بسم الله الرحمن الرحيم

MEGBI Antibiotics/Antibodies Production Pilot Plant (MEGBI-APP)

- 7th Project Report (Mar 2019- Dec 2019) -

- thin-layer chromatography to reveal presence of penicillin
- quantitative and qualitative determination of penicillin
- Supplement on large scale penicillin synthesis

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مركـز أبحـاث الشـرق الأوسـط للجينـات والتقنيـة البيولوجية

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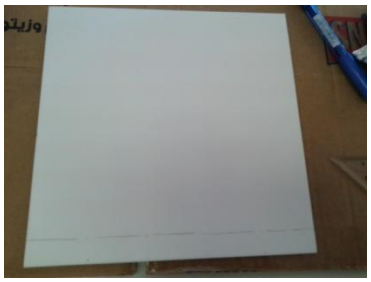
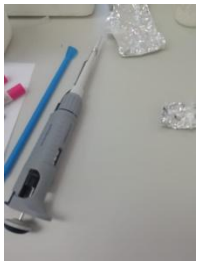
1.1 Devices


HCl ,iodine
silica gel paper
KI , ethylacetate ,ethanol
Penicilline stander
Micropipet,

2 Working methods in diagnoses to penicillin

2.1 Thin-layer chromatography to reveal presence of penicillin[1]

Purpose: The paper describes some thin layer chromatographic procedures that allow simple and rapid separation and identification of penicillins and cephalosporins from complex mixtures. ***Methods:*** Using silicagel GF254 as stationary phase and selecting different mobile phases we succeeded in the separation of the studied beta-lactamins. Our aim was not only to develop a simple, rapid and efficient method for their separation but also the optimization of the analytical conditions. ***Results:*** No system will separate all the beta-lactams, but they could be identified when supplementary information is used from color reactions and/or by using additional chromatographic systems. ***Conclusion:*** The right combination of solvent system and detection method allows the identification of the studied penicillins and cephalosporins and can be successfully used in the preliminary analysis beta-lactam antibiotics.

Materials and Methods

Instrumentation

The TLC system consisted of a Camag Nanomat III automatic sampler, a Camag Linomat IV semiautomatic sampler (Camag, Switzerland), a 2-ml Hamilton microsyringe (Hamilton, USA), a Camag Normal Development Chamber and a Camag fluorescence inspection lamp (Camag, Switzerland). As stationary phase we used 10x20 and 20x20 cm pre-coated silicagel GF254 HPTLC glass plates (Merck, Germany).

Reagents

Penicillins: amoxicillin trihydrate, ampicillin trihydrate, benzylpenicillin sodium, oxacillin sodium (Antibiotice Iaşi, Romania). Cephalosporins: cefalexin monohydrate, cefadroxil monohydrate, cefaclor monohydrate (Sandoz, Romania), cefuroxim sodium (Medochemie, Cyprus), ceftazidim pentahydrate, ceftriaxon sodium (Antibiotice Iaşi, Romania). All the studied beta-lactams were of pharmaceutical grade.

Reagents: acetone, acetic acid, benzene, butanol, ethanol, ethyl acetate, formaldehyde, methanol, sulphuric acid (Reactivul Bucureşti, Romania). All reagents were of analytical grade.

[1] Source ?

Samples

PEN and OXA, were used as sodium salts, consequently samples were prepared in water at a concentration of 0.2%. AMP and AMO, used as trihydrates, exhibit poor solubility in water; consequently samples of 0.2% were prepared in a 2% sodium bicarbonate solution. Cephalosporin samples were prepared by dissolving the substances in methanol and then diluting with water (1:1). Amounts of 0.5 ml were applied on the chromatoplates using a Hamilton syringe.

Method

The chromatographic chambers were saturated with the mobile phase for 30 minutes. The plates were developed over a distance of 15 cm in filter-paper-lined chromatographic chambers, dried in a stream of hot air, and examined under UV radiation at wavelengths of 254 and 366 nm. The spots were then visualized by placing the plates in a chromatographic chamber saturated with iodine vapors. Some specific in situ color reactions were used in order to increase specificity of the method. All experiments were carried out at room temperature. Photographs of the chromatoplates were taken with a Nikon D-3100 camera, equipped with a UV filter.

