Spectrofluorimetric Determination of Nateglinide in Pharmaceutical Preparations

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Abstract
In this study, Selective assay methods have been presented for the analysis of nateglinide in tablets. The assay involves derivatization with 5-(dimethylamino) naphthalene-1-sulfonyl chloride (dansyl chloride) which is a specific reagent in the analysis of primary and secondary amines. Optimum conditions of the reaction between nateglinide and dansyl chloride were investigated and it was found that the reaction proceeds quantitatively at pH 11.0, 40 ^{0}C in 10 min when the mole ratio of reagent to the drug was 16. After the completion reaction, the derivative was extracted with dichloromethane from the reaction medium and the fluorescence intensity was measured at 517 nm with excitation at 397nm.
The developed method was successfully applied to the assay of nateglinids in tablets and the results were compared statistically with those obtained by pharmacopeia method (USP XXXIV). There is no significant difference between the two methods in the respect of mean values and the standard deviations at 95 % confidence level.

Keywords: Nateglinide, 5-(dimethylamino) naphthalane-1-sulfonyl chloride (Dansyl Chloride), Derivatization, Determination, Spectrofluorimetric method.

1. INTRODUCTION
Nateglinide (Figure 1) is an amino acid (phenylalanine) derivative that is chemically and pharmacologically different from other antidiabetics. Early phase insulin secretion, reducing the rise of blood sugar and Hbaic after meals.
Early stage insulin secretion is a fundamental mechanism for maintaining normal glycemia control.
The methods developed for the analysis of the drug active ingredients containing the primary and secondary amine group are usually made by Derivatization. For this purpose, dansyl chloride (Figure.2) was used as a derivation agent in this thesis.(Abd El-Ghaffar et al. 2011, Aksamija et al. 2012, Wang et al. 2007, Romero et al 2006, Higashi, and Fujii 2004)
It was thought to develop a simple sensitive and reliable method of analysis for nateglinide by differentiating it with dancil chloride, validate the methods developed and apply it as a combination to the analysis of this drug in pharmaceutical preparations. A spectrofluorimetric method was developed in this direction.

2. MATERIALS AND METHODS
Chemicals
Nateglinide was supplied from Deva Pharmaceuticals (Istanbul, Turkey). Dansyl chloride was purchased from Sigma Aldrich (Missouri, USA). Starlix tablets (120 mg Nateglinide), was purchased from Novartis Pharmaceuticals Corporation (Basel, Switzerland). Methanol, Sodium bicarbonate, Sodium hydroxide, Trifluoroacetic Acid, Acetonitrile, Dehydrated sodium sulphate were purchased from Merck KGaA

(Germany). Dichloromethane, Acetone were purchased from Sigma Aldrich (Missouri, USA). All the chemicals and ultra pure water which used in the assays were HPLC grade.
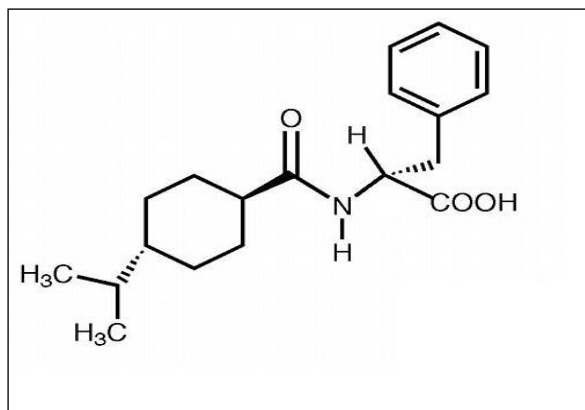
Figure 1- Chemical structure of the Nateglinide
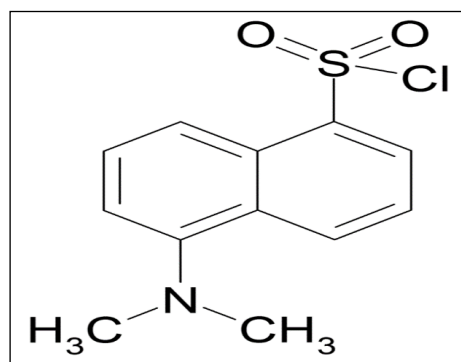
Figure 2- Chemical structure of the Dansyl Chloride

