World Journal of Applied Chemistry
2017; 2(4): 120-128
http://www.sciencepublishinggroup.com/j/wjac
doi: 10.11648/j.wjac.20170204.11

Determination of Beta-Lactamase Inactivation of Cephalexin by Validated RP-HPLC Method

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To cite this article:

Mahmoud Faysal Mshref, Hesham Mohamed Ghonemy, Amr Helmy Ali. Determination of Beta-Lactamase Inactivation of Cephalexin by Validated RP-HPLC Method. *World Journal of Applied Chemistry.* Vol. 2, No. 4, 2017, pp. 120-128. doi: 10.11648/j.wjac.20170204.11

Received: August 2, 2017; **Accepted**: August 26, 2017; **Published**: September 13, 2017

Abstract: Determination of the needed amount of liquid sterile Lactamator™ to be mixed to cephalexin, β-lactam antibiotic, for optimum deactivation of its molecule's antibacterial properties was conducted using RP-HPLC method. RP-HPLC method was validated for the parameters as linearity, accuracy, LOD, LOQ, and precision. Before the routine microbiological examination for any pharmaceutical dosage form containing β-lactam antibiotic, it is a must to make inactivate of β-lactam active pharmaceutical ingredient (API) by mixing with beta-lactamase before testing. So, the study indicated that mixing of 0.5 ml liquid sterile Lactamator™ with phosphate buffer solution pH (7.2) containing 50 mg cephalexin, with holding the test sample for 90 minutes prior to HPLC measurement will deactivation of cefalexin molecule's antibacterial properties.

Keywords: Cephalexin, Lactamator, Keflex, Method Validation

1. Introduction

β-lactam antibiotics are a class of broad spectrum antibiotics, consisting of all antibiotic agents that contain a common element in their molecular structure: a four-atom ring known as a β-lactam (Figure 1).

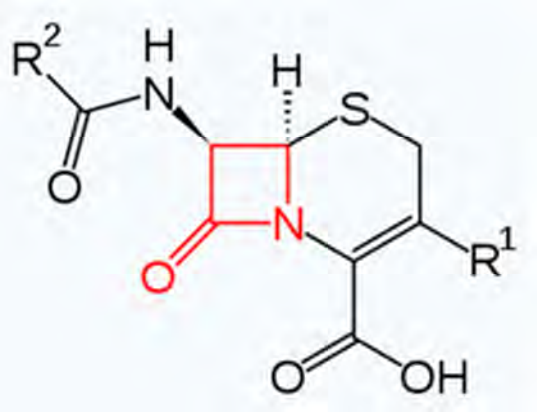

Figure 1. *Molecular structure of β-lactam antibiotics.*

This includes penicillin derivatives (penams), cephalosporins (cephems), monobactams, and carbapenems [1]. Most β-lactam antibiotics work by inhibiting cell wall biosynthesis in the bacterial organism and are the most widely used group of antibiotics. Bacteria often develop resistance to β-lactam antibiotics by synthesizing a β-lactamase, an enzyme that attacks the β-lactam ring. All β-lactam antibiotics have a β-lactam ring in their structure. The effectiveness of these antibiotics relies on their ability to reach the penicillin binding protein (PBP) intact and their ability to bind to the PBP. Hence, there are two main modes of bacterial resistance to β-lactams, one mode possessed by altering penicillin binding proteins and the another by enzymatic hydrolysis of the β-lactam ring. If the bacterium produces the enzyme β-lactamase or the enzyme penicillinase, the enzyme will hydrolyze the β-lactam ring of the antibiotic, rendering the antibiotic ineffective [2].

Beta-lactamases are enzymes produced by bacteria, that provide multi-resistance to β-lactam antibiotics such as penicillins, cephalosporins, cephamycins, and carbapenems (ertapenem), although carbapenems are relatively resistant to beta-lactamase. Beta-lactamase provides antibiotic resistance by breaking the antibiotics structure. Through hydrolysis, the lactamase enzyme breaks the β-lactam ring open, deactivating the molecule's antibacterial properties (Figure 2). Beta-lactamases are classified according to functional classification [3] and molecular classification [4].

