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Validated spectrofluorimetric method for the determination of sunitinib malate, dye complexation approach for a novel anticancer drug

Antikanser ilaç Sunitinib malat'ın boya ile komplekleştirme yöntemi kullanılarak spektroflorimetrik tayini

Mesut Arıcı[1], Emrah Kılınç[2]*

[1]University of Ege, Faculty of Pharmacy, Department of Pharmaceutical Technology, 35100 Bornova, Izmir, Turkey
[2]University of Ege, Faculty of Pharmacy, Department of Analytical Chemistry, 35100 Bornova Izmir, Turkey

Abstract

A rapid, selective, sensitive and simple spectrofluorimetric method was developed for the indirect determination of sunitinib malate (SM) in bulk and pharmaceutical preparations. Dye complexation based spectrofluorimetric quantification of SM was performed by the monitorization of emission of the free dye (Eosin Y) at 800 nm following the excitation at 350 nm. Described method was fully validated with the analytical parameters studied being; accuracy, linearity, specificity, limit of detection (LOD), limit of quantification (LOQ), precision, reproducibility and robustness. The described method displayed linearity over the studied 0.08 to 5.00 µg/mL concentration range. 0.041 µg/mL ($7.69x10^{-8}$ M) and 0.850 µg/mL ($1.59x10^{-6}$ M) were the LOD and LOQ values, respectively. Defined parameters of the method (such as medium pH, dissolved oxygen concentration, temperature, dye concentration, incubation time and etc.) were changed systematically to check robustness and the method yielded pretty good results. Reproducibility was tested by applying the proposed method to the assay of SM using the same operational conditions, in two different laboratories at different elapsed time by using different instruments. Results obtained from lab-to-lab variations were found to be reproducible, as RSD values did not exceed 2%.

Keywords: sunitinib malate, eosin Y, ion-pair complex, spectrofluorimetric determination, analytical method validation

Introduction

Sunitinib malate (SM), butanedioic acid, hydroxy-, (2S)-, compound with N-[2-(diethylamino)ethyl]-5-[(Z)-(5-fluoro-1,2-dihydro-2-oxo-3H-indol-3-ylidine)methyl]-2,4 dimethyl-1H-pyrrole-3-carboxamide (1:1), is a novel oral multitargeted tyrosine kinase

***Corresponding author:** emrah.kilinc@ege.edu.tr

inhibitor with antitumor and antiangiogenic activities. SM has been also identified as a potent inhibitor of vascular endothelial growth factor receptors (VEGFR-1 and VEGFR-2), fetal liver tyrosine kinase receptor 3 (FLT3), KIT (stem-cell factor [SCF] receptor), platelet-derived growth factor receptors (PDGFRα and PDGFRβ) in both biochemical and cellular assays (Abrams et al. 2003 and Mendel et al. 2003). It was approved by the FDA for the treatment of gastrointestinal stromal tumors (GIST) and advanced renal-cell carcinoma in January 2006 (FDA labeling information).

As a novel compound, not many methods have been employed in the determination of SM and its metabolites. Most of the few reported methods for the analysis of SM in biological fluids and tissues rely on the use of liquid chromatography/mass spectrometry and HPLC-UV-Visible method. An analytical method for the determination of SM and of its metabolite in human plasma by LC–MS/MS (LC-tandem MS) following plasma protein precipitation by filtration in the 96-well format had been developed and fully validated (Frigerio et al. 2002). Another validated approach, the quantification of SM and its metabolite in several monkey tissues (liver, kidney, brain and white fat), was a LC–MS/MS determination following semi-automated liquid–liquid extraction (LLE) (Barattè et al. 2004). In an another LC-MS/MS method, plasma concentrations of SM and its metabolite were determined with a detection limit of 0.099 ng/mL for SM and 0.088 ng/mL for its metabolite (Fiedler et al. 2005). In another study in the recent year, quantification of SM in human plasma was performed by high-performance liquid chromatography-tandem MS (Minkin et al. 2008). The obtained calibration plots were linear over the range of 0.2-500 ng/mL with the values for determination coefficient of >0.9950. Within and between day precision and accuracy were ≤10%. Regarding HPLC-UV-Visible method, Blanchet et al. (2009) proposed a HPLC-UV-Visible method for SM quantification in human plasma. The calibration plot was linear in the range 20 to 200 ng/mL and inter-day and intra-day coefficients of variation were less than 7%.

Reviewing the literature revealed that, to our best knowledge, up to the present time no paper has been reported concerning the spectrofluorimetric determination of SM. Also LC-tandem MS equipment system, which is preferred in literature for SM quantification, is not available in most clinical laboratories due to high price of the system and its operational costs. This prompted us to develop a rapid, sensitive, selective and economic spectrofluorimetric method for the determination of SM in bulk and pharmaceutical preparations. Spectrofluorimetry is a suitable technique for the analysis of active principles in bulk form and pharmaceutical preparations due to its simplicity, specificity, and sensitivity (Damiani et al. 1998 and 1999, Arancibia and Escandar 1999, Escandar 1999a and 1999b). Furthermore, for routine pharmaceutical quality control, it is more rapid and of lower cost than liquid chromatographic methods.

The method developed and validated, involves the reaction of SM with a native fluorescent dye (Eosin Y) to produce a binary complex which causes dramatic decreases in the high fluorescence intensity (FI) of the free dye emission monitorized in the range 400-800 nm, following the excitation at 350 nm. The overall mechanism underlying the determination process can simply be explained as the ion-pair complex formation between the organic dye and SM. This technique is one of the methods available for the determination of organic compounds. Suitable organic dyes including bromothymol blue, bromophenol blue, bromocresol green, bromocresol purple,

methyl orange and eosin were employed various times initially (Kelani et al. 1999, Prabhakar et al. 1999, Al-Ghannam 2006, Abdellatef 2007).

