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Effect of physical factors on the antimicrobial efficacy of amoxicillin trihydrate capsules and reconstituted suspension

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ABSTRACT

Background: Stability of drugs depends on both environmental factors and drug-related factors. Exposure of medicines to high temperatures during storage or transit could reduce their efficacy thus, shelf life and expiry dates may no longer be guaranteed. The objective of this study was to determine the effect of heat and environmental conditions on the antimicrobial efficacy of amoxicillin trihydrate.

Methods: Five brands of amoxicillin trihydrate capsules and six brands of amoxicillin trihydrate powder for suspension were purchased from retail pharmacies in Abuja, Nigeria. For the capsules, 100 µg/mL of the different brands and 25, 50 and 100 µg/mL of reference standard were prepared and subjected to heat treatment at different temperatures (25, 40, 60 and 80 °C) for 30 min. Each of the amoxicillin trihydrate powder for suspension was reconstituted in water and subjected to different storage conditions for 7 days. The UV absorbance of the reference amoxicillin trihydrate was determined while the potencies of heat-treated amoxicillin capsules and stored suspensions were evaluated by agar diffusion method.

Results: At 25 and 40 °C there was no significant change in the antimicrobial efficacy of amoxicillin, however as the temperature increased to 80 °C there was a reduction in the zones of inhibition against the test organisms. The absorbance readings of the heat-treated reference amoxicillin trihydrate powder gradually increased with increase in temperature. There was no significant ($p < 0.05$) reduction in the antimicrobial effects of the reconstituted suspensions within 7 days of testing.

Conclusion: The antimicrobial stability of amoxicillin can be affected by exposure to extreme heat

1. Introduction

Drug quality influences treatment efficacy and safety. The quality of a drug is determined by manufacturing and storage processes to which it is subjected; high-quality drugs are ensured when procured using rational buying procedures and when suppliers are reliable[1]. Stability of drugs depends on both environmental factors such as light, temperature, humidity, air, and drug-related factors such as the active ingredient itself, the dosage form (tablet, suspension, etc.) and the manufacturing process[1]. It is therefore necessary to adhere to storage instructions given in the official guide or by manufacturers (on notices and labels) if the recommendations are not identical[2].

In Nigeria, the overall annual temperature ranges from 21.4 °C to 32.8 °C [3]. However, in the savannah region which includes Abuja, the mean maximum temperature is above the overall average (32.8°C compared to nationwide average of 26 °C to 28 °C)[4]. A classification of the country based on the thermal conditions divides it into five regions, Abuja classified as a part of Region 5 which is characterized by the most thermally stressed condition[5]. There is therefore no doubt that the temperature difference can influence the efficacy and stability of these drugs.

Exposure of medicines to high temperatures in transit or in storage could reduce their efficacy. At higher temperatures, there is the risk that the efficacy of drugs will be adversely affected, and the quality of drugs stored in such temperatures is called to question. During transit and transportation, temperatures may reach levels as high as 60°C inside vehicles, shipping containers or on docks. In this case, shelf life and expiry dates may no longer be guaranteed[2]. The stability of antibiotics at different temperatures has been researched for several decades in various disciplines ranging from use of antibiotics in therapy to disease prevention and growth promotion in food-producing animals[1]. A number of studies have been carried out to ascertain the effect of cooking temperatures and higher temperatures on the stability of these antibiotics[6]. However, little information exists on the effect of high temperatures of storage (above prescribed room temperature) on the stability and efficacy of antibiotics.

Various studies have been performed to examine the heat-sensitivities of antibiotics. The results generally suggest that aminoglycosides, quinolones, chloramphenicol, clindamycin, novobiocin, trimethoprim, vancomycin, oxacillin, and sulfamethazine are heat-stable drugs, tetracyclines and erythromycin were identified as thermo-labile, while several β-lactams (penicillin G, ampicillin and amoxicillin), nitrofurantoin, polymyxin B and rifampicin were reportedly partially heat-labile[5]. However, these studies were focused on effect of cooking temperatures and cooking time and not storage temperature[7].

