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Comparative Studies of the Spectrophotometric and Microbiological Assay Methods Used for the Determination of Ampicillin Levels in Ampicillin/Cloxacillin Combinations

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Abstract: The spectrophotometric and microbiological assay methods used for the determination of ampicillin levels in ampicillin/cloxacillin combinations were compared. A total of 24 samples of ampicillin/cloxacillin suspensions from 8 batches and 3 companies (B_2, D and E) were assayed for their ampicillin content levels using the Spectrophotometric assay method. Infra-red (IR) Spectroscopy confirmed the presence of ampicillin in all the samples and the results obtained showed that the samples had a wide range of amounts of ampicillin which compared favourably with the microbiological assay results of the same products. All the products assayed had ampicillin content within acceptable ranges of the BP and USP. Spectrophotometric assay is recommended for the quick determination of the levels of ampicillin in their combinations with cloxacillin.

Key words: Ampicillin, cloxacillin, Infra red, spectrophotometric assay, spectroscopy

INTRODUCTION

The Spectrophotometer is an analytical photoelectric instrument which is used for estimating the concentrations of solutions by measuring either the amount of light passing through (transmittance) or the amount of light absorbed (Absorbance). It is known to work on the principle of the Beer Lamberts law and within the range in which the law is obeyed, it has been effectively used in the assay of pharmaceuticals when made into solutions (Eboka *et al*., 1997). The method which involves principally the use of the spectrophotometer for this purpose is termed, spectrophotometric assay. Spectrophotometric assay of pharmaceuticals is highly dependent on the chemical reactions of the active ingredients of the agent concerned e.g., the concentration of the active ingredients or its characteristic end product can be determined by comparing its light absorption with that of a solution of the same substance at the same standard concentration.

Microbiological assays are generally used in the determination of the potency of growth inhibiting substances especially antibiotics (William, 2005). This involves the use of suitable microorganisms as the biological system. 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 (William, 2005; Udobi and Onaolapo, 2010).

Ampicillin and Cloxacillin are semi-synthethic penicillins which share very close similarity in their structures due to the presence of the Beta lactam and thiazolidine rings. This explains why the design of the methods for their selective determination in combined preparations must be done with a high degree of selectivity in mind. Penicillanic and Penicilloic acids, the acid and base hydrolysis products, respectively of penicillin have been found to react with formaldehyde to give a pyrazinone derivative in acidic medium. A wavelenght of 268 nm, was initially used to determine the level of ampicillin in the presence of cloxacillin but was found to be interfered with by readings from cloxacillin and other additives in pharmaceutical preparations (Akanni and Ayim, 1992). A wavelength of 373 nm where there will be no such interference was therefore suggested. Presently, the component ampicillin in combination of ampicillin and cloxacillin can successfully be determined using microbiological assays.

This study presents the results of the comparison between spectrophotometric and microbiological assay methods for the determination of the levels of the ampicillin content of combined preparations of ampicillin/cloxacillin suspensions.

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MATERIALS AND METHODS

Materials: The basic materials used were standard ampicillin powder obtained from National Agency for Drug Administration and Control (NAFDAC) in Nigeria, Different ampicillin/cloxacillin suspensions sold within Kaduna and Zaria environs and Spectrophotometer-(Pye-unicam) from Faculty of Pharmaceutical sciences, Ahmadu Bello University, Zaria-Nigeria where the experiments were carried out.

