Review Article

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Effectiveness of Microbiological Assays as an Alternative Method to Determine the Potency of Antibiotics: A Review

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ABSTRACT

In this review, chemical and biological assays performed in the pharmaceutical industry to determine the potency and bioactivity of antibiotics are discussed. Though commonly employed chemical methods can measure the potency of antibiotics, inefficiency in estimating the bioactivity is one of their major limitations. Due to their sensitivity and cost-effectiveness, common microbiological assays can serve as alternative methods. Several factors like doses of antibiotics, homogeneity of agar medium, inoculum concentration, the chemical composition of agar media, size and solubility of samples or drug molecules, pH, relative humidity and exposure time can influence microbiological assays. Based on specific requirements and experimental targets, agar diffusion assays are designed focusing on their costs, errors, accuracy and simplicity. To avoid the misuse and overuse of antibiotics that lead to drug-resistance, parameters like zone of inhibition, minimum inhibitory concentration, minimum bactericidal concentration, mutation prevention concentration and critical concentration are also discussed in this study. Finally, microbiological and high-performance liquid chromatography methods were specifically compared for their sensitivity, accuracy and assessment of biological activity with minimal cost. Due to their advantages and disadvantages, parallel use of both bioassays and chemical methods are suggested to precisely determine the potency of antibiotics.

Keywords: Bioassay, antibiotics, alternative method, cost-effective, microbiological assay

1. Introduction

With a particular focus on pathogenic bacteria, antibiotics are powerful chemotherapeutic weapons capable of impeding the growth of microbes or completely destroying them altogether. They are used at low concentrations to minimize the undesirable effects on host cells. Antibiotics are synthesized naturally by isolating them from microorganisms or through chemical synthesis. Antibiotics have become widespread applicable drugs for bacterial infections.

Low concentrations of antibiotics are potent enough to inhibit or destroy bacteria. Irrational utilization of antibiotics can lead to the development of antibiotic resistance. The emergence and swift dissemination of multidrug-resistant and pandrug-resistant bacteria, commonly referred to as superbugs, makes it difficult to accurately quantify the actual concentration of active ingredients in antibiotic preparations. It has been reported that 60% of marketed anti-infectious drugs fail to meet the standards set by pharmacopeias in Asia and Africa [1].

Antibiotics are particularly vulnerable to counterfeiting and adulteration within pharmaceutical industry as even minor variations in the concentration of active ingredients can significantly affect their efficiency and bioactivity. The effectiveness of antibiotics depends on various factors, encompassing the patient's physiological system, routes of administration, sites of action, concentration of drugs, target pathogens and the properties of antibiotics themselves [2]. In pharmacology, ensuring the efficiency and actual potency of antibiotics is crucial for their safe and appropriate therapeutic application, although it presents significant challenges for drug regulatory agencies around the world [3,4].

Numerous chemical and biological methods are available for quantifying and determining the potency of antibiotics. Commonly employed techniques in the pharmaceutical industry include high performance liquid chromatography (HPLC), fluorescence polarization immunoassay, radioimmunoassay, fluorescence immunoassay and bioassay [5]. Chromatographic techniques and UV spectrophotometric methods are employed for determining the content of active ingredients, measuring purity and identifying impurities in antibiotics. While these chromatographic methods accurately measure potency, they do not provide a comprehensive indication of bioactivity, representing a limitation of chemical methods.

The microbiological assay accurately determines both the potency and bioactivity of antibiotics. They do not require heavy equipment or toxic chemicals [6-10]. Impurities and other subtle products do not interfere with the true concentration of antibiotics [11,12]. Microbiological assays are not dependent on specific strains of microorganisms against specific antibiotics [13]. These assays have become the gold standard method when addressing antibiotic resistance, providing estimations of the loss of active ingredients (APIs) in antibiotics. Both culture and non-culture techniques are employed to grow pure microbial strains, making microbial assays a cost-effective, easy-to-use, accurate, linear, precise and robust method for determining the potency and bioactivity of active pharmaceutical ingredients (APIs) in antibiotics. Additionally, microbiological assays are also used in the quality control and quality assurance departments of low- and middle-income pharmaceutical industries in developing countries. Compared to chemical methods, microbiological methods offer greater accuracy in measuring the true concentration of antibiotics to overcome antibiotic resistance.

