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NEW SIMPLE AND VALIDATED DENSITOMETRIC METHOD FOR DETERMINATION OF AMOXICILLIN AND CLOXACILLIN INTERACTION

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ABSTRACT

Drug-drug interaction of amoxicillin and cloxacillin was studied by this method. The proposed method depends on measuring the optical density of the inhibited zone area using digital camera and Image J software. Image J software can measure the area even when the inhibition zone is irregular in shape thus avoiding measurement errors during diameter measurement by help of ruler. Good correlations were obtained between the area and the antibiotics concentrations in the range (0.990-0.992). The limits of detection and limits of quantitation were 44.82, 14.51 and 10.86 μgmL^{-1} and 135.82, 43.98 and 32.91 μgmL^{-1} for amoxicillin, cloxacillin and their binary mixture; respectively. The proposed method was validated according to US-Food and drug administration (FDA) guidance for bioanalytical method validation and USP 31 guidelines.

KEYWORDS: Amoxicillin; Cloxacillin; Antibiotic activity; Drug drug Interactions; Minimum inhibitory concentration.

INTRODUCTION

Amoxicillin (2*S*,5*R*, 6R)-6-[[(2*R*)-2-amino-2-(4-hydroxyphenyl) acetyl] amino] -3,3-dimethyl-7-oxo-4-thia-1-azabicyclo [3.2.0] heptane- 2-carboxylic acid)) is an antibiotic (Fig. 1.a) useful for the treatment of a number of bacterial infections. It has an antibacterial spectrum similar to that of penicillin G but are more effective against gram-negative bacilli. They are therefore referred to as extended-spectrum penicillins,[1] and employed prophylactically by dentists for patients with abnormal heart valves who are to undergo extensive oral surgery. It is the first line treatment for middle ear infections and may also be used for strep throat, pneumonia, skin infections and urinary tract infections among others. It is taken by mouth and usually the drug of choice because it is better-absorbed following oral administration than other β-lactam antibiotics. Colxacillin (5*R*,6*R*) -6- {[3-(2-chlorophenyl)-5-methyl-oxazole-4-carbonyl]amino}-3,3-dimethyl-7-oxo- 4-thia-1-azabicyclo[3.2.0]heptane-2-carboxylic acid) is another antibiotic (Fig. 1.b) useful for the treatment of a number of bacterial infections. This drug has a weaker antibacterial activity than benzyl penicillin and is devoid of serious toxicity except for allergic reactions.[1] Amoxicillin and cloxacillin have been determined by RP-HPLC and spectrophotometric method.[2] Kirby–Bauer antibiotic testing (KB testing or disc diffusion antibiotic sensitivity testing)[3] is a test which uses antibiotic-impregnated discs to test whether bacteria are affected by antibiotics. In this test, discs of filter paper containing antibiotics are placed on an agar plate where bacteria have been placed (cultured) and the plate is left to be incubated. If an antibiotic stops the bacteria from growing or kills the bacteria, there will be an area around the disc where the bacteria have not grown enough to be visible (this is called a zone of inhibition). The size of this zone depends on how effective the antibiotic is at stopping the growth of the bacterium. A stronger antibiotic will create a larger zone, because a lower concentration of the antibiotic is enough to stop growth. Although the measuring of inhibition zone can be_done using ruler but still there is some errors during using this method due to that irregular zones that may develop during this method. The most important duty of the analyst is to reduce as soon as possible of the measurement errors during analysis. The objective of this research is to develop a new densitometric method for determination of antibacterial activity of amoxicillin, coloxacillin and a mixture of both drugs using digital camera and Image J software. Due to the rear drug interaction studies of both antibiotics this research also carried out to study the interaction between the two mentioned antibiotics and to validate this test.

Experimental and method

Equipment

-Toshiba Satellite laptop Intel (R) Core(TM) i3-2348M CPU @ 2.30GHz 2.30 GHz was used. Image J 1.50f software Wayne Rasband National Institutes of Health, USA, http.www. j.nih.gov/ij. Java 1.6.0-20(32- bit).

Hamilton syringe (Germany) was used. Samsung Note 4 digital camera 16 Mb. Triple divided petridishes were used for this study.