Apparatus and Other Materials
A Spectrofluorometer (Shimadzu RF-1501) HPLC system was employed for analysis. Shimadzu UV-160A A Spectrophotometer and Quartz tubes 1 cm (QS 1000) (10x10x45 mm) was used for absorbance measurement. While performing the comparison HPLC method CBM 10A system control unit, LC 10 at SPD-10 a UV-visible absorbance detector, DGU (with 2A helium gas extraction unit) was used. Chromatographic data was acquired using Shimadzu LC-Solution software. A C18 analytical column (HYPERSIL BDS 4,6 x 150 mm 5 µm particle size) was used for separation. The mobile phase consisting of Acetonitrile and 0.05% solution of trifluoroacetic acid (23:27). The flow rate of the mobile phase was 1.5 mL/min with detection at 210 nm. The column temperature was 25˚C and sample temperature was 25˚C. The injection volume was 10 µl. Diluent was Acetonitrile and water (11:9). Denver fragrance TB 2150 was used as a balance. For pH measurements wtw ph 526 model, was used. Electro-mag brand Vortex Mixer, Bransonic 221 brand ultrasonic bath, Memmert WB 10 brand water bath with thermostat, Janetzki T 5 brand centrifuge, Eppendorf 100 µl and 1000 µl brand automatic pipettes, Casio FX-3600P brand calculator, 1 cm glass tubs (OS 1000) (10x10x45 mm), ultra pure water device (Younglin instrument), reaction tubes (12mL with screw, cover), Bubbles (5, 10, 25, 50 and 100 mL) grade and transfer pipettes, filter paper (blue tape) used during analysis.

Method Development Studies: Determination of Optimum Reaction Conditions for Derivatization
Literature studies have shown that the reaction between dansyl chloride and the substances containing the primer and secondary amine group is carried out at alkaline pH, in the hot or room temperature, and the resulting derivative is measured by fluorescence intensity after being extracted with an organic solvent. In the light of the information in the literature, the following experiments were conducted to determine the best

conditions for the reaction between nateglinide and dansyl chloride (Higashi et al. 2008, Wang et al. 2007, Romero et al 2006, Higashi, and Fujii 2004). According to these literature investigations. Some experimental studies have been carried out.

Extraction Solvent

100 μL of the nateglinide stock solution at a concentration of 10 μg / mL was taken up into the reaction tubes, and 100 μL of pH 11.0 sodium bicarbonate buffer and 100 μL of dansyl chloride were added, followed by heating in a water bath at 40 ° C for 10 minutes. The resulting derivative was extracted in 5 different organic solvents, such as methanol, acetone, acetonitrile, dichloromethane and chloroform (3 x 2 mL). The anhydrous Na_2SO_4 was added to the organic phases combined in a 10 mL flask and then the organic solvent was extracted to volume. Spectra were taken with the aid of a spectrofluorimeter against the blank experiment prepared in the same conditions.

pH Effect

100 μL of the nateglinide solution at a concentration of 10 μg/mL was taken into the reaction tube. Then, 100 μL of sodium bicarbonate buffer (8.5-12.0) at various pHs was added and 100 μL of dansyl chloride was added. Heated for 10 minutes in a water bath at 40 °C. The cooled solution was extracted with 3 x 2 mL dichloromethane. Extracts was taken into the 10 mL volumetric flask. The organic phases combined in the flask were combined with anhydrous Na_2SO_4 and then the volume was diluted with dichloromethane. Spectra were taken with the help of a spectrofluorimeter against the blank experiment prepared under the same conditions.

Temperature and Reaction Time

100 μL of the nateglinide solution at the concentration of 10 μg/mL was taken up in the reaction tubes and pH 11.0 sodium bicarbonate buffer was added. Then 100 μL of dansyl chloride was added. The tubes were kept at room temperature, 40, 50, 60 ºC for 10, 20, 30, 40, 50, 60 minutes.