2. Materials and Methods

Data for this review were extracted from several clinical, pharmaceutical, microbiological and laboratory information published in various journals. Scanning of the online literature was performed between the years 1984 and 2022 through several databases including Nature, Web of Science, SpringerLink, ScienceDirect, PubMed, Google Scholar etc. using the keywords but not limited to 'Bioassay', 'antibiotics', 'alternative method', 'cost-effective' and 'microbiological assay' (Table 1).

3. Results and Discussion

3.1. Microbiological assays

Microbiological assays find extensive applications in various areas, such as antibiotic sterility testing, evaluation of the mutagenicity and carcinogenicity of chemicals, determining the susceptibility and resistance of different microbes to antibiotics, and diagnosis of diseases using clinical specimens. When chemical methods become unable to determine the potency of pharmaceutical products, microbiologi-

Table 1. Microbiological assays and HPLC methods performed to determine the potency of antibiotics

Microbiological Methods	Antibiotics	Formulation	Strain of Microbes used for Bioassay	Condition	Checked by HPLC	Reference
Agar well method	Vancomycin	Blood serum	Bacillus globigii	35ºC for 18 h	Yes	[5]
Bio diffusion assay (cylinder 3 × 3)	Azithromycin	Ophthalmic suspension	Bacillus subtilis ATCC 9372	35ºC ± 2ºC for 18 h.	No	[28]
Cylinder-plate method (3 × 3 parallel line assay design)	Besifloxacin (BSF)	Ophthalmic suspension	Staphylococcus Aureus ATCC 6458 P	35ºC ± 1ºC for 18 h	Yes	[29]
Turbidimetric assay(parallel lines 3 × 3 design)	Ciprofloxacin hydrochloride	Ophthalmic solution	Staphylococcus epidermidis ATCC 12228	35ºC for 4 h	Yes	[36]
Agar diffusion (5+1) bioassay	Cephalexin	Tablet	Escherichia coli MTCC443	-	Yes	[57]
Cylinder-plate method (3 × 3 agar diffusion)	Ceftriaxone sodium	–	Staphylococcus aureus ATCC 6538P	35 ± 2ºC aerobically for 16 h	Yes	[60]
Turbidimetric method (3×3 parallel line assay design)	Cefuroxime sodium	Powder for Injection	Micrococcus luteus ATCC 9341	35 °C ± 2°C, for 21 h	Yes	[59]
Agar diffusion method (cylinder-plate method)	Fluconazole	Injection	Saccharomyces cerevisiae ATCC 1600	35°C for 24 h	Yes	[31]
Agar well method	Gentamicin sulphate	Raw materials	Staphylococcus epidermidis and Bacillus pumilus	37˚C for 24 h	Yes	[62]
Cylinder plate assay	Gatifloxacin	Tablet and raw material	Bacillus subtilis ATCC 9372	37˚C for 18 h	Yes	[35]
One-level agar diffusion (5+1) bioassay	Levofloxacin	Tablet	Bacillus pumilus ATCC- 14884	37˚C for 24 h	Yes	[32]
Cylinder-plate agar diffusion assay	Moxifloxacin	Tablet, ophthalmic solution and human plasma	Esherichia coli ATCC 25922	37˚C for 18 h	Yes	[61]
Turbidimetric assay method (3×3 parallel)	Norfloxacin	Tablet	Staphylococcus epidermidis ATCC 12228 IAL 2150	35˚C for 4 h	Yes	[34]

cal assays can serve as alternative methods. Broadly, two main techniques are commonly employed in microbiological assays: one is the disc diffusion method which includes placing filter paper discs with antibiotics onto solidified agar plates to measure the resulting zones of inhibition around the discs to determine the bioactivity of antibiotics. And the other, entails diluting antibiotics in agar or broth. The British Pharmacopoeia (BP) and United States Pharmacopoeia (USP) provide various microbiological assay methods specially designed to estimate the potency and bioactivity of antibiotics.

Kirby-Bauer disc diffusion method is commonly used to determine the sensitivity and resistance of pathogenic and facultative aerobic microorganisms. In this method, Mueller-Hinton agar is commonly employed as the nutrient medium. Antibiotic filter paper discs with a diameter of 6 mm are placed on the surface of the agar plate at specific distances. The discs are impregnated with antibiotics and act as reservoirs of the antibiotic. The zone of inhibition around the filter paper disc is measured to determine the bioactivity of antibiotics against the tested microbes. A study by Rajia et al., revealed that the agar disk diffusion method proved to be a simple,

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sensitive and cost-effective approach for evaluating chloramphenicol 0.5% eye drops against *Staphylococcus aureus* ATCC 29213 while the conventional spectrophotometric method yielded false-positive results [14]. This finding highlights the advantages of the agar disk diffusion method over the spectrophotometric method in assessing the potency of chloramphenicol eye drops. It should be noted that this method provides information about the microbicidal activity of antibiotics but does not measure the minimum inhibitory concentration of those drugs [15].

