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VALIDATION OF FLOW INJECTION METHOD FOR DETERMINATION OF CEFOTAXIME SODIUM IN INJECTION DOSAGE USING FLUOROMETRIC DETECTOR

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ABSTRACT
A new fluorometric method was developed for the determination of important antibiotic; Cefotaxime sodium (CFT) in pharmaceutical dosage. The method was depend on the measurement of quenching fluorescence of 2,7-dichlorofluorescein as a fluorescent dye that is affected by cefotaxime in continuous system. The fluorescence and quenching values were measured by a local made fluorometer using solid state laser diode 405 nm as a source combined with flow injection technique. Chemical and physical parameters such as 2, 7-dichloroflurescein concentration, pH of carrier, sample volume, flow rate have been investigated. Linear dynamic of cefotaxime is ranged from 0.2-4.0 mmol. L^{-1} with correlation coefficient r = 0.9896 while the (% r^2) percentage linearity was 97.93%. Low limit of detection of 4.583 µg/sample depended on the dilution of minimum concentration in calibration curve. The relative standard deviation (% RSD) at 0.7 and 2.0 mmol.L^{-1} was found to be less than 1% (n = 6). Finally, no significant difference is existed between the developed and the classical method, which was confirmed by paired t-test with calculated t-value at 95% confidence interval (p > 0.05).

KEYWORD: Cefotaxime, Laser diode 405 nm fluorometric, 2, 7-dichlorofluorescein, flow injection analysis

INTRODUCTION

Cefotaxime sodium is a Sodium (6*R*,7*R*)-3-[(acetyloxy) methyl] -7- [(2*Z*)-2- (2-aminothiazol-4-yl)- 2- (methoxy imino) acetyl] amino]-8-oxo-5-thia-1-azabicyclo [4.2.0] oct-2-ene-2-carboxylate, [$C_{16}H_{16}N_5NaO_7S_2$] with mole cular weight 477.447 mol/L. It is white or slightly yellow powder, freely soluble in water, sparingly soluble in methanol[1]. Cefotaxime sodium Figure 1, is a drug of the third generation cephalosporin family wich is widely used for the treatment of Gram-negative bacteria. It is a broad-spectrum β-lactam antibiotic and treats many kinds of infections, including those of the skin, bone, stomach, brain, blood, respiratory tract, sinuses, ears, and urinary tract[2, 3].

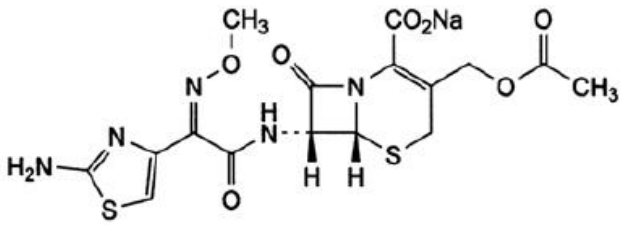

FIGURE 1: The structural formula of Cefotaxime sodium

Several of analytical methods have been reported for the quantitative determination of cefotaxime sodium in dosage forms, for instance Spectrophotometric[4-9], FIA-Spectrophotometric[10], FIA-turbidimetric[11], Spectro fluorimetric [12-14], High Performance Liquid Chromatography[15-17], and Atomic Absorption Spectro photometric method[18].

The purpose of this paper is to analyze the cefotaxime sodium in dosage form by fluorometric method combined with flow injection technique. The suggested method depended on using the 2, 7-dichlorofluorescein as a fluorescent dye which forms constant fluorescence intensity then the fluorescence is quenched by cefotaxime. The fluorescence quenching is measured via local made laser diode fluorometer combined with continuous flow injection.

MATERIALS & METHODS

Apparatus

Ismatec (ISM796) peristaltic pump with variable speed was used to fluid propulsion, a Rheodyne injection valve (2-directions, 6-port) with a sample loop was made from Teflon (0.5 mm i.d., variable length) used for sample injection.

The fluorescence was measure via Local made laser diode fluorimeter instrument. The irradiation source for excitation was used laser diode with wavelength 405 nm

while photo diode was used as the detector with a vertical position at 90° with radiation source.
Siemens recorder x-t potentiometric type KOMPENSO GRAPH C-1032 and Digital AVO-meter were used to record the output signals. Peak height was measured for each signal. Shimadzu spectrophotometer model 1800 (Japan) was used to measure the UV-Vis spectra. The manifold for flow system that used for the analysis of cefotaxime sodium was shown in Figure (2).