Amoxicillin is chemically known as (2S.5R.6R)-6-{[(2R)-2-amino-2-(4-hydroxyphenyl)-acetyl] amino}-3, 3-dimethyl-7-oxo-4-thia-l-azabicyclo [3.2.0] heptane-2-carboxylic acid. It is one of the most prescribed antibiotics in developing countries like Nigeria.

Most heat stability studies have been assessed by evaluating the degradation of parent drugs; however, the development of degradation products that may also have antimicrobial activity or potential toxicity has been less thoroughly investigated[6]. This study is thus focused on evaluating the effect of temperature on the efficacy of amoxicillin trihydrate capsules using its antimicrobial activity as a determinant of efficacy and the evaluation of its drug content and physicochemical properties.

2. Materials and Methods

2.1 Reagents and media

Muller Hinton agar (MHA; Oxoid, UK), Muller Hinton broth (MHB; Oxoid, UK), Tryptic soy agar (TSA; Oxoid, UK), Sabouraud dextrose agar (SDA; Oxoid, UK),

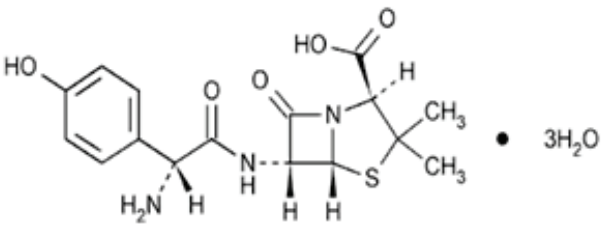

Figure1:ChemicalstructureofAmoxicillinTrihydrate

Amoxicillin trihydrate reference standard (USP, Rockville MD USA), Sodium phosphate (Sigma, UK), Citric acid (Sigma, UK).

2.2 Microorganisms

Staphylococcus aureus ATCC 25923, *Escherichia coli* ATCC 25922, clinical isolates of *Salmonella typhi* and *Bacillus subtilis*. The clinical isolates were identified by Gram staining techniques, biochemical test and morphological characteristics on culture medium according to Bergey's manual of determinative bacteriology[8]. For the purpose of the study, pure colonies of the overnight cultures of the bacteria grown on nutrient agar were suspended into 3 mL of normal saline and turbidity matched to 0.5 McFarland standard (1.5×10^8 CFU/mL)[9].

2.3 Sample collection

Five brands of amoxicillin trihydrate capsules and six brands of amoxicillin trihydrate powder for suspension were purchased from retail pharmacies within Abuja, Federal Capital Territory (FCT), Nigeria. All samples possessed NAFDAC registration numbers.

2.4 Antimicrobial analysis and heat treatment

2.4.1 Preparation of test drugs

Stock solutions at concentrations of 1 mg/mL of each brand of amoxicillin trihydrate capsule were prepared by dissolving in double distilled water (DDW) with further dilution to the desired concentration. Working standard solutions at concentration of 100 µg/mL for each antibiotic were prepared by appropriate dilution of the stock solutions with DDW. For thermal treatments, all working solutions in 10 mL vials were heated for 30 min by immersing in a water bath at 40, 60 and 80 °C.

2.5.2 Spectrophotometric analysis of heat-treated amoxicillin trihydrate reference standard

The method of Prakash *et al*.,[10] was used with some modifications. One hundred milligrams (100 mg) of standard amoxicillin powder equivalent was weighed and transferred into 100 mL volumetric flask to which 25 mL of citro-phosphate buffer pH 7.2 was added and sonicated for

10 minutes. The solution was shaken thoroughly for 15 minutes and the volume made up to 100 mL with citro-phosphate buffer (pH 7.2). A series of standard solutions containing 25, 50 and 100 µg/mL of amoxicillin trihydrate were prepared in citro-phosphate buffer (pH 7.2) and subjected to temperatures; 25 °C, 40 °C, 60 °C and 80 °C. Absorbance was measured at 295 nm using UV Spectrophotometer (Agilent Cary) against solvent blank[10].

