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Spectrofluorometric quantification of Clozapine in pharmaceutical formulations and human plasma

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Abstract: Herein, we present a simple, precise, accurate, and ultra-sensitive spectrofluorimetric method for estimation of clozapine (CLZ) in tablets and human plasma was developed and then validated. A highly fluorescent brown-yellowish fluorophore was formed (λ_{ex}=469 nm, λ_{emi}=540 nm) as a nucleophilic substitution reaction occurred between CLZ and 4-chloro-7-nitro-2,1,3-benzoxadiazole (NBD-Cl) in alkaline mcllavine buffer (pH 9.0). Optimum values of experimental parameters were carefully determined and optimized. The calibration curve was rectilinear over the concentration range of 80-900 ng mL^{-1} with a linear correlation coefficient (r =0.9984). The LOD and LOQ were determined to be 14 ng mL^{-1} and 42 ng mL^{-1}, respectively. The proposed approach has been used successfully to quantification of Clozapine in its commercial formulations and human plasma.

Key words: clozapine, NBD-Cl, spectrofluorimetric, human plasma

1. Introduction

Clozapine [3-chloro-6-(4-methylpiperazin-1-yl)-11H benzo[b][1,4]benzodiazepine], a member of the dibenzodiazepine derivatives *Fig.* 1, is considered a second-generation anti-psychotic commonly used in the treatment of both negative and positive symptoms of schizophrenic patients[1-3]. CLZ is regarded as an effective choice for patients who suffer from resistance or are unresponsive to conventional neuroleptic medications like haloperidol.[4,5] Clozapine is metabolized by liver through microsomal oxidative cytochrome to the relatively inactive metabolites clozapine-N-oxide and N-Desmethylclozapine.[6] Despite its excellent effectiveness, clozapine's usage is severely limited due to the incidence of drug-induced agranulocytosis in 1-2 % of patients.[7-10] This effect is attributed to the toxicity of one of the clozapine's metabolites, N-desmethylclozapine, that appears to be more harmful to the bone marrow than CLZ itself, leading to decreased white blood cells. Thus, Regular monitoring of the white blood cell count is recommended to reduce this risk.[11-14] It was reported that clozapine metabolism is inhibited by fluvoxamine medication, resulting in considerably higher clozapine levels in the blood.[15] The Physicochemical properties of clozapine were

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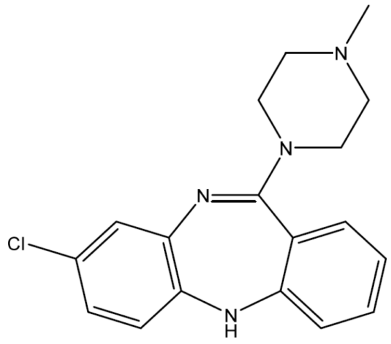

Fig. 1. chemical structure of Clozapine.

Table 1. The physicochemical properties of clozapine

Molecular weight (g/mol)	326.83
Melting point °C	182-186
Solubility in water	Practically insoluble in water
Solubility in ethanol	Soluble
pKa	3.7 & 7.6 (at 25 °C)
Log p	3.23

detailed in (*Table* 1).[16,17] Various analytical methods have been reported for determination of clozapine in several samples. Of these, Electrochemical,[18,19] Chromatographic,[20-30] Spectrophotometric,[31] and voltammetric methods.[32,33] The literature review reveals that there was no fluorometric approach was reported for estimation of clozapine, thus the suggested approach describes the first spectrofluorometric approach for quantification of clozapine in its dosage form and fresh human plasma. The suggested approach is mainly depending on a nucleophilic substitution reaction between 4-chloro-7-nitro-2,1,3-benzoxadiazole (NBD-Cl) and CLZ via meisenheimer complex in alkaline media (Mcllavine buffer pH 9.0) to produce a bright fluorescent brown-yellowish adduct measured at 540 nm when excited at 469 nm.

2. Experimental

2.1. Chemicals and reagents

clozapine and 98 % 4-chloro-7-nitro-2,1,3-benzoxadiazole (NBD-Cl) were purchased from Baoji Guokang Bio-Technology Co., Ltd (Baoji, china). A solution of NBD-Cl reagent with a concentration of 1 mg/mL was freshly prepared by dissolving 100 mg of the reagent in 100 mL of methanol. The solution was covered with aluminum foil to avoid photodegradation. A solutions of 0.2 M Mcllavine buffer of pH (7.0-11.0) were prepared by dissolving a mixture of 3 g of potassium chloride and 2.474 g of boric acid in 200 mL of distilled water. The required pH value was attained by adjust the solution with 0.2 M of sodium hydroxide. Other chemicals used were sodium hydroxide, boric acid, potassium chloride, hydrochloric acid, 1,4-dioxan, ethyl acetate, acetonitrile, methanol, acetone, ethanol, butanol, DMSO, and DMF they were all supplied from Merck. Clozapex® Tab 100 mg (Apex Pharma) was supplied from a local pharmacy.

