

SPECTROPHOTOMETRIC ANALYSIS AND IDENTIFICATION OF SOME β -LACTAM ANTIBIOTIC DRUGS USING CHROMATOGRAPHIC TECHNIQUE

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ABSTRACT

The procedures that allows easy and rapid separation and identification of beta-lactam antibiotics (penicillins and cephalosporins) from complex mixtures has been described in this work which involves the method of thin layer chromatographic (TLC) analysis and spectrophotometric techniques: Using silica gel GF254 as the stationary phase with different mobile phases. A cost effective and efficient analytical method for the separation and identification of the studied beta-lactam antibiotics was developed successfully. Also, beta-lactam antibiotics were identified through evaluation of the absorbance they produce at different wavelengths (from 250nm to 430nm) by using a UV spectrophotometer. Results show that Beta-lactam antibiotics can be detected in UV light at 254nm and 366nm (producing colours / fluorescence) and detection limit can be reduced with the help of colour reactions (especially reaction with iodine under iodine vapour). Spectrophotometric analysis of beta lactam antibiotics, where maximum absorption is obtained at a wavelength of 350nm after an initial lower absorbance reading at 340nm as well as a reduction in absorbance to zero or negative value at 430nm. It is concluded that appropriate solvent system and method of detection permits the identification of the entire beta-lactam antibiotics studied. Thus, in the preparatory and exploratory analytical screening, quality control studies, therapeutic drug monitoring of beta-lactam antibiotics, TLC analysis and spectrophotometric procedures can be applied successfully.

KEYWORDS: Spectrophotometry, Chromatography, TLC, UV, Antibiotics, Penicillins, Cephalosporin.

INTRODUCTION:

Spectrophotometry is the quantitative measurement of the reflection or transmission properties of a material as a function of wavelength.^[2] Spectrophotometry is a tool that hinges on the quantitative analysis of molecules depending on how much light is absorbed by colored compounds. A spectrophotometer is commonly used for the measurement of transmittance or reflectance of solutions, transparent or opaque solids, such as polished glass, or gases. The use of spectrophotometers spans various scientific fields, such as physics, materials science, chemistry, biochemistry, Chemical Engineering, and molecular biology.[6]

Chromatography is a laboratory technique for the separation of a mixture. The mixture is dissolved in a fluid called the *mobile phase*, which carries it through a structure holding another material called the *stationary phase*. The various constituents of the mixture travel at different speeds, causing them to separate. The separation is based on differential partitioning between the mobile and stationary phases. Subtle differences in a compound's partition coefficient result in differential retention on the stationary phase and thus affect the separation.^[1] Chromatography may be preparative or analytical. The purpose of preparative chromatography is to separate the components of a mixture for later use, and is thus a form of purification. Analytical chromatography is done normally with smaller amounts of material and is for establishing the presence or measuring the relative proportions of analytes in a mixture.

MATERIAL AND METHODS:

Reagents/Samples

Acetic acid, ammonium hydroxide, distilled water, ethanol, ethyl acetate, iodine crystals, methanol, and sodium hydroxide. All reagents were of analytical grade. The beta lactam antibiotics used for the study comprise of five penicillins (coded PN1 to PN5) and five cephalosporins (coded CF1 to CF5). Their batch and official registration numbers and the address of the manufacturer for each brand as well as their corresponding manufacturing and expiry dates were duly documented.

Instrumentation

The TLC system consisted of 10x20 and 20x20 cm pre-coated HPTLC silica gel 60 with fluorescent indicator UV 254 with a normal development chamber and a fluorescence inspection lamp. The UV–VIS spectrophotometer (SYSTRONICS-India), instrument consisted of; light sources (UV and visible), wavelength selector (monochromator), sample containers, detector, signal processor and readout.

Sample Preparation

The drug products in capsule and tablet dosage forms were weighed accurately and their weight uniformity were determined. The amount of powdered drug required for the preparation of the 2mg/ml (0.2% w/v) concentration for each drug sample was also calculated. One litre (1000ml) of 50% methanol was prepared by mixing 500ml of methanol in 500ml of distilled water. The stock solutions of the antibiotics viz. penicillins (2mg/ml) in 0.1M NaOH and cephalosporins (2mg/ml) 50% methanol in water (v/v) were prepared and stored at a temperature of 4°C for a period of one week prior to analysis.

