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How pH and Temperature Influence Ampicillin Degradation: A Zone of Inhibition Study

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Abstract

Antibiotics such as ampicillin ($C_{16}H_{19}N_3O_4S$) are essential for treating bacterial infections, and their efficacy depends on environmental conditions like pH and temperature. This study examined how ampicillin's antibacterial activity—measured via zone of inhibition assays—is affected by different pH levels (5.5, 7.2, 8.5) and temperatures (4°C, 25°C, 50°C, 80°C). Results showed that neutral pH (7.2) and lower temperatures (4–25°C) best preserved ampicillin's activity, while alkaline conditions (pH 8.5) and high temperatures (≥50°C) caused significant degradation. These findings highlight the importance of proper storage and handling to maintain ampicillin's clinical effectiveness.

Keywords

Ampicillin; Antibiotic Stability; Zone of Inhibition; Temperature Degradation; pH Denaturation.

1. INTRODUCTION

Ampicillin works by inhibiting bacterial cell wall synthesis, a vital process for bacterial survival and replication. It belongs to the β-lactam class of antibiotics and contains a β-lactam ring that mimics peptidoglycan precursors, irreversibly bind to and inhibit penicillin-binding proteins (PBPs), which are enzymes involved in the final stages of peptidoglycan synthesis—the major structural component of bacterial cell walls [1]. By binding to PBPs, ampicillin blocks the cross-linking of peptidoglycan chains, weakening the cell wall structure. This disruption is especially lethal during cell division, as bacteria are unable to maintain cell wall integrity, leading to osmotic imbalance and eventual cell lysis [1].

However, ampicillin is chemically unstable under extreme temperature and pH conditions, leading to degradation that reduces its effectiveness [2, 3]. The core structure responsible for its activity is the β-lactam ring. When this ring is broken, the molecule becomes inactive. Both highly acidic and highly basic environments can catalyze the hydrolysis of this ring, rendering the drug ineffective [2]. Under acidic conditions, ampicillin undergoes acid-catalyzed hydrolysis. This reaction involves the protonation of the carbonyl group in the β-lactam ring, making it more susceptible to nucleophilic attack by water. As a result, the ring opens and forms penicilloic acid derivatives, which are biologically inactive [4]. In basic conditions, ampicillin is also unstable. Alkaline hydrolysis occurs when hydroxide ions attack the β-lactam ring, again leading to ring opening and deactivation of the antibiotic [3]. The rate of degradation is generally faster at extreme pH values and is minimized when the solution is near neutral pH [2]. Temperature also plays a significant role in the stability of ampicillin. Elevated temperatures lead to thermal agitation, accelerate the hydrolysis reactions, and can also promote oxidation of the molecule. Prolonged exposure to heat can lead to both chemical degradation and racemization, the latter of which may convert the biologically active stereoisomer into an inactive one [5].

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To better understand the effectiveness of ampicillin when processed under different pH and temperature, acidic acetic acid-sodium acetate buffer (pH=5.5), PBS buffer (pH=7.2), and Tris-HCl buffer (pH=8.5) were each added to an ampicillin solution aliquote. Each ampicillin aliquote was then divided into four equal proportions and processed at respective temperature (4°C, 25°C, 50°C, and 80°C) for twenty minutes, and then used to prepare antibiotic susceptibility test disks for the zone of inhibition test.

The zone of inhibition test, also known as Kirby-Bauer test or the disk diffusion method, is a widely used technique to evaluate the effectiveness of antibiotics against specific bacteria [6]. In this test, small paper disks impregnated with a known concentration of an antibiotic are placed onto the surface of an agar plate that has been uniformly inoculated with the chosen bacterial strain. During incubation, the antibiotic diffuses from the disk into the agar, inhibiting bacterial growth in a circular area around the disk. This clear area, called the zone of inhibition, indicates the antibiotic's ability to prevent bacterial proliferation. By measuring the diameter of this zone, researchers can assess the susceptibility of the bacteria to the antibiotic, with larger zones generally reflecting greater antimicrobial activity [6].

In this experiment, we choose Bacillus subtilis because it is a Gram-positive model organism that grows rapidly on agar media [7]. B. subtilis is also classified as GRAS (Generally Recognized As Safe), non-pathogenic, and poses negligible risk to operators or the environment, making it particularly suitable for laboratory operations [7]. Additionally, its genetic stability ensures that the restriction zone test reflects true antimicrobial activity, not random genetic changes in the bacteria to ensure the reliability, reproducibility, and scientific validity of experimental results [8].

