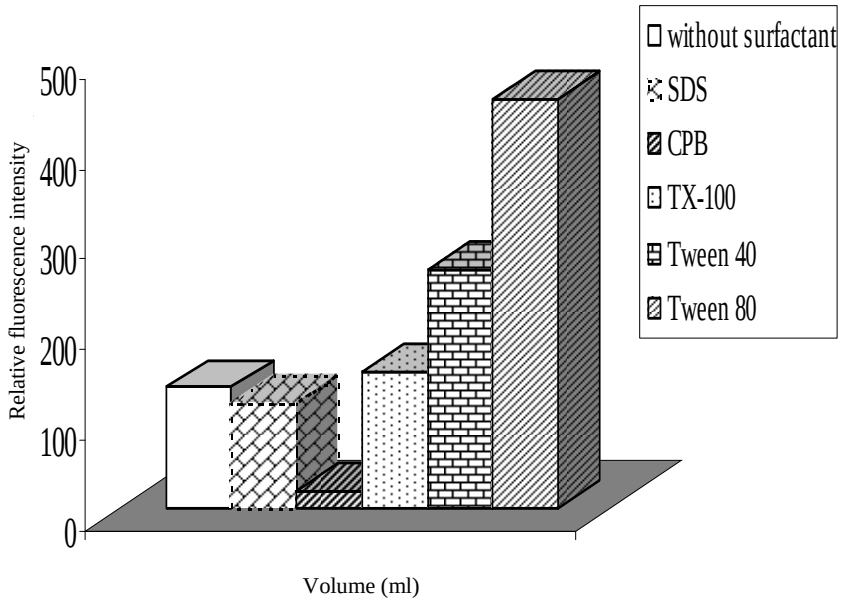


Fig-5: Effect of 1 ml of 0.04% (w/v) of different surfactants on the formation of colored reaction product of topiramate (100 ng ml $^{-1}$) with fluorescamine.

3.2.2. Optimization of Spectrophotometric Procedure: The factors affecting the derivatization reaction (the concentrations of ninhydrin and molarity of sodium bicarbonate, heating time, and the diluting solvents) were investigated by altering each variable in a turn while keeping the others constant. The studying of ninhydrin concentrations revealed that the reaction was dependent on ninhydrin reagent (Figure 6). The maximum absorption intensity was attained when the concentration of ninhydrin was 0.2 mL of 2.0 % (w/v) in the final solution. The results of investigating the effect of different molarity of $NaHCO_3$ on the reaction revealed that the optimum molarity was 0.05 M (Figure 7). The effect of heating time on the absorption intensity was studied by carrying out the reaction at 90 ^{o}C for different time intervals the highest absorption was obtained after 30 min (Figure 8). Different solvents were tested for dilution the highest fluorescence intensity was obtained when water/ 0.05 M $NaHCO_3$ (50/50) was used.