To accommodate larger sample volumes (20–100 mL) on agar plates, the agar well method is considered appropriate. This method is used to evaluate the antimicrobial activity of plant and microbial extracts. In this technique, the entire surface of a petri dish is covered with a standardized concentration of microbial inoculums. Multiple holes, typically with a diameter of 6-8 mm, are created using a sterile cork borer or a tip. Specific quantities (ranging from 20, 100 mL) of antimicrobials or experimental samples are then introduced into these wells or holes [16,17].

In a study conducted by Vaikosen et al, the agar well method was introduced to evaluate and validate the antimicrobial activity of amikacin sulfate injection [18]. In this study, agar plates were inoculated with *Staphylococcus aureus,* and a well was designated for the standard amikacin sulfate (100 μL), while other wells contained amikacin sulfate injection with concentrations of 2, 4, 8 and 16 ug/mL. The bioassay conducted in this study demonstrated linearity within the concentration range from 1 to 16 μg/mL (bioassay, r = 0.9994) and the results were compared with a spectrophotometric assay, which showed linearity within the concentration range of 10 to 50 μg/mL (spectrophotometric, r = 0.9998) [18]. This study highlights the application of the agar well method in evaluating the antimicrobial activity of amikacin sulfate injection and demonstrates its comparability to the spectrophotometric assay within their respective concentration ranges.

The antibacterial activity of copper phenyl fatty hydroxamate, derived from canola and palm kernel oils, was evaluated using the agar well diffusion method. This method was used to assess its effectiveness against *Escherichia coli (E. coli)* and *Staphylococcus aureus (S. aureus*) [19].

In addition, the agar well method was utilized to quantify the antibacterial activity of four commonly used antibiotics: ampicillin, amoxicillin, tetracycline and erythromycin that were conducted against nine microbial stains. The objective of the study was to evaluate the antibiotics according to specifications outlined in standard literature, in the absence of those recommended from reference books [13].

The dilution method is a semiautomatic system used to determine both the quantitative (minimum inhibitory concentration MIC) and qualitative (category interpretation) properties of antimicrobials. This method can be performed in two ways. The first one is the micro dilution technique where a standard test tube is used with a broth volume of 1 ml. The second is the microdilution technique, where a microtiter plate is utilized with a broth volume ranging from 0.05 to 0.1 ml [20].

The agar dilution method is employed to determine the minimum inhibitory concentration (MIC) of a particular antimicrobial agent. This method involves placing a standardized concentration of microbes onto a molten agar medium in a petri dish. The absence of growth of the inoculum indicates the susceptibility of the microorganisms to the antimicrobial agent.

The antimicrobial gradient method, also known as the E test, is a combined approach that incorporates elements of dilution and diffusion tests for determining the MIC of antibiotics, antifungals and anti-mycobacterial agents. This method relies on the concentration gradients of the antimicrobials impregnated into a strip [21]. MIC value is determined at the point where the strip intersects with the growth inhibition ellipse. The E test is renowned for its enhanced accuracy and precision compared to other methods [22].

Before initiating treatment with metronidazole, clarithromycin, and levofloxacin against *H. pylori*, it is crucial to assess the susceptibility of *H. pylori* to these antibiotics. The agar dilution method and E test are widely employed methods for determining the susceptibility of *H. pylori* and are still commonly being used [23]

The agar diffusion method is an alternative approach used to evaluate the potency and bioactivity of antimicrobials. There are two types of agar diffusion methods: the cylindrical-plate or cup-plate method. In the cylindrical-plate method, diluted antibiotics are diffused from vertical cylinders or cups onto solidified agar that already contains microbial colonies. This method enables the correlation of the size of

the zone of inhibition with the dosage of antibiotics tested [24-26].

The cylindrical–plate diffusion method has demonstrated accurate results when evaluating the efficiency of azithromycin and besifloxacin ophthalmic solutions. Test organisms used in this study were *Bacillus subtilis* ATCC 9372 and *Staphylococcus epidermidis* ATCC 12228. For azithromycin, concentrations ranging from 50 to 200 µg/ml were tested, and it was possible to measure a concentration of 1.6667 mg/ml of the ophthalmic solution [27,28]. Besifloxacin on the other hand, was tested at concentrations of 0.5, 1.0 and 2.0 mg/ml [29].

