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QUANTITATIVE ANALYSIS OF CEPHAMYCIN C IN FERMENTATION BROTHS BY MEANS OF THIN-LAYER SPECTRODENSITOMETRY

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SUMMARY

A method has been developed that consists of one-dimensional thin-layer chromatography of the samples on pre-coated silica gel $60F_{254}$ plates followed by an *in situ* spectrophotometric evaluation of the chromatograms at 273 nm. Statistical evaluations showed a linear calibration ($r^2 > 0.999$) for the range of 0.2–20 μg/spot. The relative standard deviations for broth samples containing 1.505 and 12.47 μg Cephamycin C per spot were 3.61 and 1.07%, respectively. The recovery was virtually quantitative: 97.6%. The correlation with a liquid chromatographic method was quite acceptable with $r^2 = 0.99124$ and $b = 0.998$ (slope).

INTRODUCTION

For most β-lactam antibiotics liquid chromatography (LC) is virtually the only chromatographic technique that has received widespread acceptance as far as quantitative analysis is concerned[1–4]. Thin-layer chromatography (TLC) on the other hand remained a separation method merely serving qualitative purposes[5,6]. Some earlier data[7–9], however, along with the general chromatographic and spectrophotometric properties[5] of β-lactam antibiotics, indicate that the *in situ* scanning of TLC plates could be a viable alternative to other assay methods currently in use.

Both LC and TLC serve basically identical areas in analytical methodology, namely, the separation of non-volatile compounds. However, while linear detector systems were available for high-performance LC (HPLC) from the introduction of the technique on, opinions about the same for thin-layer spectrodensitometry (TLS) are still conflicting. The primary concern is that the narrow linear concentration range of most commercially available instruments cannot meet the demand of analytical laboratories.

The development of TLS took an encouraging turn in the late sixties and early seventies, as a few isolated research groups working on the basis of the Kubelka–Munk theory[10,11] or its modified version[12,13] reported their data. Following the basic research, the commercial development of instrumentation soon began. Yamamoto *et al.*[14] described a new TLC scanner that utilized the Kubelka–Munk theory[15,16] as

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applied to TLC plates, to obtain a wide range of linear response. They tested the instrument on several chemically stable compounds. More recently, studies conducted by independent laboratories[17,18] indicate that the Kubelka–Munk theory, in appropriate form, is gaining widespread acceptance as the most reliable approach in TLS. The present paper describes the quantitative analysis of a fermentation product, Cephamycin C[19,20], used as starting material in the manufacturing of a significant new antibiotic named Cefoxitin. The assay of the active component in fermentation broths is carried out by means of a TLC scanner recently introduced to the market.

EXPERIMENTAL

The standard solutions were freshly prepared each day from the dibenzylethylenediamine salt of Cephamycin C as described by Caldwell and Houck[3]. The concentration was in the range of 2–2.5 g/l. For the TLS assays, a diluted sample (range 0.2–0.6 g/l) was also made to obtain two calibration points.

For broth samples, aliquots were taken in various stages of the fermentation for assay purposes. They were centrifuged and the supernatants were taken for further processing. The HPLC and TLS assays were separated at this point and conducted in a double blind fashion.

High-performance liquid chromatography

The method is based on reversed-phase ion-pair chromatography[6] with spectrophotometric detection. The details of the procedure are described in an internal communication[3] and will be published elsewhere.

Thin-layer chromatography

The standard size (20 × 20 cm) pre-coated silica gel $60F_{254}$ (E. Merck, Darmstadt, G.F.R.) plates were prepared for a total of 10 samples. With a sharp pencil, a line was drawn 20–25 mm above the bottom edge of the plate. This baseline was then divided into ten lanes the centers of which were marked exactly 18 mm from one another (one of the several choices the automatic lane changer of the densitometer permits within the range of 6–30 mm). Pre-scoring the silica layers was not required. The center of each lane was the starting point of a sample. A 10-μl volume of glacial acetic acid was applied onto each origin. The plate was allowed to dry for about 15–20 min, then 5.00 μl of the samples were applied onto the starting points. One plate thus accomodated two standards and eight broth samples. The plates were allowed to dry for about 10–15 min. They were subsequently developed in the solvent consisting of ethanol–glacial acetic acid–conc. ammonium hydroxide (6:3:1). The certified ACS grade solvents were obtained from Fisher Scientific (Pittsburgh, PA, U.S.A.). The chromatographic tanks were lined with filter paper and were allowed to equilibrate. The developed plates were air dried for about 1 h.

