Review Article

COMPARISON AND EVALUATION OF PHARMACOPOEIAL METHODS FOR THE ASSESSMENT OF POTENCY OF ANTIBIOTICS

ABSTRACT

The detection and assessment of potency of antibiotics is crucial for the pharmaceutics. The valid methodsfor microbiological assays in pharmacopoeias are mainly based on statistical comparison of the data obtained by measuring the cidal activity resulting from the treatment of the antibiotic active ingredient in the composition of the pharmaceutics with the target microorganism.In either turbidimetric or chromatographic methods, the statistical evaluation of the sample is compared with the standard reference material. While the data obtained in chromographic methods are linear peak areas; spectrophotometer readings etc., inhibition zone diameters or turbidimetric turbidity dataperformed for the potency of antibiotics. However it was seen that there is no validated microbiological method for some active ingredients. Due to microbiological assays are indispensable methods for determining the potency of some active ingredient groups, the calculation of the potency is performed logarithmically. In these study above mentioned microbiological assaysare compared in the context of the main pharmacopoeias EP, USP, CP, IP and BP,and evaluated in terms of the chorotographic method and classical microbiological method. It was concluded that the realization of new microbiological methods to be validated by evaluating the methods in all differences will facilitate the study.

Key Words: Pharmacopoeialmethods, antibiotics, microbiological assay, potency, pharmaceutics

INTRODUCTION

Antibiotics are therapeutic agents that have a bactericidal, fungicidal effect or inhibit the growth of microorganisms.These active ingredients are used to destroy microorganisms or to treat infection by inhibiting the growth of microorganisms without harming the host.Antibiotics are active substances produced by some microorganisms or by chemical synthesis. These substances are effective on microorganisms andpartially or completely destroy or inhibit the targeted microorganisms. Antibiotics are widely used in the treatment of bacterial diseases. Despite the global increase in antibiotic resistance, the widespread use of these drugs remains a major threat to the safety of human and animal life.For this reason, it is important to use antibiotics effectively and to determine their effectiveness on microorganisms.[1].

Quality control analysis of pharmaceutical products consists of many parameters. Microbiological quality control parameters are determination of bioburden in pharmaceutical products, sterility test, antimicrobial efficacy test and microbiological assay tests. Quantitative assay analysis, which is one of the important quality control parameters, mainly analyzed with the chromographic methods. Assay analysis is generally performed by chromatographic methods in pharmaceutical products whose active ingredient is antibiotics.As an alternative to chromatographic methods, microbiological assay analyzes can also be performed.In this context, quantitative analysis of antibiotic products that cannot be analyzed by chromatographic methods can also be performed microbiologically. Microbiological

determination of the amount of antibiotic agent in products is very important for antimicrobial efficacy and is an important analysis parameter to check the efficacy of the product. It is among the guiding analyzes for controlling the microbiological activity of these products, determining the activity on the microorganism causing the infection, adjusting the application dose and determining the amount of antibiotic active substance in the pharmaceutical product.Generally, internationally accepted pharmacopoeia methods are widely used for microbiological assay analysis. When the analysis methods applied in the past are examined, while microbiological assay analyzes are applied for many antibiotic active substances, there is a tendency to switch to chromatographic methods with method validation studies carried out today. However, there are many antibiotic active ingredients that cannot be microbiologically analyzed. Many antibiotic agents such as vancomycin, gentamicin, colistimethate sodium, teicoplaninassay analyzes are performed only as a microbiological assay method.In the European Pharmacopoeia-EP, the United States Pharmacopeia-USP, the Chinese Pharmacopoeia-CP, International Pharmacopoeia-IP and the British Pharmacopoeia-BP areroutinely used in microbiological assay analysis for antibiotics. In this study, the pharmacopoeia methods used in the microbiological analysis of pharmaceutical products containing antibiotic active substances, which are used as an alternative to the chorotographic method or that cannot be analyzed by chromatographic methods, were examined. [2-6].