3.2. Different designs for the agar diffusion assay

According to various pharmacopeias, agar diffusion assays are structured in various formats. The 2×2 and 3×3 designs are commonly used and referred to in British, European, and Brazilian pharmacopeias (Figure 1).

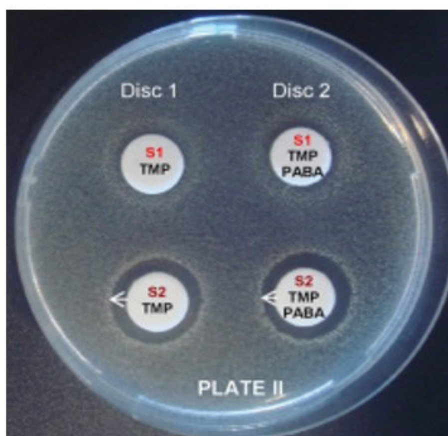
In the 2×2 assay design, a single petri dish is utilized, containing two doses of the standard and two doses of samples. The four doses of each preparation are arranged alternately within the petri dish and the number of replicates equals the number of dishes. This design is both simple and effective in its implementation [30].

The 3×3 assay design involves applying three different dose levels to both the standard and samples [31]. This design is suitable for research and development purposes. Additionally, the European pharmacopeia mentions a 3×1 assay design, which is popular and randomly used for routine analysis and potency determination in the quality control department of pharmaceutical companies. This design is simple, cost-effective and provides higher linearity, precision and accuracy. It is also suggested to be a time efficient method. In the 3×1 experimental assay, three doses of the standard and one dose of sample are impregnated, on average on each petri dish. Six dishes are used for each sample in the 3×1 experimental design.

The 5×1 experimental design is another well-known approach for bioassays. In this design, five standards and one sample with a median concentration are inoculated in a petri dish (Figure 2). Each experiment is repeated six times for each sample, allowing a robust analysis. This method is commonly employed in routine analysis within quality control laboratories. One of its advantages is the ability to evaluate a higher number of samples simultaneously, making it a time-efficient and practical choice for quality control purposes.

The 3×3 diffusion method was specially designed in accordance with the Brazilian and European pharma-

A.



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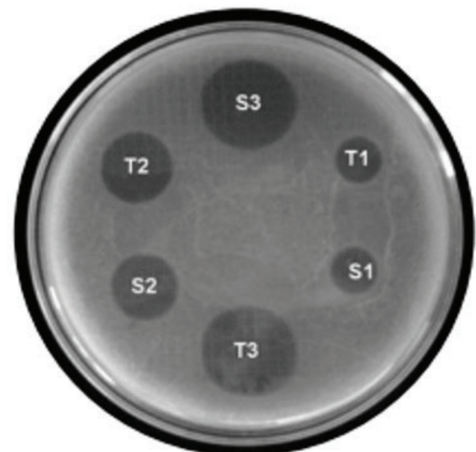

Figure 1. (A) In a 2×2 plate assay, disc 1 is loaded with 50 µg/ml of sample extract and 10 µg/ml of Trimethoprim (TMP), and disc 2 is loaded with 50 µg/ml of sample extract, 10 µg/ml of TMP and 10 µg/ml of 4-aminobenzoic acid (PABA) solution 100 µg/ml against *Bacillus subtilis*. Image Courtesy: Dang et al., 2010 [30] (B) In a 3×3 plate assay, for fluconazole standard at 25 (S1), 100 (S2) and 400 (S3) µg/ml and fluconazole injection at 25 (T1), 100 (T2) and 400 (T3) against a strain of *Saccharomyces cerevisiae* (ATCC 1600). Image Courtesy: Huratado et al., 2008 [31].