2.5.3 Assessment of antimicrobial activity

Antimicrobial activity of all antibiotic suspensions was measured using the Kirby-Bauer disk diffusion method. The study was performed following the European Committee on Antimicrobial Susceptibility Testing (EUCAST) protocol against bacteria strains; *B. subtilis*, *E. coli* and *S. aureus*. Samples were tested in duplicates using 6 mm paper disks impregnated with 20 µL sample solution. The disks were placed equidistantly on petri dishes with Mueller Hinton agar previously inoculated with a standardized suspension of the test organism. Plates were incubated at 37°C for 24 hours, after which diameters of zones of inhibition (ZOI) were measured and recorded[11].

2.6 Statistical analysis

Experiments were conducted in triplicates; results are presented as mean ± standard error of mean. Data was processed and visualized using the Microsoft excel package. Data was analysed with two-way ANOVA followed by Dunnett's multiple comparisons tests with GraphPad Prism version 6.01 for Windows, GraphPad Software, La Jolla California USA.

3. Results

3.1 The effect of heat on the antimicrobial activity of amoxicillin trihydrate capsules.

The effect of heat on the antimicrobial activity of amoxicillin trihydrate capsules is represented in Figure 2. The antimicrobial activity is measured as an average of the diameters of zones of inhibition obtained with the five brands of the drug used against the selected organisms. The antimicrobial activity obtained at 25°C (room temperature) was taken as the baseline measurement and control against which antimicrobial activity under other conditions were compared. Generally, heat treatment at 40 °C, 60 °C and 80 °C did not lead to statistically significant changes in the antimicrobial activity of the antibiotic at a 95% CI ($p > 0.05$) against all selected organisms except with *S. aureus* where a statistically significant reduction in zone of inhibition was observed after heating the antibiotic to 80 °C ($p = 0.0101$).