Described method was fully validated by using ICH guidelines through the analytical parameters; accuracy, linearity, specificity, limit of detection (LOD), limit of quantification (LOQ), precision, reproducibility, and robustness (ICH 2005). The validated method yielded good results and the obtained results of the validation process were summarized within the paper. Our proposed method was found to be suitable and accurate for the quantitative determination of SM. Therefore the present paper will be a valuable contribution to the literature, we believe.

Materials and Methods

Chemicals

Eosin Y disodium salt (EY) was obtained from Merck AG, Darmstadt, Germany (Ord. No. 1.15935.0100). Sunitinib malate (SM) was received from a pharmaceutical company, Pfizer, USA (Lot # 0001, 99.3%) and was used as received. Other solvents and chemicals were all of HPLC or ultrapure grade.

Apparatus

Spectrofluorimetric determination was performed via Shimadzu recording Spectrofluorimeter RF-540. Other spectrofluorimeter used during reproducibility studies was Shimadzu Spectrofluorimeter RF-5301PC with the personal fluorescence software version 1.40.

Method

Preparation of stock solutions

25.0 mg SM was dissolved in 1 L ultra-pure water and 18.8 mg EY was dissolved in 10 mL ultra-pure water to prepare 25 µg/mL and 2.7 mM stock solutions, respectively. Dye stock solution was diluted 1/8 times before use with SM.

Preparation of calibration solutions

10, 20, 50, 100, 200, 300, 400, 500 and 600 µL of SM stock solution (25 µg/mL) were diluted up to 2.8 mL with ultra-pure water and 200 µL dye (EY) 1/8 stock solution was added each time to make the final volume up to 3 mL.

Spectrofluorimetric determination procedure

Freshly prepared calibration solutions were sonicated for 30 seconds and excitated at 350 nm and their emissions were monitorized at 800 nm for EY. The equipment settings were as follows;

Ordinate scale: 1	Excitation range: 300-600 nm
Absis scale: 2	Emission range: 400-800 nm
Scan speed: 1	Excitation go to: 350 nm
Sensitivity: 1	Emission go to: 800 nm
Slit: 3/2	

Analytical method validation

The objective of method validation is to demonstrate that the method is suitable for its intended purpose as it is stated in ICH guidelines (ICH 2005). Thus all overall method validation studies were performed due

to the analytical parameters listed in the ICH guidelines. The method was validated for accuracy, linearity, specificity, limit of detection (LOD), limit of quantification (LOQ), precision, reproducibility and robustness.

Accuracy

According to ICH guidelines the assays to be followed for accuracy parameter are summarized in 2 subtitles; *Drug Substance* and *Drug Product*. Three different methods are available to check Accuracy through *Drug Substance* or *Drug Product*. In cases where it is impossible to obtain samples of all drug product components, it's stated that it may be acceptable either to add known quantities of the analyte to the drug product or to compare the results obtained from a second, well characterized procedure, the accuracy of which is stated and/or defined [ICH guideline 4.1.2-b]. Therefore, we proceeded our experimental procedure by adding known quantities of the analyte to the drug product. Thus, method accuracy was tested (recovery % and R.S.D. % of individual measurements) by analyzing samples of SM at three different levels (50, 100 and 150%) in stock solutions using the pharmaceutical preparation; Sutent® for each level. The results were expressed as the sampled mean fluorescence intensity (FI %), recovery % and R.S.D % values percentage of SM recovered in the samples. There were three different samples prepared; Sutent® 1 which is 50% SM spiked, Sutent® 2 which is 100% SM spiked and Sutent® 3 which is 150% SM spiked. To prepare these Sutent® samples, contents of a single capsule of Sutent® (containing 12.5 mg SM) was poured in to a 250 mL flask which was filled up to its volume by ultrapure water and then filtered through 0.45 μm filter. Later on 100, 200 and 300 μL of SM standard stock solution was added onto 200 μL of this supernatant to reach the final samples labeled as Sutent®1, Sutent®2 and Sutent®3, respectively.

Linearity

The linearity of the described spectrofluorimetric method was studied in the concentration range 0.08 to 5.00 μg/mL for SM. As emission spectrum (Fig. 1) was recorded in a less sensitive ordinate scale, in the calculations for the calibration plot all measured FI values were multiplied by 2. The calibration curves were constructed by plotting concentration versus intensities. In the overall concentration range examined, the linearity was evaluated by linear regression analysis that was calculated by the least square regression method.

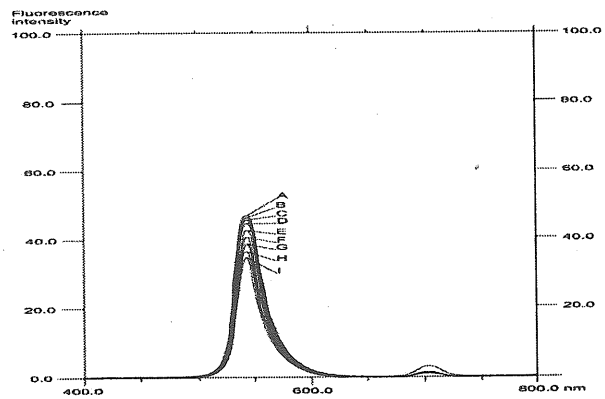

Figure 1. The dependence of fluorescence intensity of EY on the increasing concentration of SM in the range 0.08 μg/mL (A), 0.17 μg/mL (B), 0.42 μg/mL (C), 0.83 μg/mL (D), 1.66 μg/mL (E), 2.50 μg/mL (F), 3.33 μg/mL (G), 4.17 μg/mL (H) and 5.00 μg/mL (I). Experimental conditions as in the *Materials and Methods* section.

Specificity

For the specificity parameter the interference effects of the frequently used excipients in the capsule-type pharmaceutical preparations were checked with the excess ratio of the excipients to SM. Mannitol, croscarmellose sodium, magnesium stearate, glucose (dextrose) and lactose were chosen as the model excipients.