2.2. Instrumental conditions

Shimadzu RF-5301 PC Spectrofluorometer (Kyoto, japan) was used for fluorometric measurements equipped with 1 cm quartz cuvette and xenon lamp (150 watt). Both excitation and emission monochromator's slit width were adjusted at 5 nm. Thermostatically controlled water bath LCB-22D (daihan Labtech CO., Korea) has been used for warming purpose. pH measurement was performed using pH meter wtw inolab (pH 720, Germany). Sensitive balance BP 3015 (Sartorius Germany).

2.3. Standard solution

Ten milligram of clozapine powder was carefully weighed and dissolved with 10 mL acetone in beaker (20 mL), then the obtained solution was transferred into 100 mL calibrated flask and diluted with distilled water to prepare a stock solution labeled to contain 100 μg mL^{-1} of CLZ. The working standard solutions containing 10 μg mL^{-1} of CLZ were prepared from the stock solutions by dilution with distilled water.

2.4. General analytical procedure

An appropriate portion of the working standard solution (10 μg mL^{-1}) were carefully transferred to a series of 10 mL calibrated flasks and diluted to the mark to prepare a solutions with CLZ concentrations over the range (80-900 ng mL^{-1}). Followed by 1.5 mL

of reagent (NBD-Cl) together with 1 mL of Mcllavine buffer (pH 9.0). The reaction mixture was mixed well and heated in a thermostatically controlled water bath for 15 min at 80 °C, then refrigerated for 5 min. After that, 0.6 mL of concentrated HCl was added to acidify the mixture. The flask contents were then diluted to the mark using methanol. The fluorescence intensity of the resulted product was measured at 469 nm when excited at 540 nm. The same procedure was performed without CLZ to obtain blank solution. The calibration graph was produced via plotting the increment in the fluorescence intensity with the respect to CLZ concentration. The regression equation of the calibration graph was derived.

2.5. Procedure for pharmaceutical tablets

Five Clozaril® tablets have been carefully weighed and crashed into fine powder using a stoneware grinder. An accurate quantity of the powder equivalent to 10 mg pure CLZ was weighed and then dissolved using acetone. The resultant solution was filtered and diluted to the mark with distilled water into 100 mL volumetric flask to make a CLZ stock solution with 100 μg mL^{-1}. After further dilution with distilled water, a solution with concentrations of 10 μg mL^{-1} CLZ was produced. This solution has been further diluted to make a solution with a final concentration 150 ng mL^{-1} in the general analytical procedure outlined in section (2.4). The linear regression equation of calibration graph was used to calculate CLZ concentration.

2.6. Procedure for spiked human plasma

A fresh healthy human donor whole blood plasma was obtained by collect 4 mL of CLZ -free blood from four healthy human volunteers into a vacutainer sodium heparin tube, which has been subsequently centrifuged at 4000 rpm for 30 minutes. Thereafter, in centrifugal tubes, 1 mL of CLZ stock standard solution was spiked with 1 mL of fresh plasma and 3 mL of acetonitrile solvent (proteins denaturation agent). The resulting solution was diluted to 10.0 mL using acetone and centrifuged again at 4000 rpm for 20 minutes[34]. The produced supernatant was diluted with distilled water to obtain a final CLZ concentration within a range (100-300 ng mL^{-1}) in the general analytical procedure outlined in section (2.4).

3. Results and Discussion

Non-fluorescent pharmaceutical product that has primary or secondary amino group can be fluorometrically quantified using fluorescent derivatizing agent like NBD-Cl. In this work, the fluorogenic reagent (NBD-Cl) react rapidly with clozapine via meisenheimer complex in alkaline media (Mcllavine buffer pH 9.0) to produce a highly fluorescent brown-

