Digest Journal of Nanomaterials and Biostructures Vol. 9, No. 2, April – June 2014, p. 819 - 829

HIGHLY SENSITIVE SYNCHRONOUS SPECTROFLUORIMETRIC METHOD FOR DETERMINATION OF STIRIPENTOL IN CAPSULES AND HUMAN URINE: APPLICATION TO IN-VITRO DRUG RELEASE AND WEIGHT VARIATION TEST

HANY W. DARWISH[a,b*], AHMED H. BAKHEIT[a], MOHAMED I. ATTIA[a]
[a]*Department of Pharmaceutical Chemistry, College of Pharmacy, King Saud University, P.O. Box 2457 Riyadh, 11451, Kingdom of Saudi Arabia*
[b]*Analytical Chemistry Department, Faculty of Pharmacy, Cairo University, Kasr El-Aini St., Cairo 11562, Egypt*

A highly sensitive and simple spectrofluorimetric method has been developed and validated for the determination of stiripentol (STP) in its pharmaceutical formulations and human urine. The proposed method is based on the investigation of the fluorescence spectral behaviour of STP in methanol using synchronous scan technique (Δλ=80 nm, 343 nm). The fluorescence–concentration plot was rectilinear over the range 10–70 ng/mL, with lower detection limit of 2ng/mL. The proposed method was successfully applied to the assay of commercial capsules, spiked urine samples as well as weight variation testing. The application of the proposed method was extended to test the in-vitro drug release of STP capsules, according to USP guidelines.

(Received March 30, 2014; Accepted June 9, 2014)

Keywords: Stiripentol; Spectrofluorimetry; Human urine; Content uniformity; In-vitro release

1. Introduction

Stiripentol (STP; {4,4-dimethyl-1-[3,4-(methylenedioxy)phenyl]-1-penten-3-ol}) (Figure 1) is approved recently as a new antiepileptic drug (AED). STP is a racemic mixture of two enantiomers: *R*(+)-STP and *S*(–)-STP [1] due to the presence of chiral center at C-3. *R*(+)-STP displayed a 2.4-times greater anticonvulsant potency than *S*(–)-STP and it is also associated with about threefold faster elimination rate [2]. Structurally, STP is unrelated to any other AEDs that are available in the market [3, 4]. Clinical development of STP was delayed more than 40 years due to its inhibitory effect on hepatic cytochrome P450 (CYP) [5]. STP inhibits GABA metabolism *via* blocking GABA-transaminase activity [6] and reduction of synaptosomal uptake of GABA, giving rise to an increase in GABA brain concentration [7]. It has been reported that STP showed no affinity for $GABA_A$ and $GABA_B$ receptors [6]. Additionally, STP significantly enhances the mean open duration of $GABA_A$ receptor dependent chloride channels at relevant clinical concentrations (30–300 μM) through a barbiturate-like mechanism [8]. STP (Diacomit®; Biocodex Inc.) is used nowadays in Europe and Canada as an orphan drug for the treatment of severe myoclonic epilepsy in infancy (SMEI, Dravet syndrome) in conjunction with sodium valproate and clobazam, when seizures are not appropriately controlled with the latter two drugs [9].

*Corresponding author: hdarwish75@yahoo.com

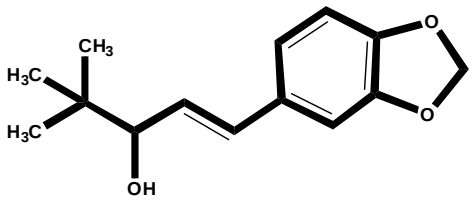

Fig. 1. Chemical structure of stiripentol (STP)

A through literature survey revealed that only one non-validated high performance liquid chromatography (HPLC)method was reported for the determination of STP to study its stereoselective pharmacokinetics [10]. Consequently, development of analytical method for determination of STP in its bulk drug and pharmaceutical capsules is essential. This paper presents a new sensitive spectrofluorimetric method for the determination of STP in capsules. Depending on the high sensitivity of the proposed method it can be applied for the determination of STP in human urine. The method allows a quick determination of STP in bulk drug and in capsules without pretreatment of the sample with high accuracy and precision, and without interference from excipients. The proposed method can be considered as a simple and fast alternative to the already existing HPLC procedure.