Calibration curve for standard ampicillin: A calibration curve for standard ampicillin was drawn using results obtained from reactions of ampicillin as described by Akanni and Ayim, (1992) (at concentrations18 g - 91 μg. A stock solution containing 1 mg/mL of standard ampicillin was made and 2,4,6,8 and 10 mL aliquots of the stock were taken into separate flasks and hydrolysed using 2 mL of 2 M sodium hydroxide. After 20 min, 1 mL of 2 M sulphuric acid was added to each mixture. 50 mL of formaldehyde containing phosphate buffer PH 2.5 was added to each flask and heated for one hour at 100°C. Each of the flasks so heated was designated solution A. Equivalent amounts of aliquots (2, 4, 6, 8 and 10 mL) of the same 1 mg/mL stock solution of standard ampicillin were taken in another set of flasks. Each sample was reacted with 50 mL formaldehyde containing phosphate buffer (PH 2.5). The flasks were each designated solution B. A 1:2 dilution of corresponding solution A and B were made with 2 M sulphuric acid and the absorbance of solution A was measured at the wavelenght 373 nm using solution B as reference. The absorbance reading so obtained were used with the corresponding concentrations to plot a calibration curve for ampicillin.

Determination of ampicillin in ampicillin/cloxacillin oral preparations:

Spectrophotometric assay: A stock solution of ampicillin/cloxacillin oral preparations containing 1 mg/mL of ampicillin was made from reconstituted samples of concentration of 250 mg/mL. Aliquots (5 mL) of the stock solution was then used to prepare reaction mixtures to make solutions A and B as done during the calibration. A 1:2 dilution of corresponding solutions of solutions A and B were made using 2M sulphuric acid and the absorbance of solution A determined with solution B as reference. This procedure was strictly followed for all the preparations tested by this method. The absorbance due to each sample is compared with that due to 5 mL of the standard ampicillin in the calibration curve.

Microbiological assay: The microbiological assay was done using the method described by Udobi *et al.* (1994, 2010) which involves the use of Bacillus megatharium NCTC 10342$A_{76\ AS}$ indicator organism.

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/ 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 0.1M phosphate buffer (usp) as diluent.

Assay procedure: The agar diffusion method was used. The 6x6(3x3) 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 1 million cells of Bacillus megatharium NCTC 10342A_{76}) 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 μL 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 37ºC for 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) were taken.

RESULTS

Up to a concentration of 91 μg/mL, the corresponding OD 373 nm absorbance readings obtained using the spectrophotometric method showed a linear relationship with concentration (Fig. 1). This means that up to this concentration and using the method of Akanni and Ayim (1992), ampicillin content can still be determined by absorbance readings. The results agree with the method and goes further to show that even at this high concentration (up to 91 μg/mL), a linear relationship between absorbance and concentration could still be obtained. (Table 1).

Table1: Absorbance readings of the concentrations of standard ampicillin (18-91 μg/mL)

Volume of standard	Concentration (μg/mL)	Absorbance at 373 nm
2	18.2	0.4
4	36.4	0.8
6	54.6	1.4
8	72.8	1.9
10	91.0	2.4

Table 2: Results for the Spectrophotometric assay of ampicillin in ampicillin/cloxacillin combinations from companies B_2, D and E

	B_2			D			E		
Companies	Absorbance	Calculated potency (%)	Corresponding concentration	Absorbance	Calculated potency (%)	Corresponding concentration	Absorbance	Calculated potency (%)	Corresponding concentration
Batch 1									
1	1.20	104.3	130.37	1.10	95.60	119.50	1.03	89.50	111.87
2	1.18	102.6	128.25	1.20	104.3	130.37	1.04	90.49	113.11
3	1.20	104.3	130.37	1.10	95.60	119.50	1.08	93.91	117.38
Batch 2									
1	1.18	102.6	128.25	1.06	92.17	115.21	1.28	113.0	141.25
2	1.14	99.0	123.75	1.10	95.60	119.50	1.30	111.0	138.87
3	1.14	99.0	123.75	1.08	93.90	117.37	1.30	113.0	141.27
Batch 3									
1	1.28	113.3	139.12	1.16	100.8	126.00			
2	1.28	113.3	139.12	1.14	99.13	123.91			
3	1.28	113.3	139.12	1.19	103.4	129.25			

Table 3: Levels of ampicillin in ampicillin/cloxacillin combinations (expressed in % age) from companies B_2, D and E determined using microbiological method

