Antibiotic determination of antibiotic susceptibility testing disks using liquid chromatography and microbiological assay

Abstract

Introduction: The aim of this work was to control the quality of some antibiotics cartridges (ampicillin, amoxicillin-clavulanic acid, ciprofloxacin and vancomycin) used for antibiotic susceptibility testing by disk diffusion method. Antibiotics were determined using disks and two techniques were compared for this purpose, chromatographic and microbiological method at constant temperature.

Methods: Chromatographic method (High-Performance Liquid Chromatography: HPLC) was used for determining ampicillin, amoxicillin-clavulanic acid and ciprofloxacin, and microbiological method for vancomycin and ampicillin.

Results: Dosage results reveal that 35% of unexpired cartridges had low content and all the expired AB's disks revealed low results.

Conclusion: This study demonstrated that the content of antibiotics in disks could be decreased if the storage and transport conditions (temperature and relative humidity) of disk cartridges were not respected. Therefore, properly performed quality control of antibiotic disks before use in laboratories would aid in providing accurate and reproducible results of dosage.

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Keywords

Antibiotic Disks; Quality Control; Antibiogram; High Performance Liquid Chromatography; Microbiological Method.

Introduction

Antibiotics are a group of drugs used to treat various infections caused by bacteria and some antibiotics are effective only against specific

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bacteria types. Therefore, antibiotic susceptibility testing (AST) is usually carried out to determine which antibiotic will be most successful in treating a bacterial infection in vivo and the results of the test are reported on antibiogram form with defined categories (susceptible, intermediate and resistant) [1]. Clinicians consider these interpretations to determine which antibiotic might be effective in treating their patients.

There are few tests used in determination of antibiogram of bacteria but the disk diffusion is the most practical method and is still the method of choice for the average laboratory.

In this method, disks impregnated with antimicrobial agents are used. The disks are placed onto agar plates, which are pre-inoculated with the suspension of the microorganism being tested. The basic principle of the method is the diffusion of the antimicrobial agent into the medium, which occurs when the disks come into contact with the moist surface of the plate. The concentration of the agent reduces logarithmically as the distance from the disk is increased. After the incubation period, the plates are observed for the circular inhibition zone created around the disk, which is due to the inhibitory effect of the antimicrobial agent on the microorganism [1].

The procedure is very sensitive to changes in test conditions. Therefore, it is crucial that each variable in the procedure should be standardized and carefully controlled.

Owing to the numerous variables that may affect the results, rigorous quality control is of utmost importance for susceptibility testing.

The objective of this study was to verify the contents of antimicrobial disks used in disk diffusion method. Four antibiotics were used ampicillin, amoxicillin-clavulanic acid, ciprofloxacin and vancomycin, and analyzed by a chromatographic technique (HPLC) and microbiological method[2, 3].

Methods

Instrumentation

The HPLC system (Perkin Elmer Flexar) with UV/Vis detector was used. The device was monitored and processed using Total Chrom software. The cold accumulators were used for transporting the cartridges, a refrigerator (Liebherr Premium) for the conservation, analytical balances (Mettler Toledo AG 285 and Shimadzu BW 4200H) and an autoclave RAYPA AE150.

For the microbiological method, we used an incubator BD BINDER 240, Water bath (Memmert) and microbiological safety cabinet with Bunsen burners.

Chemicals and reagents

All the Reagents and solvents used in our work are HPLC-R.

Antibiotic standards were of high purity grade (> 90%) and were purchased from United States Pharmacopeia (USP) Reference Standards. Distilled water was obtained by the GFL water distiller.

Phosphoric acid H_3PO_4 , chlorhydric acid HCl, acetic acid, sodium dihydrogen phosphate dihydrate ($NaH_2PO_4 \cdot 2H_2O$), monopotassium phosphate KH_2PO_4 , methanol CH_3OH , triethylamine and acetonitrile were provided by Solvachim (Casablanca, Morocco).

Samples

We used ATB disk's cartridges from four health centers provided by the laboratories of medical microbiology as shown in (Table 1).

All cartridges were stored at temperature of + 4°C in the refrigerator.

The methods for determination of ampicillin and ciprofloxacin and the microbiological technique were followed the procedures described in the European Pharmacopoeia monographs EP 8th edition (8.0). While, the method for determining amoxicillin is described in the method published in 2015 by Ramli Y et Al. [4]

Table 1. Sources of disk cartridges

Sources	Ibn Rochd University Hospital		Morocco National Institute of Hygiene	Pasteur Institute of Morocco	Ibn Sina University Hospital	
Manufacturers	Oxoid France	Bioanalyse Turkey	Oxoid France	Oxoid France	Oxoid France	Mast Diagnostics France
Amoxicillin/clavulanic acide (20/10 µg) *	E ₁	E ₂	E ₅ **	E ₈	E ₁₂	E ₁₃
Ampicillin (10µg)	-	-	E ₆ **	E ₉	E ₁₄	E ₁₅
Ciprofloxacin (5 µg)	E ₃	-	-	E ₁₀	E ₁₆	-
Vancomycin (30 µg)	E ₄	-	E ₇ **	E ₁₁	E ₁₇	-

*: Only Amoxicillin were tested. **: Expired disks.