Material and reagents

20 g of Muller Hinton agar (Tulip Diagnostic, India) was weighed and dissolved in 500 mL of distilled water and spread equally over the surface of 20 petri-dishes using a spreader. 6mm - diameter Whatman filter paper (made in England) discs were made and put on the surface of the cultured media. Amoxicillin trihydrate (purity is 100.45%) and cloxacillin sodium (the purity 98.5%) were obtained from Shafaco Pharmaceutical Ind. (Attan, Sana'a- Republic of Yemen) as gifts.

Preparation of standard solutions

Stock solution containing 800 µg mL^{-1} of amoxicillin, 800 µg mL^{-1} of cloxacillin and 800µg mL^{-1} mixture of each drug were prepared in distilled water, the binary mixture was prepared by the ratio 1:1. Working standard solutions were prepared by further dilution of suitable volumes of the stock solutions with distilled water in separated 10 mL volumetric flasks to obtain concentrations in the range of 150–750 µg mL^{-1} corresponding to the concentration range of 450–2250 ng μL^{-1}.

General procedure

After preparation of the Muller Hinton agar by dissolving suitable amount in distilled water at the boiling temperature for 5-15 minutes. A quantity of the medium poured into the petridishes to form a uniform layer 2-5 mm in thickness. Store the dishes so that no appreciable growth or death of the micro-organisms occurs before the dishes are used and so that the surface of the medium is dry at the time of use. Swapping a suitable amount of *Staphylococcus aureus* by inoculating loop. 3µL volume of standard working solution must be added to each filter paper disc. Impregnate the discs with the solutions of the reference substance or the solutions of the antibiotic to be examined and place on the surface of the agar. Arrange the solutions on triple-divided petri dishes. Incubate at 37± 2°C for night (nearly about 24 hr). A period of diffusion prior to incubation, usually 1 hr to 4 hr, at room temperature, as appropriate, may be used to minimize the effects of the variation in time between the application of the solutions and to improve the regression slope. The image of inhibition zone was taken using Samsung Note 4 (the resolution of 16 MB) or any digital camera of high resolution from a distance about 30cm, then the image (in the tiff form) treated with Image J software. The images were treated and adjusted as 8 bit and thresholded (Fig.2). The area of inhibition for each zone inhibition was measured and the inhibition zone for each dose (concentration) was measured (Inhibition zone for each dose= Total inhibition zone filter paper disc area). The calibration curve was drawn by plotting inhibition zone area as square pixels against dose concentration (Fig. 3).

RESULTS AND DISCUSSION

Method validation

The developed densitometric method was validated in accordance with US-Food and drug administration (FDA) guidance.[4] for bioanalytical method validation and USP 31 guidelines.[5] For the statistical analysis, Excel 2003 (Microsoft Office) was used. The studied validation parameters were; linearity, range, limit of detection (LOD), limit of quantitation (LOQ), precision and accuracy.

Table 1: Statistical and quantitative parameters for determination and analysis of amoxicillin, cloxacillin and the binary mixture.

Drug	LOD (µgmL^{-1})	LOQ (µgmL^{-1})	Slope ±SD	Intercept ±SD	Correlation coefficient r
Amoxicillin	44.82	135.82	1057.30± 53.15	763387.59± 14360.20	0.990
Cloxacillin	14.51	43.98	626.21± 111.77	225005.30± 2754.22	0.991
Mixtrure	10.86	32.91	987.69± 69.74	722991.52± 3250.57	0.992

Table 2: The accuracy and precision of the proposed densitometric method for determination of amoxicillin, cloxacillin and the binary mixture.

Drug	Concentration (µg.mL^{-1})	Recovery [a] %±SD	RSD%
Amoxicillin	150	97.94 ± 3.64	3.72
	450	98.41 ± 1.99	2.02
	750	100.76 ± 2.90	2.88
Cloxacillin	150	97.84 ± 1.04	1.04
	450	101.99 ± 1.63	1.60
	750	98.05 ± 2.82	2.88
Mixture	150	97.74 ± 1.03	1.05
	450	100.12 ± 1.02	1.02
	750	98.04 ± 1.65	1.68

[a] Average of three determinations.