2. Experimental

2.1 Apparatus

- Fluorescence measurements were carried out on a RF-3501 version 3.0 spectrofluorimeter (Shimadzu Corporation Kyoto, Japan) equipped with a 150 W xenon lamp and 1 cm quartz cells. The slit widths for both the excitation and emission monochromators were set at 5.0 nm. The calibration and linearity of the instrument were frequently checked with standard quinine sulphate (0.01 µg/mL). Wavelength calibration was performed by measuring λ_{ex}at 275 nm and λ_{em}at 430 nm; no variation in the wavelength was observed. All recorded spectra converted to ASCII format by RFPC software.

- Hanna pH-Meter (Romania) was used for pH adjustments.

- Automatic dissolution tester (8 cup system), Abbota Corporation, 178 Franklin Road, New Jersey07869, United States.

2.2 Reagents and Materials

All the chemicals used were of Analytical Reagents grade, and the solvents were of HPLC grade.

Stiripentol reference standard (purity ~ 99.6%) was purchased from Sigma-Aldrich Co. (St. Louis, MO, USA). Diacomit® capsules (Biocodex, Montrouge, France), labeled to contain 250 mg (as the anhydrous base) per capsule was procured from the local market.

Sodium dodecyl sulphate (SDS; 95 %) was purchased from Winlab (UK). 0.5 % aqueous solution ofeach surfactant was prepared.

β–Cyclodextrin (β–CD) and carboxymethylcellulose (CMC) were obtained from Merck (Germany) and used as 0.5 % w/v aqueous solution.

Tween–80 (Techno Pharmchem Haryana Company (INDIA), used as 0.5 % w/v aqueous solution.

Methanol (Prolabo, France), n-propanol and acetonitrile (Sigma-Aldrich Chemie GmbH, Germany).

Dimethyl sulphoxide was purchased from Riedel deHaen (Germany).

Glacial acetic acid, sodium acetate tri-hydrate, boric acid, sodium hydroxide, hydrochloric acid, and dimethylformamide were all of spectroscopic grade

Acetate buffer (0.2 M, pH 3.7–5.7) and borate buffer (0.2 M, pH 6.5–9.5) solutions were freshly prepared.

Ultrapure water of 18 $\mu\Omega$ was obtained from a Millipore Milli-Q® UF Plus purification system (Millipore, Bedford, MA, USA) was used throughout the study.

2.3 Standard Solutions

STP stock solution (0.4 mg/mL) was prepared by dissolving10 mg of STP reference standard material into 25 mL methanol in a 25mL volumetric flask and completing the volume properly. This stock solution was later diluted two times with methanol to produce a working standard solution of 1 µg/mL. The standard solutions were stable for at least 14 days when kept in the refrigerator.

2.4 Construction of the Calibration Graphs

Aliquots of methanolic STP standard solution was transferred into a series of 5 mL volumetric flasks to give final concentrations of 10–70 ng/mL. The volume was completed with methanol, the contents of the flasks were mixed well and then measured by synchronous scanning fluorescence in the range of 300–450 nm employing a $\Delta\lambda$ of 80 nm. The intense band observed at 343 nm was used for quantitative purposes. Relative fluorescence intensity (RFI) was measured at 343 nm after excitation at 263 nm. RFI was plotted vs. the final drug concentration (ng/mL) to obtain the calibration graphs. The regression equations for the data were computed.

