ISSN: 2357-0547 (Print)
ISSN: 2357-0539 (Online)

Research Article / JAPR
Attia et al., 2017, 1 (4), 209-215

Journal of Advanced Pharmacy Research

Validated Spectrofluorimetric Method For The Determination of Cefoxitin Sodium in Its Pure Form and Powder for Injection via Derivatization with 4-Chloro-7-nitrobenzo-2-oxa-1,3-diazole (NBD-Cl)

Khalid Abdel-Salam M. Attia[1], Nancy Magdy[2], Ghada F. Mohamed[1]*

[1]*Pharmaceutical Analytical Chemistry Department, Faculty of Pharmacy, Al-Azhar University, Cairo, Egypt.*
[2]*Pharmaceutical Analytical Chemistry Department, Faculty of Pharmacy, Ain Shams University, Cairo, Egypt.*

***Corresponding author*:** Ghada F. Mohamed, Pharmaceutical Analytical Chemistry Department, Faculty of Pharmacy, Al-Azhar University, Cairo, Egypt. *Tel.: +201067646034 Fax: +20225541601*
E-mail address: ghada.fmi@gmail.com

Submitted on: 12-07-2017; Revised on: 10-09-2017; Accepted on: 24-09-2017

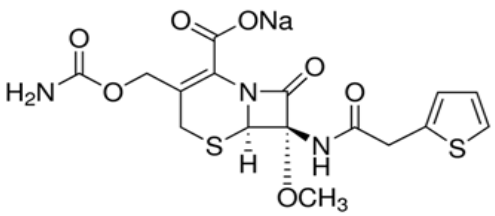
ABSTRACT

Objectives: An accurate and precise spectrofluorimetric methodwas developed and validated for the determination of β-lactam antibiotic named; cefoxitin sodiumin its pure form and powder for injection. **Methods:** Based on nucleophilic substitution reaction of target drug with 4-chloro-7-nitrobenzo-2-oxa-1,3-diazole (NBD-Cl) to form a highly fluorescent fluorophore measured at 540 nm after excitation at 460 nm. **Results:** Under optimum condition, the proposed method obeys Beer's law in range (0.5-7 µg mL^{-1}) and the reaction mechanisms were presented. **Conclusion:** The method was validated according to ICH guideline for accuracy, precision and was successfully applied for the determination of the drug in its pure form and powder for injection. The obtained results were statistically compared with those of the reported method and found to be in good agreement.

Keywords: *Cefoxitin sodium; 4-Chloro-7-nitrobenzo-2-oxa-1,3-diazole (NBD-Cl); Spectrofluorimetric method.*

INTRODUCTION

Cefoxitin sodium is a semisynthetic cephamycin antibiotics classified as a second generation Cephalosporin, chemically named Sodium 3-carbamoyloxymethyl-7-methoxy-7-[2-(2-thienyl) aceta-mido]-3-cephem-4-carboxylate[1]. The most novel of chemical feature of cefoxitin sodium is the possession of an alpha-oriented methoxyl group in place of the normal H atom at C-7. **Figure (1)**, this increased steric bulk conveys very significant stability against β-lactamases[2]. It is produced by *Streptomyces lactamdurans* and used for the treatment of infections caused by anaerobic and mixed aerobic anaerobic infections, such as pelvic inflammatory disease and lung abscess[3,4]. Literature survey reveals that HPLC methods were developed for the determination of cefoxitin sodium in pharmaceutical formulations[5] and in biological fluids[6-9], TLC method[10], LC-MS/MS[11] and a flow injection chemiluminescent method was also reported[12]. Colorimetric methods were used for the determination of cefoxitin sodium in pharmaceutical formulations and in biological fluids[13-15], first and second derivative UV spectroscopy[16,17] and a stability indicating method by spectrofluorimetric analysis[18] was also described for its analysis. Khalid et al, recently developed different spectrophotometric method for determination of cefoxitin sodium in the presence of its alkali-induced degradation product[19,20].



M. Formula: $C_{16}H_{16}N_3O_7S_2Na$ **M.Wt:** 449.4 g/mol

Figure 1. Chemical structure of cefoxitin sodium.

4-Chloro-7-nitrobenzo-2-oxa-1,3-diazole (**Figure 2**) also known as 7-chloro-4-nitrobenzo-furazan (NBD-Cl)

is a stable non-fluorescent pale-yellow solid[21]. It has been used as derivatizing reagent in devolvement of both spectrophotometric and spectrofluorimetric methods for determination of many amines[21-23], also for β-latam antibiotics[34-35] . The aim of the present work is to develop a spectrofluorimetric methodfor the determination of cefoxitin sodiumvia derivatization with4-chloro-7-nitrobenzo-2-oxa-1,3-diazole (NBD-Cl).