Effect of Dansil Chloride amount on the nateglinide-dansyl Derivative

100 μL of the nateglinide solution at a concentration of 10 μg / mL was taken up in the reaction tubes and 100 μL of the solution was added from a pH 11.0 sodium bicarbonate buffer. Then 25, 50, 100, 150, 200, 250, 300, 400, 450, 500, 550 μL of the dansyl chloride solution (0.2 mg / mL) added to the solution.

Analytical method validation

According to the results obtained from the method development study, optimum method conditions were determined and the method was validated according to these conditions.

Preparation of solutions

Nateglinide Solution

Stock solution: nateglinide 100 mg was transfered to 100 mL of bubble solution and completed in volume with methanol (1 mg/mL). 10 mL of this solution was transferred to 100 mL balloon joje and completed with water volume (100 μg/mL). In the study, a solution containing nateglinide was used in 10 μg/mL concentration diluted with water.

Dansyl Chloride Solution

In the determination of reaction conditions, a solution prepared in acetone was used for the concentration of 0.2 mg/mL of densil chloride.

Buffer Solutions

50 mL 0.1 m sodium bicarbonate solution by adding 0,1 M NaOH required amounts of pH's 8,5, 9,0, 9,5, 10,0, 10,5, 11,0, 12,0 the buffer solutions have been prepared.

Precision and Accuracy

For precision and Recovery evaluation at three different concentrations (25, 100 and 250 ng / mL), six of the nateglinide-containing solutions were prepared. In line with this aim Intraday and Interday analysis were done.

Linearity and range

To prove the linear response relation, fluorescence intensity of the standard solutions between 25.0 - 250

ng.mL^{-1} at 5 different concentrations (25, 50, 100, 150 and 250 ng.mL^{-1}) were measured and linearity curves were plotted. After solutions were analyzed, y=ax+b threshold was found and regression analysis was performed (y: area, a: slope, b: intercept, x: concentration, mg/mL).

Specificity

Specificity test was performed to demonstrate the ability of the analytical method to measure only the intended substances in a given sample. Excipients such as Lactose monohydrate, Croscarmellose sodium, Microcrystalline cellulose, Povidone, Hydroxypropyl methyl cellulose, Colloidal silicon dioxide, Magnesium stearate, Talc, Polyethylene glycol 4000, Yellow iron oxide (E 172), Titanium dioxide (E 171) in tablets containing 120 mg nateglinide The solutions in the concentrations of 100 µg.mL-1 were prepared and studied as in sample preparation.

Robustness

With the Robustness parameter the effect of changes in the method parameters and solution stability were examined. For this aim parameters which Changes in the Method Parameters and Solution Stability were performed.

Changes in the Method Parameters
-     Excitation Wave Length   : λ_{eks}=398 and λ_{eks}=396
-     Emission Wave Length   : λ_{ems}=518 and λ_{ems}=516
-     Reaction Time   : 10 min. ± 1 min.
-     Dansyl Chloride Amount   : 400 ± 5µl

Solution Stability

The effect of the nateglinide-dansyl derivative on the fluorescence intensity values of the solution was examined at room temperature in daylight, in the dark and in the dark at + 4 ° C for a period of time to examine the stability of the nateglinide-dansyl derivative in dichloromethane solution.

Results and Discussion

The spectrofluorometric method developed for the quantitative determination of nateglinide tablets is based on the reaction between dansyl chloride and this substance containing the secondary amine group. (Figure 2)

Dansil forms derivatives with strong fluorescence observed in the yellow reaction in the derivative reaction with chlorine primer and secondary amine. Progress in the aqueous medium of the reaction is of considerable advantage in that the overexpression of the reagent does not detriment to the assay and proceeds with minimal amounts of the amine.

In literature searches, it has been shown that Dansyl chloride (Figure 3) reacts with amines at alkaline pH and hot, and the resulting product is extracted from the reaction medium with an organic solvent. Optimum conditions were investigated for this information light. Optimization studies showed that the best results were obtained in pH 11.0 sodium bicarbonate buffer. In order to determine the optimum reaction temperature and reaction time, various temperatures and various reaction times were studied. The optimum reaction temperature, which is also in the direction of these studies, was determined to be 10 minutes at 40 ºC.