Chromatographic detection procedure

Three detection procedures were used; first with iodine vapors and then using in situ plate color reactions with iodine and ninhydrine, after an alkaline hydrolysis.

A few iodine crystals were placed on the base of tightly sealed chromatographic chamber, stored in a fume cupboard. After a few hours during which violet iodine vaporizes and distributes itself homogenously throughout the interior of the chamber, the chromatographic plates were introduced in the chamber. After 30 minutes the plates were sprayed with a 1% starch solution.

Chromatograms were first sprayed with a 1N sodium hydroxide solution, in order to hydrolyze the beta-lactam ring, and after 15 minutes with a solution containing 0.2 g potassium iodine, 0.4 g iodine dissolved in 20 ml ethanol and 5 ml 10% hydrochloric acid.

Chromatoplates were first sprayed with a 1N sodium hydroxide solution, in order to hydrolyze the beta-lactam ring, and after 15 minutes with a 0.1% ninhydrine solution in ethanol, and heated in an oven at 120 °Cfor 10 minutes.

Results and Discussion

The purpose of the method (simultaneous separation of a multicomponent mixture), and the information about the samples (structure, polarity, solubility, stability) were important as initial hints for the choice of the chromatographic system, using the rule of the Stahl's triangle.[2,10,11]

The most widely used stationary phase for the analysis of beta-lactam is silicagel, but if we consult the literature reversed-phase or cellulose plates have also been used. Silicagel surface bears Si-OH groups capable of hydrogen bonding with polar substances. Mobile phases for the separation of both penicillins and cephalosporins are polar, usually containing variable quantities of water.[5-7,12]

An acid (acetic acid) was added to the mobile phase in order to avoid decomposition of the beta-lactam ring on silicagel.

Around twenty solvents were tested and six mobile phases were selected (Table 1)

Table 1

The selected mobile phases

No	Mobile phases (V/V)
I	butanol – water – ethanol – acetic acid 50:20:15:15
II	butanol – water –acetic acid 60:20:20
III	ethyl acetate – water – acetic acid 60:20:20
IV	ethyl acetate – methanol – acetic acid 45:50:5
V	acetone – acetic acid 95:5
VI	acetone – benzene – water – acetic acid 65:14:14:7

All beta-lactams can be detected in UV light at 254 nm (green fluorescence) and 366 nm (blue fluorescence). Applying reagents such as ninhydrin or exposing the chromatoplate to iodine vapor can diminish the detection limit.

(Hancu et al., 2013)