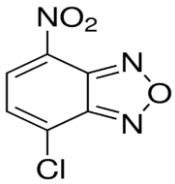


Figure 2. Chemical structure of 4-chloro-7-nitrobenzo-2-oxa-1,3-diazole (NBD-Cl). M. wt. 199.56 g/mol

MATERIALS AND METHODS

Instruments

Jasco FP6200 single beam spectrofluorometer (Japan).

Chemicals and reagents

Cefoxitin Sodium 98.8% was kindly supplied by Pharco B International Co., Cairo, Egypt. Lot no.12052036

Primafoxin® 1gm vial labeled to contain 1gm of cefoxitin sodium per vial, Batch No. (109), the product of PharcoB international Co., Egypt, were purchased from local pharmacies.

0.1% NBD-Cl (99%) (Sigma Chemical Co., St. Louis, USA) was freshly prepared by dissolving 100mg in 100 mL methanol and protected from light.

1M Hydrochloric acid, 0.2 M Sodium bicarbonate (El-Nasr Co., Egypt).

Methanol, ethanol, acetonitrile, acetone, propanol (sigma-Aldrich, USA).

Water used throughout the procedures was freshly double distilled.

Standard solutions

Stock solution (1mg mL^{-1}) was prepared by dissolving 100 mg of cefoxitin sodium in 80 mL water, and the volume was then completed to 100 mL with water. The solution was found to be stable for at least two weeks when stored at 5°C in the dark[16].

Working solution (0.1 mg mL^{-1}) was obtained by dilution of the stock solution with water.

Linearity and construction of calibration curves

Aliquots from stock standard solution of cefoxitin sodium were accurately measured and transferred into a test tube set to prepare different concentration covering the linearity range (0.5-7 µg/mL), then 1mL (0.1% NBD-Cl) was added followed by 1.5 mL of (0.2M) $NaHCO_3$. The reaction mixtures were allowed to proceed in thermostatically controlled water bath at 60 °C for 30 minutes, and then cooled to room temperature. After cooling, the reaction mixture was acidified by adding 1mL of 1M HCl, and completed to volume with water. The relative fluorescence intensity was measured at $\lambda_{em.}$= 540 nm after excitation at $\lambda_{ex.}$= 460 nm.

Application to pharmaceutical preparation

An accurately weighed quantity of well mixed powder from three vials of Primafoxin® 1gm equivalent to 100 mg of cefoxitin sodium was transferred into a 100-mL volumetric flask. The powder was dissolved by shaking with 50 mL water. Then volume was adjusted with water to obtained stock solution labeled to contain (1mg mL^{-1}) cefoxitin sodium, which was further diluted to contain (0.1 mg mL^{-1}). Cefoxitin sodium then analyzed by the corresponding regression equation for the proposed method.

RESULTS AND DISCUSSION

Cefoxitin sodium doesn't has a native fluorescence, so its derivatization with fluorigenic reagent was necessary for spectrofluorimetric determination. 4-chloro-7-nitrobenzo-2-oxa-1,3-diazole (NBD-Cl) an electroactive halide reagent, which was considered as a likely target for good nucleophiles, thus upon reaction of cefoxitin sodium with (NBD-Cl), a yellow-colored fluorescent derivative was formed, which exhibited maximum fluorescence intensity (λem.= 540 nm) after its excitation at wavelength (λex.= 460nm). The excitation and emission spectra for the reaction product of cefoxitin sodium with (NBD-Cl) was shown in Figure (3).

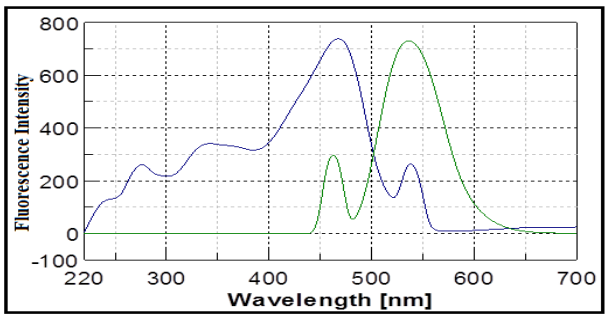


Figure 3. Excitation and emission spectra of the reaction product of cefoxitin sodium (7 µg mL^{-1}) with 0.1% NBD-Cl.