2. METHODS

The experiment started with 60 μL of 50 mg/mL ampicillin stored at -20°C. Using a pipette, 240 μL of distilled water was added to and thoroughly mixed with the freshly-thawn ampicillin, yielding a total volume of 300 μL diluted 10 mg/mL ampicillin. This diluted preparation was then divided equally into three microcentrifuge tubes (100 μL per tube), each added to 900 μL of one pH buffer (acidic acetic acid-sodium acetate buffer (pH=5.5), PBS buffer (pH=7.2), and Tris-HCl buffer (pH=8.5) respectively). The final ampicillin solution concentration is 1 mg/mL. For each pH condition (5.5, 7.2, and 8.5), the 1000 μL of ampicillin was divided into four 250 μL aliquots (total aliquot counts = 12). The samples were then incubated for 20 minutes under four temperature regimes: 4°C (refrigeration), 25°C (room temperature), 50°C (moderate heating by water bath), and 80°C (high-temperature treatment by water bath). 250 μL of unprocessed 1 mg/mL ampicillin was prepared as a positive control, and 250 μL distilled water was prepared as a negative control. For these14 prepared ampicillin samples, 20 μL of the sample were transferred to each sterile filter paper disc (8 mm in diameter) as antibiotic sensitivity discs (the final amount of each antibiotic was 20 μg). Triplicate discs were designated for each pH-temperature combination, yielding 42 test discs in total (3 pH levels×4 temperatures×3 replicates + 2 controls× 3 replicates). Allow the discs to dry at room temperature in the fume hood.

The B. subtilis slant culture was obtained from the Shanghai Bioresource Collection Center. Using a sterile pipette tip, a small visible portion of the bacterial colony was transferred from the slant into a 10 mL test tube containing 5 mL of LB liquid medium. The culture was incubated at 37°C in a shaking incubator for 16 hours.

After incubation, the optical density (OD) of the bacterial suspension was measured at 600 nm using a spectrophotometer, with LB medium as the blank. The culture was diluted with LB medium until the OD600 reached 0.1. Sterilized work surfaces, hands, and required tools with 75% alcohol to avoid contamination by bacteria and put in the fumed hood. In the fumed hood,

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a volume of 100 µL of this OD 0.1 bacterial suspension was then spread evenly across the surface of a nutrient agar plate using a disposable sterile spreader. Choose two out of three test discs from each group and use a tweezer to place the disc on the agar plate. Each labeled agar plate contains four equally distanced test discs. The plates were then inverted and incubated at 37°C for 16 hours.

After incubation, the diameters of the inhibition zones were measured using a vernier caliper. Three measurements were taken per inhibition zone, and the average readings of the two replicates and the average readings of each condition were calculated respectively. The relative antibacterial activity could be derived from calculating measured inhibition zone diameter over positive control inhibition zone diameter.

3. RESULTS

The antibacterial efficacy of ampicillin, measured by inhibition zone diameter and relative antibacterial activity (%), was significantly influenced by pH and temperature as suggested in Figure 1 and Figure 2. Under neutral conditions (pH 7.2), ampicillin exhibited the highest activity, retaining 96.1% relative antibacterial activity at 4°C, which gradually declined to 76.6% at 80°C. This demonstrates that neutral pH provides the most stable environment for ampicillin, even at elevated temperatures. In contrast, acidic conditions (pH 5.5) resulted in moderate activity, starting at 78.4% at 4°C and decreasing to 71.7% at 80°C, indicating progressive thermal degradation. The most drastic decline was observed under alkaline conditions (pH 8.5), where ampicillin lost all activity (0%) at temperatures ≥50°C. Even at milder temperatures (25°C), its efficacy was severely reduced (38.3% activity), highlighting its instability in alkaline environments.