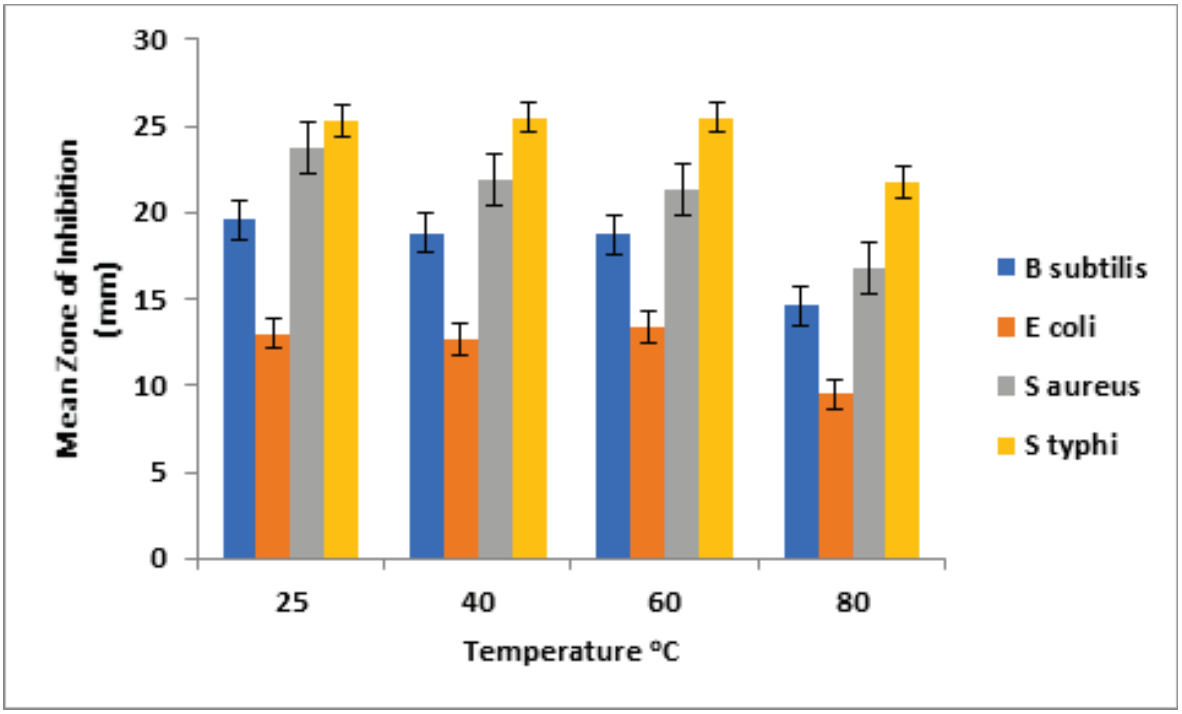

Figure 2: Effect of heat treatment of amoxicillin trihydrate on inhibition of B. subtilis, E. coli, S. aureus and S. typhi at 100 µg/mL

3.2 Effect of heat on amoxicillin trihydrate reference standard

The findings of the assessment of the effect on amoxicillin trihydrate are presented in Figure 3. The effect of heat is expressed as a function of the absorbance of the solution containing the drug at its maximum wavelength (λ_{max}= 295 nm). Taking the values obtained at 25°C as baseline and control against which other values are compared, the absorbance of amoxicillin trihydrate reference standard did not change significantly after heating at 40 °C, 60 °C and 80 °C ($p > 0.05$) at the concentrations prepared except at 25µg/mL where heating to 80 °C caused a statistically significant increase in absorbance at 95% CI ($p = 0.0099$) compared with the baseline measurement at 25 °C.

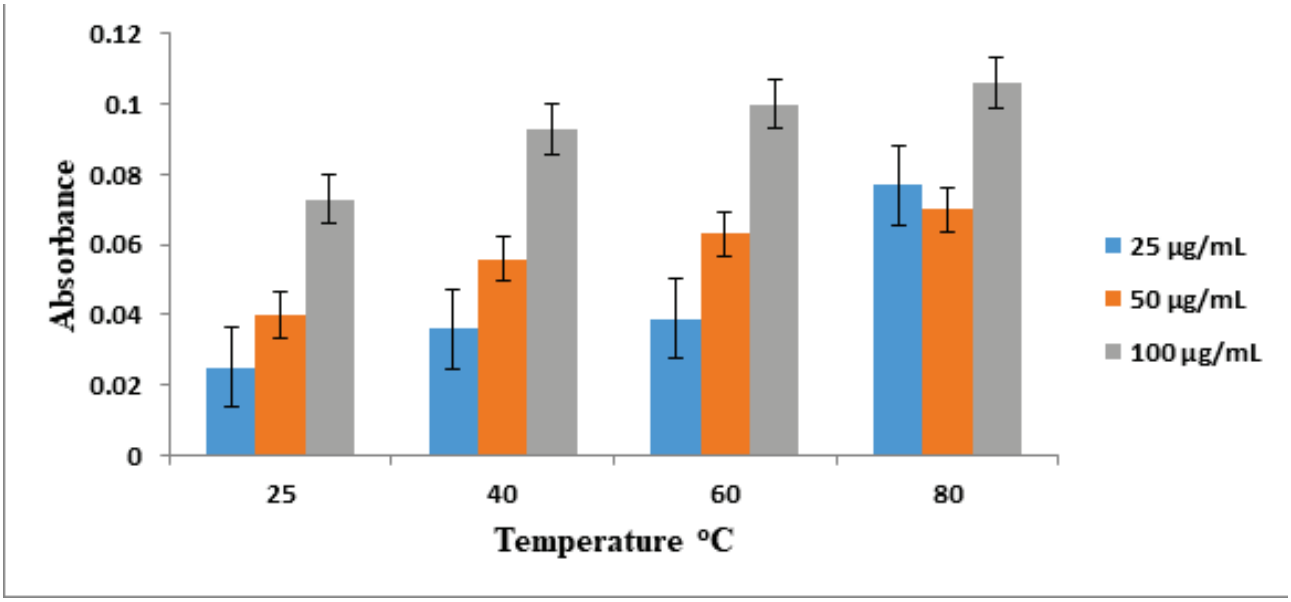

Figure 3: Effect of heat treatment on the absorbance of amoxicillin trihydrate reference standard