2.5 Assay of capsule samples

The content of ten capsules (Diacomit®250mg capsules; Batch no. 2611) was emptied and weighed. An accurately weighed portion of the powder equivalent to 250 mg of STP was transferred to a100 mL volumetric flasks. A volume of 50 mL of methanol was added, the contents were mechanically shaken for 10 min, ultrasonicated for 5 min, and the volume was diluted to 100 mL with methanol. This solution (2.5 mg/mL) was diluted as required for analysis.

2.6 Assay of human urine samples

Human drug free urine (5 mL) was spiked with a suitable amount of STP standard solution and mixed for 60 seconds, successive dilutions using methanol were made to reach to the calibration concentration range. RFI was measured at 343 nm using $\Delta\lambda$ of 80 nm and the concentration of the drug was determined from the regression equation.

2.7 Procedure for Weight Variation Testing for STP

Ten different capsules were analyzed using the same procedure applied for the analysis of the studied compound in capsules. The uniformity of their contents was tested by applying the official USP guidelines [11] (Chapter 905: Uniformity of Dosage Units).

2.8 Procedure for In-Vitro Drug Release Test (Dissolution Test)for STP

Dissolution test was performed on three capsules (Diacomit®250mg capsules). The dissolution USP apparatus II [11] using 900 mL of1 % SDS aqueous solution maintained at 37 ± 0.50C stirred at 50 rpm for 90 min. A 5 mL sample was withdrawn through a 0.45 µm syringe filter and replaced with another 5 mL of a suitable fresh dissolution medium at preselected intervals up to90 min. The procedure described under ‘construction of the calibration graphs’ was applied on the filtered samples. The release studies were performed in three replicates and mean values were taken by applying the proposed method.

2.9 Weighing uncertainty considerations

To determine the uncertainty in the weighing procedure, two sets of stock solutions of the same strength for STP were prepared. The aim is to determine whether there is a significant difference in the mean concentration between the two stock solutions in each set relative to the differences in mean concentrations over all samples. The data obtained for the two set mixtures are the average of three sample preparation replicates to reduce the non-systematic error due to volume transfer during preparation.

3. Results and Discussion

Upon severe literature survey, it was clear that only one HPLC method was reported for the determination of STP in its pharmaceutical preparations. For this reason, it is crucial to develop simple and sensitive method for STP quantitative determination. Spectrofluorimetry was adopted in this study because of the inherent high sensitivity, improved selectivity, practical simplicity, and wide availability in quality control laboratories. Different experimental parameters affecting the RFI of STP were carefully studied and optimized. Such factors were changed individually, where others kept constant.

3.1 Fluorescence spectra and characteristics of STP

The conventional fluorescence spectrum of a molecule consists of two spectra: (i) one related to the excitation process and (ii) other related to the emission of the absorbed radiation. The excitation spectrum can be obtained by varying the wavelength of the excitation monochromator (λ_{em}) in a given range and keeping the excitation monochromator at a fixed wavelength (λ_{ex}). The emission spectrum can be obtained using the same strategy, but scanning the emission monochromator and fixing the excitation monochromator at a particular wavelength. For a fluorescent molecule, a pair of wavelengths is observed where maximum intensity appears in the respective bands of emission and excitation spectra (λ_{em} and λ_{ex}).The first requirement to a compound generates a fluorescence spectrum is to absorb electromagnetic radiation. STP exhibits two excitation wavelengths of 263 and 310 nm. This is justified by the light absorption promoting electron from the ground state to two excited states. STP being also able to give a fluorescent spectrum. Its fluorescent spectrum was recorded (using a 40ng/mL) fixing the excitation monochromator at 263 nm and scanning the emission monochromator in the range of 300– 450 nm. An intense emission band with maximum at 343 nm appeared in the spectrum, evidencing the fluorescence of the STP. Afterwards, the excitation spectrum was registered, but now keeping the emission monochromator at 343 nm and scanning the excitation monochromator to ensure the excitation wavelength which appeared at 263 nm. . A good alternative for the conventional fluorescence spectrum is the use of synchronous scanning fluorescence [12]. In this technique, the excitation and emission monochromator are scanned simultaneously and the emission intensity is recorded as a function of the excitation wavelength. The $\Delta\lambda$ (difference between λ_{em} and λ_{ex}) plays an important role and is responsible for the intensity and the position of the bands in the synchronized spectrum. In general, sharper bands are obtained in the synchronous fluorescence spectrum in comparison with the conventional fluorescence spectrum, which increases the spectral resolution (Fig.2) [13]. In the present work, the optimum $\Delta\lambda$ was already determined by the interpretation of the fluorescence spectrum. The difference between maximum excitation and emission was 80 nm.