Densitometry

A Shimadzu Model CS-920 high-speed TLC scanner was used for the *in situ* evaluation of the plates. The main features of the instrument are as follows: (i) a grating monochromator for continuous wavelength selection from 200–630 nm, (ii) two-dimensional scanning in the reflectance mode with several options of stroke

width selection ranging from 3–30 mm, (iii) scan distance selector from 6–180 mm, and (iv) a microprocessor controlling two modes of baseline correction, a four-position linearizer, one and two points external and internal standard calibration modes, electronic digital integrator with a printer and analogue output for a chart recorder, and the lane-change automatic.

The scanning of the samples was carried out at 273 mn. For quantitative analysis, a 30 mm long section of the chromatogram was scanned with the main fraction located in the center, and the automatic zero suppression in the "ON" position. With both standards scanned first in the calibration mode, the instrument computes the value of every sample automatically. The use of the chart recorder is optional, but nevertheless recommended to enhance the reliability of the technique through visual display of the baseline and peak shape. The coordinates of a chromatographic zone are defined by the lane number (x) and the distance from the bottom edge of the plate (y) both printed out by the integrator.

Criteria of reliability

Calibration. A series of increasing amounts of Cephamycin C samples was applied onto a plate covering the range of 0.2–20 μg per spot. The procedure of sample application, chromatography and quantitative evaluation is described above. To study the effect of the spot size samples of constant concentration and various volumes were compared with samples with constant volumes but various concentrations. The calibrations were evaluated on several plates to determine the transferability of standard values from one plate to another.

Recovery. A series of identical amounts of authentic material was added to broth samples. The broth samples without addition as well as those with addition were chromatographed and scanned in the usual manner. The values found were compared with those expected and evaluated statistically (Table I).

TABLE I

SUMMARY OF THE RESULTS OF THE RECOVERY STUDY ON CEPHAMYCIN C

Number of samples: 10.

Sample	*Integral*		*Concentration (g/l)*	*Amount (μg/spot)*
	Mean	*S.D. (%)*		
Standard I	623.9	19.13 (3.07)	0.212	1.06
Standard II	6639	105 (1.58)	2.12	10.6
Broth I	904.3	32.65 (3.61)	0.301	1.505
Broth II	7819	83.95 (1.07)	2.494	12.47
Broth I + standard II	7404*	131 (1.77)	2.363**	11.82***

* Expected value 7588.
** Expected value 2.421.
*** Expected value 12.11.

Precision. Ten identical samples of Cephamycin C were applied onto a plate for chromatography and subsequent densitometry. The data were evaluated statistically to determine the standard deviation. This experiment was carried out on sample groups of various concentrations.

Correlation between the HPLC and TLS techniques. Approximately fifty fermentation broth samples selected to cover the concentration range of 0–2.5 g/l were assayed simultaneously by both methods in a double-blind test. The correlation was evaluated statistically using the least squares method.

RESULTS AND DISCUSSION

The solvent system for TLC is a buffer solution which has a pH of about 4.5 before being mixed with ethanol. The layer is also made weakly acidic by treating the origin with 10 μl of acetic acid. These precautions must be taken to prevent the chemical decomposition of Cephamycin[4,21], particularly as the samples dry on the adsorbent surface. Compounds of β-lactam type react with nucleophilic reagents opening the β-lactam ring as an initial event to a chain of non-specific degradation processes. The most common nucleophiles in broths are compounds containing free amino groups. Moderately low pH values cause the amino groups to become protonated losing their nucleophilic properties, without cleaving the β-lactam ring by acid hydrolysis. In a later part of this section, it will be demonstrated that indeed no detectable decomposition was observed in broth samples within the limits of experimental errors (Table I).

For the separation, the generally recommended practice of TLC was adhered to; the tanks were equilibrated and kept at reasonably constant temperature. Thus the chromatograms were always uniform. The separation allowed sufficient resolution of the main peak from contaminants present in broths. The fluorescent indicator in the adsorbent layer made it quite practical to properly align the plate for the automatic scan.