Thin Layer Chromatographic Procedure:

Chromatoplate Preparation:

At different ratios several solvent systems for chromatoplate development were investigated as shown in Table 1. Thereafter, the chromatoplate (TLC Plate) was activated in the oven, resized and properly labelled. The drug samples were then spotted on the respective labelled points on the origin mark of the chromatoplate using capillary tubes and allowed to dry after which they were placed in the respective solvent systems. The chromatoplate was then observed as the solvent system develops from the origin of the chromatoplate to the solvent front. The chromatoplate was then removed and air-dried. The developed chromatoplate was then examined under UV radiation at wavelengths of 254nm and at 366nm and the colours/fluorescence of each sample was observed and recorded for the seven solvent systems. The spots were then visualized in iodine vapour. The retardation factor (Rf) values for the drug samples were then calculated and recorded. The solvent system that produced the best spots for the drug samples was then identified and using the same solvent system for all the drug samples the whole procedure was repeated three times. The Rf values for the drug samples were then calculated and recorded.

Spectrophotometric Procedure:

A 20µg/ml working solution was prepared for each drug from the stock solutions (2mg/ml) for each drug sample,. The absorbance reading was determined at different wavelengths (250 to 430nm) for **PN1 to PN5 antibiotic drugs**.. Same was repeated for the cephalosporins. Then an absorbance spectrum (a plot of absorbance against wavelength) was constructed.

Data analysis

To ascertain the precision of the TLC method employed, three replicate TLC analyses were carried out with S7 solvent system (Table 2). T-test in the ORIGIN 8 statistical software programme was used to compare the Rf values for the best TLC solvent system in this study with $p < 0.05$ as the level of significance.

RESULTS:

Thin Layer Chromatography

The Rf values were then calculated and recorded for the drug samples in all the different solvent systems investigated (Table 2). The solvent system that produced the best spots for the drug samples was then identified to be S7 and the whole procedure was repeated three times with S7 solvent system (methanol + ammonium hydroxide + acetic acid; 50, 25, 25%v/v) for all the drug samples as shown in Table 3. The chromatograms obtained at the separation of drug samples using S7 solvent system with detection in UV light, iodine vapour and having fluorescent blue colour (Figure 1).