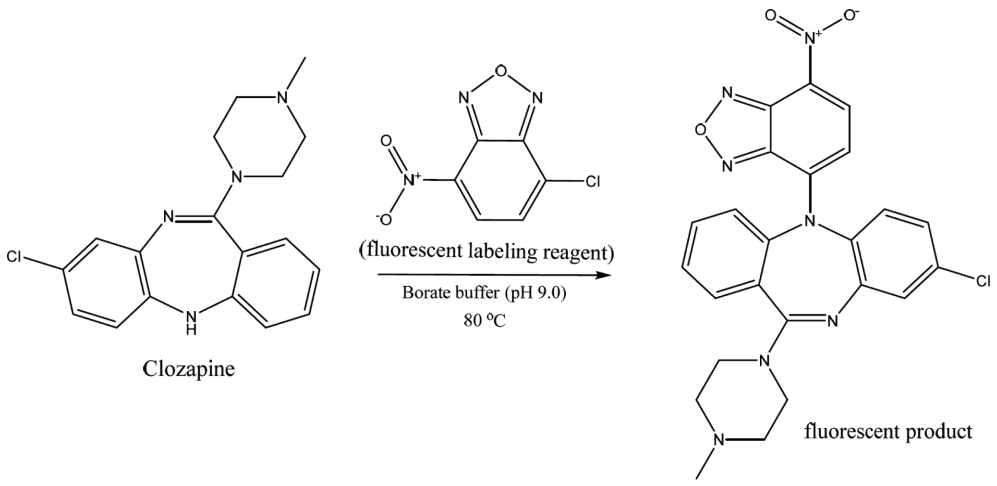

Fig. 2. The suggested reaction pathway between clozapin and NBD-Cl reagent.

yellowish adduct which measured at 540 nm when excited at 469 nm *Fig.* 2.

3.1. Experimental conditions optimization

The experimental factors of the subjected approach that enhance the stability and fluorescence intensity of NBD-CLZ product were thoroughly determined and optimized. Each Factor has been studied independently while the other factors kept constant.

3.1.1. Effect of reagent volume

The effect of reagent volume on the fluorescence intensity of reaction product has been studied in the range (0.1-2.5 mL). the fluorescence intensity improved as the reagent volume increased until reach a point (1.5 mL) and the intensity did not increase further. As a consequence, 1.5 mL of NBD-Cl has been chosen for subsequent experiments as detailed in *Fig*. 3.

3.1.2. Effect of pH and mcllavine buffer's volume

In order to study the effect of the acidity and basicity of the reaction media on the formation of the NBD-CLZ product, several Mcllavine buffer solutions with pH ranging from 7.0-12.0 have been employed. *Fig.* 4 illustrates the gradual increase in fluorescence intensity with the respect to pH value until it attained a maximum point (pH 9.0), above which there was no further increase. Instead, it diminished considerably as a result of the formation of NBD-OH compound, which increased the background signal. Thus, pH 9.0 was selected as the optimal value as detailed in *Fig.* 4. To select the volume that produces the greatest fluorescence intensity of NBD-CLZ product, several

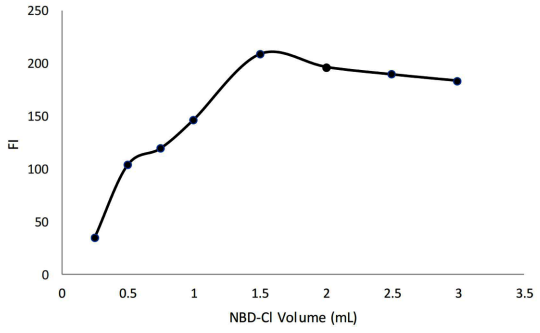

Fig. 3. Reagent volume effect on the FI of CLZ-NBD product

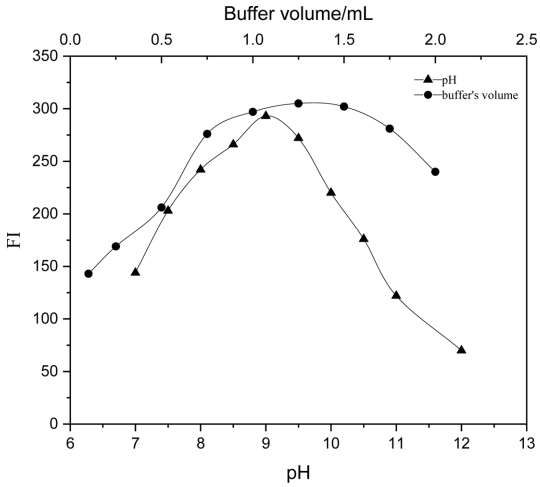

Fig. 4. Effect of pH and mcllavine buffer's volume on the FI of NBD-CLZ product.

volumes of Mcllavine buffer (pH 9.0) within the range (0.1-2 mL) were used. As shown in *Fig*. 4, the FI increases as a function of buffer volume until reach a steady state region from (1.0-1.5 mL). followed by marked decrease. Therefore, 1 mL is optimal buffer's volume.