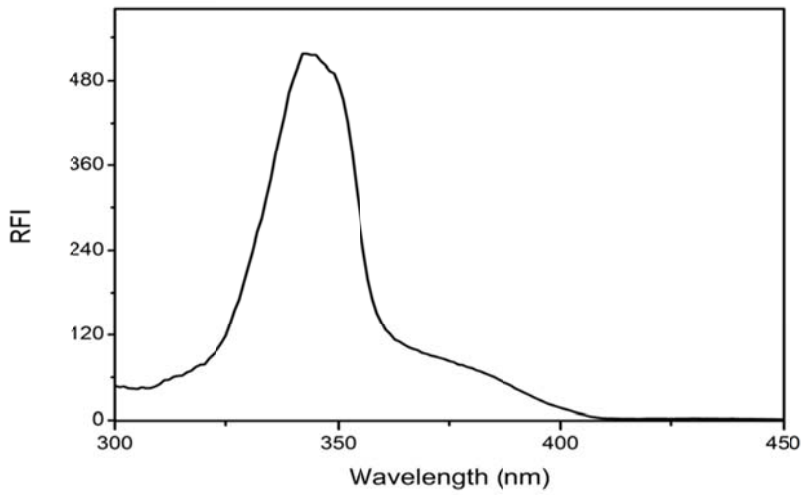

Fig. 2. Synchronous fluorescence spectrum of 40 ng/mL STP in methanol (343 nm, Δλ = 80 nm)

3.2 Optimization of the Experimental Conditions

3.2.1 Effect of Organized Media

The effect of different organized media on the RFI of STP was studied by adding 0.5 mL of an aqueous solution of each one of them to the drug solution. Different surfactants, like sodium dodecyl sulfate (SDS) [anionic surfactant], carboxymethylcellulose (CMC), tween 80 [non ionic] and macromolecules such as β-cyclodextrin were tried.

All the organized media studied caused slight decreases in the RFI of the drug as shown in Fig. 3. Therefore, no surfactant was used throughout the study

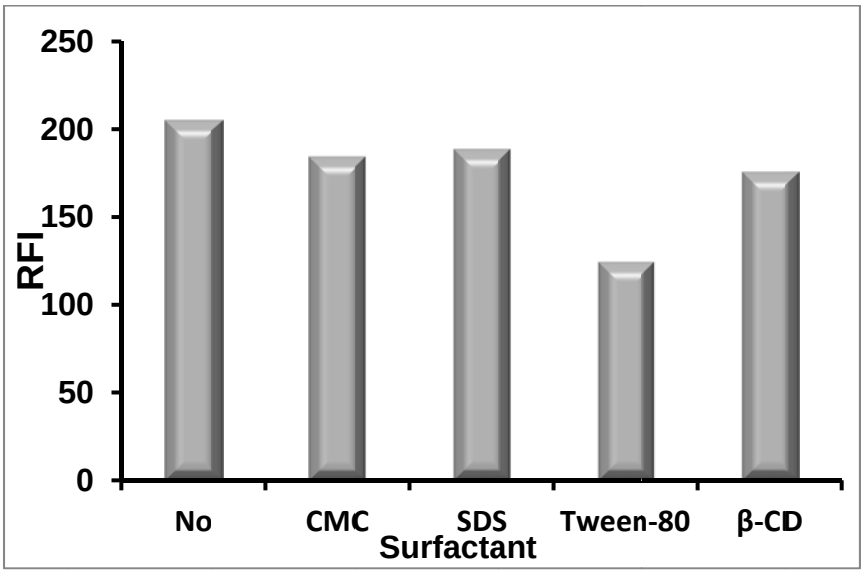

Fig. 3. Effect of the type of organized media (0.5 mL 0.5 % w/v solution of each) on RFI of STP (25 ng/mL)