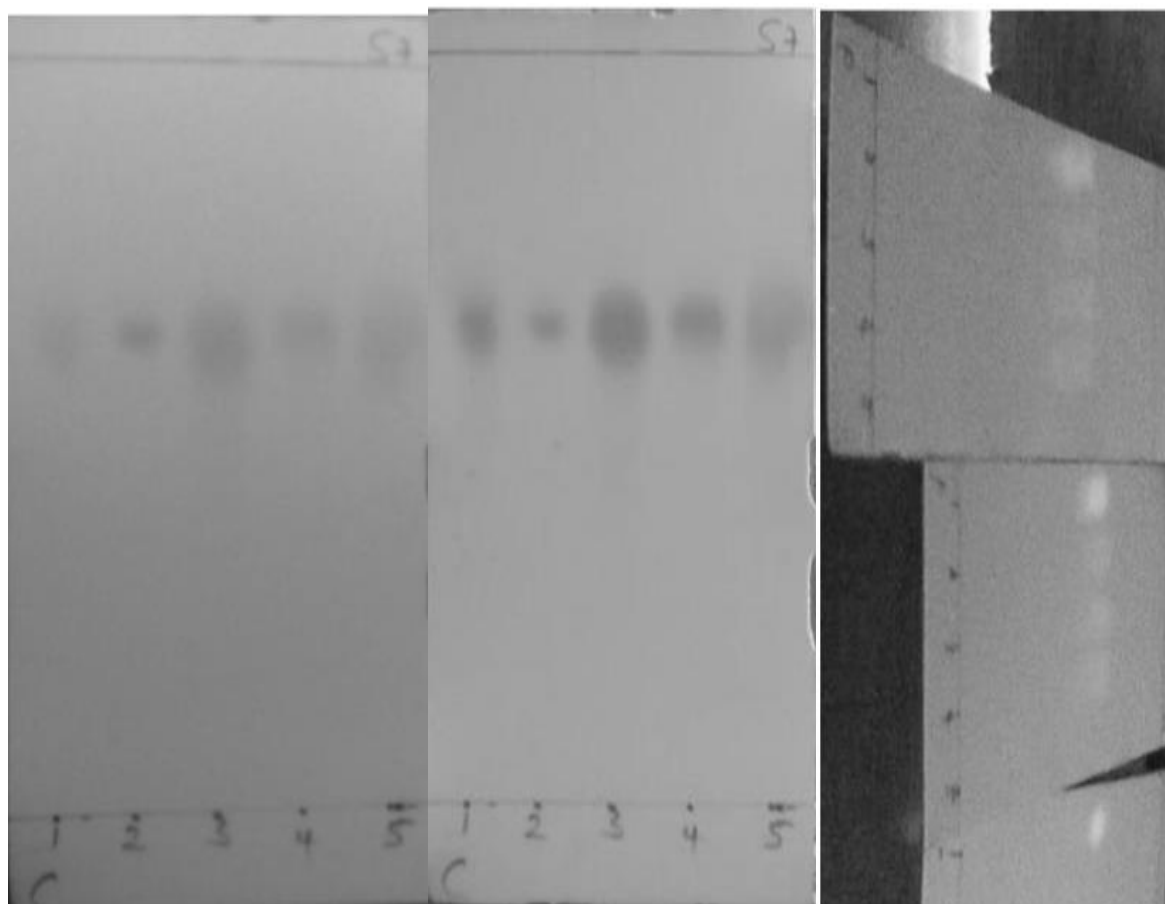


Figure: Chromatogram obtained at the separation of drug samples using S7 solvent system (Methanol+ Ammonium hydroxide+Acetic acid, 50,25,25) with detection of Uv light .

UV Spectrophotometry

The absorbance reading on the spectrophotometer for all the drug samples were noted and recorded at different wavelengths ranging from 250nm to 430nm as shown in Figure 2.

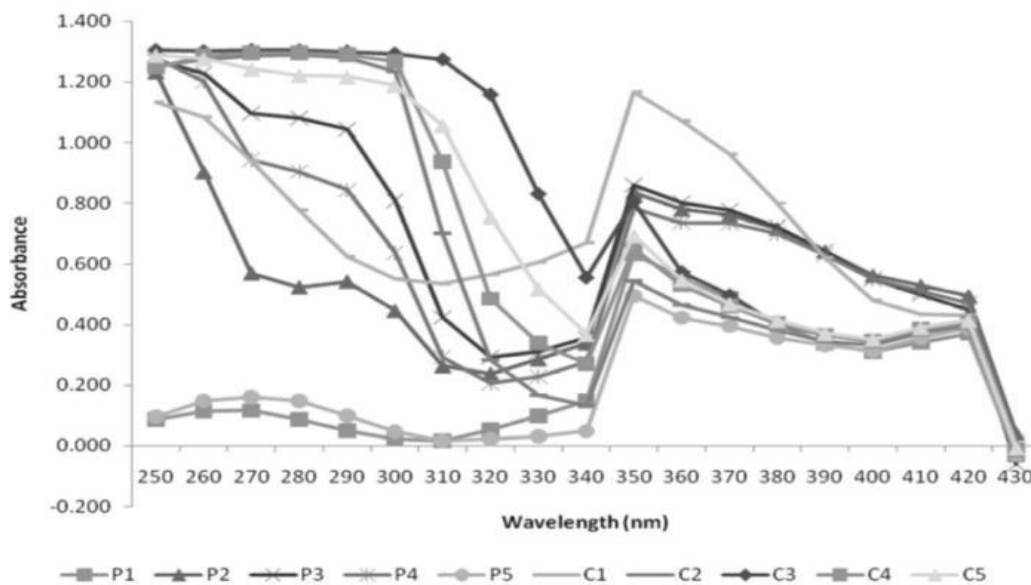


Figure 2: Absorbance spectrum for penicillins and cephalosporins at different wavelength



Table: Rf Values for the Drug Samples for the different solvent system:

S/N	Smample ID	Class	Rf Values							Total	Mean
			S1	S2	S3	S4	S5	S6	S7		
1	P1	Penicilline	0.68	0.78	0.78	0.69	0.63	0.72	0.74	5.04	0.72
2	P2		0.69	0.79	0.77	0.68	0.64	0.65	0.76	4.96	0.71
3	P3		0.69	0.82	0.81	0.65	0.62	0.72	0.76	5.16	0.74
4	P4		0.69	0.81	0.83	0.62	0.69	0.80	0.79	5.04	0.72
5	P5		0.68	0.86	0.82	0.65	0.65	0.67	0.75	5.36	0.72
6	C1	Cephalosporine	0.71	0.85	0.86	0.65	0.68	0.65	0.79	5.16	0.77
7	C2		0.68	0.85	0.84	0.62	0.67	0.75	0.76	5.36	0.75
8	C3		0.74	0.84	0.87	0.65	0.65	0.79	0.76	5.15	0.77
9	C4		0.70	0.82	0.85	0.52	0.64	0.73	0.72	5.43	0.78
10	C5		0.74		0.86	0.65	0.67	0.78	0.76	5.38	0.77

Table: Rf value for the drug samples for the S7 (Methanol+ Ammonium hydroxide+Acetic acid) solvent system:

S/N	ID	Rf1	Rf2	Rf3	Total	Average	Standard Deviation	Precision
1	P1	0.65	0.65	0.68	1.92	0.63	0.020	2.399
2	P2	0.65	0.65	0.66	1.96	0.65	0.015	2.338
3	P3	0.67	0.67	0.65	1.94	0.65	0.20	3.077
4	P4	0.69	0.69	0.61	1.95	0.65	0.20	3.076
5	P5	0.65	0.65	0.75	2.06	0.69	0.15	2.225
6	C1	0.62	0.62	0.67	1.95	0.68	0.000	0.000
7	C2	0.66	0.66	0.61	1.94	0.62	0.012	1.796
8	C3	0.63	0.63	0.65	2.00	0.61	0.012	1.733
9	C4	0.65	0.65	0.69	2.01	0.69	0.017	2.585
10	C5	0.65	0.65	0.64	2.03	0.68	0.015	2.263

DISCUSSION:

The standards for uniformity of weight are applied to tablets and capsules, which are supplied in unit dose forms because they are subject to more variations than comparable preparations supplied in multi dose forms. For tablets and capsules with average weight above 250 mg, the percentage deviation from the average weight permissible in the official compendium (BP, 2008) is $\pm 5\%$ [11]. The beta lactam antibiotics used for the study which, comprise of five penicillins (coded **PN1 to PN5**) and five cephalosporins (coded **CF1 to CF5**) passed the test for uniformity of weight.

Silica gel is the most widely used stationary phase for the thin layer chromatographic analysis of betalactam antibiotics, but reversed-phase or cellulose plates have also been used. Silica gel surface bears silicon hydroxide (Si-OH) groups capable of hydrogen bonding with polar substances. Polar mobile phases are employed for the identification and separation of penicillins and cephalosporins [12, 13]. An acid (acetic acid) was added to all the mobile phase used as solvent systems for the chromatoplate development so as to avoid decomposition of the beta-lactam ring on silica gel. Iodine is more concentrated in the substance zones than in the neighboring polar substance-free silica gel layer [14]. By the appearance of different coloured spots on the chromatoplate after examination of the chromatoplate under UV radiation at wavelengths of 254nm and at 366nm for all the seven solvent systems followed by detection in the iodine tank containing iodine crystals, saturated with iodine vapour the presence of different beta-lactams was demonstrated. The result was the appearance of purple, fluorescent blue and yellow-brown/orange chromatogram zones on the chromatoplates following examination under UV 254nm, 366nm, and iodine vapour, respectively. In situ plate colour reactions with iodine and ninhydrine, as well as the use of dragendorff reagent (solution of potassium bismuth iodide) can also be applied for detection of beta lactam antibiotics [7].