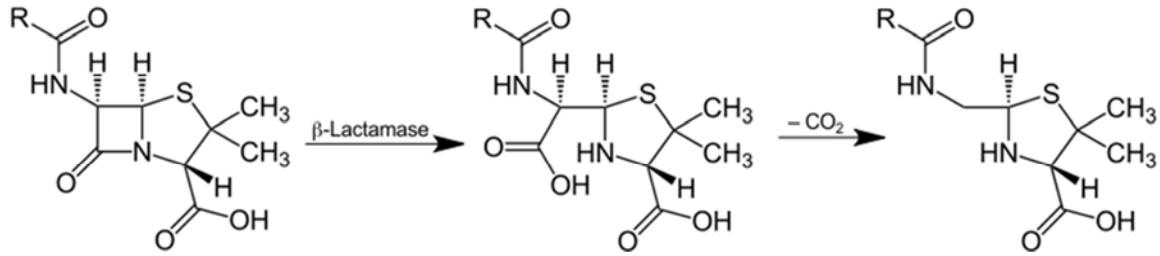

Figure 2. *Hydrolysis of β-lactam antibiotics by beta-lactamase.*

In Gram-negative bacteria, the beta-lactamase was usually produced at very high concentration constitutively or by induction via direct interaction of beta-lactam antibiotic with regulatory system [5 – 10]. In Gram-negative bacteria, the expression level of beta-lactamase is usually low; however, it has been observed that production of beta-lactamase was inducible but molecular basis for this phenomenon was not clear [11, 12]. *Staphylococcus aureus*, *Hemophilus influenzae* and *Escherichia coli* produce beta lactamases which can hydrolyze the penicillins but not all the cephalosporins. Other beta lactamases which are produced by *Pseudomonas*, *Enterobacter*, *Neisseria gonorrhoeae* and *Moraxella catarrhalis* have the ability to hydrolyze both the penicillins and the cephalosporins [13].

Cephalexin, is a semisynthetic cephalosporin antibiotic for oral administration, belongs to the group of β-lactam antibiotics. It is chemically designated as (6*R*,7*R*)-7-[[(2*R*)-2-Amino-2-phenylacetyl]amino]-3-methyl-8-oxo-5-thia-1-azabicyclo [4.2.0]oct-2-ene-2-carboxylic acid monohydrate. The chemical formula for cephalexin is $C_{16}H_{17}N_3O_4S$. H_2O and the molecular weight is 365.4 (Figure 3).

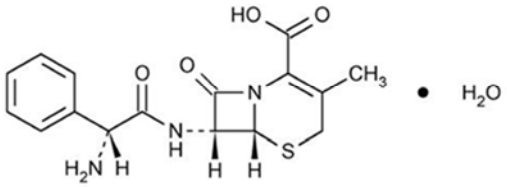

Figure 3. *Molecular structure of cephalexin.*

Cephalexin, developed under the trade name Keflex, is a first-generation cephalosporin antibiotic for the treatment of infections caused by susceptible Gram-positive and Gram-negative bacteria including infections of the respiratory and genito-urinary tracts, bones, and skin [14].

Lactometer™ is an innovative enzyme based product and specifically designed for the inactivation of a wide range of beta-lactam antibiotics. It can inactivate pencillins, cephalosporins of first, second, third, fourth and fifth generation and penems. In pharmaceutical industries, Lactamator™ is used in the inactivation of beta-lactams active pharmaceutical ingredients (APIs) found in the test samples prior to routine microbiological examination. The sterile liquid Lactamator™ is an optimized ready to use solution that can be directly added to the test samples. The amount of liquid sterile Lactamator™ to be added to the test sample should be determined and set-up case by case depending on the application, concentration of antibiotic that should be inactivated, and depending on the specific beta-lactam that should be inactivated.

Our scope in this study is to exactly determine the needed amount of liquid sterile Lactamator™ to be added to cephalexin, beta lactam antibiotic, for optimum deactivation of its molecule's antibacterial properties.

2. Materials and Methods

2.1. Materials

Cephalexin monohydrate was purchased from Dhanuka Laboratories Limited, Haryana, India. Methanol, Acetonitrile and triethylamine were of HPLC grade and were purchased from Scharlab S.L., Spain. Other reagents were of analytical-reagent grade and purchased from Scharlab S.L., Spain. Water was deionised and double distilled. Tryptone soya agar and eosin methylene blue agar were purchased from Oxoid, USA. Bacterial pathogens, *Escherichia coli* (ATCC® 8739) and *Staphylococcus aureus* (ATCC® 6538) were provided as gifts from HIKMA Group, Beni-Suief, Egypt. Lactamator™ Sterile liquid, > 100 IU cephalosporinase and > 1000 IU penase/vial, with lot no. 019. LQS.00116 and expiring on 04/2020, was purchased form CPC Biotech, S. R. L., Italy. Marketed formulations of cephalexin were provided either as gifts or were purchased after checking their batch number, production and expiry date. These were as follows:

a. Keflex 250 mg/ 5ml powder for oral suspension, designated as test sample, with lot no. 2060 and expiring on 03/2020 (HIKMA Group, Beni-Suief, Egypt).

b. Keflex 500 mg film coated tablet, designated test sample, with lot no. 2071 and expiring on 06/2020 (HIKMA Group, Beni-Suief, Egypt).

c. Keflex 1000 mg film coated tablet, designated as test sample, with lot no. 2038 and expiring on 05/2020 (HIKMA Group, Beni-Suief, Egypt).