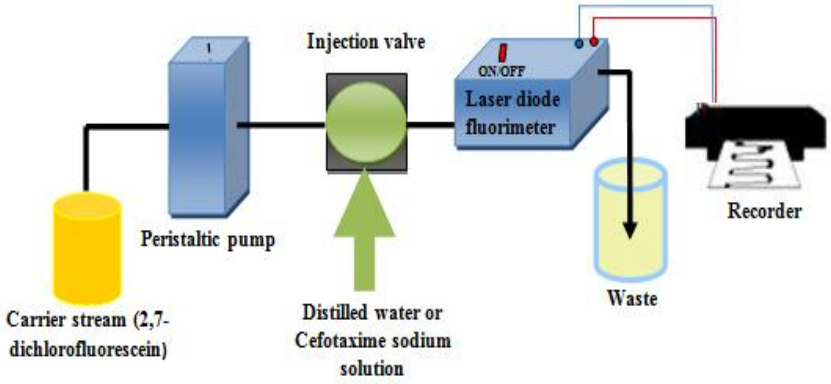

FIGURE 2: Manifold that used for determination of cefotaxime sodium based on fluorescence quenching

Chemicals
0.01 mol.L^{-1} Stock solution of cefotaxime sodium (SDI-Iraq) ($C_{16}H_{16}N_5NaO_7S_2$, 477.447 g.mol^{-1}) was prepared by dissolving 0.4774 g in 100 mL distilled water; the dark container was used to keep solution and kept in refrigerator.
Analytical grade of 2,7-dichlorofluorescein was purchase from Riedel De Haen AG Seelze Hannover, (0.001 mol.L^{-1}) standard stock solution of 2,7-dichloro fluorescein ($C_{20}H_{10}Cl_2O_5$, 401.21 g.mol^{-1}) was prepared by dissolving 0.4012 g in 0.01M Na_2CO_3 and complete with same base. A 0.01 mol.L^{-1} standard solution of sodium carbonate (BDH) (Na_2CO_3, 105.99 g.mol^{-1}) was prepared by dissolving 1.0599 g in 1000 mL distilled water. Stock solution of sodium hydroxide (BDH) (NaOH, 40 g.mol^{-1}) (0.1 mol.L^{-1})was prepared by dissolving 1.00 g of the base in distilled water and dilute to the mark with the same solvent to 250 mL calibrated flask. All chemicals that used in this paper were of analytical grade and distilled water was used in all dilution processes.

METHODOLOGY
The manifold system is composed of single line which was used for the analysis of cefotaxime by fluorescence quenching of (0.1 mmol.L^{-1}) 2,7-dichlorofluorescein after that interaction with cefotaxime sodium in aqueous media.Local made fluorometer was used for emission of the carrier stream (2,7-dichlorofluorescein, 0.1 mmol.L^{-1}) that gives a constant and continuous emission of fluorescence light at 1.6 mL.min^{-1} flow rate which lead to the injection valve to carry cefotaxime sample segment with 96 µL sample volume. The mixture was then passed throughout the measuring cell that gives fluorescence quenching response which was recorded on x-t potentiometric auto AVO-meter.

RESULTS & DISCUSSION
Variable Optimization
The effects of chemical parameters such as 2,7-dichlorofluorescein concentration and phosphate buffer as well as to the physical parameters like a flow rate, sample volume, delay coil studied were investigated using single line manifold system (Figure-2) to establish the optimum parameters.