Optimization of experimental conditions

Different experimental parameters affecting the fluorescence intensity were studied and optimized.

Effect of reagent volume

The influence of NBD-Cl concentration was studied using different volumes of 0.1% (w/v) NBD-Cl solution ranging from (0.25-2 mL), it was found that 1mL of 0.1% (w/v) NBD-Cl produce the highest FI and beyond which the FI decreased. Figure (4).

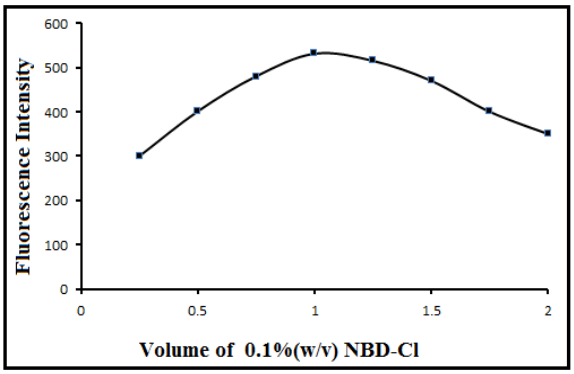


Figure 4. Effect of volume of 0.1% NBD-Cl on the fluorescence intensity of cefoxitin-NBD fluorophore at λ_{em} 540 nm.

Effect of $NaHCO_3$ concentration

The reaction of cefoxitin sodium with NBD-Cl should be carried out in alkaline medium (pH ~8.3) in order to generate the nucleophile from cefoxitin sodium. The influence of NaHCO3was studied using different volumes of 0.2 M NaHCO3 solution ranging from (0.25-2.5 mL), it was found that 1.5 mL (0.2 M) $NaHCO_3$produces the highest FI and above and beyond which the FI decreased, **Figure 5**.

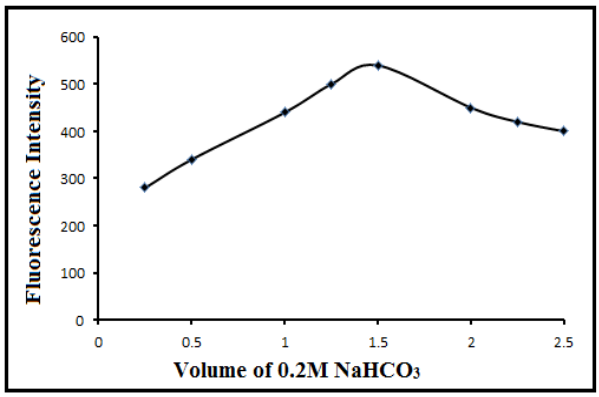


Figure 5. Effect of volume of 0.2 M $NaHCO_3$ on the fluorescence intensity of cefoxitin-NBD fluorophore at λem 540 nm.

Effect of temperature

The influence of temperature the reaction was carried out at different temperatures (25–70 °C), it was found that the reaction was dependent on the temperature and the FI increased as the temperature increased and the maximum FI was obtained at 60 °C (**Figure 6**). This result was coincident with the result reported previously by H. W. Darwish *et al* [25].

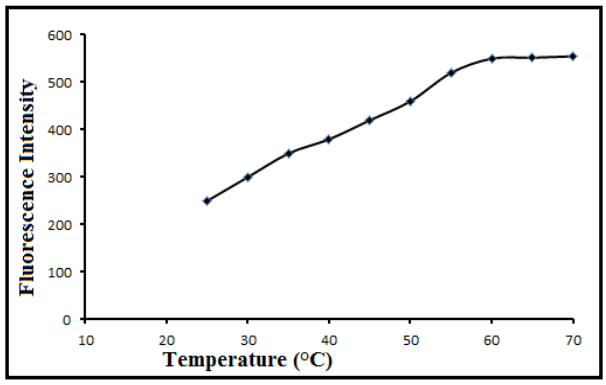


Figure 6. Effect of heating temperature (°C) on the fluorescence intensity of cefoxitin-NBD fluorophore at λem 540 nm.

Effect of reaction time

In order to determine the time required for completion of the reaction, the reaction was carried out at different reaction time interval (5-40 min.). The results indicated that the optimum time was 30 min (**Figure 7**).