3.3 Assessment of stability of reconstituted Amoxicillin trihydrate suspension.

The effects different storage conditions have on the antimicrobial efficacies of the reconstituted amoxicillin suspensions are represented in Tables 1-5. The antimicrobial activities of the samples are measured by the average sizes (diameter) of the zones of inhibition they elicit on agar media inoculated with *B. subtilis*, *E. coli*, and *S. aureus* respectively. There was a statistically significant reduction in the antimicrobial activity of the samples after storage at room temperature for 1 day ($p = 0.0096$). The antimicrobial activity recorded after storage under this condition for seven days was however not significantly different from the baseline measurement on day 0 ($p > 0.05$).

Storage in the dark did not cause any significant changes in the antimicrobial activities of the samples after one and seven days. Storage in sunlight and storage at 40°C both resulted in significant reductions in the antimicrobial activities of the samples only after seven days of storage under these conditions ($p = 0.0004$ and 0.0050 respectively).

Table 1: Effect of storage at room temperature on the antibacterial activity of reconstituted amoxicillin suspension against *S. typhi*.

Sample	Diameters of zones of inhibition (mm)		
	Day 0	Day 1	Day 7
1	26.33± 0.58[a]	26.00±0.0[a]	27.00±0.50[a]
2	24.00±0.0[a]	23.67±1.53[a]	24.50±0.50[a]
3	20.33±0.58[a]	19.33±1.15[a]	19.50±0.50[a]
4	25.00±0.0[a]	24.00±0.58[a]	24.00±1.00[a]
5	25.67±0.58[a]	24.33±0.58[b]	24.50±0.50[a]
6	25.33±0.58[a]	24.67±0.58[a]	24.00±0.0[b]

Mean ± Standard error of mean (n = 3). Values carrying superscripts different from control (Day 0) are significantly different from control values.

Table 2: Effect of storage in refrigerator (4-8°C) on the antibacterial activity of reconstituted amoxicillin suspension against *S. typhi*.

Sample	Diameters of zones of inhibition (mm)		
	Day 0	Day 1	Day 7
1	24.67±0.58[a]	24.33±0.58[a]	24.50±0.50[a]
2	25.67±0.58[a]	25.67±1.0[a]	25.50±0.76[a]
3	26.67±1.15[a]	25.67±0.58[a]	24.50±0.50[a]
4	26.00±0.0[a]	25.33±0.58[a]	24.50±0.50[a]
5	26.33±0.58[a]	25.00±0.0[a]	25.00±0.0[a]
6	24.00±0.0[a]	24.00±0.0[a]	23.67±0.58[a]

Mean ± Standard error of mean (n = 3). Values carrying superscripts different from control (Day 0) are significantly different from control values.

Table 3: Effect of storage in the dark on the antibacterial activity of reconstituted amoxicillin suspension on *S. typhi*.

Sample	Diameters of zones of inhibition (mm)		
	Day 0	Day 1	Day 7
1	24.00±0.0[a]	23.33±0.58[a]	23.50±0.50[a]
2	24.33±0.58[a]	24.00±.0.0[a]	24.0±0.50[a]
3	20.00±0.0[a]	22.00±0.0[b]	22.0±0.0[b]
4	25.00±0.0[a]	24.50±0.0[a]	25.0±0.0[a]
5	25.67±0.58[a]	24.33±0.58[b]	25.00±1.00[a]
6	24.0±0.0[a]	23.00±0.0[b]	23.00±0.0[b]

Mean ± Standard error of mean (n = 3). Values carrying superscripts different from control (Day 0) are significantly different from control values.

Table 4: Effect of storage under sunlight on the antibacterial activity of reconstituted amoxicillin suspension against *Salmonella typhi*.