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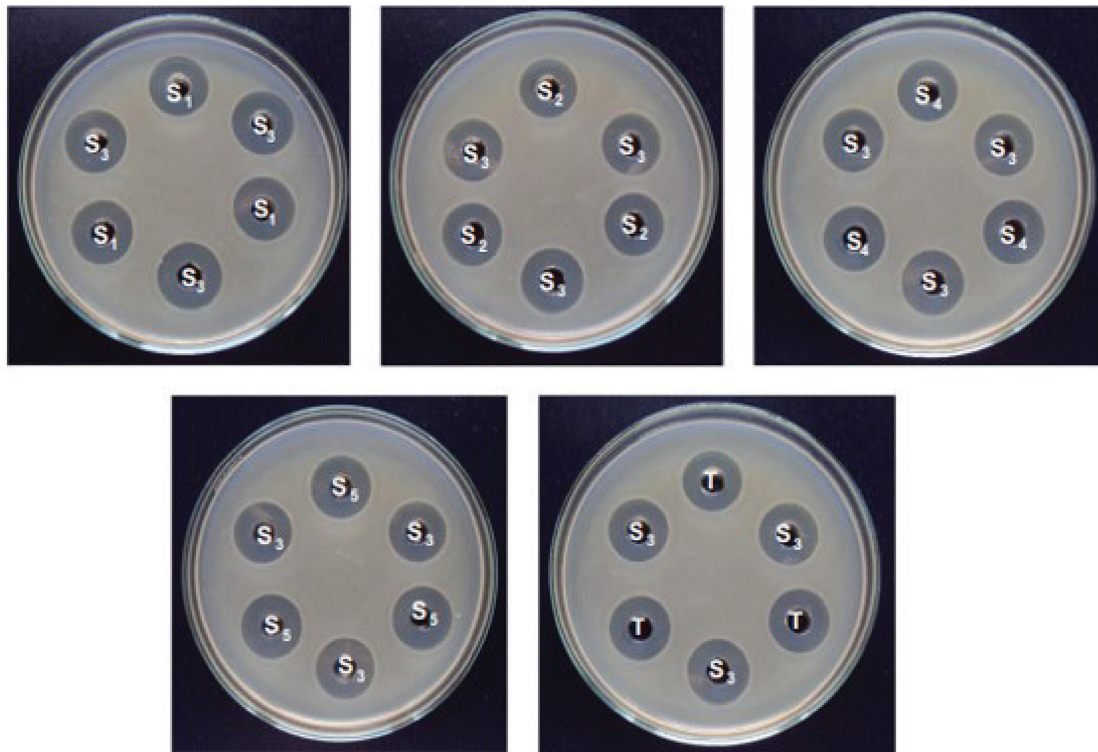

Figure 2. In a 5×1 plate assay, five petri dishes represent the reference solutions S1 (2.56 µg/ml), S2 (3.20 µg/ml), S3 (4 µg/ml), S4 (5 µg/ml) and S5 (6.25 µg/ml) and test sample Levofloxacin solution (4.0 µg/ml) against *Bacillus pumilus* (ATCC-14884). Image courtesy: Dafale et al., 2015 [32].

copeias for determining the potency of azithromycin ophthalmic solution [27]. This method compares the results of samples with a standard curve obtained from the same set of experiments. The potency of azithromycin was determined using the Hewitt equations. This approach allows for a standardized and reliable assessment of the potency of azithromycin in ophthalmic solutions.

The 3×3 parallel technique was considered convenient for the quantitative evolution of besifloxacin. This method involves six stainless steel uniformed cylinders (8×6×10 mm) onto the inoculated agar medium. Three cylinders were filled with 200 µL of standard besifloxacin (S1, S2, and S3) while the other three were filled with ophthalmic solutions (T1, T2, and T3) [29]. This method had also been utilized for the evolution of other substances such as ceftazidime injection, ciprofloxacin hydrochloride, gatifloxacin, norfloxacin in tablets, and orbifloxacin in pharmaceutical preparations [33-37].

The stability of azithromycin was also assessed using the cylinder plate 3×3 bioassay method [28]. Six plates were used for each assay, allowing for the evolution of the stability of the azithromycin sample under different conditions or over time.

The 5×1 agar well diffusion method is employed for the quantification of levofloxacin in pharmaceutical products. This method involves five petri dishes representing reference solutions with different concentrations: (S1: 2.56 mg/ mL, S2: 3.20 mg/mL, S3: 4.0 mg/mL, S4: 5.0 mg/mL and S5: 6.25 mg/mL). The sample solution (T) contains levofloxacin at concentration of 4.0 mg/ mL [38]. This method allows the comparison and determination of the levofloxacin content in the pharmaceutical preparation.

A comparative analysis was conducted between biological and ninhydrin-derivatized spectrophotometric assays for amikacin sulfate injection. The precision, linearity, coefficient and accuracy were highly appreciated, indicating its reliability and ability to provide accurate results for the quantification of amikacin sulfate in the injection [18].

These different assay designs are suggested for different purposes, taking factors like cost, errors, and simplicity of the assay into consideration. Research-

ers and analysts can choose the most appropriate design based on their specific requirements and experimental goals.