Precision

Regarding ICH guideline repeatability expresses the precision under the same operating conditions over a short interval of time. Repeatability is also termed intra-assay precision. The intermediate precision is a measure of precision between repeatability and reproducibility and it should be established according to the circumstances under which the procedure is intended to be used (Ermer and Ploss 2005), thus according to the ICH description of precision in cases where reproducibility has been performed, intermediate precision is not necessary. Therefore, the precision studies were proceeded in accordance to intra-assay precision rather than intermediate precision. Precision was assessed using 90 determinations (9 concentrations / 10 replicates each). All FI recordings and the standard deviation (SD) values are displayed in Table 1A as a measure of intra-assay precision (repeatability).

Reproducibility

Even though the reproducibility is a sub-parameter of precision and should be given there, in this paper it's discussed as a separate title of analytical method validation studies. Reproducibility is assessed by means of an inter-laboratory trial. Reproducibility should be considered in case of the standardization of an analytical procedure. In the current work, for the reproducibility parameter of the analytical method validation process, spectrofluorimetric determination was alternatively performed in another analytical research laboratory (University of Ege, Faculty of Science, Department of Chemistry, Division of Analytical Chemistry, 35100, Bornova – Izmir, Türkiye) by a different analyst using a different spectrofluorimeter (explained as in Apparatus section). The very same method was repeated in the preparation of all solutions in the same experimental conditions. The equipment settings were as follows:

Ex wavelength: 350 nm	Emission range: 400-800 nm
Recording range Low: 0.0 High: 100.0	Sampling interval: 1.0 (nm)
Scanning speed: Super	Slit width (nm): Ex 3 Em 1.5
Sensitivity: High	Response time (sec): Auto.

Robustness

Robustness was checked through the parameters pH, dissolved oxygen effect, temperature, dye concentration and incubation time, whose results were summarized in Table 1B. The effect of medium pH on the results of the defined method was summarized and discussed initially in Fig. 2. The effect of dissolved oxygen was studied at normal room, air saturated and deoxygenated conditions at the presence of 2.5 μg/mL SM and 180 μM EY. For the temperature effect, similarly 2.5 μg/mL SM and 180 μM EY were employed at 10, 25, 50 and 70°C. The effect of reagent dye (EY) concentration on the recorded FI values was discussed initially as a separate figure (Fig. 3). Incubation time was thought to be a possible parameter affecting the FI values being recorded. Therefore, t=0, 5, 10 and 15 min. were investigated in the presence of 2.5 μg/mL SM and 180 μM EY.

Analyte stability studies

25 µg/mL SM stock solution short-term stability was tested at ambient room temperature (25±1°C), under 254 nm UV radiation (at 25±1°C) and at 4±1°C. All stability monitorization was performed at t_0, 1st, 6th, and 72nd h.

Results and Discussion

Proposed mechanism of complex formation reaction

Fig. 4 displays the chemical structures of sunitinib malate (SM) and the dye Eosine Y (EY) and the proposed mechanism for the formation of the SM-EY complex. SM may be named as *N*-[2-(diethylamino)ethyl]-5-[(*Z*)-(5-fluoro-1,2-dihydro-2-oxo-*3H*-indol-3-ylidine)methyl]-2,4 dimethyl-*1H*-pyrrole-3-carboxamide and malic acid (1:1), while EY may be shortly called as 2', 4', 5', 7'- tetrabromofluorescein. Regarding the mechanism of the formation of SM-EY complex, for a acidic pH range of 2.0 to 5.5, we can propose a complexation mechanism based on the electrostatic interaction between maximally protonated 3H indol ring of SM and sodium salts of carboxylate functional group of the dye EY, in other words the formed complex is due to the electrostatic interaction between the studied drug (SM) and anionic functional group of EY at acidic pH. For the complexation, the ratio of the reactants may be 1:1 when assuming the total electrostatic charge of SM and EY to be 1+ and 1- respectively (Dinesh et al. 2002, Süslü and Tamer 2002, Al-Ghannam 2006, El-Shiekh et al. 2007). A schematic proposal for reaction between SM and EY is shown in Fig. 4.

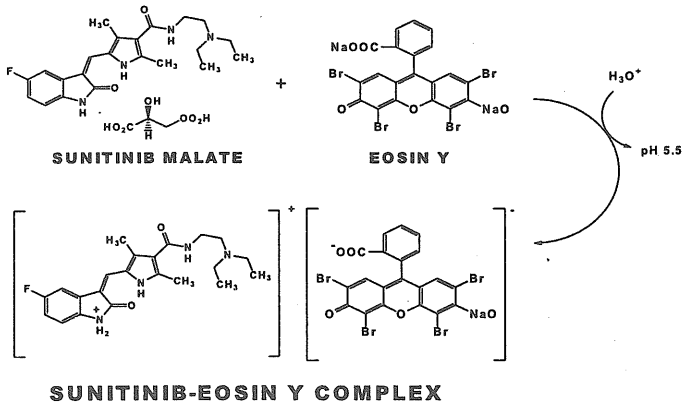

Figure 4. Chemical structures of Sunitinib malate (SM) and the dye Eosin Y (EY) and the proposed mechanism of the formation of SM-EY complex formed with the electrostatic interaction between maximally protonated 3H indol ring of SM and sodium salts of carboxylate and hydroxyl functional groups of the dye.