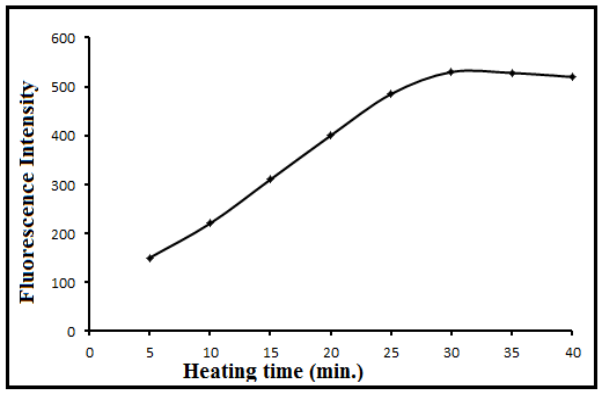


Figure 7. Effect of heating time at 60 ºC on the fluorescence intensity of cefoxitin-NBD fluorophore at λem 540 nm.

Effect of HCl concentration

Addition of HCl[21] to the reaction mixture before measurement of the FI was necessary for remarkably decreasing the background fluorescence (duo to the hydrolysis product of NBD-Cl to the corresponding hydroxyl derivative namely, 7-hydroxy-4 nitrobenzoxadiazole (NBD-OH)[32]. The fluorescence of NBD-OH was found to be quenched in strong acidic medium (pH ≤ 1), where the reaction product was not affected, the reaction was carried out using different volumes of 1M HCl ranging from (0.25-2mL). The optimum concentration of HCl required for acidification was found to be 1 mL of 1M HCl (**Figure 8**).

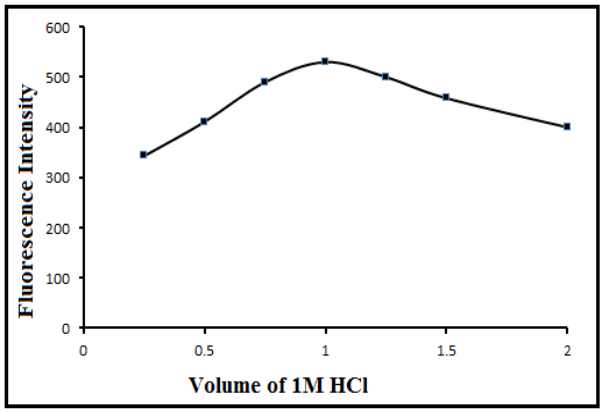


Figure 8. Effect of volume of 1M HCl on the fluorescence intensity of cefoxitin-NBD fluorophore at λem 540 nm.

Effect of diluting solvent

To select the most appropriate solvent for diluting the reaction solution, different solvents involve: water, methanol, ethanol, propanol, acetone, acetonitrile were studied. The highest FI was obtained upon using water or methanol but water was used as a diluting solvent because it is environmental friendly (**Figure 9**).

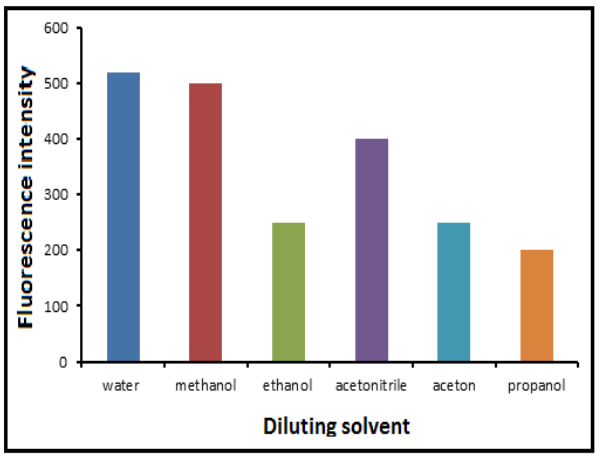


Figure 9. Effect of diluting solvent on the on the fluorescence intensity of cefoxitin-NBD fluorophore at λem 540 nm.

Stability of fluorescent fluorophore

The effect of time on the stability of the Fluorescent cefoxitin-NBD fluorophore was studied by measuring the FI at different time intervals. It was found that the FI values remain constant for at least 24 hour at room temperature.

The optimum variables affecting the reaction of cefoxitin sodium with NBD-Cl were summarized in **Table 1**.

Table 1. Optimization of variables affecting the reaction of cefoxitin sodium with NBD-Cl.