2.2. Apparatus

A HPLC system consisting of a CMB-20 Alite system controller, two LC-20AT pumps, SIL-20A auto-sampler, CTO-20 column oven and SPD-20A UV-VIS detector at a sensitivity of 0.0001 (Shimadzu, Japan). The drug analysis data were acquired and processed using LC solution (Version 1.25) software running under Windows 7 on Intel, Pentium

PC. Electronic balance, AUW-220D (Shimadzu, Japan). Autoclave, HX-150 (Systec, Germany). Incubator, BD-53 (Binder, England).

2.3. Methods

2.3.1. Validation of HPLC Method

General preparations and buffer solutions were prepared as per "Reagents Chapter" in USP 36 [15]. The mobile phase was prepared as following: dissolve 0.985 gm of sodium 1-pentanesulfonate in a mixture of acetonitrile, methanol, triethylamine, and water (100:50:15:850 v/v), adjusted with phosphoric acid to a pH of 3.0 ± 0.1 and degas. The mobile phase pumped at a flow rate of 1.5 ml/min through the column (C_{18}; 250 mm x 4.6 mm, 5µ Thermo ODS, USA) at 25ºC, ultraviolet detection at 254 nm and injection volume was 20 µL. The mobile phase was filtered through a 0.45 µm nylon membrane filter and degassed prior to use under vacuum.

Stock solutions of cephalexin (1000 µg/ml) were prepared in phosphate buffer pH (7.2) and diluted to get standard solutions of 50% to 150% of target concentration (500 µg/ml). The method was validated for the parameters as system suitability, system precision, linearity, limit of detection, limit of quantitation, and accuracy as per ICH guidelines [16].

The system suitability was assessed by five replicate analyses of standard solution at a 100% level to verify the resolution and reproducibility of the chromatographic system. This method was evaluated by analyzing the repeatability of peak area, retention time, tailing factor and theoretical plates of the column. Also, the system precision was conducted using five replicates of the standard and RSD of the injections was calculated to verify that system was precise.

Accuracy was assessed using nine determinations over three concentration levels covering the specified range of the standard. The measurements are made at different concentrations which is from 50% to 150% include 100% of the target concentration. The limit of detection, limit of quantitation and percentage recovery were calculated to verify the method accuracy. For the linearity, five solutions of the drug substance were prepared (50%, 75%, 100%, 125%, and 150%) of the target concentration then their responses measured by the same method of analysis are recorded. The criteria of good linearity were determined by obtaining correlation coefficient not less than 0.99 of concentration versus peak area graph.

2.3.2. Calculation of the Needed Amount of Liquid Sterile Lactamator™ for Optimum Deactivation of Cephalexin

Several solutions of cephalexin were prepared as follows. Transfer accurately 50 mg of cephalexin to seven volumetric flasks, 100-ml capacity, containing 70 ml phosphate buffer pH (7.2), sonicate till dissolve. Then add different concentrations of liquid sterile Lactamator™ (i.e. 0.1, 0.2, 0.3, 0.4, 0.5, 0.6, & 0.7 ml) to the prepared solutions respectively and complete to volume with the same solvent and mix well.

In all the experiments, samples were withdrawn after 30, 60, 90, and 120 minutes for analysis. The test samples, blank (solvent) and standard cephalexin (without enzyme) were filtered through membrane filter and reject the first portion of the filtrate and assayed using HPLC-UV at 254 nm. The concentration of each sample was determined from a calibration curve obtained from validated HPLC method of cephalexin in phosphate buffer pH (7.2).

2.3.3. Verification of the Calculated Amount of Liquid Sterile Lactamator™ Needed for Optimum Deactivation of Cephalexin

Verification of the calculated amount of liquid sterile Lactamator™ needed to be added to cephalexin, for optimum deactivation of its molecule's antibacterial properties was done by the validated HPLC method and agar well diffusion method.

i. HPLC Method

From the analysis results of the previous samples, the most effective concentration of liquid sterile Lactamator™ after suitable holding time was used to be added on different dosage forms of cephalexin for optimum deactivation of its molecule's antibacterial properties as follows. Take suitable quantities of different dosage forms of cephalexin (i.e. Keflex 250 mg/5ml powder for oral suspension, Keflex 500 mg film coated tablet and Keflex 1000 mg film coated tablet). Grind to fine powder. Transfer an accurately weighed from powdered products equivalent to 50 mg cephalexin into a 100-ml volumetric flask. Add about 70 ml phosphate buffer pH (7.2), sonicate till dissolve. Then add 0.5 ml of liquid sterile Lactamator™ to the prepared solutions and complete to volume with the same solvent and mix well.