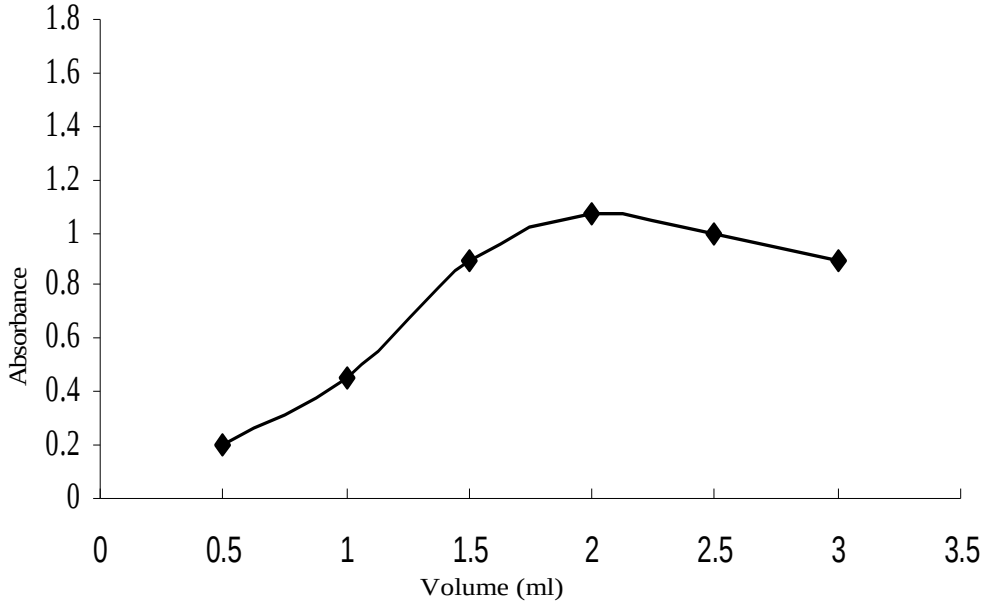


Fig-6: Effect of different volume of ninhydrin (2.0% w/v) on the formation of colored reaction product with topiramate (40 µg ml^{-1}).

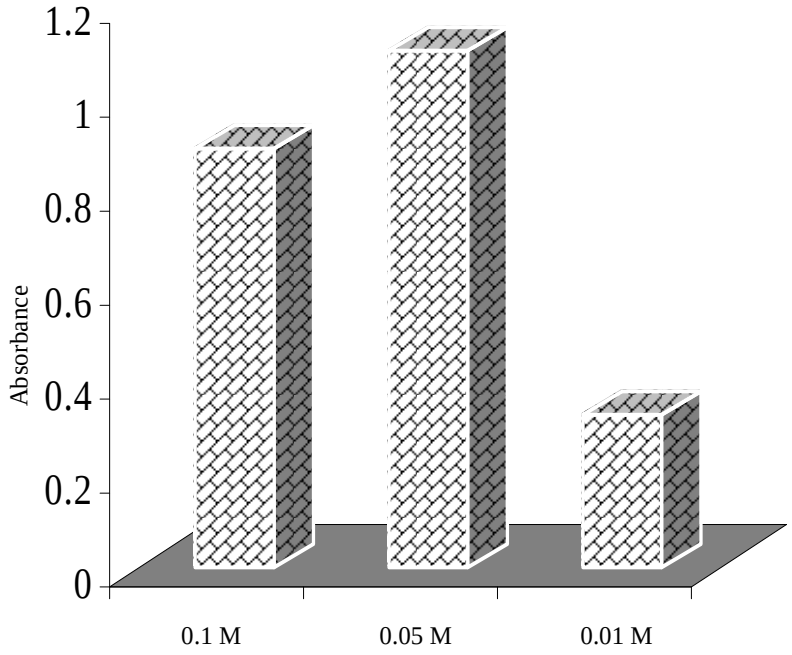


Fig-7: Effect of different molarity of sodium bicarbonate on the formation of colored reaction product with topiramate (40 µg ml^{-1}).

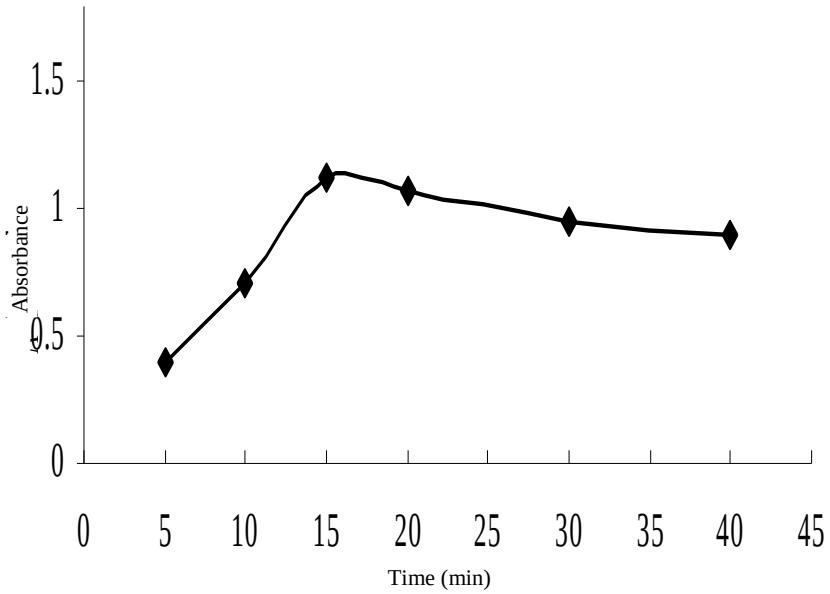


Fig-8: Effect of heating time in boiling water bath on the formation of topiramate ninhydrin colored reaction product (40 µg ml $^{-1}$).