Emission spectrum

Typical emission behavior of EY at 800 nm (initial excitation at 350 nm) is displayed with only one series of SM addition in Figure 1 (pH 5.5). Increasing SM concentration, in the range 0.08 µg/mL (A), 0.17 µg/mL (B), 0.42 µg/mL (C), 0.83 µg/mL (D), 1.66 µg/mL (E), 2.50 µg/mL (F), 3.33 µg/mL (G), 4.17 µg/mL (H) and finally 5.00 µg/mL (I), causes dramatic decreases in the fluorescence intensity (FI) of the free dye, since the remaining dye molecules are complexed by SM. The mean values (±SD) of FI were as follows; initially for 0.08 µg/mL it was around 45.5±1.22 followed by 44.37±2.61, 44.42±1.43, 43.41±1.62, 41.59±2.65, 39.16±1.27,

37.16±1.12, 35.18±1.02 and 33.33±1.39 for 0.17 μg/mL, 0.42 μg/mL, 0.83 μg/mL, 1.66 μg/mL, 2.50 μg/mL, 3.33 μg/mL, 4.17 μg/mL and finally 5.00 μg/mL, respectively. For a total increase of 4.92 μg/mL (5-0.08) SM concentration, a decrease of -12.17% FI was observed.

Calibration plot

As emission spectrum (Fig. 1) was recorded in a less sensitive ordinate scale, in the calculations for the calibration plot all measured FI values were multiplied by 2. In the overall concentration range (0.08 to 5.00 μg/mL) examined, when trend lines are used, the regression equation and R^2 value were calculated as: $y = -4.8439x + 90.681$ and linearity is observed in the overall range studied with the R^2 value of 0.9962. Regarding the precision of the calibration plot, in the overall concentration range, the first 3 highest SD values of ±2.65, 2.61 and 1.62 (n=10) were calculated for 1.66, 0.17 and 0.83 μg/mL, respectively. The lowest SD value of ±1.02 was calculated for 4.17 μg/mL. Remaining concentration values were repeated with more or less the very same SD values at around ±1.20. When SD values were taken into consideration, it can be concluded that the present method is relatively less precise in the determination of lower concentration values, which is the opposite case with the higher concentration values around 5.00 μg/mL.

Regarding similar papers of eosin-drug complex formations; Kelani et al. (1999) described an eosin ternary complex formation based spectrofluorimetric method which is applicable over the concentration range of 4.1–37.6, 11.8–47.2 and 2.4–19.1 μg/mL for astemizole, terfenadine and flunarizine hydrochloride, respectively. Belal et al. (2002) published a method for the spectrofluorimetric determination of vigabatrin (VG) and gabapentin (GB). The method was based on the reaction between the two drugs and fluorescamine, which was applicable over the concentration range of 0.20–4.00 and 0.1–1.0 μg/mL, respectively. Hefnawy et al. (1999) described a fluorimetric method for the determination of four α-aminocephalosporins (cefaclor, cefadroxil, cephalexin and cephradine), which involves the reaction of the target compounds with fluorescamine. The proposed method was linear over the concentration range of 0.05–1 μg/mL for both cefaclor and cephalexin, and 0.05–0.65 and 0.025–0.5 μg/mL for cefadroxil and cepharadine, respectively. El-Brashy et al. (2004) published a spectrophotometric method, based on the formation of a binary complex between the drugs and Eosin Y, for the determination of levofloxacin, norfloxacin and ciprofloxacin. The described method was operational over the range of 2–8 μg/mL for all drugs. Moustafa (2000) described a similar method for the determination of lansoprazole and pantoprazole sodium sesquihydrate with the operational concentration ranges of 3.69 to 16.61 μg/mL and 4.3 to 25.9 μg/mL for the mentioned compounds, respectively. When the obtained concentration range of our method (0.08 to 5.00 μg/mL) was compared with these papers, it can be concluded that even though the linear ranges look like more or less the same, we obtained a wider range. Here we must also emphasize that, 5.00 μg/mL is the studied upper limit, which means that if we had gone further with the concentration we would have obtained probably wider linear range than 0.08-5.00 μg/mL.

Effect of pH

The effect of medium pH is summarized in Fig. 2. In the far acidic region of the pH scale ($pH \leq 2.0$) highest decrease in the emission of the free dye was observed (Figure 2B). For 0.08 μg/mL

SM the FI had a decrease of -76.5% which is followed by -36.82%, -10.18%, -4.19%, -3.89% and -16.16% for pH 4.0, 5.5, 7.0, 9.0 and 11.0, respectively.

With the increasing pH values lesser decreases of FI were visible. This trend is observed in the all pH range studied with the fluctuating exceptions of pH 11 (Fig. 2A). The pH trend of acidic preference might be explained with the chemical stability of the dye (EY) and/or the increased stability constant of the SM-EY complex. With the decreasing pH value, spectrofluorimetric detection becomes more precise relative to the neutral or basic range. As mentioned previously, the structure and the stability of the formed SM-EY complex might be of interest to a separate manuscript of ours in near future. When optimum pH ranges of similar papers dealing with the complexation of eosin with various drugs were compared, optimum pH values were reported as pH 3 and 3.8–4.5 by Kelani et al. (1999), Moustafa (2000) and El-Brashy et al. (2004), respectively. These results mentioned in the literature display the same trend with our optimum pH value of pH 2.0.