3.3. Parameters used to define the potency of antibiotics

Misuse and overuse of antibiotics have led to the rise of antibiotic resistance, emphasizing the need for accurate quantification of active pharmaceutical ingredients (APIs) during production. Parameters such as Zone of Inhibition (ZOI), Minimum Inhibitory Concentration (MIC), Minimum Bactericidal Concentration (MBC), Mutation Prevention Concentration (MPC) and Critical Concentration (Ccr) are utilized to express the antibiotic concentration in microbiological bioassays. Determination of potency and bioactivity of antimicrobials often involves the measurement of zone of inhibition (ZOI). The ZOI is assessed by observing clear zones around a disk containing the antimicrobial agent on a transparent scale. The diameter of the zone of inhibition serves as a qualitative indicator of the potency of drugs. Nowadays, computer vision algorithms can be utilized to automatically calculate the zone of inhibition [38]. The potency of antimicrobials can be evaluated by measuring the zone of inhibition on Mueller- Hinton agar plate [14,29].

On the other hand, the Minimum Inhibitory Concentration (MIC) is another important parameter used to assess the effectiveness of antimicrobials. It refers to the lowest concentration of the antimicrobial agent that can inhibit the growth of bacteria after overnight incubation under standardized conditions. MIC values are expressed in micrograms per milliliter (µg/mL) or standard units [39]. Several methods including agar dilution, broth microdilution and agar diffusion can be used to determine the MIC.

MIC values serve as a valuable research tool to accurately measure the activity of pharmaceutical antimicrobials *in vitro*. They are also used to confirm microbial susceptibility, compared resistance and susceptibility, assess increased exposure, and identify resistance to specific antimicrobials using breakpoints [40-46]. It is important to note that MIC values can be influenced by factors such as the type of antibiotics used, the bacterial strain being tested, as well as host factors like serum effects and impacts on gut microbiota in patients [2]. For instance, in a study involving twelve isolates of the Bacteroides fragilis group, the true MIC of cefoxitin was determined to be 16 µg/ml, with a range of 16 to 32 µg/ml and 4 µg/ml increments [47].

Minimum Bactericidal Concentration (MBC) values indicate the lowest concentration of antimicrobials required to inhibit the growth of pathogenic microbes after subculturing them in an antibiotic free medium [48]. MBC values offer a standardized quantitative measure that indicates the concentration at which 99.9% eradication of microbial isolates takes place. MBC determination is typically performed using broth dilution methods and agar plate methods. Unlike MIC values, MBC values are more effective for assessing the potency of antibiotics or antimicrobials but less useful in determining microbial resistance [49].

In the study, MIC and MBC values were determined for a total of nine antimicrobials and three biocides against eight strains of *Listeria monocytogenes.* The MICs (ppm) for the biocides ranged between 1750 and 4500 for sodium hypochlorite (SH), 0.25 and 20.00 for benzalkonium chloride (BC), and 1050 and 1700 for peracetic acid (PAA). The corresponding MBCs (ppm) ranged from 2250 and 4500 for SH, 0.50 and 20.00 for BC, and 1150 and 1800 for PAA. For the antibiotics, the MICs (ppm) showed a range of 1 and 15 for ampicillin, 8 and 150 for cephalothin, 20 and 170 for cefoxitin, 0.05 and 0.20 for erythromycin, 4 and 50 for chloramphenicol, 3 and 100 for gentamicin, 2 and 15 for tetracycline, 2 and 80 for vancomycin, and 160 and 430 for fosfomycin. Similarly, the corresponding MBCs (ppm) were found to range from 5 to 20 for ampicillin, 9 to 160 for cephalothin, 70 to 200 for cefoxitin, 4 to 5 for erythromycin, 9 to 70 for chloramphenicol, 5 to 100 for gentamicin, 3 to 30 for tetracycline, 3 to 90 for vancomycin, and 160 to 450 for Fosfomycin [50].

Mutation Prevention Concentration (MPC) is a concept that refers to the concentration of antibiotics capable of preventing the growth of mutants or inhibiting the growth of bacterial cells with double mutants. Its purpose is to address the emergence of antibiotic-resistant strains by quantifying the potency of a compound in restricting the selection of resistant mutants. MPC measurements can be conducted on numerous clinical isolates, providing valuable insights into the effectiveness of antibiotics [51]. The determination of MPC utilizes agar dilution methods similar to MIC, but requires a higher bacterial cell count exceedingly more than 10^{10} cells [52].