Variable	Studied range	Optimum
Excitation wavelength (nm)	350 – 520	460
Emission wavelength (nm)	490 – 600	540
(0.1 %, w/v) NBD-Cl	0.25-2 mL	1 mL
0.2 M $NaHCO_3$	0.25-2.5M	1.5
Temperature (°C)	25 – 70	60
Time (min)	5-40	30
1M HCl	0.25-2 mL	1mL
Solvent	water, methanol, ethanol, propanol, acetone, acetonitrile.	Water
Stability of cefoxitin-NBD fluorophore	1-24 hr.	24 hr. at room temp.

Stoichiometry and mechanism of the reaction [36-3]

The stoichiometry of the reaction between cefoxitin sodium and NBD-Cl was investigated by the limiting logarithmic method [34], where, two sets were prepared, one of which containing variable concentration of (NBD-Cl) ranging from (6×10^{-3}-3×10^{-2} M) while, constant drug concentration containing (3×10^{-3} M), the second set contained variable concentration of the drug ranging from(1×10^{-4} -1×10^{-2} M), while constant concentration of (NBD-Cl) containing (1×10^{-3} M), **Figure 10**, A plot of log FI against log concentration of NBD-Cl and cefoxitin sodium, two straight lines were obtained. The slopes were 0.8245 and 0.9368 indicating the 1:1 ratio for the reaction (owing to the molar reactivity of the reaction is 0.8245/0.9368). This ratio means one molecule of the drug reacts with one molecule of NBD-Cl.

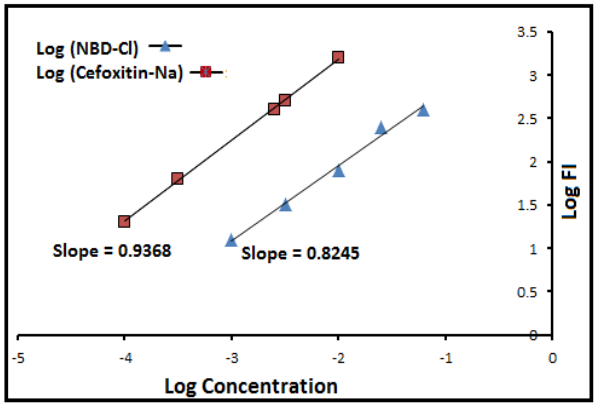


Figure 10. Stoichiometry of the derivatization reaction between cefoxitin sodium and NBD-Cl using limiting logarithmic method.

NBD-Cl is an electroactive halide reagent, which was considered as a likely target for good nucleophiles, the β-lactam cefoxitin sodium has free terminal amino group considered as good nucleophile, react with NBD-Cl through nucleophilic substitution forming highly fluorescent golden-yellow fluorophore, the suggested reaction pathway between cefoxitin sodium and NBD-Cl was shown in **Figure 11.**

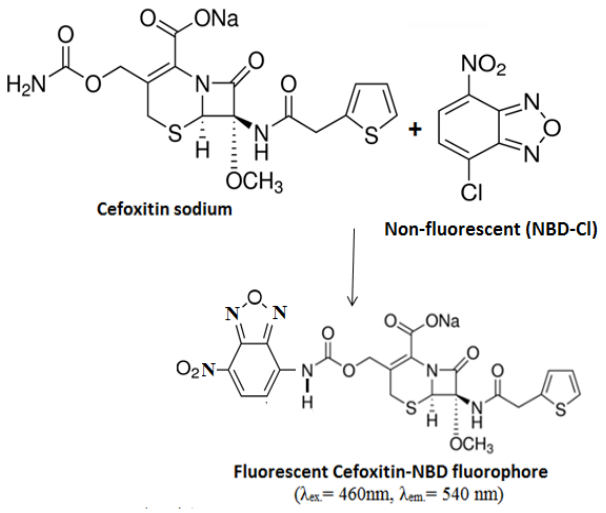


Figure 11. The Proposed reaction pathway between cefoxitin-sodium and NBD-Cl.

Methods validation [39]

The proposed method was validated according to the International Conference on Harmonization (ICH) guidelines in terms of linearity, range, LOD, LOQ, accuracy and precision.

Linearity and range

The method obeys the Beer's law in the studied range of 0.5-7 µg mL^{-1}, **Table 2,** illustrated the regression parameters of the calibration curve and correlation coefficient of the drug analyzed.

Limits of detection and quantitation

LOD was found to be 0.048µg mL^{-1}, while LOQ was found to be 0.160µg mL^{-1}, as shown in **Table 2.**

Accuracy and precision

Accuracy of the proposed procedure (%R) was found to be 99.84. Intra-day precision (repeatability day precision) as % RSD was found to be 1.551, while inter-day precision (intermediate precision) was found to be 1.036, table (2). Good %R confirms excellent accuracy.

