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Notes

Separation and Detection of Certain β-Lactam and Fluoroquinolone Antibiotic Drugs by Thin Layer Chromatography

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Introduction

The misuse of antibiotics is frequent and has several undesirable consequences. It is expensive and may obscure the nature of the illness and delay recovery. The most important example of misuse is the treatment of fever without attempting to make a specific diagnosis, an improper dose and the route of administration, the use of two or more drugs when only one is necessary, and the use of a new antibiotic without becoming familiar with its properties. Fatal cases due to penicillin injections are well known. The main reason behind this type of case is the non-performance of preallergy prior to injection. Such medico legal cases are received from modern urban areas as well as from rural areas. Cases of abuse of antibiotic drugs when submitted to laboratories cause problems to the analyst in getting rapid and correct results. Although a review of the literature has revealed that attempts were carried out by earlier workers to find some suitable methods using the TLC technique, not much in the way of a systematic approach has been done.

Ampicillin, amoxycillin, benzylpenicillin, benzathine penicillin, becampicillin, cloxacillin, cephalexin, cefotaxime, ceftriaxone, cefadroxil, cefazolin and penicillin G procaine are semi-synthetic cephalosporin β-lactam antibiotics that are active against both Gram-positive and Gram-negative bacteria[1] and are widely used for the treatment of infections.

Ciprofloxacin (CFLX), norfloxacin (NFLX), enrofloxacin (EFLX), sparfloxacin (SFLX), ofloxacin (OFLX) and pefloxacin (PFLX) are fluorinated quinolone synthetic broad-spectrum antibacterial drugs. These fluorinated quinolones are active against many Gram-positive and Gram-negative bacteria through inhibition of their DNA gyrase.[2]

A literature survey has revealed various TLC methods for the selective separation and simultaneous determination of β-lactam[3-10] and fluoroquinolone[11-16] antibiotic drugs from biological fluids and pharmaceutical formulations. Wagman and Weinstein[17] reviewed TLC procedures useful for the identification and differentiation of numerous antibiotics, including penicillins. Belal *et al*.[3] reviewed fluoroquinolone antibiotic drugs up to 1998. Sherma and Fried[18] providing more detailed information on the TLC of antibiotics. Hendrickx *et al*.[19] described the separation of 18 penicillins on silica gel and silanized silica gel using 35 mobile phases. Official microbiological methods for determining these drugs suffer from high variability and time-consuming procedures. To overcome this lack of sensitivity and selectivity, thin-layer chromatographic (TLC) and liquid-chromatographic (LC) methods have been developed, which have been reviewed by Moats.[20]

The present work was undertaken in order to use unimpregnated, thin layers of silica gel F_{254} as an adsorbent and to develop economical and convenient methods for the separation of β-lactam and fluoroquinolone antibiotic drugs.

Experimental

Materials

Silica-gel 60 F_{254} precoated commercial plates (20 × 20 cm, 0.25 mm thickness) were supplied by Merck (Darmstadt, Germany). A 10-µl Hamilton syringe calibrated at 0.1 µl intervals was used.

Reagents and chemicals

All of the solvents and reagents used were of analytical reagent grade. Deionized water was used to prepare all solutions. Freshly prepared solutions were always employed.

Preparation of reagents

Iodine azide solution. First, 1.269 g of resublimed iodine was dissolved in 2.5 ml of a solution containing 1.5 g of potassium iodide, which was free from iodate when dissolved after swirling dilute to 100 ml, to prepare a 0.1 N iodine solution. Then, 3.5 g of sodium azide was dissolved in 100 ml of a 0.1 N iodine solution (solid iodine azide is explosive). It was freshly prepared.

Starch solution (1% w/v). After dissolving 1 g of starch in 100 ml of distilled water, it was gently heated.

Ninhydrin reagent. After dissolving 0.1 g of ninhydrin in 50 ml of ethanol (96%), 10 ml of glacial acetic acid was added.

Ferric chloride reagent (0.5% w/v). We dissolved 0.5 g of anhydrous ferric chloride in 0.5 M hydrochloric acid in a 100 ml calibrated flask.

Preparation of standard solutions. Stock solutions of pure ampicillin, amoxycillin, benzylpenicillin, benzathine penicillin, becampicillin, cloxacillin, cephalexin, cefotaxime, ceftriaxone, cefadroxil, cafazolin and penicillin G procaine were prepared by dissolving exactly 0.5 g of each in 10 ml of methanol.