3.2.2 Effect of pH

The influence of pH on the RFI of the STP was studied using different types of buffers covering the whole pH range, such as 0.2 M acetate buffer over the pH range 3.7–5.7 and 0.2 M borate buffer over the pH range 6.5–9.5. It was found that no subsequent increase in the RFI upon using any of the buffers. Therefore, no buffer was incorporated throughout the study

3.2.3 Effect of diluting solvent

Dilution with different solvents including water, methanol, ethanol, and acetonitrile was employed. Of the all tested solvents methanol gave the highest RFI compared with the other solvents (Fig. 4). This may be attributed to change in the medium polarity that may result in physical interaction between these solvents and the excited singlet state of the drug molecules Thus, methanol was chosen as the diluting solvent throughout the study.

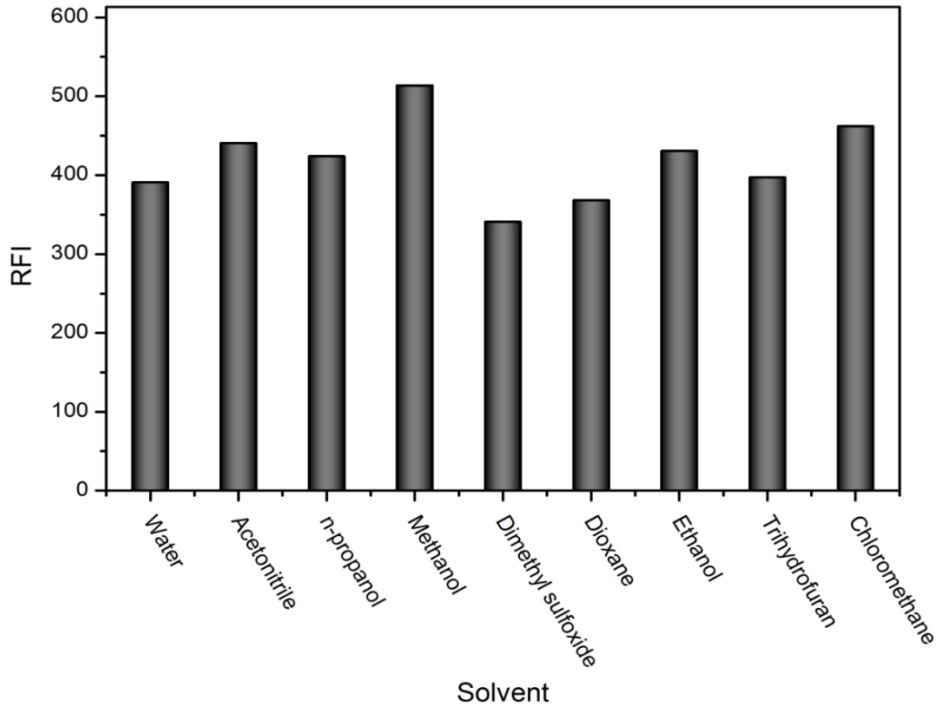

Fig. 4. Effect of the diluting solvent on RFI of STP (40 ng/mL)

3.2.4 Effect of time

The effect of time on the stability of the RFI of STP was also studied. It was found that the RFI developed instantaneously and remained stable for at least one hour.