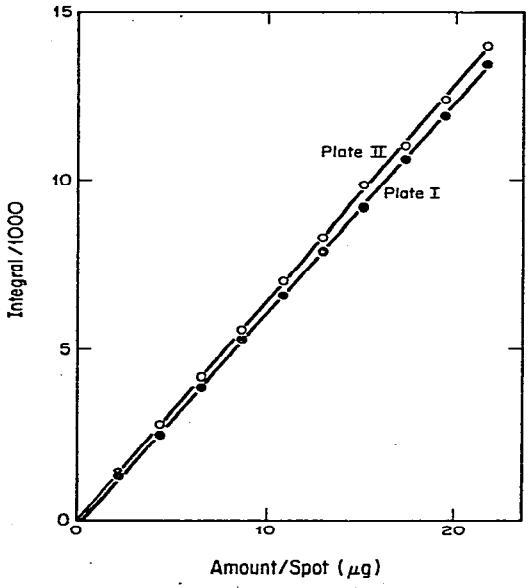

Fig. 1. Calibration of Cephamycin C on two separate pre-coated silica gel $60F_{254}$ plates. The best-fitting straight lines ($y = a + bx$) are calculated by means of the least squares method: plate I: $a = -131$; $b = 619$; $r^2 = 0.99956$; plate II: $a = 1.4$; $b = 640$; $r^2 = 0.99954$.

The Shimadzu CS-920 scanner has all the significant features that other instruments[12,13] previously used in our laboratories had. However, unlike other instruments, it scans a whole plate automatically. The operator can leave the scanner unattended for 10–60 min, depending on the length of the lanes to be scanned. The built-in digital integrator, analogue converter or linearizer are all standard features, whereas other commercial instruments had to be equipped with the same essentials[13] after the purchase was completed. It is not only the matter of inconvenience and added expenses burdening the user. The standardized instrument could at last lead to analytical methods readily transferrable from one laboratory to another. The scanner was subject to thorough testing for its reliability by criteria used for evaluating other analytical equipments and procedures. The linear range of calibration was determined and found to extend from 0.2 to above 20 μg/spot with practically zero intercept (Fig. 1).

The plate-to-plate variation is noticeable but for certain purposes it is small enough to be disregarded. The standard values determined on six different plates gave correlations (Fig. 2) acceptable for the purpose of studying the production kinetics during the fermentation.

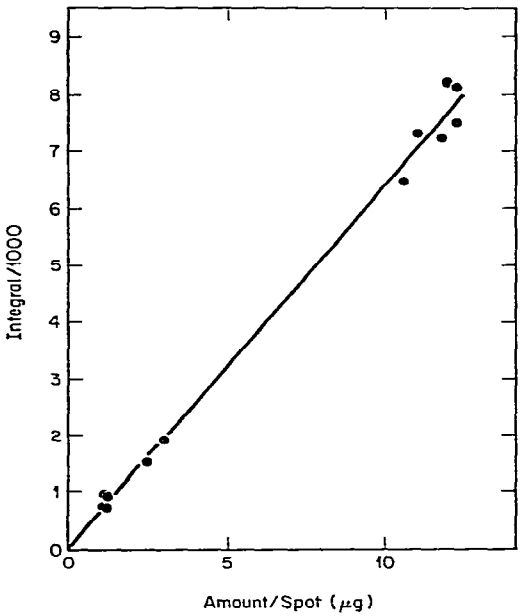

Fig. 2. Reproducibility of the calibration from plate to plate. The best-fitting straight line ($y = a + bx$) is calculated by means of the least squares method: $a = 32.74$; $b = 6.36$; $r^2 = 0.99493$.

At low concentrations (weight/surface area on the TLC plate), the precision is less than at higher levels, as a greater portion of the signals can be attributed to the noise. At 1.06 μg/spot authentic material, the relative standard deviation (R.S.D.) found was 3.07%, at 10.6 μg/spot 1.58%. Similar figures were obtained with broth samples (Table I). The recovery of 97.6% (Table I) indicates virtually no loss during the procedure. The loss is well within the expected experimental variations based on the plate-to-plate reproducibility and precision.