In Method Development Studies Optimum reaction conditions carried out such, Extraction Solvent effect on fluorescence intensity (Table 1, Figure 4, Figure 5 and Figure 6), pH Effect (Table 2 and Figure 7) Temperature and Reaction Time (Table 3 and Figure 8) Effect of Dansil Chloride amount on the nateglinide-dansyl Derivative (Table 4 and Figure 9). According to the results obtained from these studies optimum method conditions were determined.

The maximum excitation and emission wavelengths for this solvent were determined to be 397 nm and 517 nm, respectively, when the various fluorescence intensities were obtained when the various organic solvents were used for the extraction of the nateglinide-dansyl derivative from the reaction medium after completion of the derivatization reaction and consequently dichloromethane was used.

It was observed that the auxiliary substances prepared did not react with Dansil chloride and did not affect the fluorescence intensity in Specificity analysis.

The linearity between the concentration and the fluorescence intensity after the specified optimum conditions is 25-250 ng / mL Correlation coefficient between concentration and areas of more than 0.999 was sought. (Table 5, Table 6, Figure 10). The calculated correct equation is F= 3.0135C+119.37. The LOD and LOQ values were 6.17 and 18.68 ng / mL-1, respectively. (Table 7)

Table 1. Maximum excitation (λexs) and emission (λem) wave length and fluorescence intensity values of the nateglinide-dansyl derivative in various organic solvents

	$\lambda_{exs}/\lambda_{em}$ (nm)	Fluorescence Intensity
Methanol	401/532	193
Acetone	419/527	315
Acetonitrile	419/531	385
Dichloromethane	397/517	1007
Chloroform	404/502	953

Table 2. Effect of pH on the nateglinide-dansyl Derivative

pH	8,5	9,0	9,5	10,0	10,5	11,0	12,0
Fluorescence Intensity	90	188	182	280	317	482	354

Table 3. Effect of Time and Temperature on the nateglinide-dansyl Derivative

Temperature (^{0}C) \ Time (min)	10	20	30	40	50	60
Room Temperature	444	478	446	354	448	444
40	480	412	410	416	428	346
50	282	280	252	232	262	220
60	122	108	57	66	101	79
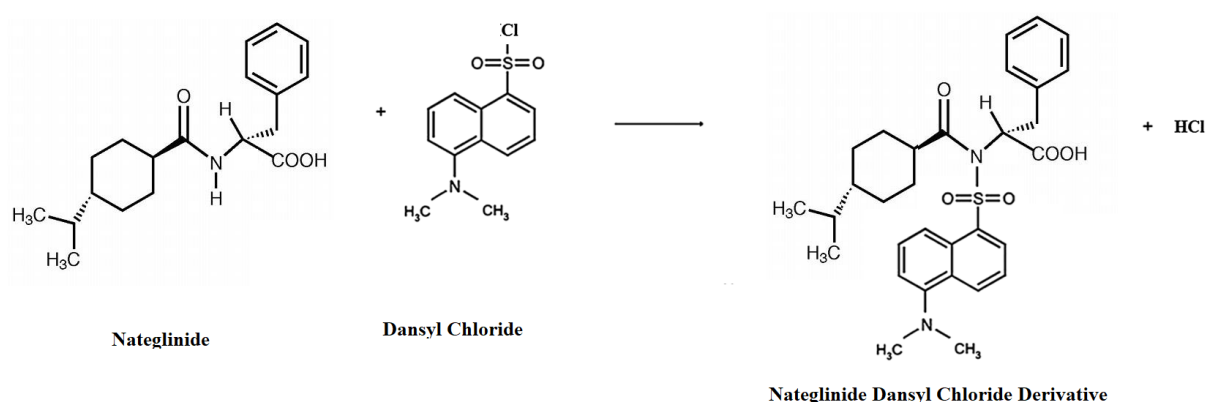
Figure 3. The derivatization reaction mechanisim between Nateglinide and Dansyl Chloride
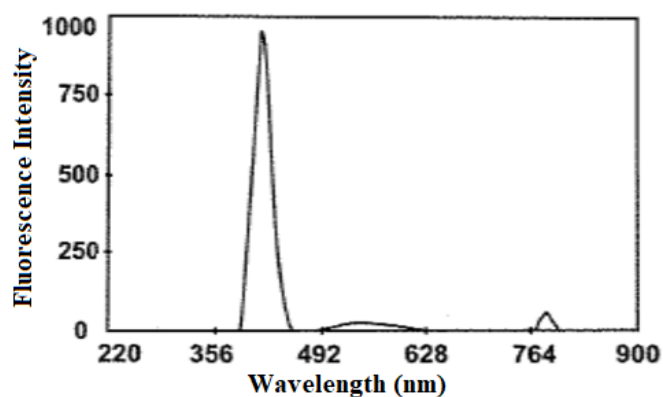
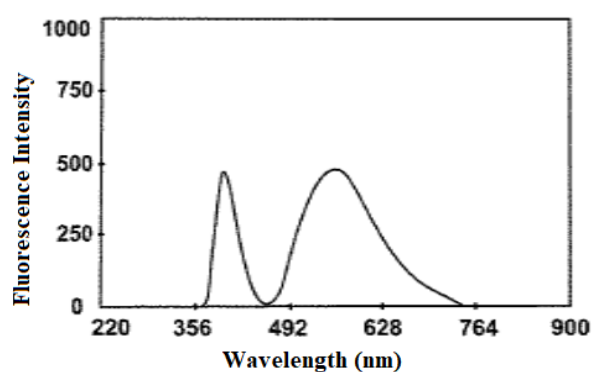**Figure 4.** Emmision Spectrum of Blank