3.3 Validation of the Method

The proposed methods were validated according to the ICH-guidelines for validation of the analytical procedures[14] in terms of the linearity, sensitivity, accuracy, specificity, repeatability and reproducibility

3.3.1 Linearity and Range

The calibration graph for the determination of STP by the proposed method was constructed by plotting the relative fluorescence intensity (RFI) versus the concentration. The graph was found to be rectilinear over the concentration range cited in Table 1.

Statistical analysis [15] of the data gave high values of the correlation coefficients (r) of the regression equations, small values of the standard deviation of residuals (Sy/x), of intercept (S_a), and of slope (S_b), and small value of the percentage relative standard deviation and the percentage relative error (Table 1). These data proved the linearity of the calibration graph for the studied drug.

Table 1:Analytical performance data for the spectrofluorimetric determination of STP

parameter	STP
Wavelength [$\lambda_{ex/}$ λ_{em}] (nm)	270/342
Linearity range (ng/mL)	10-70
Intercept *(a)*	1.456
Slope *(b)*	12.783
Correlation coefficient (*r*)	0.9985
S.D. of residuals ($S_{y/x}$)	9.999
S.D. of intercept (S_a)	7.742
S.D. of slope (S_b)	0.215
% RSD[a]	1.655
% Error[b]	0.499
LOD (ng/mL)[c]	2
LOQ (ng/mL)[d]	6.

[a] Percentage relative standard deviation for three replicate samples
[b] Percentage relative error for three replicate samples
[c] Limit of detection
[d] Limit of quantitation

3.3.2 Limit of Quantitation (LOQ) and Limit of Detection (LOD)

Limit of quantitation (LOQ) and limit of detection (LOD) were calculated according to the ICH Q2 (R1) recommendation [14].The limits of quantitation (LOQ) were determined by establishing the lowest concentrations that can be measured below which the calibration graph is nonlinear. The limits of detection (LOD) were determined by evaluating the lowest concentrations of the analytes that can be readily detected. The results are also summarized in Table 1. The values of LOQ and LOD were calculated according to the following equation:

$$LOD = 3.3\frac{\sigma}{S}, \qquad LOQ = 10\frac{\sigma}{S}$$

Where, σ was the standard deviation of the intercept of regression line and S was the slope of regression line of the calibration curve. The results are given in Table 1

3.3.3 Accuracy

The accuracy of the proposed methods was tested by analyzing triplicate samples of STP solution. The recovery percentage was 99.71 ± 1.517 (Table 2). These results revealed the excellent accuracy of the proposed method. The results obtained by applying the proposed method for determination of STP in bulk forms and in dosage form (Diacomit® capsules)

Table 2: Results of the determination of STP in pure form, Diacomit capsules (Batch no. 2611) and urine samples.

	Pure form			Diacomit capsules			Urine samples		
parameter	Amount taken (ng/mL)	Amount found (ng/mL)	% Found	Amount taken (ng/mL)	Amount found (ng/mL)	% Found	Amount added (ng/mL)	Amount found (ng/mL)	% Found
	25	25.127	100.51	25	25.018	100.07	20	19.50	97.5
	40	40.264	100.66	30	30.298	100.99	40	40.02	100.05
	60	58.779	97.96	40	40.669	101.67	60	59.81	99.68
Mean			99.71			100.91			99.08
± S.D.			1.517			0.804			1.379

3.3.4 Repeatability and reproducibility

Intra-assay precision was assessed by analyzing varying concentrations of STP (25, 40 and 60ng/mL) in triplicate in one assay batch. The inter-assay precision was assessed by analyzing the same concentrations in triplicate on 3 consecutive days. The average recovery percentages were around100% and the low relative standard deviations (RSD) indicated the high accuracy and precision of the proposed method, respectively (Table 3).

Table 3: Accuracy and precision data for the determination of STP by the proposed method

Amount taken (ng/mL)	% Found	% RSD	% Error
Intra-day			
25	99.05±2.515	2.540	1.037
40	99.87±2.165	2.167	0.885
60	97.50±1.256	1.288	0.526
Inter-day			
25	99.42±1.027	1.033	0.516
40	98.00±1.815	1.852	0.926
60	98.23±2.526	2.572	1.286

3.3.5 Specificity

The specificity of the method was investigated by observing any interference encountered from common capsule excipients. It was shown that these compounds did not interfere with the results of the proposed method (Table 2).