Sample	Diameter of zones of inhibition (mm)		
	Day 0	Day 1	Day 7
1	25.67±0.58[a]	25.33±0.58[a]	24±0.0[b]
2	24.67±0.58 [a]	24.33±0.58[a]	22.50±0.50[b]
3	21.33±1.15 [a]	22.67±0.58[b]	20.00±0.50[b]
4	25.33±0.58 [a]	24.67±0.58[a]	23.50±50[b]
5	27.67±0.58 [a]	26.00±0.0[b]	25.50±0.50[b]
6	24.33±0.58 [a]	23.67±0.0[a]	21.50±0.50[b]

Mean ± Standard error of mean (n = 3). Values carrying superscripts different from control (Day 0) are significantly different from control values.

Table 5: Effect of storage at 40 C on the antibacterial activity of reconstituted amoxicillin suspension against *S. typhi*.

Sample	Mean zones of inhibition (mm)		
	Day 0	Day 1	Day 7
1	25.67±0.58[a]	25.00±1.00[a]	24.5±0.50[a]
2	24.33±0.58[a]	24.33±0.58[a]	23.0±0.50[a]
3	24.67±0.58[a]	23.33±0.58[a]	21.50±0.50[a]
4	25.67±0.58[a]	25.67±14.43[a]	23.50±0.50[a]
5	25.67±0.58[a]	24.00±0.0[a]	23.00±1.00[a]
6	26.00±0.0[a]	22.00±0.0[a]	22.00±0.0[a]

Mean ± Standard error of mean (n = 3). Values carrying superscripts different from control (Day 0) are significantly different from control values.

4. Discussion

A drug is said to be stable if it remains within established specifications, maintaining its identity, quality, purity and strength throughout a retest or its shelf life[12]. Degradation of drugs has been linked to poor storage conditions[13]. Ideally, the heat stability of antibiotics is usually assessed microbiologically by measuring changes in their antimicrobial activity against microbes that were initially susceptible to them[14].

From results obtained, *S. aureus* was generally found to be the most susceptible organism to amoxicillin as observed by the largest zones of inhibition, while *Escherichia coli* generally was least susceptible. It was also observed that heating to temperatures up to 80 °C had no significant effect on the efficacy of amoxicillin against all strains of test organisms used except *S. aureus* against which the antimicrobial activity of amoxicillin trihydrate was significantly reduced after heating to 80 °C. This agrees with a study conducted in 2015, which reported incremental thermal degradation of antibiotics with increase in temperature[7]. According to this study, ß-lactam antibiotics like amoxicillin degraded faster than other antibiotics like flouroquinolones due to the changes in the lactam rings. In another study it was documented that the heat instability of ß-lactam antibiotic increased with temperature with about 50 % degradation at treatment beyond 100 °C. This degradation and loss in potency is linked to destruction of the ß-lactam ring[14].

The findings obtained during assessment of the effect of storage conditions on the antibacterial efficacy of reconstituted amoxicillin suspension revealed that storage at 40 °C and under sunlight for seven days respectively caused a significant reduction in the antimicrobial activities of the reconstituted amoxicillin suspension ($p < 0.05$). Reconstituted amoxicillin-clavulanic acid suspension has also been reported to undergo excessive degradation after 7 days of storage over a temperature range of 5-29 °C[15]. Storage at room temperature (25 °C) and in the dark generally did not result in a significant reduction in the efficacy of the antibiotic suspension after seven days. The results obtained after seven days of storage at room temperature make it so that the statistically significant reduction observed after 24 hours can barely be attributed to any physicochemical changes in the suspension caused by the storage condition.

5. Conclusion

Temperature has a significant effect on the efficacy of drug. It can be concluded that with the temperature ranges achievable in Nigeria with the average highest being 32.8 °C the activity of amoxicillin would be intact. It is recommended to store amoxicillin suspension after reconstitution in a refrigerator or room temperature but not

in excessive high temperature so that the antimicrobial efficacies remain intact.

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