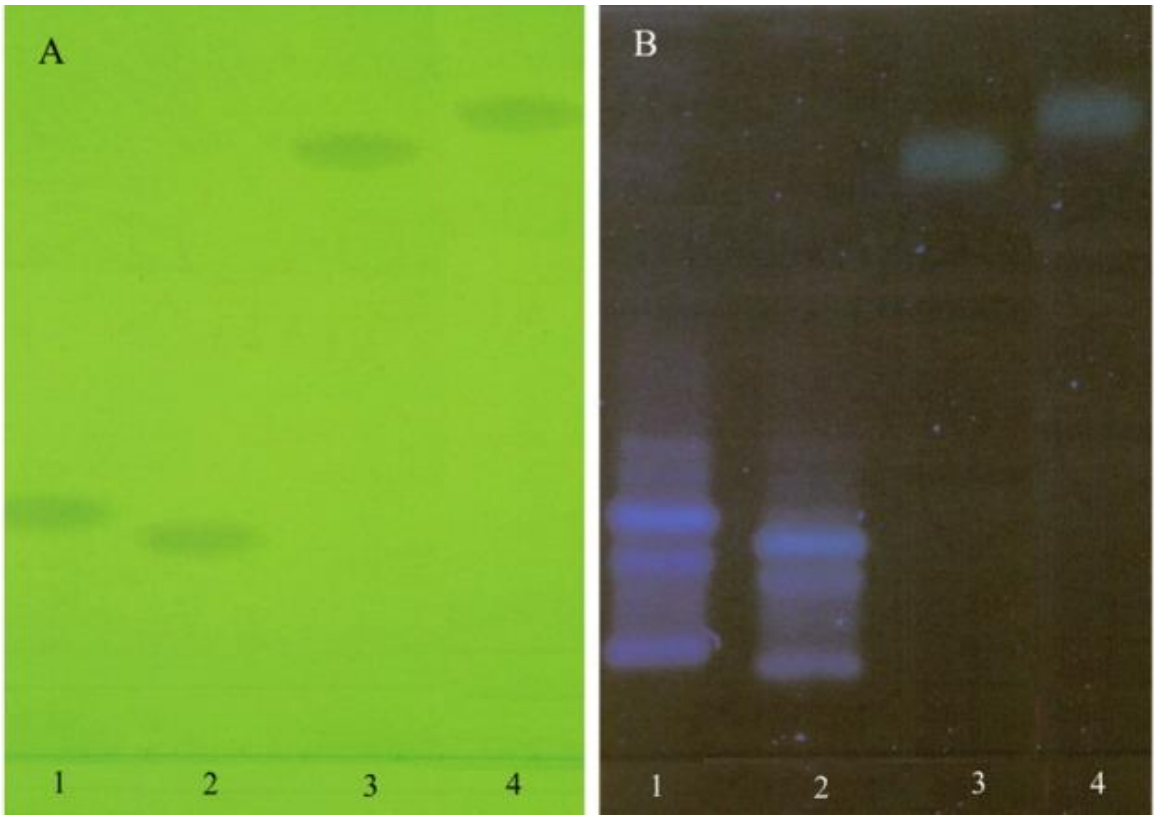


Fig 1 Chromatogram obtained at the separation of penicillins using mobile phase III (ethyl acetate – water – acetic acid 60:20:20), detection in UV light at 254 nm(A) and 366 nm(B) (1 - AMP, 2 - AMO, 3 – PEN, 4 - OXA)

(Hancu et al., 2013)

2.2 PREPARATION OF THE STOCK TEMPLATE SOLUTION

For the preparation of the stock control solution, benzyl penicillin potassium is required as a pure powder commercially available, a finished product or suitable raw material of good quality (> 85%) for reference purposes. . Place an aluminum foil on the measuring plate of the supplied electronic pocket scale, set the zero and measure approx. 0.3 g appropriately. Of benzyl penicillin potassium using a spatula. Carefully empty the aluminum foil over a 10 ml laboratory glass vial and rinse all the resulting powder with 5.7 ml of water using a graduated pipette. Record each time the exact weight obtained and adjusts the amount of water suitable for dissolution using, for example, 5.5 ml of water for 0.29 g or 6.1 ml of water per 0.32 g of water. Control substance collected from the main container respectively. Close the laboratory bottle and shake until

Dissolution the solids. The final solution obtained should contain 50 mg of benzyl penicillin sodium equivalents per ml and be labeled as a Penicillin G Stock Control Solution. Prepare this solution only just before each test. Important Note: The scales supplied cannot weigh exactly less than 0.25 g. The relative standard deviation of +/- 2% is considered too high. For the measurement of higher quantities, the difference is only about +/- 1%. The scale will not record changes of a few milligrams added or subtracted approaching the target weight of 0.3 g. Then remove the aluminum foil or lightly pat the scale pan with a pencil or spatula whenever a few milligrams have been added or subtracted to compensate for dynamic inertia and ensure correct readings.

(Jähnke, s. d.)