Microbiological assay analysis can be performed using the bactericidal and fungicidal effects of pharmaceutical products containing antibiotics as active and / or auxiliary substances on microorganisms.For these microbiological assays, the internationally recognized methods specified in The European Pharmacopoeia-EP, the United States Pharmacopeia-USP, the Chinese Pharmacopoeia-CP, International Pharmacopoeia-IP and the British Pharmacopoeia-BP are routinely used. Some of the microbiological assay methods are similar in principle to chromatographic methods. In both methods, the amount of active substance is determined by statistical evaluation of the sample with the standard reference substance. Dose-response data obtained in the analyzes are peak areas or spectrophotometric data in chromatographic methods, while inhibition zone diameters or turbidity in the medium in microbiological methods. With the microbiological assay analysis, the antimicrobial activity of the antibiotic agent, ie its potency, is determined.However, it is not possible to analyze some antibiotics by chromatographic methods and to detect a decrease in antimicrobial activity by chemical methods. For this reason, it is important to determine the microbiological potency of the product, especially in pharmaceutical products containing antibiotics, in order to measure the antimicrobial activity. Controlling the efficacy of pharmaceutical products containing antibiotics as active or excipients can be accomplished by microbiological potency determination rather than chemical methods. Therefore, it is an indisputable fact that the amount of antibiotic to be added to the pharmaceutical product will be efficient by determining its antimicrobial effectiveness [7].

The potency (activity) of an antibiotic product is expressed as the ratio of the dose that inhibits the growth of a suitable susceptible microorganism to the dose of an International Biological Standard, an International Biological Reference Preparation, or an International Chemical Reference Substance.Secondary reference materials that have been properly validated can also be used in testing.In order to carry out the experiment, the rate of inhibition of the growth of microorganisms is compared with known concentrations of the reference material and measured dilutions of the test substance.This response can be measured by the diffusion method, as described below, or in a liquid medium by the turbidimetric method. The decrease in antimicrobial activity may not be sufficiently demonstrated by chemical methods. Therefore, the potency of antibiotics can be demonstrated by microbiological methods. Reference substances used in microbiological determinations must be those that have been

precisely determined with reference to the relevant international standard or international reference preparation. The test design to be carried out should be designed in a way that allows it to be examined mathematically. Accordingly, the antibiotic concentrations to be chosen should be chosen linearly. The concentrations selected for the reference substance and the sample should be parallel to each other [7-10].
Many methods specifically for the active ingredient have been approved for microbiological quantification. These methods can be examined under two headings.

METHODS FOR MICROBIOLOGICAL ASSESSMENT OF PHARMACEUTICS

Agar diffusion method

A known concentration of antibiotic-sensitive microorganisms to be examined is inoculated into the medium by liquefying a medium suitable for the test conditions and at a suitable temperature (eg, 48°C to 50°C for vegetative forms). With the effect of antibiotic concentrations used in the test on microorganisms, it is aimed to produce clearly defined inhibition zones of appropriate diameter. The medium used in the analysis should consist of a uniform layer 2-5 mm thick.Alternatively, the medium may consist of two layers and the microorganism may be inoculated only on the topsheet.It is especially necessary to use a plate containing two-layer medium in the USP method, but the plates to be used in EP and BP methods do not need to be composed of two layers.The agar diffusion method is defined as cylinder plate assay in USP. The roller plate experiment is based on diffusion of the antibiotic solution from a vertical cylinder through a solidified agar layer in a petri dish.The growth of specific microorganisms that are inoculated as the antibiotic solution formed diffuses in the agar is prevented in a circular area or zone around the cylinder or other materials. EP and BP methods cover USP methods, while the USP enforces strict rules. The methods are similar with respect to the type of medium and pouring double-layered, use of a cylinder disc, and the test microorganisms.The concentration of the inoculum should be chosen such that the most acute inhibition zones and appropriate dose response at different concentrations of the standard are obtained. When preparing the inoculum, an inoculated medium containing 1 mL of suspension per 100 mL of culture medium is usually suitable.Inoculum volumes of target microorganisms to be transfered to the medium in USP methods are clearly specified. Sterile cylinders made of suitable material such as glass, porcelain or stainless steel can be used to apply the test and standard solution to the medium. Instead of cylinders, 8-10 mm diameter wells can be drilled into the medium with a pre-sterilized puncher. Test and reference solutions can be transferred to the medium with cylinders or wells. Alternatively, sterile absorbent paper discs of suitable quality can be used. The discs are impregnated with reference and test solutions and placed on the agar surface. It is the purpose of all methods to diffuse the antibiotic on the agar medium.While it is necessary to use a cylinder to transfer the antibiotic to the agar in the USP method, other systems can also be applied in EP and BP methods.Reference material solutions of known concentration and the theoretical test solution assumed approximately the same concentration are prepared in a sterile buffer with an appropriate pH value. To ensure test validity, the analysis is performed with an equal number of doses of the test substance with the same theoretical activity as the solutions of the reference material. Generally, at least three different doses of reference material are used. This number goes up to five in USP methods.he dose levels used should be in geometric progression (eg in a 2: 1 ratio) by preparing a series of dilutions, and an equivalent number of sample doses should be prepared according to the EP, BP methods. In the USP method, an unknown sample solution can be prepared against reference solutions in five geometrical advances and this number can be increased.The relationship between the logarithm of the