In all the experiments, samples were withdrawn after 90 minutes for analysis. The test samples, blank (solvent), tested finished product (without enzyme) and standard cephalexin (without enzyme) were filtered through membrane filter and reject the first portion of the filtrate and assayed using HPLC-UV at 254 nm. The concentration of each sample was determined from a calibration curve obtained from validated HPLC method of cephalexin in phosphate buffer pH (7.2).

ii. Agar Well Diffusion Method

Agar well-diffusion method is widely used to evaluate the antimicrobial activity of plants or microbial extracts [17, 18]. Similarly to the procedure used in disk-diffusion method, the agar plate surface is inoculated by spreading a volume of the microbial inoculum over the entire agar surface. Then, a hole with a diameter of 6 to 8 mm is punched aseptically with a sterile cork borer or a tip, and a volume (20–100 µL) of the antimicrobial agent or extract solution at desired concentration is introduced into the well. Then, agar plates are incubated under suitable conditions depending upon the test microorganism. The antimicrobial agent diffuses in the agar medium and inhibits the growth of the microbial strain tested. Suitable quantities of tryptone soya agar (TSA) and eosin methylene blue agar (EMB) were prepared and sterilized as per chapter <62> in USP 40 [19]. The previously described method was performed using cephalexin as beta-lactam antibiotic, and *Escherichia coli* (gram negative bacteria) and *Staphylococcus aureus* (gram positive bacteria)

as bacterial pathogens. Plates containing microorganisms with cephalexin were evaluated against that containing microorganisms, cephalexin and liquid sterile Lactamator™.

3. Results and Discussion

3.1. Test Method Validation

The experiment was carried out according to the official specifications of USP, ICH- 1995, and Global Quality Guidelines. Table (1) represents system suitability tests results of this method. The system was found suitable as the % RSD of injection precision was less than 1.0 %, mean theoretical plate count was more than 2000 and the mean tailing factor was less than 1.5.

Table 1. *System suitability study results of cephalexin in phosphate buffer pH (7.2).*

Validation Parameter	Results ± SD [a]
% RSD [b] of peak area	0.729 ± 41635
% RSD of retention time	0.368 ± 0.022
Average tailing factor	0.938 ± 0.005
Average theoretical plate	7437.82 ± 48.738

[a] SD = Standard deviation.
[b] RSD = Relative standard deviation.

Results of the validation parameters of cephalexin in phosphate buffer pH (7.2) were summarized in table (2).

Table 2. *Linearity, accuracy, LOD, LOQ and precision results of cephalexin in phosphate buffer pH (7.2).*

Validation Parameter		Results
System Precision	% RSD	0.031
Linearity	R^2 (Regression coefficient)	0.9995
	Y-intercept	68541
	Slope	55963
Accuracy	% Recovery (mean ± SD [a])	99.8 ± 1.01
	% RSD [b]	0.01
LOD [c]	µg/mL	3.5
LOQ [d]	µg/mL	10.61

[a] SD = Standard deviation.
[b] RSD = Relative standard deviation.
[c] LOD = Limit of detection.
[d] LOQ = Limit of quantitation.

It was shown that, the system was precise in the buffer used with % RSD not more than 1.0 %. The method was found linear as regression coefficient of calibration curve was more than 0.99 as shown in figure (4).

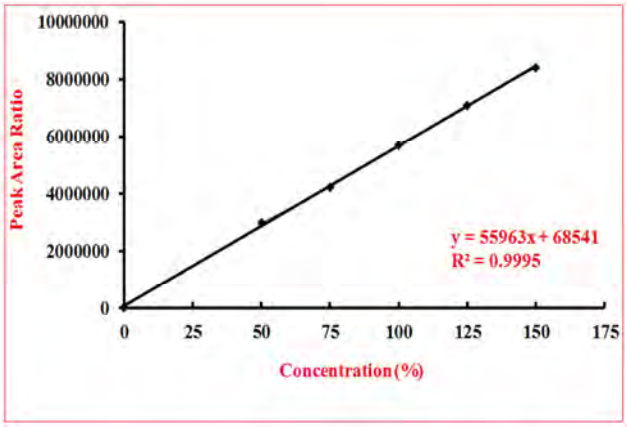

Figure 4. *Calibration curve of cephalexin in phosphate buffer pH (7.2) at λ_{max} 254 nm using HPLC method.*

Detection and quantitation limits are based on the standard deviation of the response and the slope. A specific calibration curve should be studied using samples containing an analyte in the range of LOD and LOQ. The residual standard deviation of a regression line or the standard deviation of y-intercepts of regression lines may be used as the standard deviation. LOD=3.3 × σ /slope and LOQ =10 × σ /slope, where σ = the standard deviation of the response. Limit of detection (LOD) and limit of quantitation (LOQ) were 3.5 µg/ml and 10.61 µg/ml respectively. Also, average % recovery was within the limit (98 – 102%). Regarding the system precision, the % RSD was within the limit, (0.031 %). So, the method is highly accurate, and precise.

3.2. HPLC Analysis of Drug Content

Table (3) represents the remaining amounts of cephalexin from the prepared solutions after adding of different concentrations of liquid sterile Lactamator™ with different holding time before HPLC analysis.