TABLE 1: Variation of 2, 7-dichlorofluorescein concentration on the total quenching of fluorescence expressed as an average peak heights (n=3)

2,7-dichloro fluorescein concentration (mmol.L^{-1})	Continnuous of fluorescence response (n=3) $\bar{y}i$ in mV	Total Fluorescence quenching (n=3) $\bar{y}i$ in mV	σ_{n-1}	RSD%	Confidence interval of the average at (95%) $\bar{y}_i \pm t_{(\alpha=0.05/2)} \frac{\sigma_{n-1}}{\sqrt{n}}$	Fluorescence quenching by distilled water in mV	Fluorescence quenching of cefotaxime $\bar{y}_{Qi}$ (n=3) in mV	Fluorescence remaining $\bar{y}_{Ri}$ (n=3) in mV
0.01	120	68	0.46	0.67	68±1.14	66	2	52
0.03	418	374	1.73	0.46	374±4.30	325	49	44
0.05	703	656	1.11	0.17	656±2.77	580	76	47
0.1	1651	1308	4.36	0.33	1308±10.83	1163	145	343
0.2	2934	1826	5.29	0.29	1826±13.15	1311	515	1108
0.3	3706	2411	3.61	0.15	2411±8.96	1548	863	1295
0.5	5743	1923	2.65	0.14	±6.571923	1084	839	2434

Chemical Parameters

1- Variation in 2,7-dichlorofluorescein Concentration

A series of 2,7-dichlorofluorescein solutions (0.01-0.5 mMol.L^{-1}) were prepared, using preliminary trial sample volume of 96µL, a carrier stream of 2,7-dichloro fluorescein at 1.6 mL/min flow rate.

The relation between fluorescein dye concentration and the continuous of fluorescence response is expressed as an average response (n=3) ȳi in mV as shown in Table (1). The obtained results showed that there is an increasing in fluorescence with increasing 2,7-dichlorofluorescein concentration also increasing in the quenching effect with blank (distilled water) or cefotaxime. Table (1) shows the variation of 2,7-dichlorofluorescein and fluorescence quenching for both of water and cefotaxime. Therefore; 0.3 mmol.L^{-1} 2,7-dichlorofluorescein concentration was chosen as the optimum concentration that applied for aftertime tests.

2. **Variation of pH of Phosphate Buffer Solution**

The study was achieved by dilution of the stock solution of 2,7-dichlorofluorescien in different phosphate buffer solution using the optimum concentration (0.3 mMol.L-1) of 2,7-dichlorofluoresein, and preliminary 3 mMol.L^{-1} of cefotaxime with 65 µL sample volume. Figure (3) showed that the distilled water was used as the only solvent; no other chemicals were used. While the buffer solutions at pH (3.5-9) were caused an effective which led to lossing of drug concentration, due to the formation of precipitate and consumption of drug during the reaction of drug with the buffer.

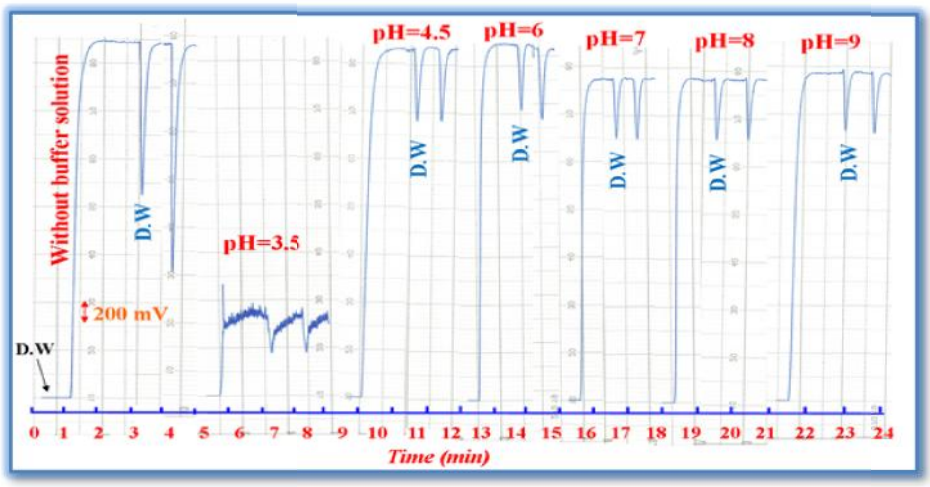

FIGURE 3: Effect of pH variation on the measurements of fluorescence

Physical Parameter

1- Flow rate

Using the optimum concentration of 2,7-dichloro fluorescein 0.3mmol.L^{-1} was studied and sample volume 65 µL with a variable range 0.6 to 2.5 mL.min^{-1} flow rate for the carrier stream controlled by the peristaltic pump for determination of cefotaxime at preliminary concentration 4 mmol.L^{-1}. The results obtained were summarized in Table (2). It can be recognized that at low flow rates, there were an increasing in peak base width (Δt_B), a response peak broadening, which occurred with little increasing in peak height this mostly attributed to the dispersion and dilution, while at higher flow rate (> 1.3 mL.min^{-1}) for the carrier stream, although caused in obtaining regular response and maxima get sharp, but it was not very high because of departure at relatively higher speed, thus the does has not enough time to detect. Therefore, the best flow rate 1.3mL.min^{-1} was chosen as the optimum flow rate for the carrier stream.