concentration of the antibiotic for the system used and the diameter of the inhibition zone should be demonstrated to be approximately linear. The reference material on the plates and the test solutions should be placed on the plates by creating a design. This design is not explicitly specified in EP and BP methods, but the assay design is defined in USP.These assay designs to be determined are carried out in order to ensure the neutrality in measuring the inhibition zones formed as a result of the analysis and to keep the interaction of the obtained inhibition zones at a minimum level. Plates are incubated at a suitable temperature and incubation time that is usually about 16 hours. The diameters or areas of the inhibition zones produced by the various concentrations of the standard and test substance are measured. The zones are measured with a precision of 0.1 mm and the strength of the tested substance is calculated from the results [8,11,12,13,14].Differences and similarities between pharmacopoeial methods are shown in Table 1.

Turbidimetric method

The test is performed by inoculating a suspension of the target microorganism into a suitable medium in order to create microbial growth inhibition under test conditions. A known amount of the selected suspension is used to obtain an easily measurable opacity after an incubation period of about 4 hours.

The inoculated medium should be used immediately after preparation. Using the solvent and buffer solution specified in the pharmacopoeia methods, the solutions of the reference substance and the sample solution with equal activity are prepared.In order to evaluate the test validity, at least three doses with the same theoretical activity as the doses of the reference substance should be prepared.For the analysis, it is preferred to use a series of doses with the doses of the test solutions of the reference material and the sample in geometric progression.Alsoto provide the required linearity, it may be necessary to choose between approximately three consecutive doses for the reference substance and test substance to be examined. For this reason, the number of doses can be increased. An equal volume of each solution is dispensed into the test tubes, and an equal volume of inoculated medium (e.g. 1 mL of solution and 9 mL of medium) is added to each tube. Two antibiotic-free control tubes are prepared at the same time as the test set. Both control tubes contain inoculated medium and 0.5 mL of formaldehyde R should be added to one of them. All tubes should be prepared randomly or in a Latin square or the plates should be prepared in random block pattern. All tubes are quickly placed in a water bath or other suitable apparatus to bring them to the proper incubation temperature. The tubes arekept at this temperature for 3 seconds to ensure stabilization and then incubated for 4 hours at homogeneous temperature.After incubation, the growth of microorganisms is inhibited by adding 0.5 mL of formaldehyde R to each tube or by heat treatment. Opacity in the tubes is measured up to three significant numbers with the appropriate optical apparatus. Alternatively, a method that allows the opacity of each tube to be measured after exactly the same incubation time should be used. In the USP method, the wavelength is defined as 530 or 580 nm.[8,11,12,13,14].Differences and similarities between pharmacopoeial methods are shown in Table 1.

Preparation of microorganisms

Preparation of target microorganisms to be used in tests varies according to the characteristics of microorganisms. Preparation of microorganisms such as *Bacillus cereus, B. subtilis, B. pumilus* are achieved by the formation of spore concentrations. These microorganisms are incubated at 35-37°C for 7 days or at 26°C for 4-6 dayson their surface in a suitable medium supplemented with 0.001 g / L manganese sulphate R. The spore forms formed are suspended using sterile water R. This suspension is kept in a 70°C water bath for 30 minutes to kill the