pH	Temperature (°C)	Inhibition Zone Reading (mm)			Standard Deviation	Coefficient of Variation (%)	Average Reading for each replicate (mm)	Average Reading for each condition (mm)	Relative Antibacterial Activity (%)
		1	2	3					
pH=5.5	4	20.58	21.40	22.54	0.98	4.58	21.51	20.60	78.40
		20.78	18.54	19.76	1.12	5.70	19.69		
	25	20.54	20.46	20.98	0.28	1.36	20.66	20.37	77.50
		19.86	20.44	19.92	0.32	1.59	20.07		
	50	20.66	19.98	19.84	0.44	2.18	20.16	19.70	75.00
		19.52	19.22	18.98	0.27	1.41	19.24		
	80	19.68	18.58	19.78	0.67	3.44	19.35	18.85	71.70
		19.36	17.62	18.08	0.90	4.91	18.35		
pH=7.2	4	27.92	26.90	27.26	0.52	1.89	27.36	25.26	96.10
		24.40	23.20	21.90	1.25	5.40	23.17		
	25	24.92	24.82	25.32	0.26	1.06	25.02	24.89	94.70
		25.03	24.34	24.92	0.37	1.50	24.76		
	50	23.26	23.44	24.12	0.45	1.92	23.61	23.22	88.30
		22.42	23.58	22.48	0.65	2.86	22.83		
	80	21.88	20.58	19.72	1.09	5.25	20.73	20.12	76.60
		19.66	18.74	20.12	0.70	3.60	19.51		
p=8.5	4	18.88	17.74	18.88	0.66	3.56	18.50	19.63	75.00
		19.72	19.22	19.94	0.37	1.88	19.63		
	25	9.42	9.92	9.52	0.26	2.75	9.62	10.07	38.30
		10.24	10.58	10.76	0.26	2.51	10.53		
	50	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
		0.00	0.00	0.00	0.00	0.00	0.00		
	80	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
		0.00	0.00	0.00	0.00	0.00	0.00		
+ve control		25.62	26.30	26.26	0.38	1.46	26.06	26.28	1.00
		27.02	26.32	26.18	0.45	1.70	26.51		
-ve control		0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
		0.00	0.00	0.00	0.00	0.00	0.00		

Figure 1. Effect of pH and temperature on the antibacterial activity of ampicillin, as measured by inhibition zone diameters.

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Ampicillin solutions were incubated at specified pH values (5.5, 7.2, 8.5) and temperatures (4 °C, 25 °C, 50 °C, 80 °C) for 20 minutes before being applied to agar plates inoculated with bacteria. Inhibition zones were measured in millimeters after 16 hours incubation, with two replicates per condition. The first average inhibition zone diameter reading was calculated from three independent readings of each replicates, and the. Standard deviation (SD) reflects the variability among the three replicates. Coefficient of variation (CV) was calculated as: CV (%) = SD/Mean*100. Relative antibacterial activity (%) was normalized to the average inhibition zone diameter of the positive control (unprocessed ampicillin, set as 100%).

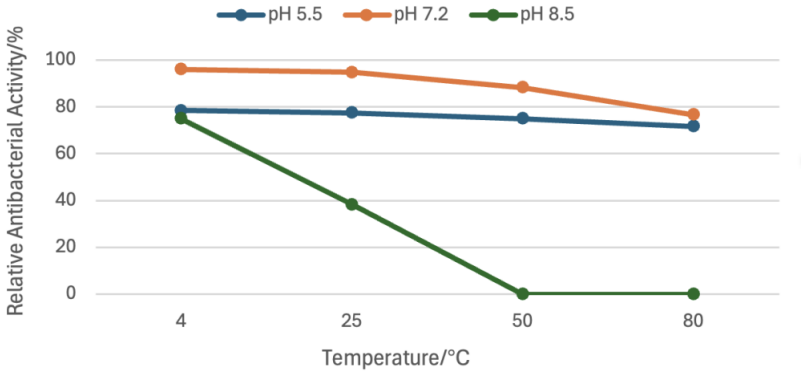

Figure 2. Relative antibacterial activity of ampicillin under different pH and temperature conditions

Relative antibacterial activity (%) was calculated by normalizing the average inhibition zone diameter of each condition to that of the positive control (ampicillin at pH 7.2, 4 °C), which was set at 100%. The graph shows that ampicillin retained the highest activity at neutral pH across all temperatures, while significant loss was observed under alkaline conditions, especially at elevated temperatures. It also showed a general trend of decreasing activity at higher temperature across all pH.

Temperature played a critical role in ampicillin's performance across all pH levels. For all pH values, increasing temperature correlated with reduced activity, which was more steep at higher temperature. Additionally, the decline was most pronounced under alkaline conditions, followed by under neutral conditions, and least pronounced under acidic conditions. Notably, at pH 7.2, ampicillin maintained >90% activity up to 25°C, whereas at pH 5.5, activity dropped below 80% even at 4°C. The most extreme sensitivity was observed at pH 8.5, where temperatures ≥50°C led to complete inactivation, suggesting that alkaline conditions exacerbate thermal degradation. The positive control (untreated ampicillin) confirmed expected efficacy (100% activity, 26.06–26.51 mm inhibition zones), while the negative control (no antibiotic) showed no activity, validating the experimental setup.