TABLE 2: Effect of variable flow rate via quenched continuous fluorescence for determination of Cefotaxime sodium

Pump speed (rpm)	Flow rate (mL. min^{-1})	Total Fluorescence quenching (n=3) ȳi in (mV)	σ_{n-1}	RSD%	Confidence interval of the average at (95%) ȳi(mV) ± $t_{(0.05/2,n-1)}\sigma_{n-1}/\sqrt{n}$	Fluorescence quenching by distilled water in mV	Fluorescence quenching of Cefotaxime $\bar{y}_{Qi}$ (n=3) in mV	Fluorescence remained $\bar{y}_{Ri}$ (n=3) in mV	Δt_B (sec)	V_{add} (ml)	Conc. (mM)
10	0.6	2740	21.79	0.79	2740±54.14	620	2120	1000	123	1.326	3.453
15	1	2580	11.36	0.44	2580±28.22	900	1680	1160	72	1.296	3.375
20	1.3	2680	10.00	0.37	2680±24.84	960	1720	1060	54	1.266	3.297
25	1.6	2560	13.23	0.52	2560±32.86	1080	1480	1180	42	1.216	3.167
30	1.9	2520	11.00	0.44	2520±27.33	1280	1240	1220	30	1.046	2.724
35	2.2	2500	5.29	0.21	2500±13.15	1460	1040	1240	24	0.976	2.542
40	2.5	2460	7.00	0.28	2460±17.39	1500	960	1280	18	0.846	2.203

Response of continuous fluorescence: 3740mV, V_{add}: addition volume

2-Sample Volume

The injected volume of sample was variable from 46 to 440µL using different length of the sample loop in the injection valve, while the other chemical and physical parameters were remained constant (2,7-dichlorofluorescein 0.3 mmol.L^{-1} and flow rate 1.3 mL.min^{-1}). The results are tabulated in Table (3), which indicate that 96 µL was the optimum sample volume for Cefotaxime, which is characterized by sharpness and smooth response profile. It was noticed that an increase in sample volume led to an increase in the heigh of response, there was an increase in dilution and dispersion which may cause an increase in the base width Δt_B of response. Therefore, 96µL was chosen to be the optimum sample volume range. Larger volume (> 96 µL) led to a broadening at the peak maxima and an increase in the base width (Δt_B) which was most probably attributed to continuous long time duration of segment in front of the detector, Figure (4).

TABLE 3: Effect of the variation of sample volume in the measurement of fluorescence response

Length of sample loop (cm) Diameter (0.5mm)	Injected sample loop volume (µl)	Total Fluorescence quenching (n=3) $\bar{y}i$ in mV	σ_{n-1}	RSD%	Confidence interval of the average at (95%) $\bar{y}i(mV)\pm t_{(0.05/2,n-1)}\ \sigma_{n-1}/\sqrt{n}$	Fluorescence quenching by distilled water in mV	Fluorescence quenching of cefotaxime $\bar{y}_{Qi}$ (n=3) in mV	Fluorescence remained $\bar{y}_{Ri}$ (n=3) in mV	Base width Δt_B (sec.)
12	46	2340	6.24	0.27	2340±15.51	800	1540	1400	51
17	65	2680	8.19	0.31	2680±20.34	960	1720	1060	54
25	96	3080	9.64	0.31	3080±23.96	1020	2060	660	59
35	135	3140	11.36	0.36	3140±28.22	1520	1620	600	66
40.9	157	3200	15.13	0.47	3200±37.59	1660	1540	540	72
53.6	206	3320	18.52	0.56	3320±46.01	2020	1300	420	84
56(r=0.7)	440	3340	16.46	0.49	3340±40.89	2580	760	400	90