Stock solution of pure CFLX, NFLX, EFLX, SFLX, OFLX

Table 1 Developing solvent system for β-lactam & fluoroquinolone antibiotic drugs

Sl. No.	System	Developing solvent	Composition[a](v/v)
1	A	Acetone–methanol	50 + 50
2	B	Isopropanol–methanol	30 + 70
3	C	Butanol–ethanol–water	35 + 35 + 30
4	D	Butanol–pyridine–water	35 + 35 + 30
5	E	Butanol–acetic acid–water	40 + 10 + 50
6	F	Ethylacetate-acetone–water–acetic acid	50 + 25 + 15 + 10
7	G	Acetone–chloroform–DMF–acetic acid	40 + 35 + 15 + 10
8	H	*n*-propanol–water	70 + 30
9	I	Methanol–chloroform–water–pyridine	45 + 40 + 15 + 5
10	J	Butanol–water–DMSO–acetic acid	60 + 15 + 20 + 5
11	K	Acetone–acetic acid	80 + 20
12	L[b]	Dichloromethane–methanol–ammonium hydroxide–acetonitrile	40 + 40 + 20 + 10
13	M	Chloroform–methanol–toluene–diethylamine	40 + 40 + 20 + 14
14	N	Acetonitrile–2% acetic acid	15 + 85
15	O	Methanol–water–diethylamine	75 + 25 + 10
16	P	Chloroform–ethanol–toluene–triethylamine–dichloromethane	40 + 40 + 20 + 10 + 10
17	Q	Methanol–acetonitrile–5N acetic acid–dichloromethane	30 + 10 + 50 + 10
18	R	Butanol–acetic acid–water	40 + 10 + 50
19	S	Chloroform–methanol–ethylacetate–THF–aq.ammonia	50 + 30 + 20 + 10 + 10
20	T	Chloroform–methanol–ethylacetate–THF–aq.ammonia	50 + 40 + 10 + 20 + 10

DMF, dimethylformamide; DMSO, dimethyl sulfoxide; THF, tetrahydrofuran.

a. The developing solvents are expressed as parts, not percentages.

b. Developer containing volatile materials such as ammonium hydroxide should be prepared just prior to use and not stored for future. Ammonium hydroxide has been prepared as 1.5 ml aq. ammonia in 3.5 ml of distilled water.

and PFLX were prepared by dissolving exactly 0.5 g of each in 10 ml of methanol.

Working solutions were prepared as required by dilution. These solutions were stored in well-closed vessels and direct contact with light was avoided.

Procedure

Silica gel 60 F_{254} precoated commercial plates (size, 20 × 20 cm and a layer thickness of 0.25 mm) from E-Merck, (Darmstadt, Germany) were used. Each plate was activated at 110˚C for about 30 min before use. The plates were divided into strips 1.5 cm wide, and solute spots were applied using a calibrated Hamilton Syringe. Then, 2 – 10 µl each of the drug solutions were spotted on the TLC plates as separate spots. The plates were developed in a closed glass sintered chamber containing developing solvents (listed in Table 1), having 30 min prior saturation at 25 – 30˚C temperature. The solvent in the chamber was allowed to reach the lower edge of the adsorbent, though the spot points were not allowed to be immersed. The cover was put in place, and the system was maintained until the solvent ascended to a point 10 to 15 cm above the initial spots; this usually required about 30 to 90 min. After a plate had been run, the plate was removed from the developing chamber and dried in air. Non-destructive procedures, such as the use of ultraviolet light (both 254 nm and 356 nm) were then used for the localization of separated spots. After that plates were sprayed by two different methods using chromogenic reagents: (i) iodine azide reagent followed by a 1% starch solution (Table 2, systems A to I) and (ii) ninhydrin reagent heated at 110˚C for the best color development (Table 2, systems J and K) for the detection of β-lactam antibiotic drugs. Ferric chloride spray reagent was used for the detection of fluoroquinolone antibiotic drugs. The R_F values for the separated drugs were recorded. All experiments were carried out at room temperature.

Results and Discussion

Silica gel 60 F_{254} was used as an adsorbent. The use of adsorbents containing fluorescent inorganic pigments is advisable for the detection of substances which absorb in the UV-region. The great advantage of this method is that the separated materials can be detected without being chemically modified in any reaction.[21] There is virtually no difference in the performance between fluorescent and non-fluorescent plates, though the former has the advantage of visibility of a spot under ultraviolet light.