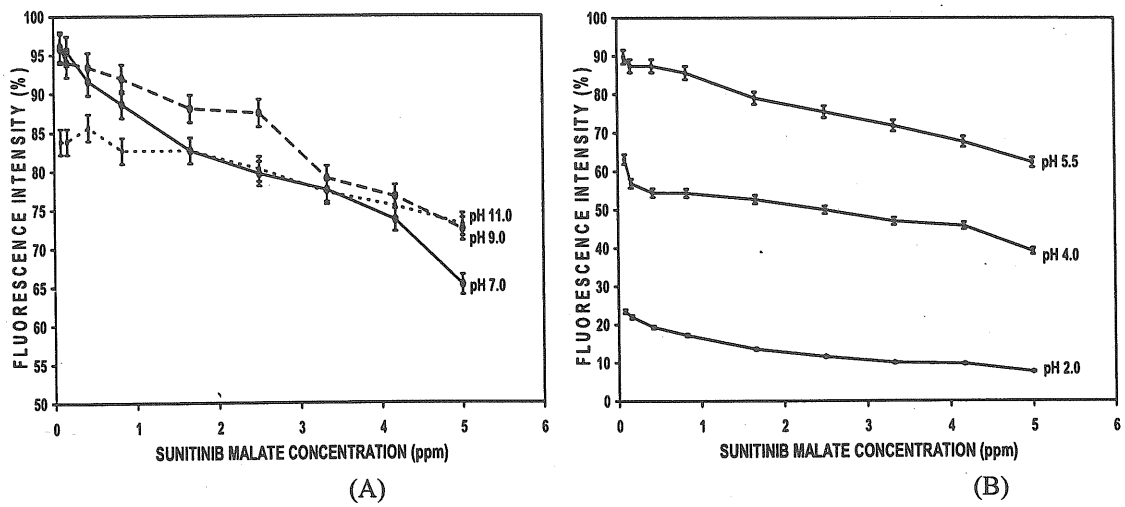

Figure 2. Effect of medium pH values; pH 11.0, 9.0, 7.0 (A) and 5.5, 4.0, 2.0 (B); on the dependence of intensity of EY on the increasing concentration of SM. Experimental conditions as in the *Materials and Methods* section.

Optimum concentration of the dye

The effect of EY concentration on the emission recorded in the presence of 2.5 μg/mL (4.69×10^{-6} M) SM is summarized in Figure 3. The aim of this study was to determine the optimum concentration of the reagent dye (EY). The FI was sampled with twice less sensitivity than the graphs in Fig. 1 and 2, therefore, FI must be accepted as twice before comparison of any kind. The first two EY concentrations studied were 22.5 and 45 μM, which were repeatedly increased in 45 μM steps till 360 μM later on. In comparison to the constant SM concentration of 4.69 μM, the EY concentration range started with approximately 4.79 times higher value such as 22.5 μM. With the increasing EY concentration the ratio between EY and SM concentrations even grew higher such as 9.59, 19.18, 28.78, 38.37, 47.98, 57.56, 67.16 and 76.75 which correspond to the decreasing percentages of (SM compared to eosine concentration) 20.84%, 10.42%, 5.21%, 3.47%, 2.61%, 2.08%, 1.74%, 1.49% and 1.30%, respectively. When EY concentration was 22.5

μM, the constant SM concentration was up to 20.84% of it and the fluorescence intensity (FI) recorded was 7.34, but when EY concentration was increased 10 folds, up to 225 μM, FI value increased only app. 6 folds up to 45.06. With the continuous increase of EY concentration the FI value recorded for 4.69 μM SM also increased (not with the very same ratio, but slower) as expected. This behavior may be explained with the increasing free dye content, as the measurement of SM is completely based on the monitoring of the free dye emission at 800 nm. 180 μM ($0.18x10^{-3}$ M) was assigned as our optimum eosin concentration because of the dramatically increasing FI values with higher EY concentrations (as mentioned at the beginning of this paragraph, the FI values of Figure 3 must be multiplied by 2 before taken into consideration). With such higher EY concentrations it would probably be a problem to use the same y-axis scale as FI values would began to excess 100%. Shortly, 180 μM ($0.18x10^{-3}$ M) was the optimum eosin concentration as the median value of the entire dye concentration range (22.5 to 360 μM) studied. Similar optimum EY concentration values were reported by Kelani et al. (1999), El-Brashy et al. (2004) and Moustafa (2000) as $0.02x10^{-3}$ M, $0.5x10^{-3}$ M and $2.0x10^{-3}$ M, respectively.

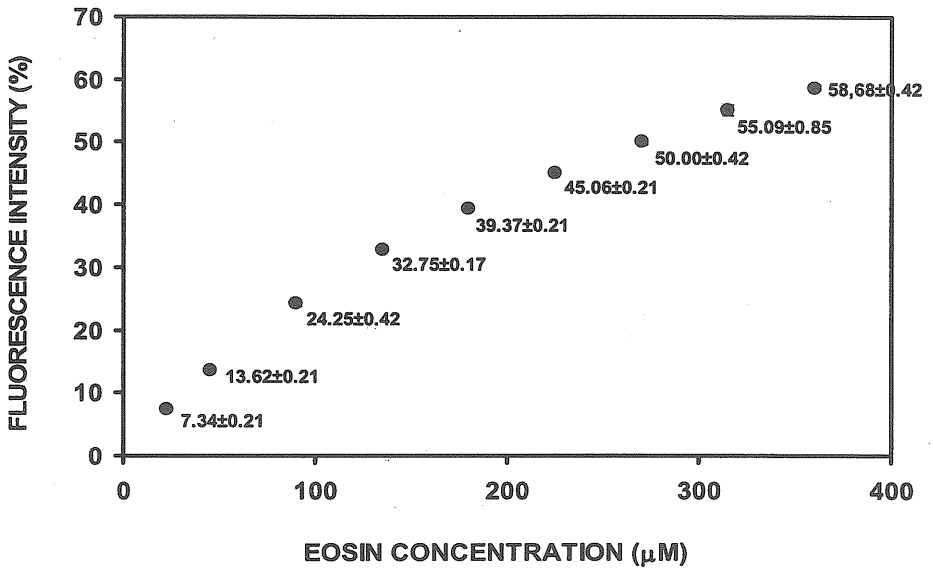

Figure 3. Effect of EY concentration on the emission recorded in the presence of 2.5 μg/mL SM. Experimental conditions as in the *Materials and Methods* section.

Analytical method validation

Regarding the analytical method validation, obtained data is displayed in Table 1A, 1B and 1C. The validation parameters studied were; accuracy, linearity, specificity, LOD, LOQ, precision (Table 1A), robustness and reproducibility (Table 1B and 1C).

Table 1A. Analytical Method validation data, Part 1 linearity, precision, selectivity (specificity), LOD and LOQ as the studied parameters.