Response of continuous fluorescence: 3740Mv

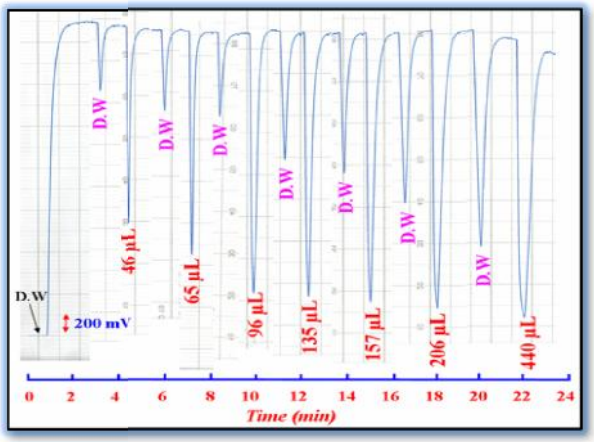

FIGURE 4: Response profile of the sample volume on fluorescence measurements

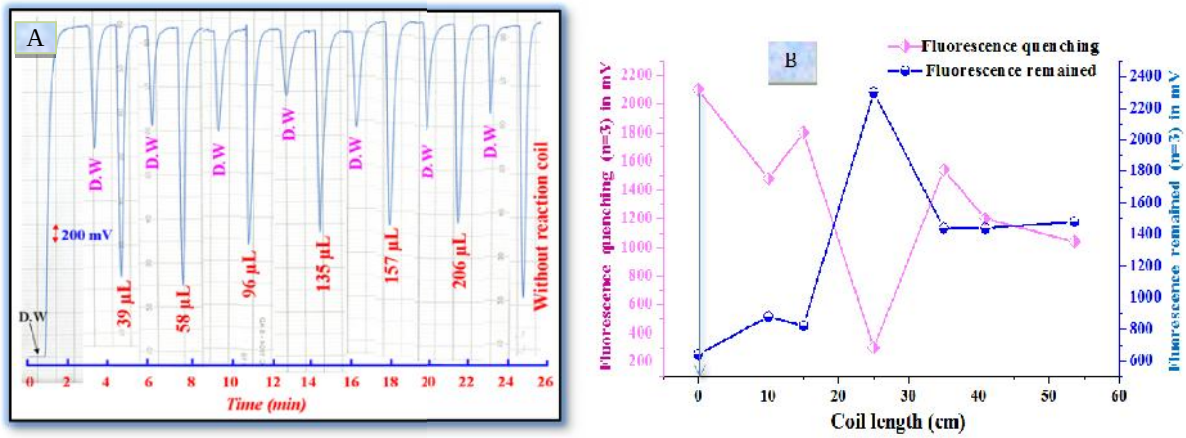

FIGURE 5: Variation of reaction coil on; A- Response time profile, B- quenching and remained of fluorescence

3- **Reaction Coil**

Variable length (0-53.6) cm of reaction coil was studied at preliminary concentration of cefotaxime sodium (4 mmol.L^{-1}) via using the optimum chemical and physical parameters which were chosen. The results are obtained from the effect of reaction coil on fluorescence response and Δt_B were shown in Figure (5). It was noticed that there is no coil was used in manifold that gave a high and repeatable response profile for the quenching of 2,7-dichlorofluorescein by cefotaxime. While increasing of coil length gave a broadening of the peak maxima which most probably attribute to the increased effect of dilution and dispersion on sample segment.

Variation of Cefotaxime Sodium Concentration

A series of Cefotaxime solutions ranging from 0.2 to 6 mmol.L^{-1} were prepared and each one of the obtained values was repeated successively three times, using 96 µl as an injected sample volume with fixed optimum parameters. Linear dynamic range was obtained from 0.2 to 4 mmol.L^{-1}, while Figure (6) showed the calibration graph using laser diode fluorometer and classical method[19] respectively. Table (4) illustrates the brief results of determination the cefotaxime sodium using laser diode fluorometer-flow injection, while the classical method spectrophotometer shows the values of correlation coefficient, linear percentage, straight line equation and the calculated t-value at 95% confidence.