3.3. Stoichiometry of Derivatization Reaction: Under the optimum conditions, the stoichiometry of the reaction between Top and ninhydrin was investigated by Job's method [24] and was found to be 1 : 1 because Top molecule contains only one center (primary amino group) available for this reaction.

3.4. Method Validation:

3.4.1. Linearity, Limits of Detection and Quantitation: In the proposed methods, linear plots (n = 6) with good correlation coefficients (0.9991 and 0.9997) were obtained in the concentration ranges of 0.01 – 0.1 µg ml^{-1} and 4 – 40 µg ml^{-1} for the spectrofluorometric and the spectrophotometric methods, respectively (Table 1). The limits of detection (LOD) and quantitation (LOQ) were determined [25] using the formula LOD or LOQ = κSDa/b, where

κ = 3.3 for LOD and 10 for LOQ, SDa is the standard deviation of the intercept, and b is the slope. The LOD and LOQ values were 1.09 and 3.60 ng ml^{-1} for the spectrofluorometric and spectrophotometric methods, respectively, While from 0.93 - 3.07 µg ml $^{-1}$ (Table 1).

Table-1: Results of assay validation obtained by applying the proposed spectrofluorimetric and spectrophotometric methods for the determination of topiramate in drug substance.

Parameters	Spectrofluorimetric method	Spectrophotometric method
Linearity range	10 - 100 ng ml^{-1}	4 - 40 µg ml^{-1}
LOD	1.09 ng ml^{-1}	0.93 µg ml^{-1}
LOQ	3.60 ng ml^{-1}	3.07 µg ml^{-1}
Precision		
Repetability [a] RSD%	100.33 ± 1.70	98.50 ± 1.06
Intermediate precision[a] RSD%	99.00 ± 2.00	98.76 ± 1.54
Accuracy mean[b]±RSD%	101.50 ± 1.87	100.50 ± 1.33
Specificity mean±RSD%	102.40 ± 2.02	99.50 ± 1.10
Regression		
Slope	0.20	0.024
SE of slope	0.095	2. 9 x 10^{-4}
Intercept	5.45	-1.9 x 10^{-2}
SE of intercept	27.02	0.70 x 10^{-3}
Correlation coefficient	0.9991	0.9997
SE of estimation	7.66	9.1 x 10^{-2}

[a] n=9, [b] n=5.

3.4.2. Specificity: To assess the stability indicating efficiency of the proposed methods, the degradants acid, base and thermal were mixed with its intact drug substance in different ratios and the mixtures were analyzed by the proposed methods. The results of interferences study showed that no interferences from 1 - 10 % and 5 - 60% for FC and ninhydrin methods respectively as presented in Table 2.

Table-2: Specificity of the proposed spectrofluorimetric and spectrophotometric methods for analysis of topiramate in laboratory prepared mixtures with their corresponding degradants.

Degradants %	% Recovery[a] of intact drug	
	Spectrofluorimetric method	Spectrophotometric methods
1	101.50	-
5	100.50	98.65
10	99.00	99.06
20	-	100.50
30	-	98.00
40	-	99.87
60	-	100.06
Mean ± RSD%	100.33 ± 1.25	99.36 ± 0.95

[a]Average of three different experiments.

3.4.3. Precision and Accuracy: The precision of the proposed methods was determined by triplicate analysis of three separate sample solutions at three concentration levels of Top. The relative standard deviations (RSDs) were 1.7–2.0 and 1.06 – 1.54% for the spectrofluorometric and spectrophotometric methods, respectively (Table 1), indicating the good reproducibility of the proposed methods. The accuracy of the proposed methods was evaluated by the recovery percentages. It is also assessed by statistical comparisons between the results obtained by applying the proposed procedures and the official method [19] of drug substance (Table 3).