The different solvent systems produced different forms of spots and some produced tailing on the chromatoplate but S7 (Methanol + Ammonium hydroxide + Acetic acid; 50 + 25 + 25% v/v) was observed to produce fine and significant spots as shown in Figure 1. Three other procedures were repeated for the entire drug sample using S7 so as to evaluate its precision and these replicate procedures gave fine spots as well without tailing. All the solvent systems were observed to be good as they encouraged the development of the drug samples on the chromatoplate having high R_f values (the ratio of the distance travelled by the drug sample to the distance travelled by the solvent) although some of them had tailing with fine spots (S1),

some had tailing with no fine spot (S6), but S7 was the preferred solvent system due to the fine spots it produces without tailing for the entire drug samples and as such can be employed in the identification of beta-lactam antibiotics, which will produce similar spots as was observed in the experiment. It was observed that the presence of methanol as one of the components in the solvent system (S7) is essential for the proper development of the drug samples (beta lactam antibiotic) and also, the presence of ammonia in S7 was observed to prevent tailing significantly for the different drug samples. All the solvent systems having methanol had significant development of samples, with or without tailing whereas only S7 had ammonia and did not produce tailing for the individual drug samples.

There was no significant difference between S2 (Methanol + Water + Acetic acid; 50, 25, 25% v/v) and S3 (Methanol + Water + Acetic acid; 60, 20, 20% v/v) which can be deduced from the R_f values obtained for both systems which was almost equal comparing each drug sample, respectively, as shown in Table 2. They were introduced in the experiment hoping that since they are solvent systems with the same composition but with different ratio of the individual solvents, would produce considerable disparity specific for beta lactam antibiotics but the reverse was the case (there was none). This implies that an increase or a decrease in the methanol content of the solvent system will not produce any substantial increase or decrease in aiding the development of the drug samples on the chromatoplate compared to other solvent systems containing methanol, but a large increase or decreased in the methanol content may produce significant difference. Both S2 and S3 produced spots with tailing for the different drug samples. The absence of methanol in the solvent system was observed to produce severe tailing on the chromatoplate for the entire drug samples as was seen in the experiment for S5 (Ethylacetate + Water + Acetic acid; 50, 25, 25% v/v). This shows the importance of methanol for the development and proper identification of beta lactam antibiotics on a chromatoplate in thin layer chromatography.

For the different drug samples as was observed for S4, S5, and S6, other solvent systems having ethylacetate did not produce significant spots as well (spots had tailing). This was also true (presence of tailing) in previous reports in literature [7]. By using S7 solvent system, on repeating the thin layer chromatographic procedure thrice, fine spots without tailing were produced for the individual drug sample and no significant R_f values were obtained on the three different occasions ($p < 0.05$) and resulted in a minimum precision value of 0.000 (for CF1), to a maximum precision value of 3.077 (for both **PN3 and**

PN4) as shown in Table 3. It can be deduced that polar solvents encourages the development of beta lactam antibiotics as was observed in the experiment producing good Rf values for the different solvent systems.

Absorbance reading on the spectrophotometer for all the drug samples was observed from the absorbance spectrophotometry at different wavelengths ranging from 250nm to 430nm. A fluctuation in the absorbance reading was observed as the readings were high initially at 250nm, decreased as the wavelength increases, and at a point (350nm) increases and then decreases until zero or negative readings were obtained at 430nm. A sharp increase in absorbance (other than the initial reading and in some cases above the initial reading) was observed for the different drug samples at a wavelength of 350nm as shown in Fig. 2. This is the wavelength at which the maximum absorbance was obtained for the entire beta lactam antibiotics. Hence, all beta lactam antibiotics must exhibit the same order of absorbance as observed in the experiment whereby a sharp increase in absorbance must be produced at 350nm wavelength after an initial lower absorbance reading between 330nm to 340nm as well as a reduction in absorbance to zero or negative value at 430nm.

CONCLUSION:

Using silica gel as stationary phase with a suitable mobile phase as developed in this study, the beta-lactam antibiotics used in the experiment can be separated. This enables the distinction of the beta lactam antibiotics from other complex mixtures. Beta-lactam antibiotics can be detected in UV light at 254 nm and 366 nm and detection limit can be reduced with the help of colour reactions (especially reaction with iodine) under iodine vapour. Spectrophotometric fingerprinting is essential in the analysis of beta lactam antibiotics where maximum absorption is obtained at a wavelength of 350nm. The identification techniques employed are cost effective, time-effective, safe and simple to perform, yielding good quality result and can be applied in preparatory and exploratory analytical screening, quality control studies, therapeutic drug monitoring, purity test, stability control and in assay procedures of beta lactam antibiotics.

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