3.1.3. Effect of the heating temperature and heating time

The influence of heating temperature on the FI of NBD-CLZ product has been checked at different

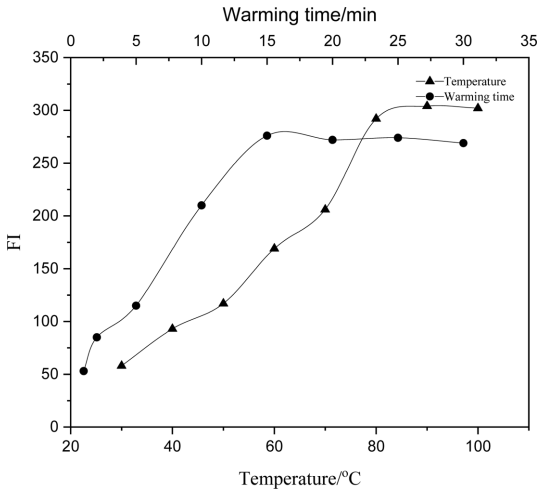

Fig. 5. Effect of temperature and warming time on the FI of NBD-CLZ product.

temperature within the range (40-90 ºC) through a certain time interval. *Fig.* 5 illustrate that the highest FI has been achieved at 80±2 ºC. At this temperature, the sufficient time required to complete the reaction has been determined using different heating time (1-30 min). As detailed in *Fig.* 5, the sufficient heating time was found to be 15 minutes.

3.1.4. Effect of concentrated HCl volume

According to the literature survey, the high hydroxyl ion concentration of the experimental condition (pH 9.0) causes an interfering problem by reacting with NBD-Cl to form NBD-OH compound, which is responsible for increasing the background signal. This problem can be overcome by lowering the pH of the reaction mixture to less than 1 without influencing the NBD-CLZ product by adding concentrated HCl volume following the cooling step. Various HCl volumes has been used within the range (0.1-1.0 mL). The optimal HCl volume was found to be 0.6 mL.

3.1.5. Diluting solvents Effect

To select the solvent that produces the maximum FI of NBD-CLZ product, various diluting solvents have been examined, such as acetonitrile, 1,4-dioxan acetone, methanol, butanol, ethyl acetate, DMF, DMSO, ethanol, and D.W. The highest FI was attained when using acetone as dilution solvent, as illustrated in *Fig.* 6.

3.2. Validation of method

The suggested approach has been validated in accordance with (ICH) recommendations.[35]

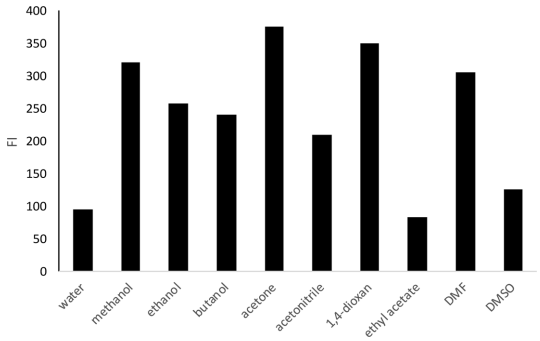

Fig. 6. Diluting solvents effect on the FI of NBD-CLZ product.

3.2.1. Linearity and range

The Calibration graph of the suggested approach was created via plotting the concentration of CLZ versus the FI of NBD-CLZ product. The linear concentration of the calibration graph ranged from 80-900 ng mL^{-1} of CLZ. The resulted data were summarized in (*Table* 2).

3.2.2. limit of detection (LOD) and Limit of quantitation (LOQ)

LOD and LOQ of the suggested approach were obtained by calculating the standard deviation (S.D) of seven blank solution. The obtained S.D value was substituted in the equations supplied by the ICH Q2 (R1) guidelines, where

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

where S denotes to S.D, while b denotes to slope of the calibration graph's regression line. As mentioned in (*Table* 2), The small LOD and LOQ values indicate that the suggested approach is highly sensitive.

3.2.3. Accuracy

The suggested approach's accuracy has been determined by measuring the fluorescence intensity of the NBD-CLZ product using three concentration level within the calibration graph (100, 200, 300 ng mL^{-1}) by performing five replications of each selected

Table 2. Analytical parameters of the suggested approach

Parameter	Suggested method
λ_{exc} (nm)	469
λ_{emi} (nm)	540
Concentration range (ng mL^{-1})	80-900
Slope	0.5175
SD of Slope	0.0146
Determination coefficient (r^2)	0.9969
Correlation coefficient (r)	0.9984
Intercept	31.6
SD of intercept	8.74
LOD* (ng mL^{-1})	14.16
LOQ** (ng mL^{-1})	42.92