Recovery study by standard addition technique:

Validity of the proposed method was performed by adopting standard addition technique with mean recovery of added ± SD of 100.78 ± 0.610 %. Results are presented in **Table 3**.

Table 2. Linearity studies and regression equation of the proposed spectrofluorimetric method.

Parameters		Spectrofluorimetric method
$\lambda_{ex.}$&$\lambda_{em.}$		460 & 540 (nm)
Linearity range (µg ml^{-1})		0.5─ 7
LOD (µg ml^{-1})		0.048
LOQ (µg ml^{-1})		0.160
- Regression Equation - Slope (*b*) ± S.D - Intercept (*a*) ± S.D		**F* = 98.542 C** +38.314** 98.542 ±0.610 38.314± 1.577
Regression coefficient (r^2)		0.9998
Accuracy (mean ± SD)		99.84 ± 1.017
Precision	Intra-day	1.551
	Inter-day	1.036

F* *is the fluorescence intensity.*
C* *is concentration in µg ml^{-1}*

Table 3. Recovery study of cefoxitin sodium in Primafoxin® vials by the proposed spectrofluorimetric method by adopting standard addition technique

Pharmaceutical taken, equivalent to cefoxitin sodium (µg ml^{-1})	Added standard (µg mL^{-1})	% R of added
Primafoxin® vials	1	**101.41**
	2	**101.01**
3	3	**100.20**
Mean ± %RSD		**100.87 ± 0.616**

Statistical analysis

Statistical comparison between results obtained by applying the proposed procedure and those obtained by applying the reported method[17] showed less calculated t and F values than the tabulated ones revealing no significant difference in accuracy and precision, **Table 4**.

Table 4. Statistical comparison between the results obtained by applying the proposed spectrofluorimetric method and reported method for determination of cefoxitin sodium in Primafoxin® 1gm vial.

Parameter	Primafoxin® 1gm vials	
	Spectrofluorimetric Method	Reported Method[17]
Mean*	101.19	99.02
S.D.	1.058	0.915
n**	5	5
Variance	1.119	0.837
t*-test**	1.860 (2.306)	----
F*-value**	1.338 (6.338)	-----

CONCLUSION

Because cefoxitin sodium has no native fluorescence, this work introduced an accurate spectrofluorimetric method for the determination of cefoxitin sodium in its pure form and powder for injection based on nucleophilic substitution reaction with 4-chloro-7-nitrobenzo-2-oxa-1,3-diazole (NBD-Cl) to form a highly fluorescent yellow fluorophore. The proposed method is suitable for the routine analysis of cefoxitin sodium in quality control and clinical laboratories.

Acknowledgement

Deepest thanks and appreciation to all the Staff Members and Colleagues in analytical chemistry department, faculty of pharmacy –boys' branch, Al-Azhar University for their useful cooperation and for providing facilities for performing experimental work.

Conflict of Interest

The authors declare that they don't have any conflict of interest.