Validation parameters	Method	Results
1. Accuracy	Accuracy was tested (recovery% and R.S.D.% of individual measurements) by analysing samples of SM at three different levels (50, 100 and 150%) in stock solutions using the pharmaceutical preparation; Sutent® for each level (n=3).	Sutent® 1 (50% SM spiked): 43.85 (mean FI), 108.3 recovery%, 1.52 R.S.D.% Sutent® 2 (100% SM spiked): 40.96 (mean FI), 100.8 recovery%, 0.71 R.S.D.% Sutent® 3 (150% SM spiked): 36.47 (mean FI), 103.5 recovery%, 0.71 R.S.D.%
2. Linearity	10, 20, 50, 100, 200, 300, 400, 500 and 600 µL of SM stock solution (25 µg/mL) were diluted up to 2.8 mL with H_2O and 200 µL dye (eosin) 1/8 stock solution was added each time to make the final volume up to 3 mL of calibration solutions. Obtained final concentrations were as: 0.08, 0.17, 0.42, 0.83, 1.66, 2.50, 3.33, 4.17, 5.00 µg/mL. Freshly prepared calibration solutions were excitated at 350 nm and their emissions were monitorized at 800 nm for eosin (n=10).	$y = -4.8439x + 90.681$ $R^2 = 0.9962$
3. Precision (intra-assay)	Calibration solutions were prepared and emission was recorded as described above (n=10). Peak heights of the corresponding concentration were read and related standard deviation was calculated.	for 0.08 µg/mL Y_{AVE} = 90.99 ± 1.22 (n=10) for 0.17 µg/mL Y_{AVE} = 88.74 ± 2.61 (n=10) for 0.42 µg/mL Y_{AVE} = 88.83 ± 1.27 (n=10) for 0.83 µg/mL Y_{AVE} = 86.83 ± 1.62 (n=10) for 1.66 µg/mL Y_{AVE} = 83.17 ± 2.65 (n=10) for 2.50 µg/mL Y_{AVE} = 78.31 ± 1.27 (n=10) for 3.33 µg/mL Y_{AVE} = 74.31 ± 1.12 (n=10) for 4.17 µg/mL Y_{AVE} = 70.36 ± 1.02 (n=10) for 5.00 µg/mL Y_{AVE} = 66.66 ± 1.39 (n=10)
4. Specificity	Samples containing a fixed amount of SM (1.66 µg/mL) and variable concentrations of excipients were measured.	Lactose, glucose, mannitol, croscarmellose sodium and magnesium stearat do not cause interference at weight ratios of excipient/SM ≤ 5000.
5. Limit of Detection (LOD)	$LOD = [(B_{AVE} + 3\ SD) - b]\ a^{-1}$ B_{AVE} = Baseline SD= Standart deviation of baseline (n=3)b= Intercepta= Slope of the calibration plot	[8.2 + (3 x 0.56) –9.68)] / – 4.8439 = 0.041 µg/mL = 7.69×10^{-8} M
6. Limit of Quantification (LOQ)	$LOQ = [(B_{AVE} + 10\ SD) - b]\ a^{-1}$	[8.2 + (10 x 0.56) –9.68)] / – 4.8439 = 0.850 µg/mL = 1.59×10^{-6} M

Table 1B. Analytical Method validation data, Part 2 robustness and reproducibility as the studied parameters.

Validation parameters	Method	Results
6. Reproducibility	Linearity, precision, LOD and LOQ parameters were studied as in Table 1A.	• Linearity: $y = -2.766x + 76.953$ and $R^2 = 0.9427$ • Precison: for 0.08 ppm $Y_{AVE} = 70.65 \pm 0.85$ (n=6) for 0.17 ppm $Y_{AVE} = 71{,}26 \pm 1.52$ (n=6) for 0.42 ppm $Y_{AVE} = 71.08 \pm 1.66$ (n=6) for 0.83 ppm $Y_{AVE} = 67.79 \pm 2.50$ (n=6) for 1.66 ppm $Y_{AVE} = 64.86 \pm 2.00$ (n=6) for 2.50 ppm $Y_{AVE} = 60.34 \pm 1.90$ (n=6) for 3.33 ppm $Y_{AVE} = 57.53 \pm 0.94$ (n=6) for 4.17 ppm $Y_{AVE} = 53.75 \pm 1.35$ (n=6) for 5.00 ppm $Y_{AVE} = 50.88 \pm 1.25$ (n=6) • LOD: $[1.21 + (3 \times 0.51) - 0.769)] / -2.766 = 0.71$ ppm = 13.3×10^{-7} M • LOQ: $[1.21 + (10 \times 0.51) - 0.769)] / -2.766 = 2.00$ ppm = 3.74×10^{-6} M
7. Robustness	Defined parameters of the validated method (such as pH, deoxygenation, air saturation, temperature, eosin concentration, incubation time, etc) were changed systematically on purpose.	• The effect of medium pH was summarized in Figure 2. • The effects of air saturation and deoxygenation were as follows for 2.5 ppm SM; $Y_{AVE} = 75.46 \pm 0.34$ (n=4) (Normal conditions) $Y_{AVE} = 75.81 \pm 1.66$ (n=5) (Air saturated) $Y_{AVE} = 72.65 \pm 0.79$ (n=4) (Deoxygenated) • The effects of operating temperature were as follows; $Y_{AVE} = 79.79 \pm 1.49$ (n=4) (10°C) $Y_{AVE} = 78.74 \pm 1.04$ (n=5) (25°C) $Y_{AVE} = 76.35 \pm 0.12$ (n=4) (50°C) $Y_{AVE} = 72.46 \pm 0.69$ (n=4) (70°C) • The effect of eosin concentration on emission recorded in the presence of 2.5 ppm SM is displayed in Figure 3. • The effect of incubation time was studied for t=0, 5, 10 and 15 minutes with the fluorescence intensity being 78.22, 78.43, 78.21 and 76.79, respectively.

Table 1C. Summary of the reproducibility data obtained in two different laboratories.