3.4 Weighing uncertainty considerations

The optimized models obtained were used to determine weighing errors for training set as presented in Table 5.Theresults for the two sets of stock solutions for STP were statistically compared using built in function of Matlab, ‘ttest 2’ for performing the t-test for comparison of means of two sets of data of unequal length. The mean and distribution of the dataset pairs are expected to be the same assuming the null hypothesis that the weighing error is not significant (H=0) as shown in Table 4.

Table 4: Detection of weighing errors by the proposed method

Parameter	S1	S2
No. of samples	13	10
Mean	100.20	98.70
	H=0	
	P=0.1862	

S1 and S2 are two different standard solutions of STP, P is the probability value

3.5 Applications

3.5.1 Application of Procedure to Analysis of STP in Capsules

The proposed method was successfully applied to STP assay in its capsules. The average percent recoveries of different concentrations were based on the average of three replicate determinations. The recovery percentage was 100.91 ± 0.804 (Table 2). These results revealed the excellent accuracy of the proposed method.

3.5.2 Application of Procedure to Analysis of STP in urine

The high sensitivity of the proposed method allowed the determination of the studied compound in spiked human urine. For STP, about 1-2% of the dose (which is around 3 gm daily) is found in the urine unchanged.

Therefore, the drug level in urine (40-60 mg/mL) is above the working range of the proposed method by 1000 times. The interference arising from the endogenous amino acids has been minimized by the vast dilutions (1000 times) to reach the working range for our method (40 - 60 ng/mL). Calibration graph prepared from data obtained from the analysis of spiked urine were linear and the percentage recovery results of STP were shown in Table 2.

3.5.3 Weight Variation Test for STP

Due to the high sensitivity of the proposed method and its ability to rapidly measure the RFI of a single capsule extract with sufficient accuracy, the method is ideally suited for weight variation testing which is a time consuming process when using conventional assay techniques. The steps of the test were adopted according to the USP [11] (Chapter 905: Uniformity of Dosage Units) procedure. The acceptance value (AV) was calculated and it was found to be smaller than the maximum allowed acceptance value (L1). The results demonstrated excellent drug uniformity as shown in Table 5.

Table 5: Results of weight variation testing of STP capsules using the proposed method

Parameter	Capsule no	Percentage of the label claim
Data	1	102.31
	2	101.92
	3	102.22
	4	100.35
	5	98.85
	6	98.51
	7	100.84
	8	99.75
	9	98.67
	10	102.28
Mean	100.57	
S.D.	1.567	
% RSD	1.558	
Acceptance value (AV) [11]	3.761	
Max. allowed AV (L1) [11]	15	

3.5.4 In-Vitro Drug Release (Dissolution Test) for STP

Dissolution test was performed on three Diacomit® capsules (250 mg). The amount of drug released was then determined with the help of the calibration curve and the percentage of drug released was calculated (Table 6). Results showed that not less than 60 % of the labeled amount of STP is dissolved in 60 min (Fig. 5).

Table 6: Results of in-vitro dissolution test data for STP capsules using the proposed method

Time (minutes)	% Drug release [11] (Mean± SD)
5	5.24
10	14.99
15	23.57
30	43.05
45	55.93
60	66.44