The standard deviations (SD) of inhibition zone diameters across replicates were generally low, ranging from 0.26 mm to 1.25 mm. The coefficient of variation (CV), which expresses variability relative to the mean, mostly remained below 6%, with the lowest at 1.06% and highest at 5.70%, indicating high consistency between measurements. These low SD and CV values suggest that the experimental data are reliable and the variation between replicates is minimal.

Overall, these findings underscore that neutral pH and low temperatures (4–25°C) are optimal for preserving ampicillin's antibacterial efficacy, while alkaline conditions and high temperatures (≥50°C) should be strictly avoided in storage and clinical applications to prevent degradation.

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4. DISCUSSION

The markedly greater loss of ampicillin activity at pH 8.5 compared to pH 5.5 can be attributed to the pH-dependent chemical stability of the antibiotic. Ampicillin, like other β-lactam antibiotics, relies on an intact β-lactam ring for its antibacterial function. Under alkaline conditions, this ring is particularly susceptible to hydrolysis due to nucleophilic attack by hydroxide ions, leading to irreversible inactivation of the molecule. Several studies have reported that β-lactam antibiotics degrade significantly faster at elevated pH values [2, 3]. In contrast, while mildly acidic environments such as pH 5.5 may slightly reduce the drug's efficacy—possibly by altering membrane permeability or target binding—the β-lactam ring remains relatively stable, preserving much of its antibacterial activity, explaining its relative acid-resistence and thus it can be administered orally [1]. Therefore, the observed results are consistent with the known chemical behavior of ampicillin, where alkaline-induced structural degradation plays a more dominant role in reducing activity than the moderate physiological effects seen under acidic conditions.

The observed instability of ampicillin under alkaline conditions also carries important implications for its medical application. Since ampicillin's β-lactam ring is more susceptible to hydrolysis in basic environments, its efficacy may be compromised in infections that occur in naturally or pathologically alkaline sites. For instance, urinary tract infections in patients with alkaline urine—such as those caused by Proteus species or those taking urinary alkalinizing agents—may see reduced antibiotic effectiveness [9, 10]. Similarly, chronic wounds or abscesses with elevated local pH due to bacterial ammonia production may create conditions unfavorable for ampicillin activity [11]. Additionally, oral administration alongside antacids or proton pump inhibitors, which increase intestinal pH, could accelerate degradation before systemic absorption. Improper dilution in alkaline intravenous solutions or unbuffered topical use may further exacerbate this loss of potency. These findings underscore the importance of considering local pH conditions and patient medication profiles when prescribing ampicillin, and may warrant the selection of more pH-stable alternatives in certain clinical contexts.

The steeper decline in ampicillin activity observed between 50 °C and 80 °C, as compared to the more gradual decreases from 4 °C to 25 °C and 25 °C to 50 °C, can be attributed to the temperature-dependent kinetics of chemical degradation. Ampicillin undergoes thermal degradation primarily through hydrolysis of the β-lactam ring, a reaction that is significantly accelerated at higher temperatures. While mild increases in temperature may only modestly affect molecular stability, the transition from 50 °C to 80 °C likely crosses a thermal threshold at which degradation becomes exponentially more rapid, consistent with the Arrhenius equation [2]. This sharp loss of activity suggests that the β-lactam ring becomes highly unstable in this temperature range, leading to rapid inactivation of the antibiotic. Additionally, at such elevated temperatures, the risk of denaturation or breakdown of other functional groups essential for antimicrobial action also increases. These results emphasize the importance of maintaining cold-chain storage for ampicillin and avoiding exposure to high heat during sterilization, preparation, or transportation.

5. CONCLUSION

This experiment investigated the changes in the antibacterial activity of ampicillin when exposed to different temperatures (4 °C, 25 °C, 50 °C, and 80 °C) and pH levels (5.5, 7.2, and 8.5) using inhibition zone assays. The results indicate a significant decrease in antibacterial activity with increasing temperature and extreme pH conditions, providing valuable insights into the optimal storage and usage of ampicillin. Future studies could explore smaller increments of temperature and pH, as well as varying treatment durations with different antibiotics, to further refine our understanding of antibiotic stability and efficacy.

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