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Full Length Research Paper

Microbiological assay of the active component of ampicillin in ampicillin and ampicillin/cloxacillin suspensions using *Bacillus megatharium* NCTC 10342A$_{76}$ as indicator organism

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51 different samples from 5 different manufacturers of ampicillin and ampicillin/cloxacillin suspensions were tested for their levels of active components of ampicillin using the microbiological assay method. *Bacillus megathariumNCTC10342A*$_{76}$ was selected from amongst a wide range of organisms screened for this purpose because of its detected suitability. Thin layer chromatography and infra red spectrophotometric analysis confirmed the presence of ampicillin in all the samples and the results obtained showed that the percentage ampicillin content ranged from 57 - 162. Products of MAN-A07 had ampicillin levels less than that recommended by the reference books while one batch from MAN A06 had ampicillin levels higher than the recommended levels. This work is discussed in the context of the proliferation and misuse of ampicillin especially for children.

Key words: Microbiological assay, *Bacillus megatharium,* ampicillin, cloxacillin, chromatography.

INTRODUCTION

Among all pharmaceutical products, the most commonly faked and adulterated are antibiotics. This is probably because of the frequency of their use which is very high. Antibiotics are drug preparations which contain some chemical substances that are produced by micro-organisms. These substances in very low concentrations are known to totally destroy or partially inhibit micro-organisms (Stephen et al., 2004). Some antibiotics can also be produced by chemical synthesis. The problem of faking, adulteration and standard compromise of antibiotics applies to those which are synthesized chemically and those produced by microorganisms. Checks on the quality of these antibiotics are done by a number of ways which include microbiological assays. These assay methods help in estimating active Constituents, biological activity and in monitoring their stability. These checks are very necessary because these drugs most times are the line that separate life and death (Hewitt, 1977). The estimation of active ingredients or determination of potency of antibiotics using bioassay methods has for long being an acceptable method for determining how good a drug. Microbiological assay methods, employs the use of the biological properties of medicinal agents in the estimation of their activity. The method uses the basic principle of comparing a sample of known activity or potency (standard) and one of unknown activity at the same time and under very strict comparable conditions. The method of comparison may be macrobiological as in the assay of insulin where mice are used or microbiological as in the assay of ampicillin where a suitable micro-organism like *Bacillus megatharium* is used.

Ampicillin belongs to the penicillins which is a group of naturally occurring and semi-synthetic antibiotics. They have high activity against both Gram-positive and Gram negative organisms although, their activity against most Gram negative bacilli is usually too low to be of any clinical significance (Foye, 1976). Their mode of action is by the inhibition of the mucopeptide component of the cell wall which the organisms need for strength and rigidity. This is done by preventing the incorporation of N-acetyl

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muramic acid into the mucopeptide layer (Burger, 1970).

Resistance to penicillin has been observed in some organisms especially those that produce the enzyme B-lactamase. The need for compounds (penicillins) which are superior to the original penicillin G in their physical, pharmacological and microbiological properties has led to the synthesis of a wide range of penicillins referred to as semi-synthetic penicillins e.g. ampicillin and cloxacillin. They are more acid stable, more B-lactamase resistant and have wider spectrum of activity than the original penicillin G. Ampicillin for instance is more effective against Gram positive organisms than penicillin G and has a broader spectrum of activity due to its amino group while cloxacillin is not readily inactivated by the penicillinase enzyme like the original penicillin G.

Ampicillin/ cloxacillin combinations are used for the treatment of most infections in preference to using them singly for obvious reasons. The cloxacillin therein has the ability to block the production of the penicillinase enzyme (B-lactamase) which makes the organism resistant because it destroys the ampicillin; the ampicillin is therefore saved from it and can then act on the organism. It is also known that that the combination gives an even broader activity than penicillin alone. The high rate of use of this combination especially for children has increased the need for making sure that the ampicillin levels in the combinations meet the required standards. This work is also interested in describing a method for the selective determination of the levels of ampicillin in ampicillin/ cloxacillin (syrup) preparations.

MATERIALS AND METHODS

Selection of indicator organism

Efforts was made to identify an organism which shows selective susceptibility only to penicillin and not to cloxacillin as well as showing good growth as recommended by BP and USP.

Different organisms were grown in the shaker bath at 120 throws per minute and monitored by turbidity measurement until they entered the logarithmic phase. Different concentrations of the standard ampicillin trihydrate powder obtained from NAFDAC Kaduna, Nigeria were tried on a wide range of standard organisms provided by Professor Onaolapo of A.B.U Zaria for their susceptibility using the cup plate method. The same was done using pure cloxacillin powder.

Inoculum standardization

The inoculum was standardized to ensure that equal number of the selected organism was used during every assay in compliance with the requirement that the conditions of the assay must be strictly comparable. The method described by Udobi et al. (1994) was used.