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In another study, MPC and MIC values of ceftiofur, enrofloxacin, florfenicol, tilmicosin and tulathromycin were determined against swine pathogens 67 *A. pleuropneumoniae*, 73 *P. multocida* and 59 *S. suis*. These values serve as a guide for dosing concentrations that can potentially reduce the amplification of bacterial subpopulations with reduced susceptibility *to* antimicrobials. The determination of MPC and MIC values provides valuable information regarding the effectiveness of these antibiotics against the specified pathogens [53]. Critical Concentration (Ccr) is the minimum concentration of an antibiotic that is required to prevent microbial growth and inhibit the concentration from reaching a specific critical point. It serves as a sensitivity parameter expressed under specific conditions [54].

3.4. Factors influencing the variability and errors in microbiological assays

Microbiological assay is used to investigate the effective dose of antibiotics and determine the potency of pharmaceutical products. However, several factors can influence the accuracy of the assay. For example, in disc diffusion methods, the zone diameter may vary due to uneven exposure of plates in the stack, the time interval between pouring and seeding agar media, and the application of discs. To measure the zone of inhibition, a measuring scale or vernier callipers are used. It is crucial to minimize human errors and parallax errors that can affect the accuracy and reliability of the assay.

Other factors, such as the thickness of agar (standard thickness is 4 mm, and thicker layers result in smaller zone diameters), homogeneity of agar medium, inoculum concentration, chemical composition of agar media, size and solubility of samples or drug molecules, pH, relative humidity, and exposure time, have an important impact the microbiological assay [7,8,32]. pH levels can influence the activity of disinfectant molecules and the cell surface of microbes. Relative humidity can affect the gaseous disinfectant, thereby influencing the microbiological assay.

Several factors like water hardness and the presence of organic and inorganic matters can interfere with the disinfection and serialization in Healthcare Facilities [55]. It was also found that ultraviolet light, different pH ranges, germicide light, acidic and basic solvent solution can affect the stability of azithromycin on *Bacillus subtilis*, ATCC 9372 [28]. Therefore, it is important to consider these factors during the experimental period. To ensure accurate results and maintain quality and quantity estimation of drugs, it is essential to follow good hygiene practices, handle samples properly, and maintain a clean environment to prevent microbial contamination.

3.5. Comparison of microbiological assay and HPLC methods

Microbiological assays are valuable for assessing biological activity, particularly when dealing with complex samples. HPLC offers high sensitivity, specificity, and the ability to analyze individual components within a mixture. The choice between the two methods depends on the specific analysis requirements, sample type, and desired information about the target substance.

Both methods are used for potency determination, concentration of antibiotics in body fluids and therapeutic applications of new drugs. Microbiological methods are simple, accurate and economical that makes them suitable for routine potency determination and detection of resistant microbes. When immediate measurement is not required, the microbiological assay method is recommended as the first choice for routine potency determination. However, potency determination of antimicrobials against microorganisms is based on the minimum inhibitory concentration under suitable conditions.

The automated HPLC method is more attractive than classical microbiological assay due to its speed, accuracy and precision and it is used as an alternative method to detect antimicrobials concentrations in body specimens such as blood, urine and serum. But the high price of columns and the requirement for HPLC grade solvents make HPLC operations more expensive and time- consuming. Additionally, HPLC provides wrong information about potency and does not always yield realistic measurements [56]. Validated microbiological assays are promptly used for potency determination of active pharmaceutical ingredients (APIs) in quality control for pharmaceutical and research in medicinal chemistry. Microbiological assays can detect even slight changes in the structure of antibiotics providing more comprehensive information about potency and susceptibility patterns of pathogenic microbes to specific antimicrobials (Figure 3) [4].