Figure 5. Emmision Spectrum of 100ng/mL Nateglinide Dansil Chloride Derivative (λ_{em}=517nm)
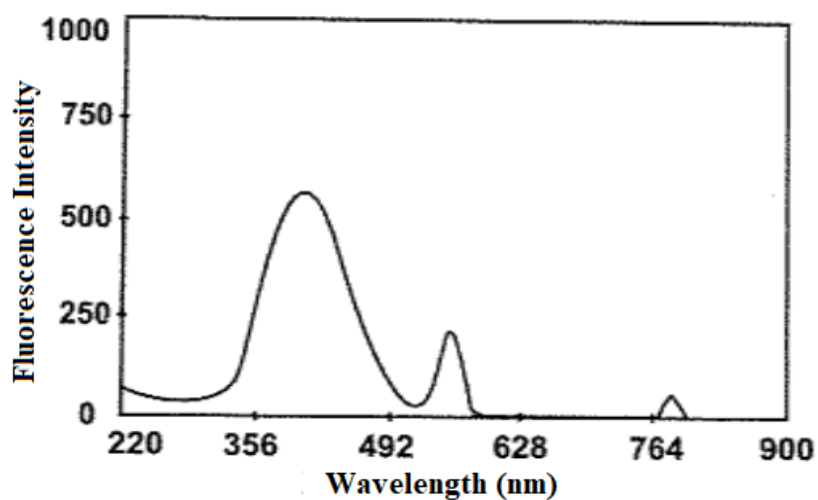