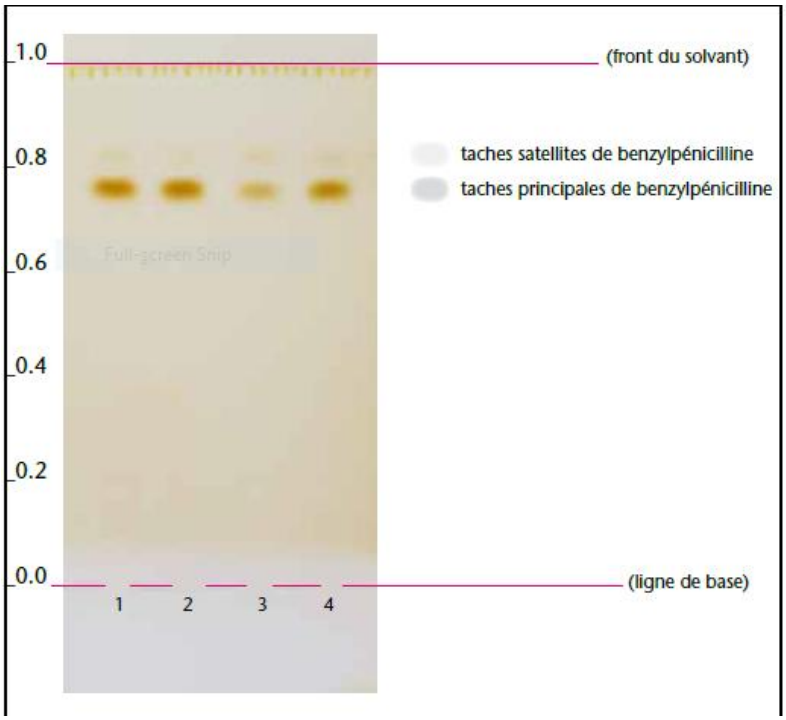


Fig3 : chromatoplaque observed in the light of the day after coloring in iode (Jähnke, s. d.)

2.3 Thin layer chromatography: the Rf or retention factor

In given conditions

nature (and composition) of the solvent
nature of the adsorbent
thickness of the absorbent layer
amount of sample deposited

the Rf of a substance is a characteristic constant as well as a melting temperature for example. His determination can therefore be valuable for identification.
Rf is determined by the ratio Rf in which

$$Rf = \frac{d}{d_s}$$

d represents the distance covered by the substance
and ds the distance traveled by the solvent.

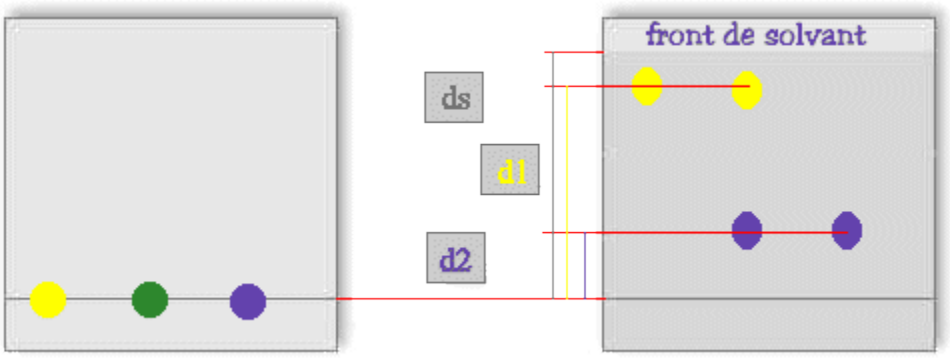
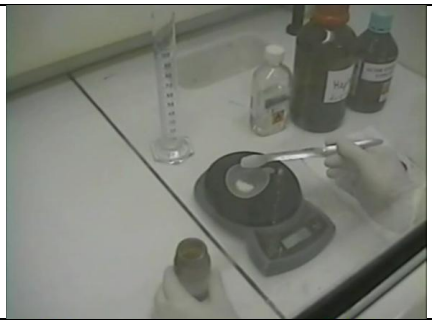
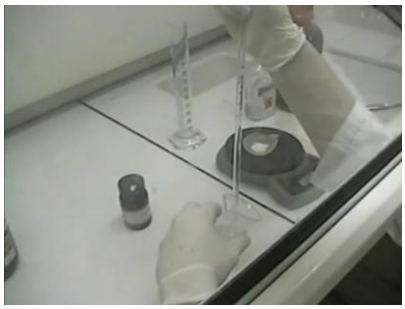
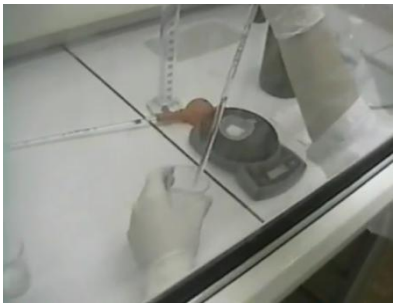
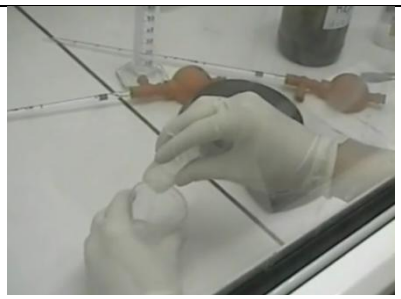
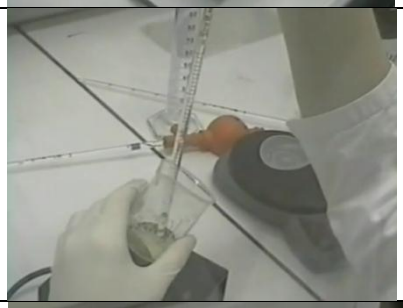
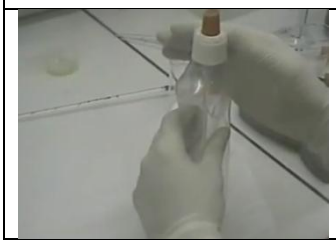