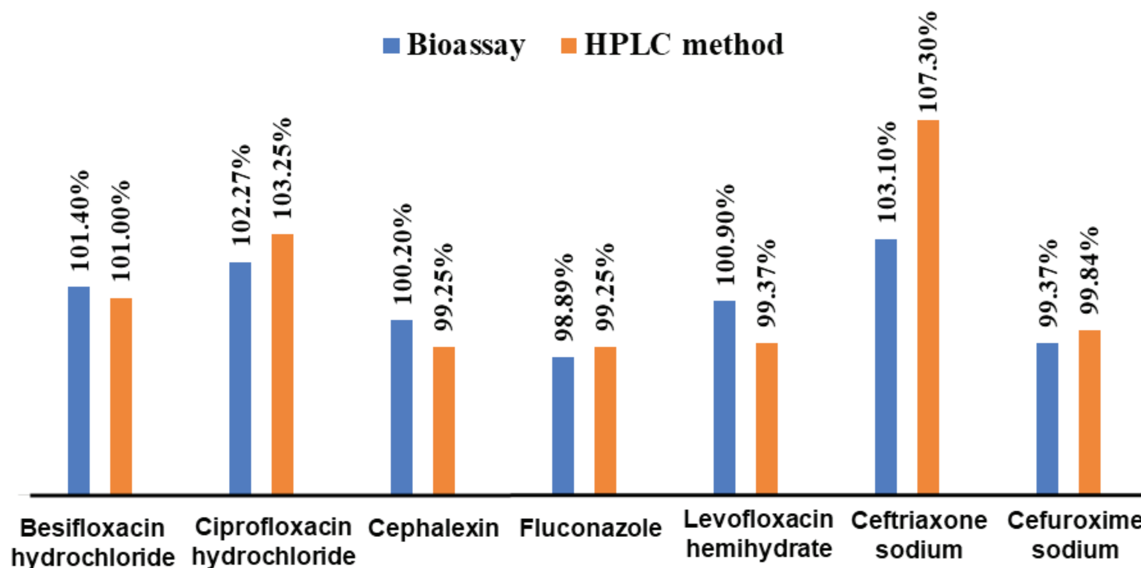

Figure 3. Comparison of potency between the bioassay and HPLC method for different antibiotics. This figure is recreated based on the data of published articles [8, 29, 33, 35, 57-59].

Several studies provided evidence by comparing the performance of both microbiological assays and HPLC methods. For example, Bisofloxacin ophthalmic suspension showed a high degree of linearity in both HPLC methods (0.9998) and microbiological assays (0.9974), indicating that both methods were able to accurately determine the potency of the suspension. A comparison between bioassay and HPLC methods: a statistical analysis using a student t-test revealed no significant differences between the two methods [29].

A study on ceftazidime sodium injection and chloramphenicol eye drops demonstrated that after degradation with sunlight, the degraded products showed false results when using UV- spectrophotometric methods [14,33]. This highlighted the importance of considering the limitations and potential sources of errors in any specific analytical techniques. It was found that ciprofloxacin HCl ophthalmic solution exhibited altered microbiological results compared to chemical methods like HPLC and UV-Spectrophotometry [36]. This suggests that certain compounds or formulations may have different responses or behaviours when assessed using different analytical methods.

Comparing the potency of amoxicillin capsules, injections and granular formulations by microbiological assay and HPLC method, it was observed that the correlation coefficients between the two methods for amoxicillin capsules, injections and granular doses were 0.996, 0.997 and 0.998, indicating a strong agreement with the result.

It is important to consider the nature of compound, its formulation, degradation factors, and the purpose of the analysis to select the most appropriate method for assessing potency and concentration of pharmaceutical formulations.

4. Conclusion

To assess the actual concentration of antibiotics in body fluid specimens and determination the potency of pharmaceuticals ingredients, HPLC is a widely used analytical technique that offers advantages in terms of accuracy, sensitivity, robustness and precision, making it a preferred method for analyzing a wide range of substances. However, it is important to note that HPLC may not always be the most suitable method for analyzing antibiotics due to factors such as the chemical properties of the antibiotic, its degradation products, or the complexity of the sample matrix. Microbiological assay is indeed a preferred choice for detecting the potency and bioactivity of antimicrobials. These assays involve testing the activity of the antimicrobial agents against specific microorganisms, providing valuable information about microbial resistance, potency of active pharmaceuti-

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cal ingredients (APIs) and therapeutic doses of antibiotics. Moreover, bioassays are simple, sensitive, accurate, precise methods and can serve as alternative methods to HPLC. But it is noted that to obtain a more realistic and precise measurement of antibiotic potency, it is advisable to utilize both bioassays and chemical methods like HPLC in parallel.

Conflict of Interest

The author/editor has no conflicts of interest, financial or otherwise, to declare.

Statement of Contribution of Researchers

Concept – S.R.; Design – S.R., Y.F., S.M.A.K.; Supervision – Y.O., S.R.; Resources – S.R., I.H.; Data Collection and/or Processing – S.R., S.J., I.H.; Analysis and/or Interpretation – S.J., I.H.; Literature Search – S.R., S.J.; Writing – S.R., I.H.; Critical Reviews – Y.F., S.M.A.K., Y.O., I.H.)

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