Figure 6. Excitation Spectrum of 100ng/mL Nateglinide Dansil Chloride Derivative (λ_{ex}=397nm)
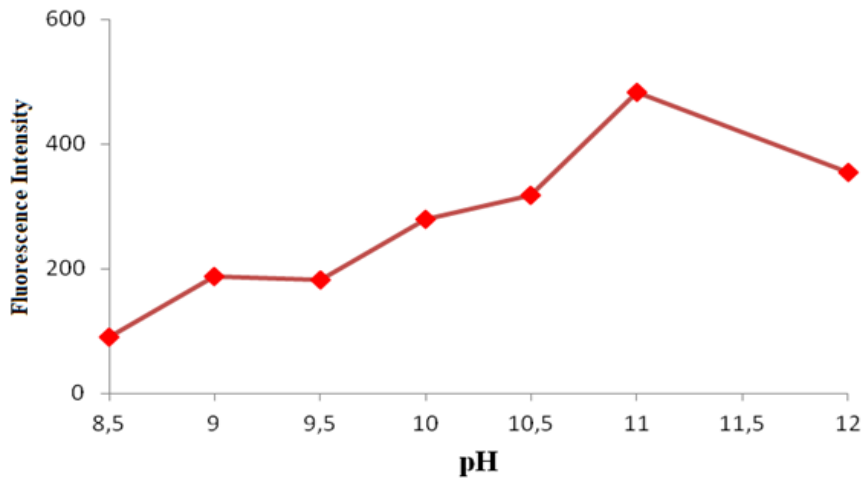
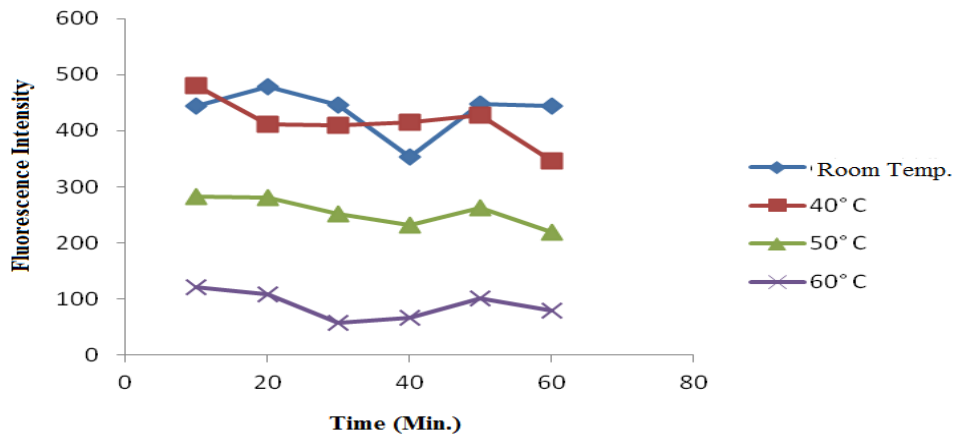**Figure 7.** Effect of pH on the nateglinide-dansyl Derivative

Figure 8. Effect of Time and Temperature on the nateglinide-dansyl Derivative
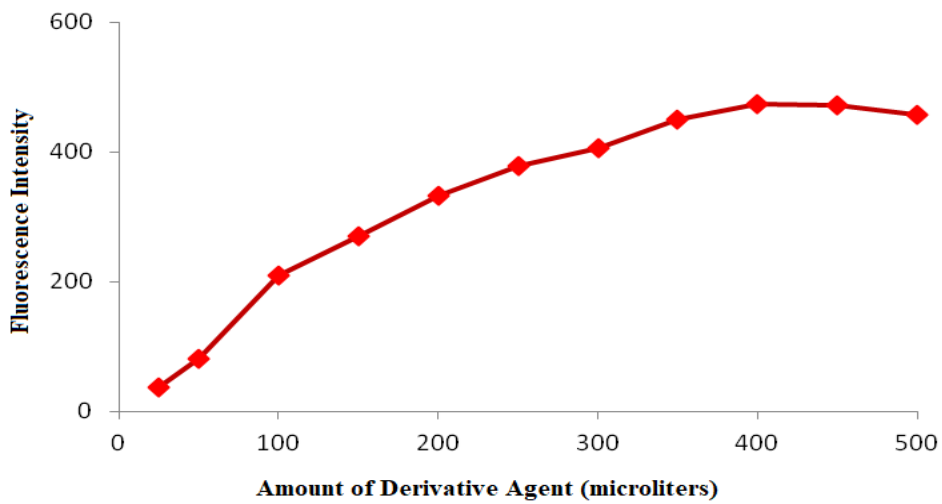