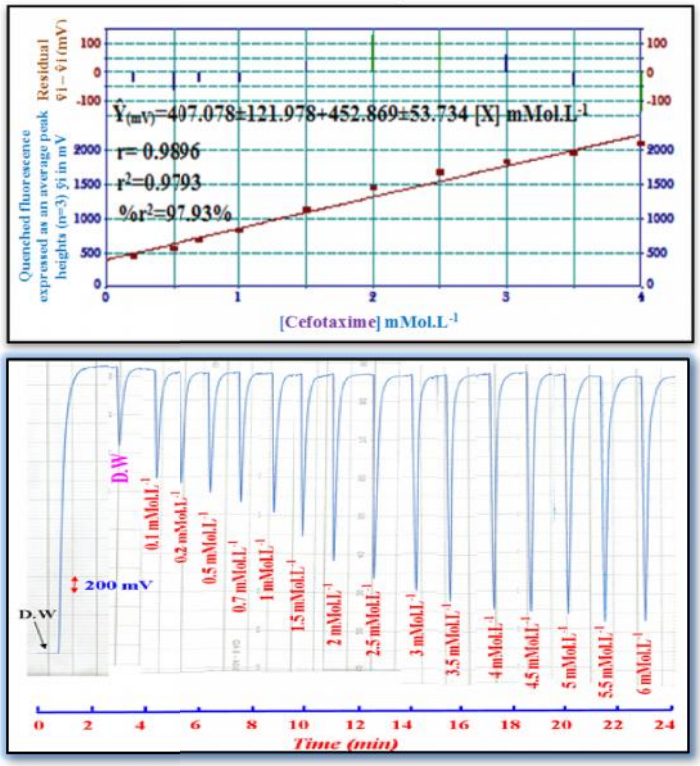

FIGURE 6: A- Calibration graph for the variation of Cefotaxime sodium concentration using laser diode fluorometer, B-Response profile of concentration variation of Cefotaxime sodium

TABLE 4: Summary of linear regression for the variation of fluorescence response with Cefotaxime concentration at optimum conditions using developed method (laser diode fluorimeter) and classical method spectrophotometer[20-21]

Measured cefotaxime mmol.L^{-1}	Liner dynamic range mmol.L^{-1}	Type of measurement	$\hat{Y}_{(mV)}=(a\pm S_a t)+(b\pm S_b t)$ [Streptomycin] mMol.L^{-1} at confidence level 95%,n-2	r r^2 $\%r^2$	t_{tab} at 95% confidence level, n-2	Calculated t-value $t_{cal}=\frac{r}{\sqrt{1-r^2}}\sqrt{n-2}$
		Total quenched fluorescence	$\hat{Y}_{(mV)}$=1601.114±218.979+323.043±64 .169 [X] mMol.L^{-1}			
0.2-6 n=14	0.2-4 n=10	Quenched fluorescence	$\hat{Y}_{(mV)}$=407.078±121.978+452.869±53. 734 [X] mMol.L^{-1}	0.9896 0.9793 97.93%	2.306 < 19.451	
		Remained fluorescence	$\hat{Y}_{(mV)}$=2332.922±121.978-452.869±53.734 [X] mMol.L^{-1}			
0.034-0.336 n=10	0.034-0.336 n=10	Absorbance	$\hat{Y}$=0.063±0.018+0.535±0.088[X] mMol.L^{-1}	0.9809 0.9621 96.21%	2.306 < 14.226	

Repeatability

The repeatability of the efficiency of local made Continuous Flow Injection Analysis-Laser Diode Fuorometer were studied at fixed concentrations of Cefotaxime (0.7 and 2 mmol.L^{-1}) using the optimum parameters. The measurements were repeated for six successive injections. The obtained results were tabulated in Table (5) and Figure (7) was shown response profile of repeatability at 0.7 and 2mmol.L^{-1} respectively. The %RSD is less than 1% that display the trustiness of the newly methodology.