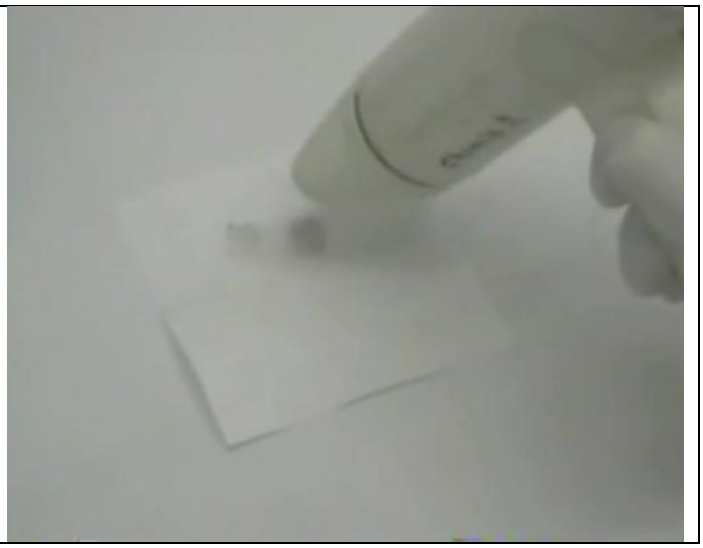
Fig 2: This technique has limitations related to the difficulty of obtaining reproducible conditions

(« CCM : Calcul du facteur de retention Rf », s. d.)

2.4 Preparation de colorant ninhydrine

On commence par peser 1g de ninhydrine	
Puis on prélève 2ml d'acide acétique cristallisant	
Ensuite on prélève 4ml d'éthanol	
On l'ajoute à l'acide acétique	

On ajoute la ninhydrine à la solution de travail	
On homogénéise la solution avec un agitateur magnétique	
On prélève 2ml de la solution de travail	
On ajoute 3,33ml d'éthanol à la solution de travail	
	