Figure 9. Effect of Dansil Chloride amount on the nateglinide-dansyl Derivative
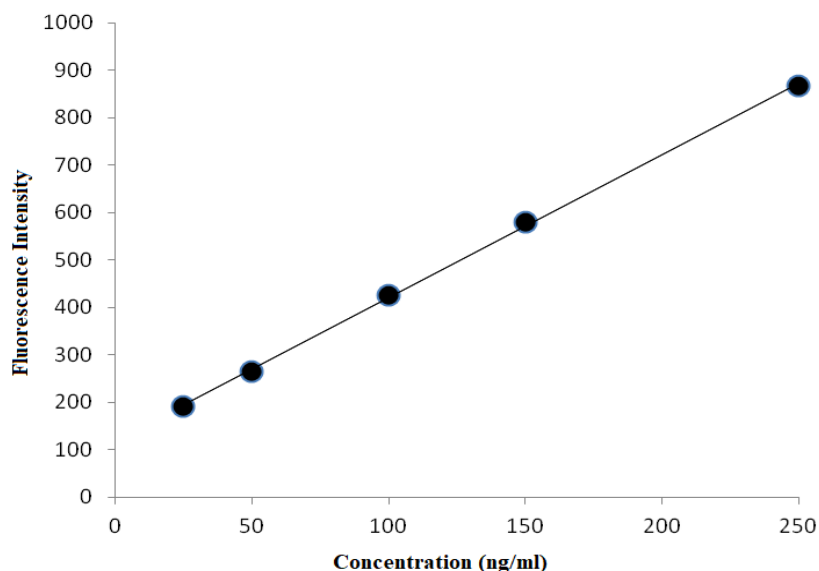
Figure 10. Calibration Curve of Nateglinide (25-250 ng/mL)

Table 4. Effect of Dansil Chloride amount on the nateglinide-dansyl Derivative

Dansil Chloride (µL)	25	50	100	150	200	250	300	350	400	450	500	550
Fluorescence Intensity	36	81	210	270	333	379	406	450	474	472	457	411

Table 5. Linearity and range **-** The fluorescence intensity values and the statistical data of the calibration curves prepared in the aqueous solution at the concentration range of 25-250 ng / mL

No	C (ng.mL^{-1})	F_1	F_2	F_3	F_4	F_5	$F_{Average}$	SD	%RSD
1	25	190	191	191	200	185	191.4	5.41	2.83
2	50	257	257	257	280	275	265.2	11.37	4.29
3	100	419	419	419	450	425	426.4	13.45	3.15
4	150	570	570	570	600	590	580.0	14.14	2.44
5	250	846	846	846	900	895	866.6	28.26	3.26

Equation of line calculated by $F_{Average}$

F =3,0135C+119.37

r= 0.999

Table 6. Parameters of regression analysis of calibration curves

	1	2	3	4	5	Average
a	2.9397	2.9369	2.9369	3.1109	3.143	3.0135
b	118.34	118.86	118.86	128.24	112.56	119.37
r	0.9995	0.9995	0.9995	0.9996	0.9998	0.9996

Table 7. LOD and LOQ values prepared according to the values of parameters of regression

Parametreler	Değerler
Equation of line	F =3.0135C+119.37
Slope ± SD	3.0135 ± 0.10
Intercept ± SD	119.37 ± 4.63
LOD ng/mL	6.17
LOQ ng/mL	18.68
r	0.9996

Nateglinide was analyzed at three different concentrations (25, 100 and 250 ng / mL) within the same day and between days to determine the precision of the developed method. Relative standard deviations for the same day and inter-day analyzes were between 0.11-1.33% and 0.06-0.85%, respectively (Table 8 and Table 9). We investigated the robustness of the method by making minor changes in parameters such as excitation, emission duration, wavelength response time, and the amount of marker. The RSD values found are 0.027-0.026%, 0.022-0.041% and 0.024-0.029%, respectively. It was observed that the values found were too small to be considered. Results showed that there was no significant change in Assay results. The method is robust in view of changes to Excitation Wave Length, Emission Wave Length, Reaction Time, Dansyl Chloride Amount. (Table 10)

When the stability of the derivative in dichloromethane was examined it was found to be stable at +4 [deg.] C. for 48 hours in the dark. (Table 11)