A simple, fast, economical, accurate, precise and reproducible RP – HPLC method with ultra-violet detection at 220 nm, a valid method for the simultaneous determination of Amoxicilline and Clavulanique acid with a good chromatographic separation between both compounds using a reversed phase C18 column and a mobile phase, consisting of Acetonitril/NaH₂PO₄. buffer pH = 4.4). The method was validated in terms of specificity, linearity, precision, accuracy, and robustness. This fully validated method, which allows the simultaneous measurement of amoxicillin and clavulanic acid in different formulations, is rapid (total run time <7 min).

Chromatographic conditions

The mobile phase of chromatographic method was as follow:

For amoxicillin

(90 V; 10V) buffer and acetonitrile R. The buffer was prepared by dissolving 5.15g of sodium dihydrogen phosphate dihydrate (NaH₂PO₄ · 2H₂O) in 1l of H₂O R and adjusted at pH 6.35 (instead of 4.4) for a better separation of the peaks. The solution was mixed, degassed and filtered [4].

For ampicillin

(0.5 ml; 50ml; 50ml; qf 1000ml) diluted acetic acid R, monopotassic phosphate solution 0.2 M R, acetonitrile R and H₂O R. Diluted acetic acid was prepared by dissolving 12 g of acetic acid in 100ml

of H₂O and monopotassic phosphate solution by dissolving 2.72g of monopotassium phosphate in 100ml of H₂O.

For ciprofloxacin

(13 V; 87 V) Acetonitrile R; phosphoric acid R. Phosphoric acid R was prepared by dissolving 2.45 g of phosphoric acid in 1l (one liter) of distilled water and the pH was adjusted to 3.0 using triethylamine R solution.

The microbiological technique includes a buffer was prepared with 50ml of monopotassic phosphate solution 0.2 M R and 29.63 ml sodium hydroxide 0.2 M R for a pH of 7.0 [8].

Preparation and the range of calibration

The chromatographic method was prepared to obtain working standards by the following points of the range:

Amoxicillin: 0.0012 – 0.0024 – 0.0048 – 0.012 – 0.024 – 0.12 mg/ml

Ampicillin: 0.0025 – 0.005 – 0.01 – 0.02 – 0.04 – 0.1-1 mg/ml

Ciprofloxacin: 0.001875 – 0.003 – 0.00325 – 0.005025 – 0.0084 mg/ml

In the microbiological technique, the following points of the range was prepared:

Ampicillin: 2.5 – 5 – 7.5 – 10 – 12.5 µg/ml

Vancomycin: 10 – 20 – 30 – 35 µg/ml

Preparation of samples

We had proceeded as follows for the determination of antibiotics by HPLC:

Amoxicillin

In a volume of 25 ml of H₂O R, we had immersed 25 disks of Amoxicillin/Clavulanic acid with an intense agitation and taking samples successively at 15, 30 and 45 min.

Ampicillin

In a volume of 20 ml of H₂O R, we had immersed 20 disks of ciprofloxacin with an intense agitation and taking samples successively at 15, 30 and 45 min.

Ciprofloxacin

In a volume of 20 ml of H₂O R, we had immersed 15 disks of ciprofloxacin with an intense agitation and taking samples successively at 15, 30 and 45 min.

The microbiological method included five disks of every type of ATB were introduced into a volume of 5 ml with an intense agitation during 45 minutes, and Mueller-Hinton culture medium was used in these experiments.

Results

HPLC analysis

The reference solutions were injected under the previous chromatographic conditions and the retention times were determined: amoxicillin 3.50 min, ampicillin 6.20 min and ciprofloxacin 4.08 min. The antibiotics concentrations in samples (Tables 2, 3 & 4), respectively, were determined using prepared calibration curves for amoxicillin, ampicillin and ciprofloxacin. Figure 1 shows one example for preparation of antibiotic standards.

Microbiological analysis

The antibiotics concentrations in samples (Tables 5 & 6) were determined by microbiological method using the calibration curves Diameter = f (c); for ampicillin and vancomycin.

Figure 1: Standard calibration curve of ampicillin (microbiological method).

Table 2. Amoxicillin concentrations in samples.

	AUC	Concentration		Content/disk	
	U	mg/ml	µg/ml	µg	%
E ₅ *	122 226	0.0086	8.6	8.6	43
E ₁₂	85 380	0.006	6	6	30
E ₁₃	166 895	0.0118	11.8	11.8	59
E ₁	191 249	0.0135	13.5	13.5	67.5
E ₈	85 836	0.006	6	6	30
E ₂	62 150	0.0044	4.4	4.4	22

*: Expired disk

Table 3. Ampicillin concentrations in samples.