Conc. (ppm)	% Norm. response ± SD		RSD	
	Shimadzu RF-540 (n=8)	Shimadzu RF-5301PC (n=4)	Shimadzu RF-540 (n=8)	Shimadzu RF-5301PC (n=4)
0.08	100.00 ± 1.05	100.00 ± 0.57	1.15	0.81
0.17	97.52 ± 1.16	100.86 ± 0.70	1.31	0.98
0.42	97.62 ± 1.15	100.61 ± 1.16	1.29	1.63
0.83	95.42 ± 1.13	95.95 ± 0.24	1.30	0.35
1.66	91.41 ± 1.38	91.80 ± 1.10	1.66	1.69
2.50	86.06 ± 1.20	85.41 ± 1.09	1.53	1.81
3.33	81.66 ± 0.96	81.15 ± 0.12	1.29	0.21
4.17	77.33 ± 0.91	76.07 ± 0.56	1.29	1.04
5.00	73.26 ± 1.11	72.02 ± 0.35	1.67	0.69

Accuracy

Experimental procedure of accuracy was proceeded by adding known quantities of the analyte to the drug product. Thus method accuracy was tested (recovery % and R.S.D. % of individual measurements) by analyzing samples of SM at three different levels (50, 100 and 150%) in stock solutions using the pharmaceutical preparation; Sutent® for each level. The corresponding mean FI%, recovery %, and R.S.D. % values were as 43.85 (mean FI), 108.3% recovery, 1.52 R.S.D. %; 40.96 (mean FI), 100.8% recovery, 0.71 R.S.D.% and 36.47 (mean FI), 103.5% recovery, 0.71 R.S.D.% for Sutent®1, Sutent®2 and Sutent®3, respectively. Obtained results of analytical method validation studies of this method revealed that the accuracy parameter showed good results as it reached far beyond 85% recovery.

Linearity

The linearity of the described spectrofluorimetric method was studied in the concentration range 0.08 to 5.00 μg/mL, where a satisfying linearity was observed with a R^2 value of 0.9962. The possible calibration graph based on the experimental data obtained looks like the graph in Fig. 1.

The limit of detection (LOD) and limit of quantification (LOQ) values were calculated as; 0.041 μg/mL (7.69×10^{-8} M) and 0.850 μg/mL (1.59×10^{-6} M), respectively. As there are no papers published on the spectrofluorimetric determination of SM, the LOD and LOQ values calculated may be compared with the liquid chromatographic determination of SM (LC-MS/MS and HPLC-UV-Visible) (Minkin et al. 2008, Blanchet et al. 2009) or the EY complex formation based spectrofluorimetric determination of some other drug compounds (Hefnawy et al. 1999, Kelani et al. 1999, Moustafa 2000, Belal et al. 2002, El-Brashy et al. 2004).

Among the limited number of LC papers dealing with the quantification of SM; Minkin et al. (2008) reported a dynamic linear range of 0.2 to 500 ng/mL with the lower limit of quantification (LLOQ) to be 0.6 ng/mL for their developed LC-MS/MS method. In another LC

approach, Blanchet et al. (2009) reported their dynamic range as 20 to 200 ng/mL and LOQ as 10 ng/mL for their described HPLC-UV-Visible method.

Regarding the comparison with the EY complex formation based spectrofluorimetric determination of some other drug compounds; Kelani et al. (1999) made a spectrofluorimetric determination of astemizole, terfenadine and flunarizine with 0.94–7.1 μg/mL as the LOD values. Belal et al. (2002) published another method with LOD of 0.05 μg/mL (2.9×10^{-7} M) and 0.06 μg/mL (2.3×10^{-7} M) for vigabatrin and gabapentin, respectively. Hefnawy et al. (1999) proposed a method with the LOQ of 25–50 ppb and LOD of 5 ppb for all drugs studied (cefaclor, cephalexin, cefadroxil and cepharadine). El-Brashy et al. (2004) calculated LOD values of 0.1475, 0.1402 and 0.1369 μg/mL for levofloxacin, norfloxacin and ciprofloxacin, respectively. Even though it will not be totally true to compare our EY assay with the mentioned fluorimetric EY papers (as they quantify other drug substances rather than SM); when LOD and LOQ values were taken into consideration, our LOD; 0.041 μg/mL ($7.69\text{x}10^{-8}$ M) and LOQ; 0.850 μg/mL ($1.59\text{x}10^{-6}$ M) values were pretty much acceptable.

Specificity

For the selectivity (specificity) parameter, the interference effects of the frequently used excipients in the capsule-type pharmaceutical preparations were checked with the excess ratio of the excipients to SM. Mannitol, croscarmellose sodium, magnesium stearate, glucose (dextrose) and lactose were chosen as the model excipients and there was no interference observed at weight ratios of excipient/SM ≤ 5000.

Precision

Regarding the precision (reproducibility), the standard deviation (SD) values are displayed in Table 1A. All recordings of the FI value were repeated 10 times (n=10) and according to the SD values calculated, it can be concluded that the present method is relatively less precise in the determination of lower concentration values, which is the opposite case with the higher concentration values around 5.00 μg/mL. The overall precision data were discussed in Table 1A.

Reproducibility

Even though the reproducibility is a sub-parameter of precision and should be given there, in this paper it's discussed as a separate title of analytical method validation studies. Reproducibility parameter was studied with a different spectrofluorimeter in a different analytical research laboratory as described in the *Materials and Methods* section. For the reproducibility, all analytical method validation parameters, except robustness, displayed in Table 1A were re-studied and results were summarized in Table 1B. The LOD and LOQ values of the very same method were obtained as 0.71 μg/mL ($1.33\text{x}10^{-6}$ M) and 2.00 μg/mL ($3.74\text{x}10^{-6}$ M) in the alternative laboratory, which were initially 0.041 μg/mL ($7.69\text{x}10^{-8}$ M) and 0.850 μg/mL ($1.59\text{x}10^{-6}$ M), respectively in our laboratory. When operational concentration ranges were compared, both laboratories obtained linear calibration plots, while R^2 values were 0.9427 < 0.9962, respectively indicating that our results were relatively more linear. Regarding the precision of the two laboratories, when obtained SD values for the overall concentration range our results (n=10) were less precise than the alternative laboratory (n=6). Finally reproducibility

of the proposed method was summarized in Table 1C. The FI data obtained for various SM concentrations with the two Shimadzu fluorimeters; RF-540 (our laboratory) and RF-5301PC (alternative laboratory) were displayed as % normalized responses (±SD) and the RSD values were calculated out of them. Results obtained from lab-to-lab variations were found to be reproducible, as RSD values did not exceed 2%. Therefore the described and fully validated spectrofluorimetric method for the determination of SM, based on ion pair complexation with EY, was found to be reproducible.