Table 3. *Determination of percent cephalexin after mixing with different concentrations of Lactamator™ and different holding time before determination using HPLC method.*

Lactamator™ Quantity (ml)	% Cephalexin remained after the following holding time in minutes			
	30	60	90	120
0.1	41.63	37.84	37.12	17.05
0.2	14.42	7.23	5.71	4.32
0.3	7.47	5.02	3.99	3.04
0.4	7.2	4.25	3.32	3.31
0.5	5.61	4.25	3.31	3.3
0.6	5.59	4.34	3.56	3.31
0.7	5.58	4.5	3.34	3.33

Figure (5) illustrated the HPLC chromatograms of cephalexin assay before and after addition of 0.5 ml liquid sterile Lactamator™ with 90 minutes as a holding time before determination by the analytical method

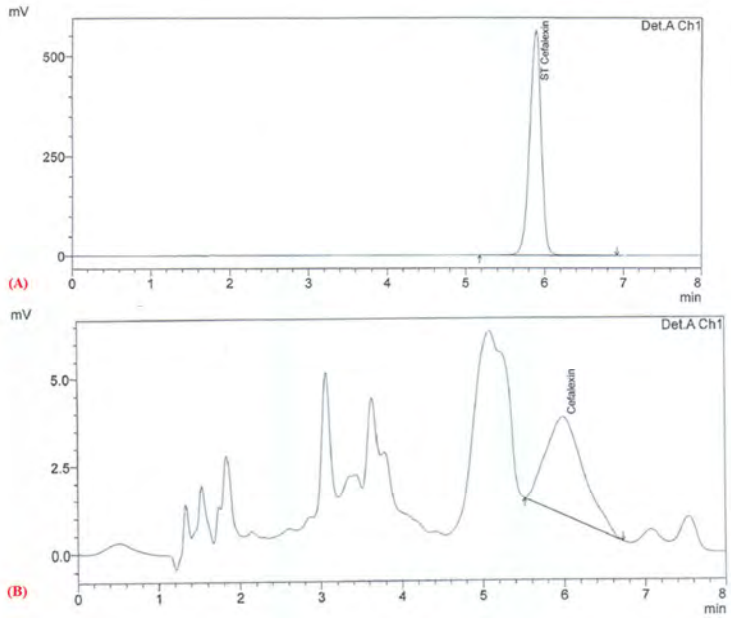

Figure 5. *HPLC chromatograms of cephalexin assay for (A) standard solution of cephalexin without enzyme addition and (B) standard solution of cephalexin after addition of 0.5 ml liquid sterile Lactamator™ with 90 minutes as a holding time before determination by the analytical method.*

From all concentrations used and holding time applied, the maximum decrease and constantly in the drug concentration was observed using 0.5 ml liquid sterile Lactamator™ with holding time of 90 minutes before HPLC analysis as shown in figure (6).

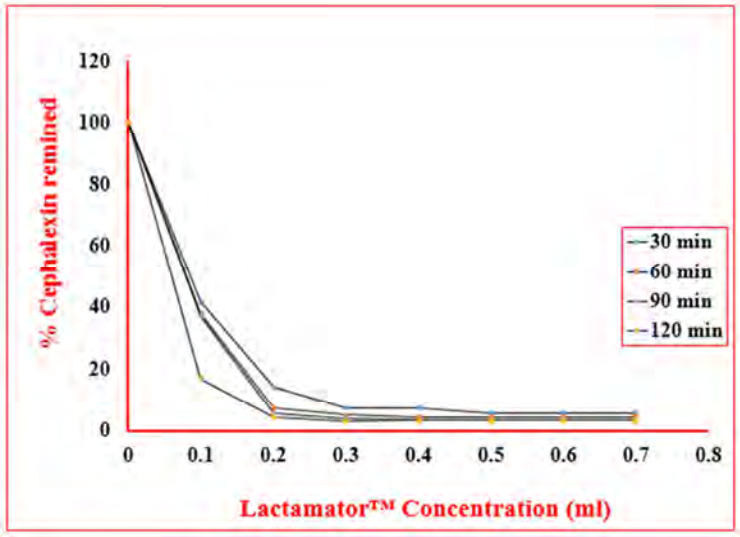

Figure 6. *Percent cephalexin remained after mixing with different concentrations of Lactamator™ and different holding time using HPLC method.*

3.3. Method Application

The validated method is applied for the determination of cephalexin content in the commercially available Keflex 250 mg/5ml powder for oral suspension, Keflex 500 mg film coated tablet and Keflex 1000 mg film coated tablet. The results of the cephalexin assay in these formulations before and after adding of liquid sterile Lactamator™ were shown in table (4).

Table 4. *Determination of percent cephalexin from different pharmaceutical formulations before and after mixing with Lactamator™ using HPLC method.*

Test Name	% Cephalexin	
	Before mixing	After mixing
Cephalexin	99.92	2.21
Keflex 250 mg/5 ml POS [a]	103.31	2.36
Keflex 500 mg film coated tablet	102.4	3.95
Keflex 1000 mg film coated tablet	96.81	4.39

[a] POS = Powder for oral suspension

Figures (7-9) illustrated the HPLC chromatograms of cephalexin assay from different dosage forms before and after liquid sterile Lactamator™ addition.