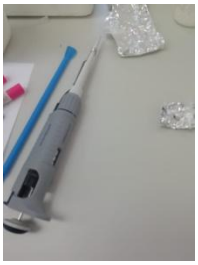
Enfin on obtient des empreintes colorées au pourpre de Ruhemann	

https://youtu.be/Gz6Vlu4h72M

3 Results: Diagnostic

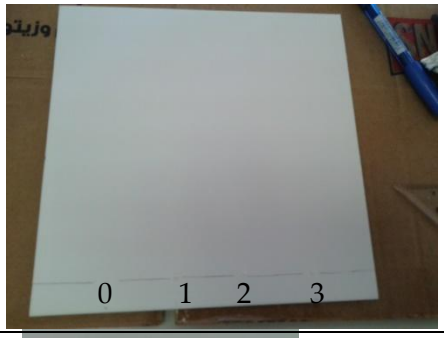
Done in the laboratory of chemistry in Lebanese university (LU)

3.1 Experiment 1: Preparation of TLC silica gel



echantion of penicilline 0 1 2 et 3

micropipette

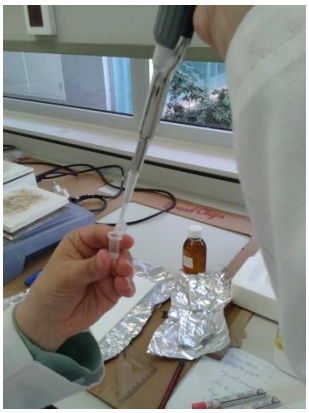


penicilline solution 0 stander

penicilline solution 1

penicilline solution 2

penicilline solution 3

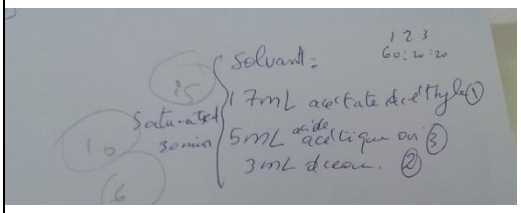


prelevation of penicilline solution

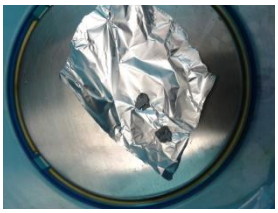
depot of penicilline

0 1 2 3

3.2 Experiment 2: preparation of solvant



3.3 Experiment 3: Preparation of color revelution



containing 0.2 g potassium iodine, 0.4 g iodine dissolved in 20 ml ethanol and 5 ml 10% hydrochloric acid.

hydrochloric acid

ethanol

4 Discussion

- Experiment n 1

We left the silicagel paper two days and this led to the rise of the solvent in a zigzag line also due to the lack of equivalent a quantity of solvent with the size of the silicagel paper

تركنا ورقة السيليس يومين وهذا ادى الى صعود المذيب بخط متعرج السبب يعود ايضا الى عدم تكافئ كمية المذيب مع حجم ورقة السيليس

This penicillin that we used as a reference wasn't powder, but it was a small, thin disc that was used for microbiology (antibiograme)

هذا والبنسلين الذي استعملناه ك مرجع لم يكن بودرة ولكن كان عبارة عن ديسكات صغيرة ورفيعة ك التي تستعمل للميكروبيولوجي

- Experiment n 2

The lack of equivalent a quantity of solvent with the size of the silica gel paper

- Experiment n 3

The quantity of HCl used was too much.
I didn't spray the paper with it
Because the acid damages the silicagel

We can use uv rays instead of color. Examined under UV radiation at wavelengths of 254 and 366 nm.

5 Explication from synthese on large scale

1 incubation 7 days

Inoculum Penicillium + medium (peptone lactose glucose eau distillee KH2PO4 MgCl)

Materials used for the manufacture of liquid medium:

2g glucose + 2g lactose (milk) + 1g Amino

0.1g MgCl2 + 0.1g kCl + 0.5g KH2PO4 + 100ml distilled water (small scale)

4

filtration

5

Incubation with éthyle acétate (vinaigre +éthanol)

Dumping the content

incubation in refrigerator some days

Following the protocol we put (5gsmall skill) of sodium bicarbonate

2

Charcoal treatment

Incubation 1 hour

0.43 g charcoal treatment+ 0.5g KH2PO4 acid such as phosphoric acid are introduced as pH will be as high as 8.5. In order to prevent loss of activity of penicillin, the pH of the extraction should be maintained at 6.0-6.5.

3

pumping

References

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