TABLE 5: Repeatability measurements of the determination of Cefotaxime Sodium

Cefotaxime mmol.L^{-1}	Number of injection	Total fluorescence quenching (n=3) $\bar{y}i$ in mV	Fluorescence quenching of Cefotaxime $\bar{y}_{Qi}$ (n=3) in mV	σ_{n-1}	RSD%	Confidence interval of the average at (95%) $\bar{y}_i \pm t_{(\alpha=0.05/2)} \frac{\sigma_{n-1}}{\sqrt{n}}$
0.7	6	1680	680	1.41	0.08	1680±3.51
2	6	2443	1443	0.63	0.03	1443±1.57

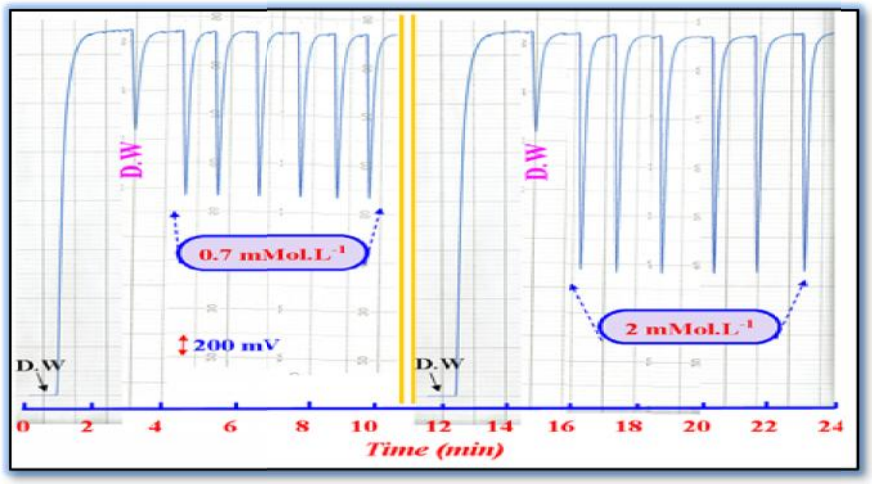

FIGURE 7: Response profile of six successive repeatable measurements of cefotaxime sodium using laser diode fluorometer

Limit of detection (LOD)

Limit of detection for the determination of Cefotaxime Sodium using a laser diode fluorometer was invetigated. The measurement was depended on the gradual dilution of lowest concentration in the calibration curve, the detection based on the numerical value of slope and from the linear regression plot. The obtained results were tabulated in Table (6) by using a sample volume of 96 µL.

TABLE 6: Limit of detection for Cefotaxime sodium at optimum parameters

Minimum concentration (mmol.L^{-1})	Practically based on minimum concentration in calibration graph	Theoretical based on the value of slope LOD=$3S_B$/slope	Based on the linear equation $\hat{Y}=Y_B+3S_B$
0.1 mmol.L^{-1}	4.583 µg/sample	0.106 µg/sample	88.019 µg/sample

$\mathbf{y_B}$= average response for the blank solution (equivalent to intercept in straight line equation)

The Applications

The adopted procedure is employed the laser diode fluorometer- Continuous flow injection to some dosage forms containing cefotaxime in three different pharmaceutical preparations and is compared with spectrophotometry method.

The standard addition method was applied by preparing a series of solution from Cefotaxime sodium used in cleaning devices by transferring 0.5 mL to five separate volumetric flasks (10 mL), followed by the addition of different aliquots of 0, 0.7, 1, 1.5, and 2 mL from 10 mmol.L^{-1} of standard Cefotaxime sodium in order to have the concentration describing the range 0.0-2.0 mmol.L^{-1}, while a spectrophotometric method[19] carried out by preparing a series solution from Cefotaxime sodium by transferring 0.32 mL to five separate volumetric flasks (10 mL), followed by the addition of 0, 0.48, 0.64, 0.8 and 0.96 mL from 2.1 mmol.L^{-1} which have a concentration range from (0.0 to 0.202) mmol.L^{-1} of standard solution. Table (7) showed the names of the supplier companies, drug dose and results at 95% confidence interval; paired t-test was used as shown in Table (8). The obtained results indicated that clearly there were no significant differences between developed and spectrophotometric method at 95% confidence interval since the calculated t-test value is less than tabulated t-test value.