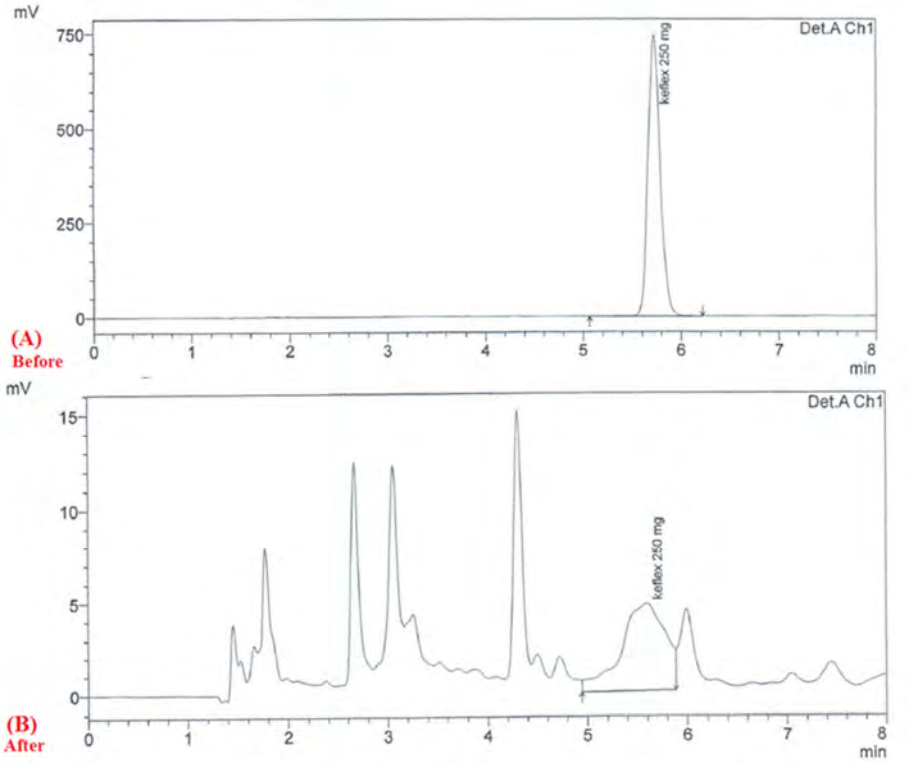

Figure 7. *HPLC chromatograms of Keflex 250 mg/ 5 ml powder for oral suspension (250 mg cephalexin): (A) before enzyme addition and (B) after enzyme addition.*

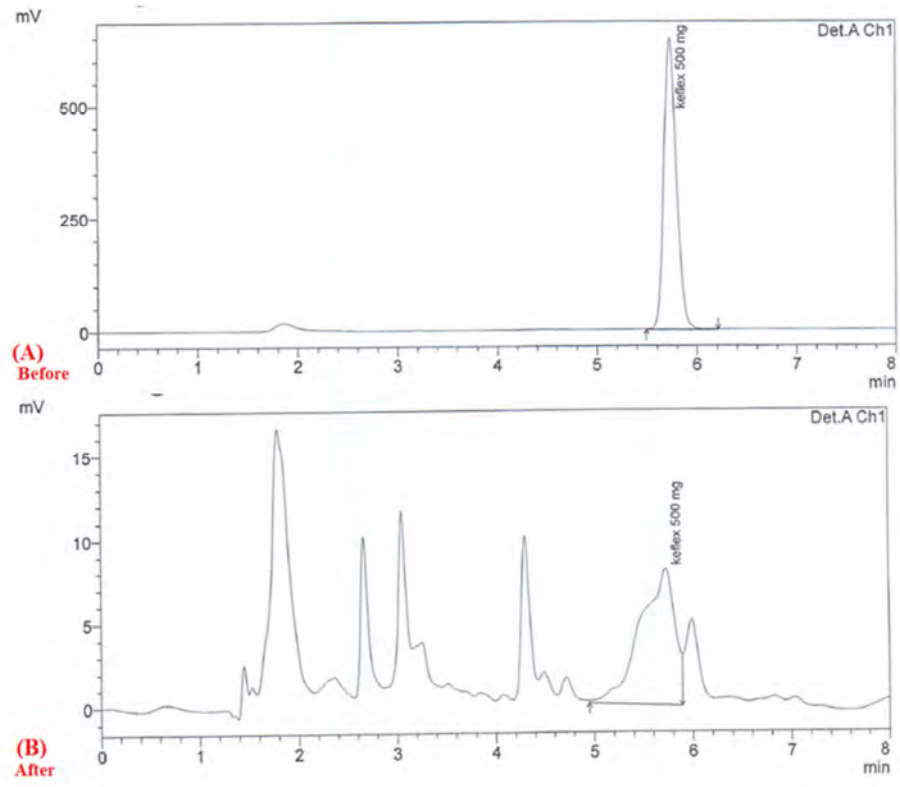

Figure 8. *HPLC chromatograms of Keflex 500 mg film coated tablet (500 mg cephalexin): (A) before enzyme addition and (B) after enzyme addition.*