REFERENCES

1- O'Neil, M. J. *The Merck index: an encyclopedia of chemicals, drugs, and biologicals*. 15th Edn.; RSC Publishing; Cambridge, UK, **2013**.
2- William, O.F.; Thomas, L. L.; David, A. W. *Principles of Medicinal Chemistry*, 4th Edn.; **1995**, pp. 759-802.
3- Laurence, L. B.; John, S. L.; Keith, L. P. Goodman and Gilmans. *The Pharmacological Basis of Therapeutics*. 11th Edn.; McGraw-Hill, USA, **2006**, pp. 1127-52.
4- Martindale. The complete drug reference 37thed. Pharmaceutical Press. INC., London, volume A, **2011**, pp. 249-250 and 311-312.
5- Jitendra, K. P.; Murthy, T.; Ryali, J.; Venkata, P.; Deepthi, B.; Nagaraju, P. Development and validation of chromatographic method for the determination of cefoxitin sodium in pharmaceutical dosage forms. *Res. J. Pharm. Biol. Chem. Sci*. **2011**, *2* (4), 604-610.
6- Charles, B.G.; Ravenscroft, P.J. Rapid HPLC analysis of cefoxitin in plasma and urine. *J. of Antimicrobial Chemo therapy* **1984**, *3*, 291-94.
7- Purser, C.; Baltar, A.; Ho, I.K.; Hume A. C. New rapid method of analysis of Cefoxitin in serum and bone by high performance liquid chromatography. *J. Chromat B: Biomed. Sci. Appl.* **1984**, *311*, 135-140.
8- Martinez, L. G.; ns-Falcó, P. C.; Sevillano-Cabeza, A.; Herráez-Hernández, R. Improved solid phase extraction procedure for assay of cephalosporins in human urine samples. *J. Liq. Chromat. Related Technol*. **1998**, *21* (14), 2191-2203.
9- Robbs, J.V.; Salisbury, R. T.; Elson, K. I.; Brockutne, J.G. Measurement of Cefoxitin levels in tissue high-pressure liquid chromatography. *SAMT* **1989**, *75*, 420-421.
10- Gerald, S.B. Analytical Profiles of Drug Substances Academic Press INC., London, 11, Chap. 5:**1982**, pp.169-195.
11- Partani, P.; Gurule, S.; Khuroo, A.; Monif, T.; Bhardwaj, S. Liquid chromatography/ electrospray tandem mass spectrometry method for the determination of cefuroxime in human plasma: Application to a pharmacokinetic study. *J. Chromat. B: Biomed. Sci. Appl.* **2010**, *878* (3-4), 428-434.
12- Thongpoon, C.; Liawruangrath, B.; Liaewruangrath, S.; Wheatley, R.A.; Townshend, A. Flow injection chemiluminescence determination of cephalosporins in pharmaceutical preparations using tris (2,22-bipyridyl) ruthenium (II)-potassium permanganate system. *Analytica Chimica Acta*. **2005**, *553*, (1-2), 123-133.
13- Abdel Halek, M. M.; Mahrous, M. S. Use of ammonium molybdate in the colorimetric assay of cephalosporis. *Talanta* **1984**, *31* (8), 635-37.
14- Mahrous, M. S.; Abdel-Khalek, M. M. Spectrophotometric determination of certain cephalosporins with ninhydrin. *Analyst* **1984**, *109*, 611-613.
15- Issopoulos, P. B.; Salta, S.E. Analytical investigation of beta-lactam antibiotics in pharmaceutical preparations. IX. Colorimetric determination of six cephalosporins of second and third generation in the range of micromolar concentrations. *Acta Pharm Hung* **1996**, *66* (2), 89-94.
16- Murillo, J.A.; Lemus, J.M.; Garcia, L.F. Analysis of binary mixtures of cephalothin and cefoxitin by

using first-derivative spectrophotometry. *J. Pharm Biomed. Anal.***1996,** *14* (3), 257- 266.

17- Korany, M. A.; Elsayed, M. A.; Galal S.M. Use of second derivative spectrophotometry for the determination of certain cephalosporins and their acid-induced degradation products in combination. *Analyt. Lett.***1989,** *22* (1), 159-175.

18- Murillo, J.A.; Lemus, J. M.; Garcia, L.F. Spectrofluorimetric analysis of cefoxitin in pharmaceutical dosage. *Talanta* **1994,** *41* (4), 557-563.

19- Attia, K. A.; Abdel-Aziz, O.; Magdy, N.; Mohamed, G.F. Determination of cefoxitin sodium in presence of its alkaline degradation product using two wavelengths manipulating methods. *Anal Chem. Lett.* **2016**, *6*, (6), 738-747.

20- Attia, K.A.; Abdel-Aziz, O.; Magdy, N.; Mohamed G.F. Determination of cefoxitin sodium in the presence of its alkali-induced degradation product through different ratio spectra manipulating methods. *Anal. Chem. Lett.* **2017**, *2*, (7), 201-214.

21- Ghosh, P.M.; Whitehouse, M.W. 7-Chloro-4-nitrobenzo-2-oxa-1,3-diazole: a new fluorigenic reagent for amino acids and other amines. *Biochem. J.* **1968**, *108*, 155-156.

22- Imai, K.; Toyo'oka , T.; Miyano, H. Fluorigenic reagents for primary and secondary amines and thiols in high-performance liquid chromatography. A review .*Analyst***, 1984,** *109*, 1365-1373.

23- Moneeb, M. S. Spectrophotometric and spectrofluorimetric methods for the determination of saxagliptin and vildagliptin in bulk and pharmaceutical preparations. Bulletin of Faculty of Pharmacy, Cairo University **2013,** *51* (2), 139–150.

24- Kassem, M.G.; Darwish, I. A.; Darwish, H. W. Highly sensitive fluorimetric method for determination of varenicline in its bulk and tablets via derivatization with 7-Chloro-4-nitrobenzoxadiazole. *Digest. J. Nanomater. Biostruct.* **2014**, *9* (3), 1065–1075.