vegetative forms according to CP method.This suspension is diluted to the proper concentration. The concentration determined in the two suspensions was set at 10×10^6 to 100×10^6 per concentration. *Bordetella bronchiseptica, Staphylococcus aureus, Klebsiella pneumoniae, Escherichia coli, Micrococcus luteus* are incubated at 35-37°C for 16-18 hours in suitable media.*Saccharomyces cerevisiae, Candida tropicalis* are incubatedat 30-37°C for 24 hours in suitable media. Suspension is prepared from growing colonies with a sterile 9 g/L solution of sodium chloride R and Diluted to a suitable opacity with the same solution. Preparation step of microorganisms are similar in all pharmacopoeia methods. Glass beads and Roux bottles can be used to suspend the microorganism in the USP method. Suspension concentrations of microorganisms to be inoculated into the medium in EP, BP and IP methods are not specified. In the USP method, approximately 25% at 580 nm transmitance concentration is used excluding spore microorganisms. In addition, the volume of microorganism suspension to be transferred to the medium in the USP method is also specified. This will facilitate the standardization of inhibition zones. If the microorganism and dose concentrations given in the USP method are complied with, the median concentration is likely to form an inhibition zone with a diameter of 14-16 mm [8,11,12,13,14].Differences and similarities between pharmacopoeial methods are presented in Table 1.

COMPARISON OF THE METHODS FOR THE POTECY OF ANTIBIOTICS

Comparison of microbiological assay methods are presented in Table 1.

Table 1. Comparison of microbiological assay methods [8,11,12,13,14].

Microbiological Assay Methods of Pharmacopoieas		
EP- BP-IP	USP	CP
For agar diffusion analysis, well paper disc, steel cylinder can be used in plates.	Only steel cylinders can be used for agar diffusion analysis.	For agar diffusion analysis, well paper disc, steel cylinder can be used in plates.
A single layer or double layer plate can be used.	A double layer plate should be used.	A single layer or double layer plate can be used.
There should be at least 3 geometrically ordered dose concentrations. The reference substance concentration and the test substance concentration should be equivalent.	The 5 geometrically sorted reference dose concentrations should be 1 dose sample concentration. The median reference substance concentration and the test substance should be equivalent	There should be at least 3 geometrically ordered dose concentrations. The reference substance concentration and the test substance concentration should be equivalent.
The concentrations of the reference substance to be used in test are not specified. These concentrations are determined by the analyst.	Concentrations of the reference substance are determined for each active substance. Specifically, the concentration of the median dose was determined and the concentration of the reference dose set was determined.	Concentrations of the reference substance are determined for each active substance.
Concentrations of the microorganism to be transferred to the medium are not specified. These concentrations are determined by the analyst.	The concentrations of the microorganism to be transferred to the medium are determined for each active ingredient.	The concentrations of the microorganism to be transferred to the medium are determined for each active ingredient.
The medium volumes and the volume of the microorganism to be transferred to the medium are not specified. These volumes are	The medium volumes and the volume of the microorganism to be transferred to the medium are determined for each active ingredient.	The medium volumes and the volume of the microorganism to be transferred to the medium are determined for

determined by the analyst.		each active ingredient.
Assay designs are determined by the analyst.	Assay designs have been determined and the distribution of 5 reference doses has been defined.	Assay designs are determined by the analyst.
There is no definition to regulate the zone variations between media.	Zone variations between the medium are controlled by the median reference substance (S3) concentration.	There is no definition to regulate the zone variations between media.
The properties of petri dishes, cylinders and tubes to be used in the analysis are not defined. These situations are determined by the analyst.	Petri dish, cylinder and tube properties to be used in the analysis are defined.	The properties of petri dishes, cylinders and tubes to be used in the analysis are not defined. These situations are determined by the analyst.
In the turbidimetric assay analysis, spectrophotometer features and wavelength are not defined.	In the turbidimetric assay analysis, spectrophotometer properties and wavelength are defined.	In the turbidimetric assay analysis, spectrophotometer features and wavelength are not defined.