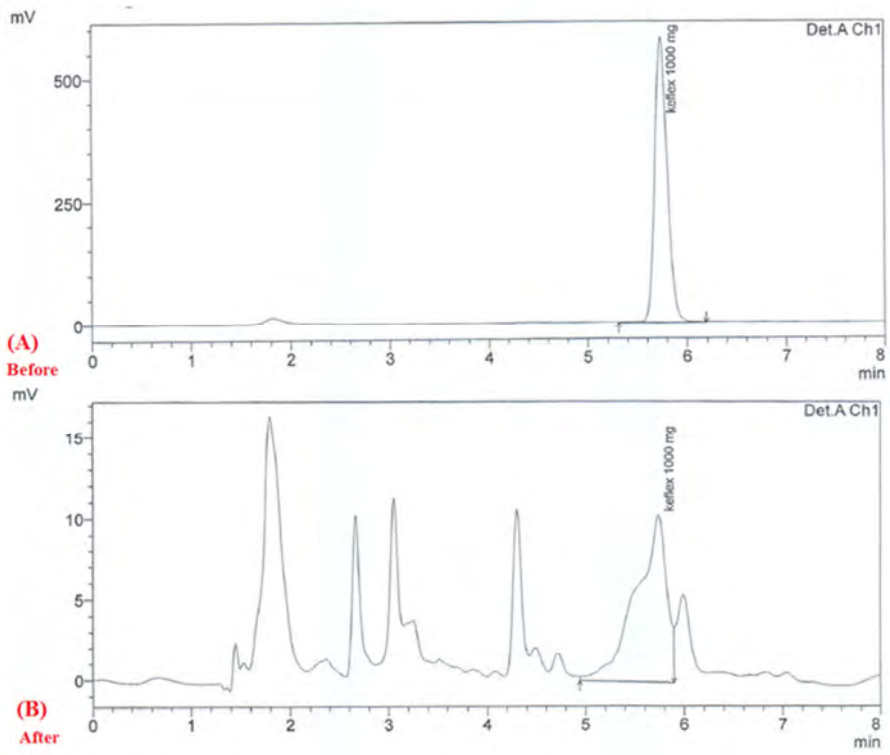

Figure 9. *HPLC chromatograms of Keflex 1000 mg film coated tablet (1000 mg cephalexin): (A) before enzyme addition and (B) after enzyme addition.*

Great efficiency of the enzyme in breaks the β-lactam ring open, deactivating the molecule's antibacterial properties of cephalexin was observed as shown in figure (10).

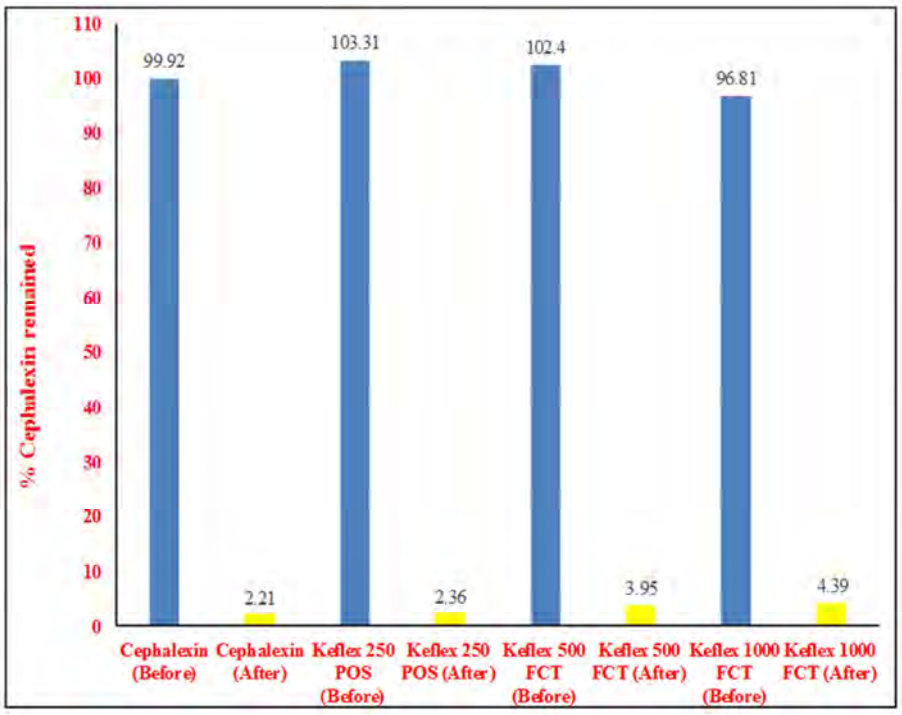

Figure 10. *Histogram of cephalexin assay from different dosage forms before and after addition of liquid sterile Lactamator™.*

Also, test verification was done using agar well diffusion method as shown in figure (11). All plates of microorganisms with cephalexin showed clear inhibition zones regarding the antibacterial activity of the drug. While plates containing microorganisms, cephalexin and liquid sterile Lactamator™ have no inhibition zones due to deactivation for beta-lactam ring of the active pharmaceutical ingredient (API) by mixing with the enzyme.