The R_F values in various solvents and the colors developed at each stage for β-lactam and fluoroquinolone antibiotic drugs are given in Tables 2 & 3. The developing solvent systems are given in Table 1. Solvent systems A to I were proposed for β-lactam antibiotic drugs and visualizations of the separated drugs were performed by both short and long wavelength UV (254 nm & 356 nm) and also sprayed with iodine azide reagent followed by a starch solution. The iodine azide reagent is decolorized by sulfur containing penicillins and cephalosporins; white spots or white spots with a brown or yellow background are formed. In solvent systems J and K, the separated spots on the TLC plate were visualized by spraying ninhydrin reagent followed by heating the plate at 110˚C for 10 min for best color development. All of the drugs gave a pink spot in solvent system J, whereas in solvent system K all of the drugs gave a rose color spot. It has been observed that out of the eleven solvent systems for β-lactam antibiotic drugs, a minimum of three solvent systems is sufficient for separation and identification.

Solvent systems L to T were used for fluoroquinolone antibiotic drugs. All of the fluoroquinolone antibiotic drugs were located by both short and long wavelength UV (254 nm & 356 nm) and the R_F values have been recorded. The separated spots on TLC plate were also visualized by spraying 0.5% ferric chloride in hydrochloric acid in solvent systems L to P. It was observed that out of the nine solvent systems (L to T), the best separation was in seven solvent systems, except N and T. The solvent systems studied in this work were not reported earlier.

Conclusions

Concerning the results, it is apparent that TLC is the method of choice to confirm the presence of β-lactam and fluoroquinolone antibiotic drugs. Thus, this method is well suited to analyze these antibiotic drugs. The separation and simultaneous detection of six fluoroquinolone antibiotic drugs by TLC was achieved in this work, which has not been previously reported.

The purpose of this investigation was to report on the separation and detection of twelve types of β-lactam and six types of fluorinated quinolone antibiotic drugs using various solvent systems that would rapidly separate these drugs. Hence, this approach could also be applied to detection in drug abuse cases in forensic science laboratories as well as pharmaceutical formulations.

Table 2 R_F-values of certain β-lactam antibiotic drugs on silica gel 60 F_{254}

Drug	$R_F \times 100$					System D			System E		System F		
	System A	System B	System C	Color in UV-short (254 nm)	Color in NaN_3-I_2/ starch	$R_F \times 100$	Color in UV-short (254 nm)	Color in NaN_3-I_2/ starch	$R_F \times 100$	Color in NaN_3-I_2/ starch	$R_F \times 100$	Color in NaN_3-I_2/ starch	Color in UV-short (254 nm)
Benzyl penicillin	0.65	0.63	0.70	Brown	White spot with brown background	0.83	Yellow	White spot with yellow background	0.50(0.58)	White	0.76	White	Brown
Benzathine penicillin	0.69(0.83)	0.64(0.75)	0.69	Brown	-do-	0.64 (0.84) (0.89)	Yellow	-do-	0.52(0.58) (0.74) (0.90)	White	0.79	White	Brown
Amoxycillin	0.68(0.82)	0.61(0.75)	0.80	Brown	-do-	0.84	Yellow	-do-	(0.55(0.65) (0.82)	White	0.85 (0.98)	White	Brown
Ampicillin	0.72(0.83)	0.61(0.75)	0.75	Brown	-do-	0.84	Yellow	-do-	0.64(0.82)	White	0.86 (0.98)	White	Brown
Cloxacillin	0.84	0.75	0.72	Brown	-do-	0.82	Yellow	-do-	0.48	White	0.96	White	Brown
Cephalexin	0.64	0.5(0.57)	0.67	Brown	-do-	0.69 (0.82)	Yellow	-do-	0.50	White	0.68 (0.97)	White	Brown
Cefotaxime	0.81	0.71	0.57	Brown	-do-	0.84	Yellow	-do-	0.56	White	0.90	White	Brown
Ceftriaxone	0.64	0.63	0.45	Brown	-do-	0.78	Yellow	-do-	0.42	White	0.09 (0.85)	White	Brown
Cefadroxil	0.68	0.58	0.47	Brown	-do-	0.82	White	-do-	0.44(0.92)	White	0.70	White	Brown
Cefazolin	0.81	0.71	0.55	Brown	-do-	0.85	White	-do-	0.49	White	0.92	White	Brown
Penicillin G. Procaine	0.83	0.71	0.60	Brown	-do-	0.94 (0.66) (0.48)	Intense Yellow	-do-	0.86	White	0.72	White	Brown
Becampicillin	0.72	0.71	0.58	Brown	-do-	Nil	NCD	NCD	0.86	White spot with yellow background	0.96	White	Brow