TABLE 7: Summary results for the determination of Cefotaxime by fluorescence system using developed and spectrophotometric method

Sample No.	Commercial name, Country, Content, Company	Confidence interval for the average tablet weight $\bar{W} \pm 1.9 \frac{\sigma_{n-1}}{\sqrt{n}}$ (g)	Sample weight equivalent to 0.477 g (10 mmol.L^{-1}) of the active ingredient (g)	Theoretical content of active ingredient at 95% (g)	Development method using Laser diode Fluorimeter Classical method using UV-Spectrophotometric at 520 nm Equation of standard addition curve at 95% for n-2 $\hat{Y}_{(mV)}=(a\pm S_a t)+(b\pm S_b t)$ [Cefotaxime] mmol.L^{-1}	r r^2 r^2%	Practical concentration mmol.L^{-1} in 10 ml , 100 mL
1	Cefotaxime sodium, U.A.E, 1.0 g, PRIMOCEF	1.057±0.014	**0.505**	1.0±0.013	$\hat{Y}_{(mV)}$=327.993±240.553+626.929±193.342 [X] mmol.L^{-1}	0.9862 0.9726 97.26%	**0.523** **10.463**
					$\hat{Y}$=0.057±0.060+0.859±0.442 [X] mmol.L^{-1}	0.9626 0.9266 92.66%	**0.066** **2.074**
2	Cefotaxime sodium, India, 1.0 g, BRAWN	**1.085**±0.025	**0.518**	1.0±0.023	$\hat{Y}_{(mV)}$=315.437±170.409+608.233±136.966 [X] mmol.L^{-1}	**0.9926** **0.9852** **98.52**%	**0.519** **10.372**
					$\hat{Y}$=0.058±0.019+0.841±0.134 [X] mmol.L^{-1}	**0.9962** **0.9925** **99.25**%	**0.069** **2.155**
3	Cefotaxime sodium, Spain, 1.0 g, LDP	**1.038**±0.009	**0.495**	1.0±0.009	$\hat{Y}_{(mV)}$=344.391±349.826+665.009±281.171 [X] mmol.L^{-1}	**0.9745** **0.9497** **94.97**%	**0.518** **10.357**
					$\hat{Y}$=0.055±0.022+0.842±0.172 [X] mmol.L^{-1}	**0.9939** **0.9879** **98.79**%	**0.065** **2.041**

TABLE 8: Paired t-test for laser diode fluorimeter method with spectrophotometric method for the determination of cefotaxime sodium

	Development method using Laser diode-Fluorometer Classical method using ((UV-Spectrophotometric method)) at 520 nm Practical concentration (mMol.L^{-1}) in 10 ml Practical concentration (mMol.L^{-1}) in 100 ml Wt. of Cefotaxime in (g)	Weight of Cefotaxime in each sample (g) Weight of Cefotaxime in vials $\bar{W}$±4.303 $\sigma_{n-1}/\sqrt{n}$ (g)	Efficiency of determination (%Rec)	Individual t-test (X-μ) $\sqrt{n}$ / σ_{n-1}	Paired t-test $X_d\sqrt{n}$ / σ_{n-1}
1	0.523 10.463 0.499	0.499 1.046±0.0008	104.554%	199.180 > 4.303	12.706 > -0.999
	0.066 2.074 0.099	0.099 1.046±0.0013	104.638%	117.047 > 4.303	
2	0.519 10.372 0.495	0.495 1.037±0.0009	103.718%	124.881 > 4.303	
	0.069 2.155 0.103	0.103 1.116±0.004	111.633%	96.515 > 4.303	
3	0.518 10.357 0.494	0.494 1.037±0.0009	103.688%	128.168 > 4.303	
	0.065 2.041 0.097	0.097 1.012±0.0009	101.153%	40.502 > 4.303	

CONCLUSION

The fluorescence measurement using the Laser Diode fluorometer-CFI analyser is proposed a new method for the determination of cefotaxime sodium which can be characterized by accuracy, sensitivity and speed. The developed method is based on the creation of constant level intensity for 2, 7-dichlorofluorescein, then measuring the fluorescence quenching after injection the cefotaxime that can lead to reducing in fluorescence intensity. The obtained value of %RSD is found to beless than 1%, which is an indication of a good precision of the proposed method. The standard addition method was used to cancel matrix effects. The statistical analysis is in good agreement with those of spectrophotometric method[19]. Therefore, the fluorescence method can consider as an alternative method for determination of cefotaxime sodium in pharmaceutical dosage.

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