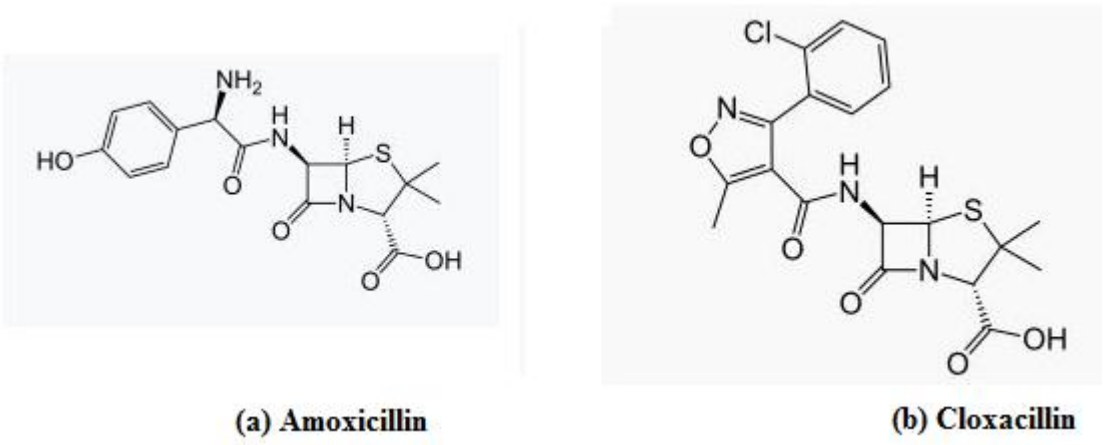

Fig. 1: The chemical structures of amoxicillin and cloxacillin.

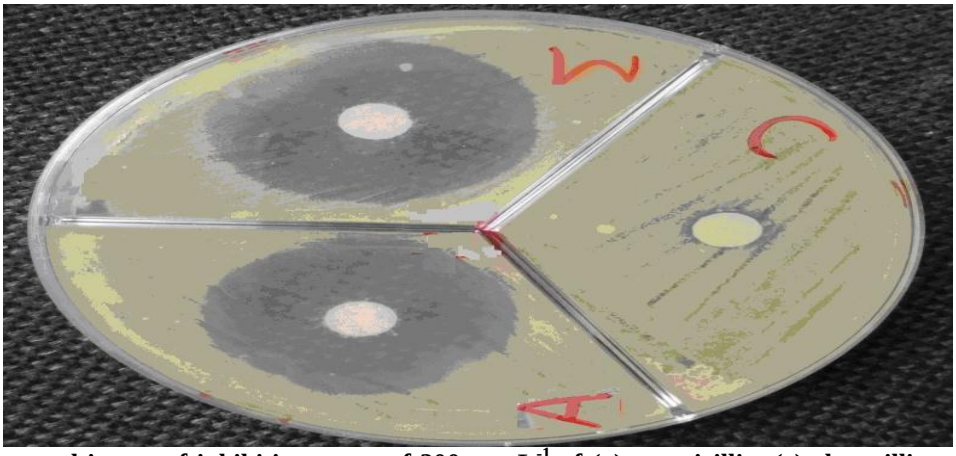

Fig. 2: The captured image of inhibition zones of 300µg.mL^{-1} of (a) amoxicillin, (c) cloxacillin and (M) binary mixture against *Staphylococcus aureus*.