1 ml of an overnight broth culture of Bacillus megatharium NCTC10342A76 was grown in a sterile nutrient broth overnight in a static condition. The organisms were washed once with sterile nutrient broth and the optical density (O.D) reading at a wavelength of 470 nm taken. Subsequent O.D470 readings were taken at 30 min intervals. Each reading and viable counts were taken after appropriate serial dilutions of the culture and plating out on nutrient agar plates. The plates were then incubated at 37°C between 18 - 24 h. A graph of O.D470 against viable count (cfu/ml) was plotted and the O.D470 of the culture which contains one million cells per ml was thus obtained by extrapolation from the graph.

Preparation of standard test doses

Three concentrations of standard ampicillin trihydrate powder 10, 20 and 40 mg/ml were prepared on the day of assay using sterile 0.1 M phosphate buffer (usp) as diluent bearing in mind that 1.15 g of pure ampicillin trihydrate contains 1 g of ampicillin.

Preparation of sample test doses

Samples of ampicillin and ampicillin/cloxacillin suspensions were first reconstituted according to the instructions of the manufacturers. Three concentrations of 10, 20 and 40 mg/ml of each sample were then prepared on the day of the assay using sterile 0.1 M phosphate buffer (usp) as diluent.

Assay procedure

For the assay, the cup plate method (BP, 1988) was used. The 6 × 6(3 × 3) dose level latin square design using large plates was employed. 200 ml of sterile nutrient agar inoculated with 1 ml of the test culture of OD470 of 0.17(this contains approximately 1million cells) was poured on top of the basal layer aseptically and was also allowed to set. Using a sterile No.4 cork borer, 36 cups were cut in the agar in a random manner referred to as the latin square design. 20 microlitre of each concentration (10, 20 and 40 mg/ml) of the sample and agents were then applied into each cup as prescribed by the latin square design method. The plates were left to stand for two hours to allow for effective diffusion. They were then incubated at 37°C between 18 - 24 h. Zones of inhibition produced by both the standard and test concentrations were measured to the nearest millimeter. The experiments were duplicated and the average of the zones of inhibition (ZOH) was taken.

Thin layer chromatography (TLC)

This was carried out according to the method described by the BP and it was used as a means of ascertaining the presence or absence of ampicillin in the market samples assayed.

Infra red spectroscopy

This was also used to ascertain the presence or absence of ampicillin in the market samples. A little quantity of the sample was finely ground in a small mortar with a few drops of liquid hydrocarbon (Nujol). The mull was then pressed between flat plates of sodium chloride and fed into a Perkin Elmer infra–red spectrophotometer. The spectrum for each sample so treated was produced by the instrument within 3 min.

RESULTS

Thin layer chromatography

Results obtained confirmed the presence of active ampicillin component in all the samples assayed. This was

This was confirmed by the presence of a principal spot due to each sample on the chromatogram with R_f value ranging from 0.435 - 0.445 and this compares very favourably with that of standard ampicillin which is 0.425.

Infra red spectroscopy

The Infra Red spectroscopy results of the samples showed characteristic peaks similar to those of standard ampicillin. Peaks were observed at 1770, 1682, 1600, 1570, 1370 and 1308 cm^{-1}. These peaks are known to be used in the identification of ampicillin.

Selection and standardization of indicator organism

Bacillus megatharium NCTC 10342A_{76} was selected as the indicator organism on account of its selective sensitivity to ampicillin and not cloxacillin ,Its ability to grow well (up to O.D of 8.0 at 470 nm before entering the stationary phase) Figure 1 as well as showing best measurable zone of inhibition from a concentration of 10 mg/ml. A plot of OD against viable count shows that a broth culture of OD.17 contains one million cells per ml.

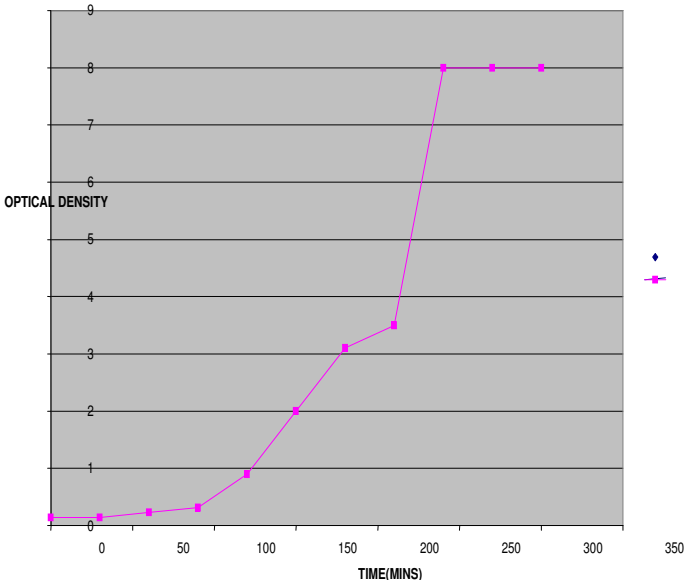

Figure 1. Graph of optical density against time showing the growth pattern of *Bacillus megatharium*.