Robustness

Robustness was checked through the parameters pH, dissolved oxygen effect, temperature, dye concentration and incubation time, whose results were summarized in Table 1B. The effect of medium pH on the results of the defined method was summarized and discussed initially in Fig. 2.

The effect of dissolved oxygen was studied at normal room, air saturated and deoxygenated conditions at the presence of 2.5 μg/mL SM and 180 μM EY. The average FI values (with ± SD values) were as follows; 75.46±0.34, 75.81±1.66 and 72.65±0.79 for normal room, air saturated and deoxygenated conditions, respectively. Therefore it may be concluded as the dissolved oxygen having a negative effect on the complex formation, probably due to possible oxidative degradation of SM and/or EY.

For the temperature effect, similarly 2.5 μg/mL SM and 180 μM EY were employed at 10, 25, 50 and 70°C and recorded FI values (±SD) were 79.79±1.49, 78.74±1.04, 76.34±0.12 and 72.46±0.69, respectively. Shortly it may be concluded that heating at high temperatures had no significant effect on the rate of reaction (SM-EY complex formation); conversely, it caused complex breakdown due to deformation of EY and weakened the FI of the blank as well. Therefore, room temperature was chosen as optimum for the overall assay procedures. Similar findings for the temperature effect were also recorded by El-Brashy et al. (2004).

The effect of reagent dye (EY) concentration on the recorded FI values was discussed initially as a separate figure (Fig. 3).

Incubation time was thought to be a possible parameter affecting the FI values being recorded. Therefore t=0, 5, 10 and 15 min were investigated in the presence of 2.5 μg/mL SM and 180 μM EY and obtained FI values (±SD) were 78.22±1.08, 78.43±0.91, 78.21±1.98 and 76.79±1.35, respectively. According to the obtained data, the formation of drug–dye color complex was instant; color development was complete within a few seconds after adding the dye reagent (EY). An additional advantage was that the intensity of the final color was stable for pretty long period of time, such as 10 min, with no precipitation of the complex. There was no significant difference between FI values obtained at t=0, 5 and 10 min, while at 15 min relatively a slight decrease occurred. Thus optimum incubation time was chosen as the shortest period of time such as 30 sec for the overall assay procedures.

Analyte stability studies

In the period of first 6 hours no evidence of decomposition of SM was determined as the sampled mean FI values showed insignificant variation. At 72nd hour no decomposition was

observed in the sample kept at 4±1°C whereas significant decomposition was observed at ambient room temperature (25±1°C) and under 254 nm UV radiation (at 25±1°C) as SM% determined values decreased to 95.38% ± 4,92 and 87,68% ±1,544, respectively. Therefore it can be concluded that SM stock solution can be kept at 4±1°C safely for at least 72 hours.

Conclusions

Complex formation reaction among SM and EY was examined. Spectrofluorimetric method for the determination of SM was established by using ion pair complex formation. The complex formed in this reaction system was regarded as the ion-association complex between drug cation and EY anion. The present method has the advantages of simplicity, sensitivity and reproducibility. The present procedure is useful and convenient for quality control and routine determination of drugs in pharmaceutical dosage forms where precision, time and cost effectiveness of analytical methods are important. Obtained results of the full analytical method validation were also summarized within this paper of ours.

In conclusion, the proposed fluorimetric method can simply be used for determining SM based on complexation with EY.

Özet

Saf maddeden ve farmasötik preparatlardan dolaylı yolla sunitinib malatın tayini için hızlı, seçici, duyarlı ve basit bir spektrofluorimetrik yöntem geliştirilmiştir. Sunitinib malatın boya kompleksleşme bazlı spektrofluorimetrik tayini, 800 nm'de serbest boyanın (Eosin Y) emisyonu ve 350 nm'de eksitasyonunun izlenmesi yoluyla gerçekleşmektedir. Yöntem, doğruluk, doğrusallık, özgünlük, gözlenebilme sınırı (LOD), tayin sınırı (LOQ), kesinlik, tekrar yapılabilirlik ve sağlamlık parametreleri ile tümüyle valide edilmiştir. Yöntem, 0.08 – 5.00 µg/mL konsantrasyon aralığında doğrusallık göstermiştir. LOD ve LOQ, 0.041 µg/mL ($7.69x10^{-8}$ M) ve 0.850 µg/mL ($1.59x10^{-6}$ M)'dir. Belirli yöntem parametreleri (ortam pH'ı, çözünmüş oksijen konsantrasyonu, boya konsantrasyonu, inkübasyon zamanı gibi) sağlamlığı test etmek amacıyla sistemetik bir şekilde değiştirilmiş ve oldukça iyi sonuçlar alınmıştır. Tekrar yapılabilirlik; önerilen yöntemin aynı çalışma koşulları altında, iki farklı laboratuvarda, farklı zamanlarda, farklı cihazlar kullanılarak Sunitinib malat tayininde uygulanması yoluyla gerçekleştirilmiştir. Laboratuvarlar arası değişimlerden elde edilen sonuçlar tekrar yapılabilir olarak bulunmuştur, RSD değerleri %2'yi aşmamıştır.

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