	B_2		D		E	
Companies	Calculated potency (%)	Equivalent concentration µg/5 mL	Calculated potency (%)	Equivalent concentration µg/5 mL	Calculated potency (%)	Equivalent concentration
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		
2	109.80	137.25	95.71	119.63		
3	108.00	135.00	103.10	128.75		

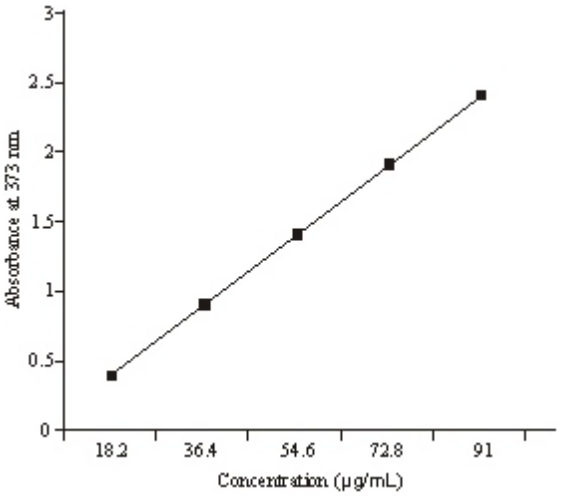

Fig. 1: Calibration curve for ampicillin at 373 nm, the calibration curve gave a straight line of reproducible linearity

DISCUSSION

Pharmaceutical quality checks is an important aspect of its manufacture which no manufacturer is willing to compromise for obvious reasons. For these same reasons, no consumer will knowingly accept a compromise on the quality of drugs he or she takes (Udobi *et al*., 1994).

The combination of ampicillin and cloxacillin is known to have a synergistic effect (Ayim *et al*., 1990). Its mode of action has also been suggested to be by a combination effect especially when penicillinase producing organisms are involved (Akanni and Ayim, 1992). Here, cloxacillin is believed to block the production of penicillinase while the ampicillin goes to destroy the organism. Whatever the method, the fact remains that the combination as a dosage form will not have any desired effect when either of the combinants is below the standard recommended level. The synergy between the two penicillins, makes the determination of the level of one in the combination tedious especially when using the age long and reliable microbiological method.

The result of all the samples assayed using the spectrophotometric method showed that they all contained acceptable levels of ampicillin in the combination by USP and BP standards (Table 2). The same samples were also assayed using the microbiological method and the results obtained are showed the same (Table 3). The highest difference in the calculated percentage potency of

different bottles of the same batch using the spectrophotomertric method, is that obtained from the first batch of the products of company D which is 9% and that obtained from the 2nd batch of company E using the microbiological assay method which is also 9%. Other batches showed differences within 0-7%. If these errors are either from the weighing, mixing or other manufacturing process, they are within reasonably accepted ranges. The spectrophotometric method as described by Eboka *et al.* (1997) can therefore, be said to be very reliable for the determination of ampicillin levels in its combination with cloxacillin due to its specificity among others. It can very well be applied in ampicillin determination up to a concentration of 91 μg/mL (Fig. 1) which covers the recommended dosage of ampicillin in its combination with cloxacillin and compares very favourably with the microbiological assay method using Bacillus megatharium NCTC $10342A_{76}$ as indicator organism as described by Udobi *et al.* (1994).

CONCLUSION

The spectrophotometric assay method used is not tedious and takes less time when compared with the microbiological method. Generally, the two methods compare favourably. One problem that may be encountered with the spectrophotometric method however, is its high specificity. This is because, a little change in absorbance reading which can result from any mistake in timing or weighing will make an appreciable difference in the corresponding percentage potency. It requires a very high degree of accuracy in all the steps and very skilled personnel if reproducible results are to be obtained. However, the simplicity, straight forward nature and the short time this method takes can be exploited at entry posts for on the spot checks of the levels of ampicillin content in this kind of combinations entering a country or a region.

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