Table 8: Repeatability of Intraday Analyzes

	Concentration (ng/mL)		
	25	100	250
	24.39	99.72	248.27
Calculated	24.58	99.5	248.5
Concentration	24.28	99.46	248.86
(ng/mL)	23.96	99.43	249.27
	24.58	99.43	249.13
	24.91	99.48	249.05
Average	24.45	99.50	248.85
Standard Deviation	0.32	0.11	0.39
% RSD	1.31	0.11	0.16

Table 9: Repeatability of Interday Analyzes

	Concentration (ng/mL)		
	25	100	250
	24.62	99.67	249.48
Calculated	24.66	99.49	249.48
Concentration	24.58	99.52	249.47
(ng/mL)	24.37	99.56	250.24
	24.85	99.59	250.13
	24.97	99.47	250.05
Average	24.67	99.55	249.81
Standard Deviation	0.21	0.07	0.37
% RSD	0.85	0.07	0.15

Table 10. Robustness

	Excitation Wave Length $\lambda_{eks}=398$	Excitation Wave Length $\lambda_{eks}=396$	Emission Wave Length $\lambda_{ems}=518$	Emission Wave Length $\lambda_{ems}=516$	Reaction Time (10-1min)	Reaction Time (10+1min)	Dansyl Chloride Amount (400-5µl)	Dansyl Chloride Amount (400+5µl)
	100.03	100.09	100.06	100.89	100.00	100.10	100.01	100.02
Calculated	100.01	100.06	100.02	100.75	100.03	100.00	100.02	100.09
Concentration	100.00	100.04	100.30	100.66	100.01	100.08	100.06	100.08
	100.05	100.02	100.04	100.02	99.99	100.07	100.07	100.10
	100.07	100.07	100.45	100.30	100.05	100.06	100.02	100.06
	100.01	100.08	100.28	100.10	100.01	100.12	100.03	100.09
Average	100.03	100.06	100.19	100.45	100.02	100.07	100.04	100.07
Standard Deviation	0.027	0.026	0.177	0.363	0.022	0.041	0.024	0.029
RSD	0.027	0.026	0.176	0.361	0.022	0.041	0.024	0.029

Table 11. Solution Stability

	Fluorescence Intensity				
	Başlangıç	6 hours later	24 hours later	48 hours later	72 hours later
Room Temperature Darkness	423	417	400	364	321
Room Temperature Day Light	423	410	393	253	117
At (+)4 ⁰C Darkness	423	422	420	420	418

The developed method was applied in the analysis of tablets containing nateglinide. The recovery was 101.20% and the RSD was 0.43%. In tablet analysis analyzed by pharmacopoeia, recovery was found to be 101.24% and RSD to be 0.64%. Statistical evaluation of the obtained results was done by calculating the standard deviation, relative standard deviation and confidence limits at the 95% probability level over 6 trials. It was observed that all the data obtained according to these results meet the requirements of "ICH Q2 (R1)". (Table 12)

Table 12. Analysis of Tablets Containing 120 mg nateglinide and Evaluation of the Results Statistically

	Pharmacopeia Method		Developed Method	
Sample	**mg/Tablet**	**Recovery (%)**	**mg/Tablet**	**Recovery (%)**
1	121.01	100.84	121.23	101.03
2	120.96	100.80	122.19	101.82
3	122.54	102.12	121.46	101.21
4	121.03	100.86	121.80	101.50
5	122.45	102.04	120.68	100.57
6	120.95	100.79	121.27	101.06
x	121.49	101.24	121.44	101.20
SD	0.78		0.52	
% RSD	0.64		0.43	
Confidence Interval	0.82		0.54	

$T_{Calculated}= 0.13$ $t_{table}= 2.23$

$F_{Calculated}= 2.25$ $F_{table} = 5.05$

$n_1= n_2 = 6$ P=0.05

In conclusion, this study is economical because the method reported for nateglinide determination in tablets is highly reproducible, simple, sensitive, and very low in quantities. A relative advantage of the method is that the derivative can remain durable for 48 hours in dichloromethane because the relative standard deviations are as small as 1%. The derivatization and extraction processes do not take much time. For this reason, it is thought that biological liquids can be a reliable method for nateglinide analysis and bioavailability studies, as is easily suggested for routine pharmaceutical analyzes.

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