****LOQ:** Limit of quantitation. ***LOD:** Limit of detection

Table 3. Accuracy data of the suggested approach

Sample	Concentrations ng mL^{-1}	Recovery%*± SD
1	100	100.09 ± 1.51
2	200	99.13 ± 1.48
3	300	99.45 ± 1.58

*Mean of five determinations

Table 4. Intra- and inter-day precisions of the suggested approach

Concentration ng mL^{-1}	Intra-day precision		Inter-day precision	
	Recovery%*	R.S.D.%	Recovery%*	R.S.D.%
100	100.87	1.77	100.48	1.60
200	99.32	0.84	99.71	1.42
300	99.83	1.04	100.22	1.02

*Mean of five determinations

concentration. As detailed in (*Table* 3), the obtained results were analyzed to obtain percent recovery (Re%) whose values indicate the suggested approach is highly accurate.

3.2.4. Precision

The precision of the suggested approach has been represented as inter-day precision (Reproducibility) and intra-day precision (Repeatability). Three CLZ concentration (150, 200, and 300 ng mL^{-1}) was selected to test the precision of the suggested approach. Each concentration was analyzed at consecutive time during the day to obtain repeatability and at different day to obtain reproducibility. As illustrated in (*Table* 4), the low value of relative standard deviation (R.S.D.% less than 2) indicate that the suggested approach is highly precise.

Table 5. Robustness evaluation for the suggested approach.

Experimental parameters	Recovery%* ± R.S.D.%
Warming time (min)	
13	97.77±1.44
15	100.29±1.12
17	98.55±1.13
Temperature (°C)	
77	100.29±1.23
80	99.71±1.42
83	100.48±1.52
Buffer pH	
8.8	98.35±1.55
9	99.32±1.45
9.2	97.19±1.45
Reagent volume (mL)	
1.3	99.71±0.96
1.5	100.09±0.96
1.7	99.32±1.35

*Mean of five determinations.

3.2.5. Robustness

The robustness describes the stability of the analytical method under a slight change in some experimental factors. In this work, four experimental factors (warming time, Temperature, pH, and reagent volume) was slightly changed to test the robustness of the suggested approach. As mentioned in (*Table* 5), the obtained values of percent recovery and R.S.D.% implies that slight changes in experimental conditions

Table 6. Statistical comparison of data resulted by suggested approach with those of the reported one for determination of CLZ in pharmaceutical tablets

Pharmaceutical preparation	Proposed method		Reported method	
Clozapex (100 mg) tablets	Added conc. (ng mL^{-1})	Found%	Added conc. (ng mL^{-1})	Found%
	20	99.6	20	98.94
	40	101	30	99
	60	97.9	60	102
	80	98.5	70	101.4
	100	100.7	80	98.45
Mean±S.D		99.54±1.34		99.95±1.61
Variance		1.81		2.62
t value		0.44 (2.30)*		
f value		1.44 (6.38)*		

*The value between bracket represent the corresponding f and t tubulated values

Table 7. Application of the suggested approach for determination of CLZ in human plasma

Added conc. (ng mL^{-1})	Found conc. (ng mL^{-1})	Recovery% * ± SD
100	101.25	101.25±1.58
150	150.34	100.22±1.81
200	194.78	97.39±2.30
250	248.50	99.40±2.38
300	294.49	98.16±0.85

*Mean of five determinations.

have no considerable effect on the quantification of CLZ.

3.3. Application

3.3.1. Application in the pharmaceutical vial

The suggested approach has been successfully used for quantifying of clozapine in pharmaceutical tablets. The resulted data have been compared with the result produced from reported method using student's t and f test at confidence level equal to 95 %.[36] (*Table* 6) illustrate the t and f values of the suggested approach were less than relevant tabulated values, implying that the official or reported method and the suggested approach were not statistically different.

3.3.2. Application in the spiked human plasma

Five CLZ concentration (100, 150, 200, 250, 300 ng mL^{-1}) were successfully determined in spiked fresh human plasma. The obtained values of recovery percent (Re%) were mentioned in (*Table* 7).

4. Conclusions

The prescribed spectrofluorometric approach provides a simple, rapid and precise method for the estimation of clozapine in its pharmaceutical tablet and spiked fresh human plasma without interferences from plasma matrix or common excipients. Moreover, the developed method showed promising selectivity, sensitivity, a good linear range, excellent quantitative recoveries and low detection limit for the estimation of CLZ. Unlike chromatographic techniques, the proposed method has less run time, low cost and less solvent consuming. Further, the values of relative standard deviation (R.S.D%) were less than 2 % for reproducibility and repeatability analysis. Therefore, the method was considering an effective tool for analysis of CLZ in clinical and quality control laboratories.

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