Microbiological assay of antibiotics varies according to certain antibiotic active substances. In particular, the quantitation of active ingredients such as vancomycin, colistimemtat sodium, colistin, and gentamicin can be performed only by microbiological quantification. In this respect, microbiological determinations appear as the primary test method in addition to the alternative method in the analysis of the quantitation of active substances. When the standard methods described in the pharmacopoeias are examined we come across the agar diffusion method, cylinder plate method, rectengular plate method, and turbidimetric method. These methods specified in the pharmacopoeias may differ in terms of analysis steps and general requirements. Quantitative analysis comparisons of active ingredients in the study conducted for the quantitation of antibiotic active ingredients are stated below. The European Pharmacopoeia-EP, the United States Pharmacopeia-USP, the Chinese Pharmacopoeia-CP and the British Pharmacopoeia-BP arepharmacopoeias were screened for the assay analysis of pharmaceutical products with antibiotics. The comparison of analysis methods of antibiotic active ingredients is given in Table 2.

Table 2. Comparison of assay methods according to active ingredients [15-18].

Active Ingredients	Chemical Methods				Microbiological Method			
	EP	USP	BP	CP	EP	USP	BP	CP
Acetylspiramycin	NAM	NAM	NAM	NAM	NAM	NAM	NAM	ADM / TM
Amikacin	CM	CM	CM	CM	NAM	NAM	NAM	ADM / TM
Amoxicilline	CM	CM	CM	CM	NAM	CPM	NAM	NAM
Amphotericin B	NAM	NAM	NAM	CM	ADM	CPM	ADM	ADM
Ampicillin	CM	CM	CM	CM	NAM	NAM	NAM	NAM
Azithromycin	CM	CM	CM	CM	NAM	NAM	NAM	ADM / TM
Aztreonam	NAM	CM	NAM	CM	NAM	NAM	NAM	NAM
Apramycin	NAM	NAM	NAM	NAM	NAM	NAM	TM	NAM
Bacitracin	NAM	NAM	NAM	NAM	ADM	CPM	ADM	ADM / TM
Bleomycin	NAM	NAM	NAM	CM	ADM	CPM	ADM	NAM
Capreomycin	NAM	NAM	NAM	NAM	NAM	TM	NAM	ADM
Cefaclor	CM	CM	CM	CM	NAM	NAM	NAM	NAM
Cefadroxil	CM	CM	CM	CM	NAM	NAM	NAM	NAM
Cefalotin	CM	NAM	CM	CM	NAM	NAM	NAM	NAM
Cefamandole	CM	CM	CM	CM	CM	NAM	NAM	NAM
Cefapirin	CM	NAM	CM	NAM	NAM	NAM	NAM	NAM
Cefazolin	CM	CM	CM	CM	NAM	NAM	NAM	NAM