25- Darwish, I. A.; Amer, S. M.; Abdine, H. H, Al-Rayes, H. L. Spectrofluorimetric Determination of Fluvoxamine in Dosage Forms and Plasma Via Derivatization with 4-Chloro-7-Nitrobenzo-2-Oxa-1,3-Diazole. *J. Fluoresc*. **2009**, *19*, 463.

26- Walash, M. I.; El-Enany, N.; Askar, H. Validated spectrofluorimetric method for the determination of carbamazepine in pharmaceutical dosage forms after reaction with 4-chloro-7--nitrobenzo-2-oxa-1,3-diazole (NBD-Cl). *Luminescence* **2015**, *30*, 1119–1124.

27- Martinc, B.; Roskar, R.; Grabnar, I.; Vovk, T. Simultaneous determination of gabapentin, pregabalin, vigabatrin, and topiramate in plasma by HPLC with fluorescence detection. *J. Chromatogr B* **2014**, *962*, 82–88.

28- Mahmoud, A.M.; Darwish I.A.; Khalil, N.Y. Fluorimetric study for the reaction between sertraline and 7-chloro-4-nitrobenzo-2-oxa-1,3-diazole: kinetics, mechanism and application for the determination of sertraline in tablets *J. Fluoresc* **2010**, *20*, 607–613.

29- Mahmoud, A.M.; Abdel-Wadood, H.M.; Mohamed, N.A. Kinetic spectrophotometric method for determination of amlodipine besylatein its pharmaceutical tablets. *J Pharm Anal*. **2012**, *2*, 334–341.

30- Basheir, B. E. A; Elbashir, A.A. Spectrophotometric method for determination of L-dopa in pharmaceutical formulation using 7-chloro-4-nitrobenzoxadiazole (NBD-CL) as a chromogenic reagent. *EJPMR* **2015**, *2* (1), 304-316.

31- Mohammed, T.O.; Elbashir, A.A. Spectrophotometric method for determination of gabapentin in pharmaceutical formulation by derivatization with 4-chloro-7-Nitrobenzo- 2-oxa-1,3-diazole (NBD-Cl). *Int. J. Drug Dev. Res.* **2015**, *7* (4),1-4.

32- Miyano, H.; Toyo'oka, T.; Imai, K. Further studies on the reaction of amines and proteins with 4-fluoro-7-nitrobenzo-2-oxa-1,3-diazole. *Anal. Chim. Acta*. **1985**, *170*, 81–7.

33- El-Kosasy, A.M.; Abdel-Aziz, O.; Magdy, N.; El Zahar, N. M. Screening and Optimization of the Reaction of Polymyxin B Sulphate with NBD-Cl for the Synchronous Spectrofluorimetric Determination of Polymyxin B Sulphate in Human Plasma. *J. Fluoresc.* **2015**, *25* (3), 695-705.

34- Rageh, A.H.; El-Shaboury, S. R.; Saleh, G.A.; Mohamed, F.A. Spectrophotometric method for determination of certain cephalosporins using 4-chloro-7-nitrobenzo-2-oxa-1, 3-diazole (NBD-Cl). *Nat. Sci.* **2010**, *2* (8), 828-840.

35- Sabry, S.M.; Belal, S.F.; Khamis, E.F.; Gawad, D. A. Spectrofluorimetric and spectrophotometric determination of cephradine and cefaclor. *ACAIJ* **2007,** *5* (1-6), 102-107.

36- J. Rose, Advanced Physico-chemical Experiments, Pitman: London, **1964**, pp. 67.

37- Walash, M.I.; Belal, F.; El-Enany; Elmansi, H. Determination of paroxetine HCl in pharmaceuticals via derivatization with 4-chloro-7-nitrobenzo-2-oxa-1,3-diazole (NBD-Cl). *J. Fluoresc*. **2011**, *21*, 105–120.

38- Ibrahim, F.; El-Enany, N.; El-Shaheny, R. N.; Mikhail, I. E. Validated spectrofluorimetric and spectrophotometric methods for the determination of brimonidine tartrate in ophthalmic solutions via derivatization with NBD-Cl. Application to stability study. *Luminescence* **2015**, *30*, 309–317.

39- ICH Harmonized Tripartite Guidelines, Q2(R_1) Validation of Analytical Procedures: Text and Methodology, **2005**.