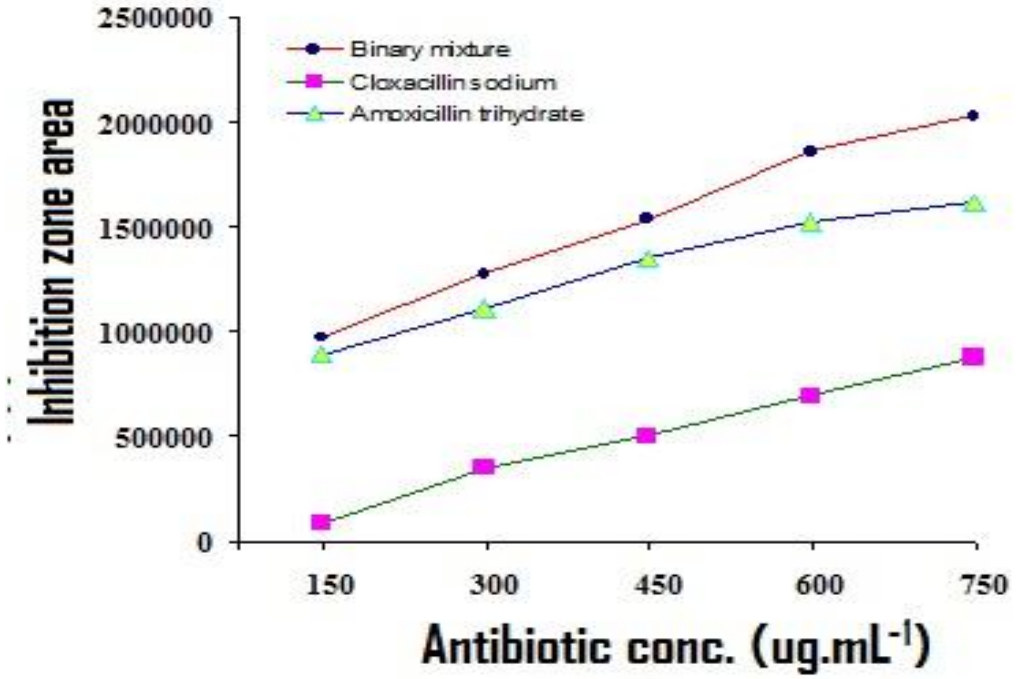

Fig. 3: The relationship between antibiotic conc. (µg.mL^{-1}) and the inhibited zone area using (150,300,450,600 and 700 µg.mL^{-1} of cloxacillin sodium,amoxicillin trihydrate and binary mixture of both drugs.

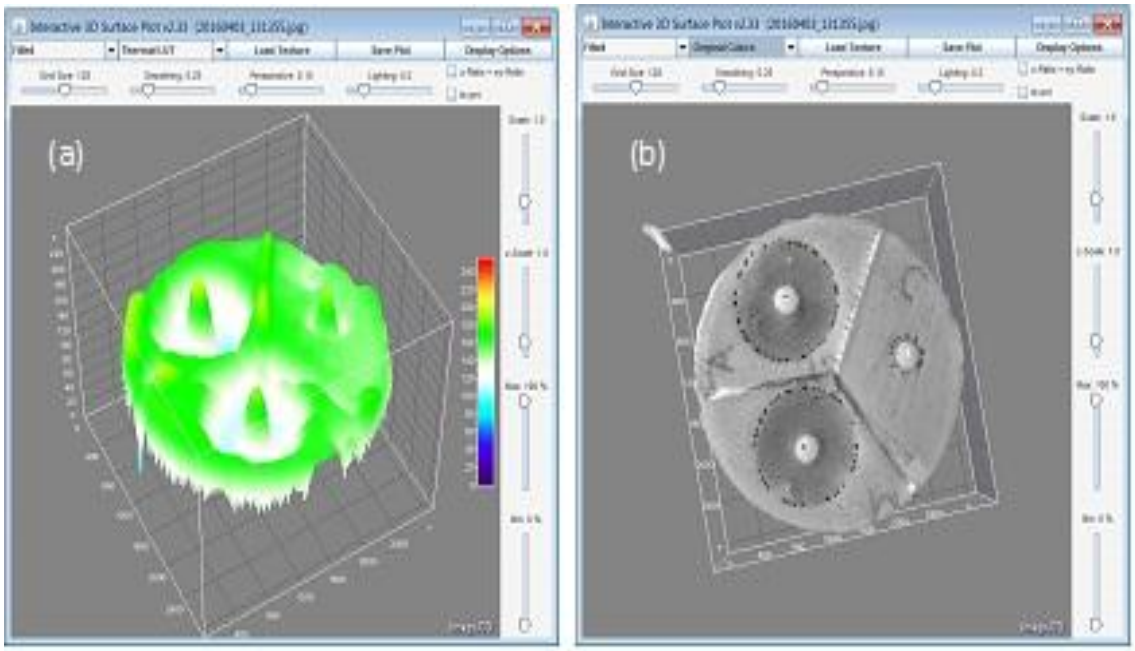

Fig. 4: (a) Interactive 3D surface plot for antibiotic activity of 300 µg.mL^{-1} amoxicillin, cloxacillin and the binary mixture of both antibiotics against *Staphylococcus aureus* using Image J software. and (b) original colors of interactive 3D surface plot for antibiotic activity of 150 µg.mL^{-1} amoxicillin, cloxacillin and the binary mixture of both antibiotics against *Staphylococcus aureus* using Image J software.