Cefdinir	NAM	CM	NAM	CM	NAM	NAM	NAM	NAM
Cefepime	CM	CM	CM	CM	NAM	NAM	NAM	NAM
Cefixime	CM	CM	CM	CM	NAM	NAM	NAM	NAM
Cefmenoxime	NAM	CM	NAM	CM	NAM	NAM	NAM	NAM
Cefuroxime	CM	CM	CM	CM	NAM	NAM	NAM	NAM
Cefmetazole	NAM	CM	NAM	CM	NAM	NAM	NAM	NAM
Cefonicid	NAM	CM	NAM	CM	NAM	NAM	NAM	NAM
Ceforanide	NAM	CM	NAM	NAM	NAM	NAM	NAM	NAM
Cefotaxime	CM	CM	CM	CM	NAM	NAM	NAM	NAM
Cefotetan	NAM	CM	NAM	NAM	NAM	NAM	NAM	NAM
Cefotiam	NAM	CM	NAM	NAM	NAM	NAM	NAM	NAM
Cefoxitin	CM	CM	CM	CM	NAM	NAM	NAM	NAM
Cefpiramide	NAM	CM	NAM	NAM	NAM	NAM	NAM	NAM
Cefpodoxime	CM	CM	CM	CM	NAM	NAM	NAM	NAM
Cefprozil	CM	CM	CM	CM	NAM	NAM	NAM	NAM
Ceftazidime	CM	CM	CM	CM	NAM	NAM	NAM	NAM
Ceftizoxime	NAM	CM	NAM	CM	NAM	NAM	NAM	NAM
Cephalexin	NAM	CM	NAM	NAM	NAM	NAM	NAM	NAM
Chloramphenicol	CM	CM	CM	CM	NAM	TM	NAM	ADM / TM
Cloxacillin	CM	CM	CM	CM	NAM	CPM	NAM	NAM
Chlortetracycline	CM	NAM	CM	CM	NAM	TM	NAM	ADM
Ciprofloxacin	PT	CM	PT	CM	NAM	NAM	NAM	NAM
Clarithromycin	CM	CM	CM	CM	NAM	NAM	NAM	ADM
Clindamycin	CM	CM	CM	CM	NAM	NAM	NAM	NAM
Colistimethate	NAM	NAM	NAM	NAM	ADM / TM	CPM	ADM / TM	NAM
Colistin	NAM	NAM	NAM	NAM	ADM / TM	CPM	ADM / TM	ADM
Cycloserine	NAM	CM	NAM	NAM	NAM	NAM	NAM	NAM
Dihydrostreptomycin	CM	NAM	CM	NAM	NAM	CPM / TM	NAM	NAM
Doxycycline	CM	CM	CM	CM	NAM	NAM	NAM	NAM
Erythromycin	CM	CM	CM	NAM	NAM	CPM	ADM / TM	ADM / TM
Fosfomycin	CM / TTM	CM	CM / TTM	NAM	NAM	NAM	NAM	ADM / TM
Framycetin	NAM	NAM	NAM	NAM	ADM / TM	NAM	ADM / TM	NAM
Fusidic acid	CHM	NAM	PT	NAM	NAM	NAM	NAM	NAM
Gramicidin	NAM	NAM	NAM	NAM	TM	TM	TM	NAM
Gemifloxacin	NAM	CM	NAM	NAM	NAM	NAM	NAM	NAM
Gentamicin	NAM	NAM	NAM	NAM	ADM / TM	CPM	ADM / TM	ADM / TM
Idarubicin	NAM	CM	NAM	NAM	NAM	NAM	NAM	NAM
Josamycin	NAM	NAM	NAM	NAM	ADM / TM	NAM	ADM / TM	ADM / TM
Imipenem	CM	CM	CM	NAM	NAM	NAM	NAM	NAM
Kanamycin	NAM	CM	NAM	CM	ADM / TM	NAM	ADM / TM	ADM
Kitasamycin	NAM	NAM	NAM	NAM	NAM	NAM	NAM	ADM / TM
Levofloxacin	PT	CM	NAM	CM	NAM	NAM	NAM	NAM
Lincomycin	CM	CM	CM	CM	NAM	NAM	NAM	NAM
Lymecycline	CM	NAM	CM	NAM	NAM	NAM	ADM	NAM
Meleumycin	NAM	NAM	NAM	NAM	NAM	NAM	NAM	ADM / TM
Meropenem	CM	CM	CM	CM	NAM	NAM	NAM	NAM
Metronidazole	PT	CM	PT	PT	NAM	NAM	NAM	NAM
Mezlocillin	NAM	CM	NAM	CM	NAM	NAM	NAM	NAM
Micronomycin	NAM	NAM	NAM	NAM	NAM	NAM	NAM	ADM / TM
Minocycline	CM	CM	CM	CM	NAM	NAM	NAM	NAM
Moxifloxacin	CM	CM	CM	NAM	NAM	NAM	NAM	NAM
Natamycin	NAM	CM	NAM	NAM	NAM	NAM	NAM	NAM
Neomycin	NAM	NAM	NAM	NAM	ADM / TM	CPM / TM	ADM / TM	ADM / TM
Netilmicin	CM	CM	CM	NAM	ADM	NAM	ADM	ADM / TM
Nafcillin	NAM	CM	NAM	NAM	NAM	CPM	NAM	NAM
Nystatin	NAM	NAM	NAM	NAM	ADM	CPM	ADM	NAM
Norvancomycin	NAM	NAM	NAM	NAM	NAM	NAM	NAM	ADM
Novobiocin	NAM	NAM	NAM	NAM	NAM	CPM	NAM	NAM
Oxytetracycline	CM	CM	CM	CM	NAM	TM	NAM	ADM
Paromomycin	NAM	NAM	NAM	NAM	NAM	CPM	NAM	ADM
Penicillin	CM	CM	CM	CM / SM	NAM	CPM	NAM	NAM
Penicillamine	PT	CM	PT	PT	NAM	NAM	NAM	NAM
Polymyxin B	NAM	NAM	NAM	NAM	ADM	CPM	ADM	ADM
Ribostamycin	NAM	NAM	NAM	NAM	NAM	NAM	NAM	ADM
Rifampicin	SM	NAM	SM	CM	NAM	NAM	NAM	NAM
Rifamycin	NAM	NAM	NAM	NAM	ADM / TM	NAM	ADM / TM	NAM
Roxithromycin	CM	NAM	CM	CM	NAM	NAM	NAM	ADM
Sisomicin	NAM	NAM	NAM	NAM	NAM	NAM	NAM	ADM / TM
Spectinomycin	CM	CM	CM	CM	NAM	NAM	NAM	ADM / TM
Spiramycin	NAM	NAM	NAM	NAM	ADM / TM	NAM	ADM / TM	NAM