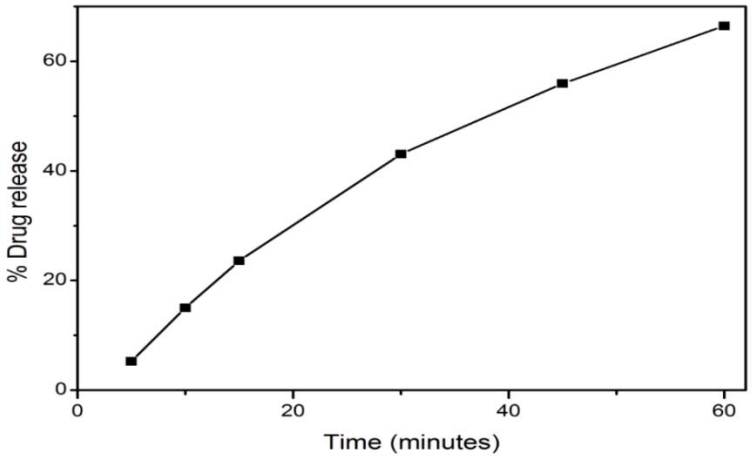

Fig.5. Dissolution profile for STP capsule according to USP guidelines

4. Conclusion

A simple and sensitive synchronous spectrofluorimetric method was developed for the determination of STP. The present study is considered the first spectrofluorimetric method for STP analysis. The proposed method is rapid, less time consuming and does not require elaborate treatment compared to the chromatographic techniques. By virtue of its simplicity, rapidity and sensitivity, the proposed method could be applied to the analysis of the studied drug in its capsules and human urine. The proposed method is very suitable to be applied in weight variation testing. Additionally, it has been adapted for dissolution testing of STP capsules as a rapid and simple method. From the economical point of view, the method involved the native fluorescence property of STP, rather than expensive derivatizing analytical reagents.

Acknowledgments

The authors would like to extend their sincere appreciation to the Deanship of Scientific Research at the King Saud University for funding this work through the Research Group Project No. RGP-VPP-196.

References

[1] D. F. Chollet, J Chromatogr B Analyt Technol Biomed Life Sci, **767**, 191 (2002).
[2] D. D. Shen, R. H. Levy, J. L. Savitch, A. V. Boddy, F. Tombret, and F. Lepage, Epilepsy Res, **12**, 29 (1992).
[3] M. K. Trojnar, K. Wojtal, M. P. Trojnar, and S. J. Czuczwar, Pharmacological Reports, **57**, 154 (2005).
[4] M. F. El-Behairy, E. Sundby, Tetrahedron: Asymmetry, **24**, 285 (2013).
[5] J. L. Fisher, Epilepsia, **52**, 76 (2011).
[6] M. Poisson, F. Huguet, A. Savattier, F. Bakri-Logeais, G. Narcisse, Arzneimittelforschung, **34**, 199 (1984).
[7] R. Wegmann, A. Ilies, M. Aurousseau, F. Patte, Cell and Molecular Biology, **23**, 455 (1978).
[8] P. P. Quilichini, C. Chiron, Y. Ben-Ari, H. Gozlan, Epilepsia, **47**, 704 (2006).
[9] E. C. Wirrell, L. Laux, D. N. Franz, J. Sullivan, R. P. Saneto, R. P. Morse, O. Devinsky, H. Chugani, A. Hernandez, L. Hamiwka, Epilepsia, **54**, 1595 (2013).
[10] R. H. Arends, K. Zhang, R. H. Levy, T. A. Baillie, D. D. Shen, Epilepsy Res, **18**, 91 (1994).

[11] M. Rockville, "The United States Pharmacopoeia 30, the National Formulary 25 US Pharmacopeial Convention," ed: Electronic version, 2007.
[12] Á. Andrade-Eiroa, G. de-Armas, J.-M. Estela, and V. Cerdà, TrAC Trends in Analytical Chemistry, **29**, 885 (2010).
[13] D. Patra, A. Mishra, TrAC Trends in Analytical Chemistry, **21**, 787 (2002).
[14] ICH, "Validation of Analytical procedures," in Methodology (Q2AR1), International Conference on Harmonization, USA:, November 1996 and November 2005.
[15] J. N. Miller, J. C. Miller, Statistics and chemometrics for analytical chemistry: Pearson Education, 2005.