Drug	System G			System H			System I		System J		System K	
	$R_F \times 100$	Color in NaN_3-I_2/ starch	Color in UV-short (254 nm)	$R_F \times 100$	Color in NaN_3-I_2/ starch	Color in UV-short (254 nm)	$R_F \times 100$	Color in NaN_3-I_2/ starch	$R_F \times 100$	Color in ninhydrin reagent	$R_F \times 100$	Color in ninhydrin reagent
Benzyl penicillin	0.01	White	Brown	0.91	White	Pink	0.63 (0.73)	White spot with yellow shade	0.26	Pink	0.22	Rose
Benzathine penicillin	0.07	White	Brown	0.87 (0.62)	White	Pink	0.75	-do-	0.25	Pink	0.05 (0.89)	Rose
Amoxycillin	0.01(0.02) (0.04) (0.06)	White	Brown	0.84 (0.92)	White	Pink	0.67 (0.73)	Yellow spot (intense)	0.10	Pink	0.86 (0.91)	Rose
Ampicillin	0.23(0.38) (0.88)	White	Brown	0.80 (0.90)	White	Pink	0.73	White spot with yellow shade	0.06	Pink	0.88	Rose
Cloxacillin	0.13	White	Brown	0.91	White	Pink	0.75	-do-	0.23	Pink	0.88	Rose
Cephalexin	0.20	White	Brown	0.64 (0.75) (0.94)	White	Pink	0.67	-do-	0.13	Pink	0.33	Rose
Cefotaxime	0.20(0.63)	White	Brown	0.57 (0.87)	White	Pink	0.75	-do-	Nil	NCD	0.77	Rose
Ceftriaxone	0.03	White	Brown	0.62 (0.87)	White	Pink	0.64	-do-	0.09	Pink	Nil	NCD
Cefadroxil	0.13	White	Brown	0.76	White	Pink	0.60	-do-	0.10	Pink	Nil	NCD
Cefazolin	0.57	White	Brown	0.86	White	Pink	0.70	-do-	Nil	NCD	0.69	Rose
Penicillin G. Procaine	0.25(0.88)	White	Brown	0.26 (0.91)	White	Pink	0.75	-do-	Nil	NCD	0.11 (0.91)	Rose
Becampicillin	0.89	White	Brown	0.72 (0.96)	White	Pink	0.65	-do-	Nil	NCD	Nil	NCD

NCD, no color development.

Table 3 R_F values of certain fluoroquinolone antibiotic drugs on silica gel 60 F_{254}

Drug	System L			System M			System N			System O		System P	
	$R_F \times 100$	Color in UV-short (254 nm)	Color in 5% $FeCl_3$	$R_F \times 100$	Color in UV-short (254 nm)	Color in 5% $FeCl_3$	$R_F \times 100$	Color in UV-short (254 nm)	Color in 5% $FeCl_3$	$R_F \times 100$	Color in 5% $FeCl_3$	Color in UV-short (254 nm)	$R_F \times 100$	Color in UV-short (254 nm)
Ciprofloxacin (CPLX)	0.56	Pink	Yellow	0.54	Violet	Brownish Yellow	0.24	Violet	Purple	0.32	Yellow	Violet	0.28 (0.83)	Brown
Enrofloxacin (EFLX)	0.32	Pink	Yellow	0.36	Violet	Brownish Yellow	0.26	Violet	Purple	0.28	Yellow	Violet	0.15 (0.83)	Brown
Sparfloxacin (SFLX)	0.74	Pink	Yellow	0.70	Violet	Brownish Yellow	0.20	Violet	Purple	0.48	Yellowish brown (streaking)	Violet	Nil	NCD
Norfloxacin (NFLX)	0.78	Pink	Yellow	—	NCD	NCD	0.18	Yellow	Brown	0.38	Brown	Yellowish Brown	0.83	Brown
Ofloxacin (OFLX)	0.58	Pink	Yellow	0.58	Violet	Brownish Yellow	0.18	Light violet	Yellow	0.30	Yellowish brown	Violet	0.60	Brown
Pefloxacin (PFLX)	0.65	Pink	Yellow	0.52	Violet	Brownish Yellow	0.16	Violet	Purple	0.24	Yellow	Violet	0.53	Brown

Drug	$R_F \times 100$				Color in UV-short (254 nm)
	System Q	System R	System S	System T	
Ciprofloxacin (CPLX)	0.57	0.42	0.35(0.60)	0.23	Brown
Enrofloxacin (EFLX)	0.52	0.36	0.22	0.09	Brown
Sparfloxacin (SFLX)	0.50	0.32	0.62	0.23(0.57)	Brown
Norfloxacin (NFLX)	0.56	0.46	0.63	0.67	Brown
Ofloxacin (OFLX)	0.47	0.27	0.45	0.64	Brown
Pefloxacin (PFLX)	0.43	0.26	0.40	0.23(0.49)	Brown

NCD, no color development.

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