Streptomycin	NAM	CM	NAM	NAM	ADM / TM	NAM	ADM / TM	ADM / TM
Sulbactam	CM	CM	CM	CM	NAM	NAM	NAM	NAM
Sulbenicillin	NAM	NAM	NAM	NAM	NAM	NAM	NAM	ADM
Sulfacetamide	DPAN	CM	DPAN	DSTM	NAM	NAM	NAM	NAM
Sulfadiazine	DPAN	CM	DPAN	DSTM	NAM	NAM	NAM	NAM
Sultamicillin	CM	NAM	CM	CM	NAM	NAM	NAM	NAM
Tazobactam	NAM	CM	NAM	CM	NAM	NAM	NAM	NAM
Teicoplanin	NAM	NAM	NAM	NAM	ADM	NAM	ADM	ADM
Thiostrepton	NAM	NAM	NAM	NAM	NAM	TM	NAM	NAM
Tetracycline	CM	CM	CM	CM	NAM	TM	NAM	ADM / TM
Tobramycin	CM	CM	CM	NAM	NAM	NAM	NAM	ADM / TM
Tylosin	NAM	NAM	NAM	NAM	ADM / TM	TM	ADM / TM	NAM
Tyrothricin	NAM	NAM	NAM	NAM	TM	TM	TM	NAM
Vancomycin	NAM	NAM	NAM	NAM	ADM / TM	CPM	ADM / TM	ADM

NAM: There is No Analysis Method
CM: Chromatographic Method
CPM: Cylinder-plate Method
ADM: Agar diffusion Method
CHM: Chemical Methods
TM: Turbidimetric Method

PT: Potentiometric Titration
SM: Spectrofotometric Method
TTM: Titration Method
DPAN: Determination of Primary Aromatic Amino-Nitrogen
DSTM: Dead-stop Titration Method

Potency calculation

As stated above, the potency of the antibiotic active ingredient analyzed should be calculated by an appropriate statistical method.The statistical methods to be chosen tend towards many different calculations in EP and BP methods. In EP and BP methods,the linearity of the dose-response relationship can generally be obtained within a limited range. This linear range is statistically significant. For this reason, at least three consecutive doses selected should be included in the test design to prove the existence of linearity. The presence of a three-point linearity in the test can be accepted as two points depending on the approval of the competent authority. However, a three-point test should be applied in all conflict situations. Sufficient number of replicates per dose should be provided to ensure the required accuracy and precision with each test.The test is reproducible and the results can be combined statistically to obtain the required accuracy and precision and to determine if the strength of the antibiotic to be studied is less than the minimum required. EP and BP methods are used for statistical calculation in the "Statistical Analysis of Results of Biological Assays and Tests" section. In the theoretical model, it is essential to transform the dose-response relationship into a linear function over the widest possible dose range to make the effect of dilution apparent. Two statistical models are of interest as models for the bioassays prescribed: the parallel-line model and the slope-ratio model [10,19].

Antibiotic assay designs according to EP and BP are defined as follows;

- Latin square design
- Completely randomised design
- Randomised block design
- Cross-over design

Antibiotic assay statistical calculation models according to EP and BP are defined as follows;

- Slope-ratio model
- Extended Sigmoid Dose-Response Curve
- Parallel-line model
- Three-dose latin square design
- Four-dose randomised block design
- Slope-ratio model

Calculation of antibiotic potency according to the USP method can be accomplished by generating a standard curve of log-transformed responses of reference doses. The analyst must consider three basic concepts when interpreting antibiotic potency results.