Calibration and linearity

The linearity of the method was in the range 150–750 µgmL^{-1} (equivalent to 450 to 2250 ngµL^{-1}) for amoxicillin, cloxacillin and their binary mixture. Good correlations were obtained between drug concentration and the inhibition zone area, the correlation coefficients for amoxicillin, cloxacillin and mixture of both antibiotics were ranged from (0.990–0.992).

Limit of detection LOD and limit of quantitation LOQ

In microbiology, the minimum inhibitory concentration (MIC) is the lowest concentration of an antimicrobial that will inhibit the visible growth of a microorganism after overnight incubation. MIC was determined for amoxicillin, cloxacillin and the binary mixture of both drugs. MIC was 150µgmL^{-1}. Mathematically the zone of inhibition of the growth of the bacteria is near zero when the concentration of the antibiotic is close to the zero so at very small concentration the antibiotic will kill bacteria even in small amount so the MIC can be calculated according to the linear equation Y= a+b X,since the Y is the area of the inhibited zone of bacteria growth a the intercept b is the slope of the calibration curve and X is the concentration of the antibiotic. The calculations of LOD and LOQ for each drug separately and for the binary mixture of both drugs the statistical data were as shown in **Table 1**.

Accuracy

The accuracy of the proposed method was determined by investigating the recovery percentages of the studied drugs at three concentration levels (150, 450 and 750µgmL^{-1}) (three repli-cates of each concentration). The results were shown in **Table 2**. The accuracy data revealed good accuracy and recovery percentages ranging from 97.94 to 100.76%, from 97.84 to 101.99% and from 97.74 to 100.12% for amoxicillin, cloxacillin and their mixture; respectively.

Precision

The precision of the developed method was assessed by assay (n = 3) at low (LQC), medium (MQC) and high (HQC) concentration levels (150, 450 and 750µg.mL^{-1}) for amoxicillin cloxacillin and mixture (**Table 2**). The precision of the method was expressed as relative standard deviations (RSD). RSD% values were ranged from 2.02 to 3.72% for amoxicillin, from 1.04 to 2.88 for cloxacillin and from 1.02 to 1.68% for the mixture of both antibiotics indicating good repeatability and precision. The obtained precisions were satisfactory for quality control measurements.

Amoxicillin trihydrate and cloxacillin sodium interactions

In general, *Streptococcus, Bacillus subtilis, Enterococcus, Haemophilus, Helicobacter* and *Moraxella* are susceptible to amoxicillin whereas *Citrobacter, Klebsiella* and *Pseudomonas aeruginosa* are resistant to it. Some *E. coli* and most clinical strains of *Staphylococcus aureus* have developed resistance to amoxicillin to varying degrees. Cloxacillin is semisynthetic and in the same class as penicillin. Cloxacillin has less potent antimicrobial activity against microorganisms that are sensitive to penicillin G but are the agents of first choice for treatment of penicillinase-producing *S. aureus* and *S. epidermidis* that are not methicillin-resistant,[6] In general the drug interaction

(alteration of drug pharmacological activity by the concomitant use of another drug). Amoxicillin and cloxacillin are both bactericidal drugs that act by inhibiting bacterial cell wall synthesis The combination of cloxacillin and amoxicillin thus widens the antibacterial spectrum of either drug given alone. Additionally, cloxacillin being a penicillinase-resistant penicillin is the drug of choice for penicillinase-producing staphylococcus aureus or staphylococcus epidermidis. Synergistic effect (Greek *syn-ergos*, "working together"[7] also the two drugs with same mechanism action are given together)[8] of amoxicillin when admixed with cloxacillin can be observed by increasing the area of inhibition zone of the cultured medium with *S. aureus* . The increased inhibited zone area when binary mixture of both antibiotics is used more than amoxicillin and cloxacillin separately. (**Fig.4)**

CONCLUSION

The proposed densitometeric method represents a new simple method for accurate determination of amoxicillin, cloxacillin and binary mixture. The reagents and solvents are available in any quality control laboratory so this method can be used for quality control of both antibiotics and their binary mixture accurately without measurement errors specially that accompanied using the traditional diameter measurement of inhibition zone when the inhibition irregular in shape.

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