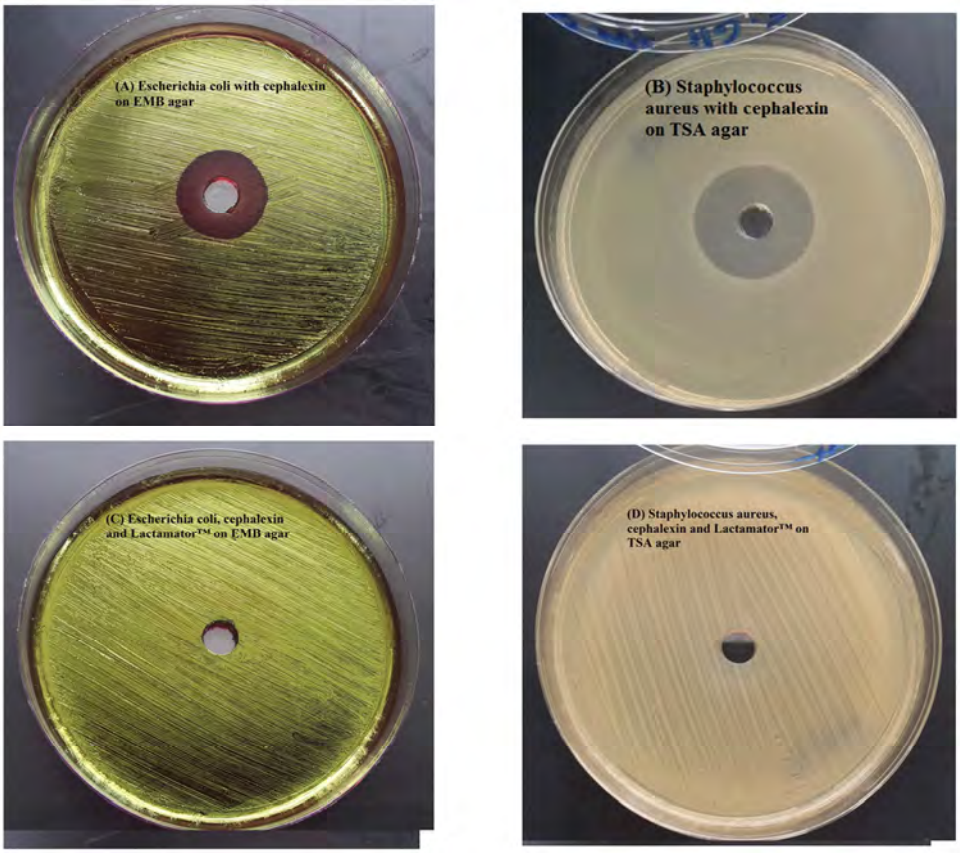

Figure 11. *Antimicrobial activity of cephalexin by agar well diffusion method. (A, B) Plates containing Escherichia coli with cephalexin on EMB agar and Staphylococcus aureus with cephalexin on TSA agar respectively. (C, D) Plates containing Escherichia coli, cephalexin and Lactamator™ on EMB agar and Staphylococcus aureus, cephalexin and Lactamator™ on TSA agar respectively.*

4. Conclusion

According to USP pharmacopeia regarding the microbial limit tests for all cephalosporin pharmaceutical preparations, it is a must to make inactivation of beta-lactam active pharmaceutical ingredients (APIs) found in the test samples prior to routine microbiological examination. Liquid sterile Lactamator™ was used for breaking the antibiotic structure. Through hydrolysis, the enzyme breaks the β-lactam ring open, deactivating the molecule's antibacterial properties. Different concentration of the enzyme at different holding time periods was used in this study to calculate the exact amount needed to give optimum inactivation of cephalexin.

In conclusion, the present study was undertaken with an aim to calculate of the needed amount of liquid sterile Lactamator™ for optimum deactivation of beta-lactam antibiotic, cephalexin using a validated analytical HPLC method. Before the routine microbiological examination for any cephalexin pharmaceutical dosage form, it is a must to make inactivate of beta-lactam active pharmaceutical ingredient (API) by mixing with beta-lactamase before testing.

So the study indicates that mixing of 0.5 ml liquid sterile Lactamator™ with phosphate buffer solution pH (7.2) containing 50 mg cephalexin, with holding the test sample for 90 minutes prior to HPLC measurement will deactivation of cefalexin molecule's antibacterial properties.

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