DISCUSSION

The non-availability of recommended organism necessitated the search for another organism which will meet the standards set by the reference books for such organism to be used for the microbiological assay of ampicillin. *B. megatharium* NCTC10342A_{76} showed reasonable sensitivity to the agents assayed. This is in conformity with the BP on the criteria for selection of alternative indicator organism for assay in the absence of that recommended by the reference books. The organism also showed very good growth under the conditions which it was grown. This is confirmed by its ability to grow up to an OD reading of about 8.0 at 470 nm before entering the stationary phase.

The results obtained gave information of very reasonable interest. Samples from batch 1 of products from company (MAN A06) had higher contents of ampicillin than officially recommended (Table 1). This can only be explained by lack of proper in-process control during manufacture, carelessness on the part of production staff or improper weighing. The point stands out however that quality control was compromised during the production of this batch of product. The high content of active medicament is potentially dangerous, may be even more dangerous than when the concentration is low because it is more likely to lead to a toxicity problem. Considering the immunity level of the children who take these drugs, this high concentration is not acceptable.

The two other batches from the same company (MAN A07) gave percentage potencies between 94 and 105 which fall within the standard recommended ranges of 80 - 120% by the BP and 90 - 110 by the USP. Interestingly, this company is an indigenous one whose product was found in almost all the pharmacy and patent medicine shops visited during the sampling. The product of company (MAN A05) tested showed percentage potency within acceptable limits of both the BP and USP (Table 1). The product of the third company (MAN A07) had so much similarities with that of MAN A06. They were made in an Asian country for a Nigerian company that markets them. These products were sold for half of the price of the other ampicillin suspensions found in the same shops. Their assay results showed that all nine bottles from the 3 batches fell bellow the USP and BP recommended levels. It is not impossible that the product was knowingly and intentionally produced to this specification because the concentration of ampicillin was just about half of the manufacturers claimed potency 125 mg/5 ml.

The microbiological assay results of products of companies MAN B07, MAN B08 and MAN BO9 (Table 2) shows that the ampicillin content in the combination products were within the BP and USP acceptable levels of 80 - 120% and 90 -110.

In conclusion, It is confirmed that Bacillus megatharium NCTC10342A76 can be effectively used for the microbiological assay of ampicillin active components in ampicillin products in the absence of organisms recommended by reference books like the USP and BP.

Table 1. Percentage potency of ampicillin syrup from manufacturers (MAN) AO5, BO6 and CO7.

	MAN.AO5		MAN. B06		MAN.C07	
	Calculated Potency (%)	Equivalent Concentration g/5ml	Calculated Potency (%)	Equivalent Concentration g/5 ml	Calculated Potency (%)	Equivalent concentration g/5 ml
Batch 1						
1	103.3	129.12	156.30	195.00	71.60	89.50
2	97.70	122.37	161.00	201.25	59.80	74.75
3	101.80	127.25	162.18	202.72	57.00	71.25
Batch 2						
1	95.0	118.75	94.40	118.00	67.80	84.75
2	99.03	123.78	97.05	121.31	68.50	85.62
3	97.20	121.50	97.20	121.50	61.90	77.37
Batch 3						
1	100.80	126.00	105.40	131.75	63.80	79.75
2	101.10	126.37	103.10	128.87	61.80	77.25
3	104.00	130.00	101.80	127.25	60.82	76.02

Table 2. Levels of ampicillin in ampicillin/cloxacillin combinations (expressed in percentage) from manufacturers (MAN) BO7, DO8 and CO7.

	BO7		DO8		CO7	
	Calculated potency (%)	Equivalent concentration g/5 ml	Calculated potency (%)	Equivalent concentration g/5 ml	Calculated potency (%)	Equivalent concentration g/5 ml
			Batch 1			
1	104.80	131.00	89.20	111.50	91.80	114.75
2	97.80	122.25	91.16	113.95	92.50	115.62
3	102.40	128.00	90.15	112.68	90.20	112.75
			Batch 2			
1	90.80	113.50	89.50	111.80	98.80	123.50
2	89.10	111.37	90.20	112.75	107.50	134.37
3	92.90	116.12	89.12	111.40	109.20	136.50
			Batch 3			
1	112.20	140.25	92.60	115.75	NT	NT
2	109.80	137.25	95.71	119.63		
3	108.00	135.00	103.10	128.75		

NT = Not tested.

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