	AUC	Concentration		Content/disk	
	U	mg/ml	µg/ml	µg	%
E ₉	4683	0.0029	2.9	2.9	29
E ₆ *	991	0.00073	0.73	0.73	7.3
E ₁₄	19859	0.0118	11.8	11.8	118
E ₁₅	16256	0.0097	9.7	9.7	97

*: Expired disk

Table 4. Ciprofloxacin concentrations in samples .

	AUC	Concentration		Content/disk	
	U	mg/ml	µg/ml	µg	%
E ₃	554628	0.00414	4.14	5.52	110.4
E ₁₀	658196	0.0051	5.1	6.8	136
E ₁₆	656761	0.00509	5.09	6.78	135.6

Table 5. Vancomycin concentrations in samples (Microbiological method).

	Diameter	Concentration	Content/disk	
	mm	µg/ml	µg	%
E ₇ *	16.3	29.18	29.18	97.3
E ₁₁	15.1	23.87	23.87	79.6
E ₁₇	16.7	30.95	30.95	103.17
E ₄	16.7	30.95	30.95	103.17

*: Expired disk

Table 6. Ampicillin concentrations in samples (Microbiological method).

	Diameter	Concentration	Content/disk
	mm	µg/ml	%
E ₆ *	14	0.5	5.1
E ₁₄	23.6	11.62	116.2
E ₁₅	21.8	9.54	95.4
E ₉	15.7	2.48	24.77

*: Expired disk

Table 7. Ampicillin concentrations in samples determined by chromatographic and microbiological techniques.

	HPLC	Microbiology	Difference of content	
	µg	µg	µg	%
E ₁₄	11.8	11.62	0.18	1.8
E ₁₅	9.7	9.54	0.16	1.6
E ₉	2.9	2.48	0.42	4.2
E ₆	0.73	0.5	0.23	2.4

Discussion

Amoxicillin - Clavulanic acid (20/10 µg)

Only amoxicillin was determined. All the results gave values lower than the reference indicated on the cartridges (20µg). The decrease in content varied between 32.5 and 78% (Table 2). It could be attributed to the hydrolysis of betalactamin, particularly of penicillin due to their known fragility.

Ampicillin (10µg)

We obtained two results with normal deviations (3 and 18%) and for the two others, E9 and E6 (expired cartridges), we had very low values and they contents were respectively 29% and 7.3%.

(Table 3)

For E9, the decrease of the content could be caused by the non-respect of the conditions of the conservation and transport of the cartridges.

The expired cartridges were included in our work in the aim to prove their nonconformity for antibiogram tests.

Ciprofloxacin (5 µg)

One sample gave normal content and the two others values were superior to the normal content (5µg). These values were 35.6 and 36% superior to reference. (Table 4)

Vancomycin (30 µg)

In the European Pharmacopoeia 8th Edition, there is no monograph for physicochemical determination of vancomycin; therefore, our reference in this work was the microbiological determination monograph.

The results were conform for three samples (E4, E11 and E17) and out of range for the fourth corresponding to expired cartridge E7 (Table 5). This technique was able to give us valuable results and prove that the expired cartridges are not valid.

Expired disks

All the results gave lower values than the reference. That is logically normal. Moreover, the results show that the content of expired disks decrease proportionally to the expired date.

Unexpired disks

In this work, the results of determination of antibiotics were diverse, ranging from normal to lower values.

The decrease of contents can be explained by poor or inappropriate storage conditions (tempera-

ture and relative humidity), during the transport, the delivering circuit or during their use.

It is also important to have a desiccant in cartridges because -after opening- the disks could be exposed to an inappropriate atmosphere and an eventual absorption of moisture that could deteriorate their quality and decrease the antibiotic content. The same consequences could be observed when the disks were having a long stand in a non-sterile area (microbiological hood, near a Bunsen burner), that is why it is indicated on some of the notices to not exceed one week of use after opening the cartridges.

Comparison of ampicillin determination by the two methods

The physicochemical method (HPLC) has been used since 1970's and it is a technique for high precision determination [5, 6, 7].

The difference between the results obtained by physicochemical and microbiological technics in the ampicillin determination is not significant (**Tables 6 & 7**).

The microbiological test was done ten days after chromatography (HPLC). Moreover, a slight decrease of the content was observed in the microbiology results and confirm the change in the quality of disks after seven days opening, although the storage conditions were respected.

These methods can be complementary [8], and the HPLC, which is more precise, can be used to confirm or to cancel microbiology results in case of doubt.

Conclusion

The present work reveals the risk to find decrease content of antibiotic in the disks of antibiogram. Therefore, we must always apply strictly the storage conditions; temperature and relative humidity as well as conditions of the transport. In addition, a standard control bacteria strains should be used

each time when susceptibility test done in vitro [9]. The cartridges should be used according to the expire date of the supplier recommendations. The expired disks must not be used, since our study demonstrated a clear decrease of their contents and they could give consequently false results. This study also shows the importance of correlation between chromatographic and microbiological methods and how it is important to encourage regular disk controls in all laboratories.

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