Table-3: Statistical comparison between the results obtained by the proposed methods and official methods for analysis of topiramate in drug substance.

Values	Spectrofluorimetric method	Spectrophotometric method	Official method[a]
Mean	98.5	99.20	100.90
SD	1.60	1.08	1.32
Variance	2.56	1.17	1.74
SE	0.70	0.48	0.59
n	5	5	5
t(2.306)[b]	2.2	2.00	
F(6.39)[b]	1.47	1.50	

[a]HPLC-RI detector (BP 2010).
[b]The values between parenthesis are the theoretical values of t and F at (p=0.05).

3.4.4. Robustness and Ruggedness: Robustness was examined by evaluating the influence of small variation of method variables, including concentration of analytical reagents and reaction time on the performance of the proposed methods. In these experiments, one parameter was changed whereas the others were kept unchanged, and the recovery percentage was calculated each time. It was found that small variation of method variables did not significantly affect the procedures. This provided an indication for the reliability of the proposed method during its routine application for the analysis of Top. Ruggedness was also tested by applying the proposed methods to the assay of Top using the same operational conditions but using two different instruments at two different laboratories and different elapsed time. Results obtained from lab-to-lab and day-to-day variations were found to be reproducible.

3.5. Application of the Proposed Methods to Analysis of Top in tablets: It is evident from the above-mentioned results that the proposed methods gave satisfactory results with Top in drug substance. Thus, its tablets were subjected to the analysis of active ingredient by the proposed methods. The percentage recoveries were found to

be 98.70±1.79 and 98.91±1.93% of claimed amount for the spectrofluorometric and spectrophotometric methods, respectively (Table 4). These results were compared with those obtained from the official HPLC method [19] with respect to accuracy and precision. Also the accuracy of the methods was assessed by application of the standard addition technique (Table 5).

Table-4: Comparison between the proposed methods and the reported method for the determination of topiramate in its drug product.

Preparation	Spectrofluorimetric method Mean recovery[a] of claimed amount ± RSD%	Spectrophotometric method Mean recovery[a] of claimed amount ± RSD%	Reported method Mean recovery[a] of claimed amount ± RSD%
Delpiramate 100mg Top /tab.	100.80 ± 1.97	100.50 ± 1.63	100.30 ± 1.61

[a]Average of five different experiments.
[b] HPLC -RI detector, procedure of the manufacturer supplied by Delta Pharma Co., Egypt, by personal communication.

Table-5: Results of application of standard addition technique for the determination of topiramate by the proposed methods in drug product.

Preparation	Spectrofluorimetric method		Spectrophotometric method	
	Pure added ng/ml	Found recovery[a]% ±RSDS	Pure added µg/ml	Found recovery[a]% ± RSD
Delpiramate 100mg Top /tab.	20 40 60	101.20 ± 1.65 102.50 ± 1.96 100.80 ± 1.88	8 16 32	98.85 ± 1.18 100.5 ± 1.46 99.65 ± 1.86

[a]Average of four different experiments.

Conclusion:

The present study described the use of FC and ninhydrin reagents for the development of selective, sensitive, and accurate spectrofluorometric and spectrophotometric methods for the determination of Top in drug substance,

drug product, and in the presence of its acid, base and thermal degradants. To our knowledge no spectrofluorimetric or spectrophotometric methods have been reported before for analysis of Top.

The proposed procedures are with comparable analytical performance devoid from any potential interference. This gives the advantage of flexibility in performing the analysis on any available instrument. Furthermore, all the analytical reagents are inexpensive, have excellent shelf life, and are available in any analytical laboratory. Therefore, these methods can be recommended for the routine analysis of Top in quality control laboratories.

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Corresponding Author:
Dr Nahla Nour El Din Ahmed Salama*,
Ph D in Pharmaceutical Sciences (Pharmaceutical Analytical Chemistry),
Head of Raw Materials Dept. and Associate Prof of Pharmaceutical Analytical,
Chemistry, National Organization for Drug Control and Research (NODCAR),
Address : 6-Abu Hazem Street, Pyramids Ave. P.O. Box 29, 35521 Giza, Egypt.
Email: *Salama_nahla2004@hotmail.com*
Telephone : 202-35851299
Fax : 202-35855582