The actual comparison with the standard HPLC method[3] gave satisfactory results with correlation coefficient $r^2 = 0.99124$ and slope $b = 0.998$. About 50 broth samples obtained from different batches and various stages of fermentation were compared (Fig. 3). The TLS results were generally higher but the difference appeared to be quite consistent. The intercept ($a = 0.085$) with a slope of $b = 0.998$ indicate that the TLS method has a higher background. The systematic error can be explained by the two modes of integration available for the TLC scanner. In the automatic zero suppression mode (AZS "ON"), the instrument establishes a constant baseline as the scan begins. All peaks are integrated on this baseline. In the AZS "OFF" mode, the instrument resets the baseline in every zig-zag stroke on both sides of the spot. In an ideal case, when the adsorbent layer is perfectly uniform, both modes give the same integral. If the sample is tailing, or highly contaminated causing a "smear" along the track, both integrals are too high (Fig. 4A and B). In the latter case the AZS "ON" mode gives a better correction, provided that the scanning begins just before the chromatographic zone to be quantitated (Fig. 4C), while the integral in the AZS "OFF" mode remains virtually unchanged regardless of where the scan started (Fig. 4D). As the integration is always carried out over a horizontal baseline, the resolution of neighboring peaks will effect the integration. The background of this method is somewhat variable depending on the arbitrary selection of the start of the scan and minute differences in R_F values quite common in TLC. However, the practical utility of the method remains just as appreciable whenever large numbers of samples have to be analyzed.

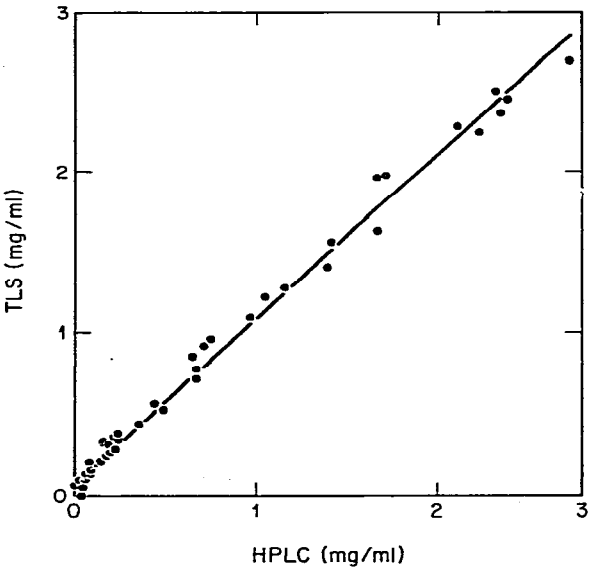

Fig. 3. Correlation between the HPLC and TLS methods. The best-fitting straight line ($y = a + bx$) is calculated by means of the least squares method: $a = 0.08513$; $b = 0.99819$; $r^2 = 0.99124$.

The potential cost efficiency of TLS can be demonstrated as follows: In the routine analytical work, TLS can replace HPLC any time when the sensitivity of the scanner is sufficient. In addition, while HPLC instruments are usually dedicated to

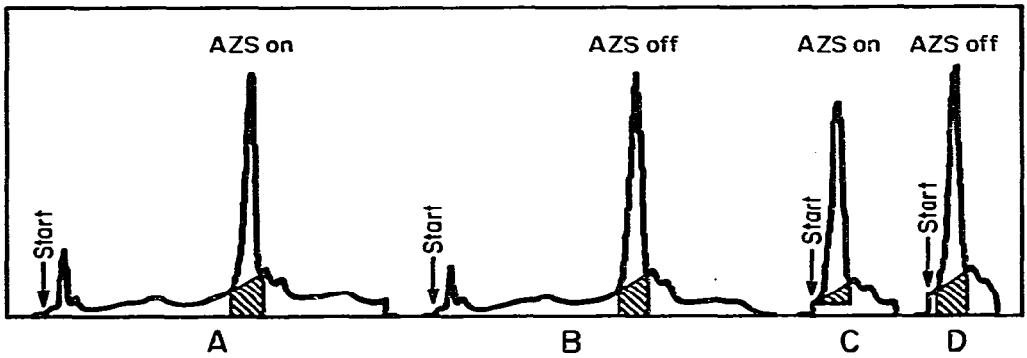

Fig. 4. The mode of integration at different settings of the instrument. The shaded portions under the peaks indicate the excess area reported by the integrator.

one single task, the same TLC scanner can be used in a practically unlimited variety of methods. For the introduction of a new assay procedure by TLS, an additional developing tank with the appropriate solvent system is usually sufficient. In order to do the same based on HPLC technique, normally the installation of a complete instrument is required.

Preliminary results indicate that similar methods can be used for the assay of other β-lactams (*e.g.* thienamycin) and a number of biologically active natural products of various classes (macrolide antibiotics, avermectins, etc.). The detailed description of these methods will be reported later in future publications.

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