- The logarithmically transformed value of the dose-response relationship should provide linearity. This linearity should be within statistically acceptable limits. For deviated values to ensure the linearity, the necessary appropriate concentrations should be deactivated in the test. These concentrations are generally determined in USP methods. If linearity is not achieved in the test, the test should be repeated.
- The number of assays required to obtain a reliable estimate of antibiotic potential depends on the required specification range and assay variability. The confidence limit calculation is determined from several estimated daily potencies that are approximately equal in sensitivity. If the calculated value for the confidence interval width is too wide, a useful decision cannot be made as to whether the potency meets its specification.
- The most effective way to reduce the variability of the measured dose-response value is by calculating the geometric mean potency between runs and replicates. The combined result of a number of smaller independent tests provides a more reliable calculation than a single large test with the same total number of plates or tubes. Three or more independent experiments are required for antibiotic potency determinations.

For the USP agar diffusion method (cylinder-plate), converted zones for 5 different standard doses are calculated and evaluated. Transformed zones are calculated according to the median concentration (S3) dose used in plate design. The responses of 5 different reference doses calculated as converted and the logarithmic values of the doses are compared. The theoretical potency of the sample can be calculated according to the linear curve obtained by this comparison. Comparison of the zone diameter with the sample-verified center with the standard curve line is used as the calculation method. For the USP turbidimetric method, a condition must be met in the analysis that the tubes are randomly distributed within the heat block or other temperature controller. For this reason, if the device providing the temperature has a non-uniform temperature profile, a random block design may be preferred. In such a design, the shelf must be divided into areas of relatively uniform temperature. At least one tube should be placed from each standard concentration and each unknown area. Statistical calculation should be made as specified in the agar diffusion method in the test design prepared as specified. Besides these calculation models If the concentrations are equally spaced in the logarithmic scale, the calculations can be performed using the following Table 3.

Table 3. Formula for manual calculations of regression and sample concentration [12].

Formula
$b = (4S5 + 2S4 - 2S2 - 4S1)/[5(L5 - L1)]$
The log of the concentration of the sample is found using: $LU = Lreference + [(U - S)/b]$

- Sk:mean corrected zone measurement (cylinder-plate assay) or average absorbance value (turbidimetric assay) for standard set k.
- K: 1, 2, 3, 4, 5
- S: mean of the five Sk values
- Lk: logarithm of the kth concentration
- U: unknown
- LU: log concentration of the sample
- b = (Yhigh − Ylow)/(Xhigh − Xlow)
- Yhigh = ⅕(3S5 + 2S4 + S3 − S1)
- Ylow = ⅕(3S1 + 2S2 + S3 − S5)
- Xhigh = L5
- Xlow = L1

In CP methods, statistical calculation methods similar to EP, BP and USP calculation methods are used. The accuracy of the data obtained by the calculation of the standard line curve is also important for this calculation.In this method, calculation is made by statistical comparison of the data obtained with the logarithm value of the reference concentrations. The steps to be applied in this calculation process are defined in the "Statisticial Method for Biological Assay" section. The definitions stated in this section are generally compatible with EP and BP methods. Assay designs and statistical calculations are similar to the methods and requirements specified in EP Statistical Analysis of Results of Biological Assays and Tests section [20].

CONCLUSION

This current examination showed that there are mostly chemical methods existed for the active substance groups included in this reseach. However, due to pharmacopoeia methods cannot be implemented for some antibiotic active ingredients, microbiological methods are still indispensable for some active ingredient groups. It should be noted that these methods have been validated. It has known that many pharmacopoeia methods can be used when performing potency analysis of pharmaceutical products containing antibiotic active ingredients.The comparison of the microbiological methods in EP, USP, CP, IP and BP pharmacopoeias showed that they are similar methods in many ways. However, criteria in other pharmacopoeias can also be evaluated when establishing test requirements and assay designs. Our evaluation showed that it is an emegous need to desing and valide new microbiological methods that are not on available for the antibiotics that are existed in the pharmacopoeias.

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Conflict of interest

The authors declare no conflicts of interest.

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