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QUANTITATIVE THIN-LAYER CHROMATOGRAPHY OF EVERNINOMICIN D

PETER KABASAKALIAN and SAMI KALLINEY
Antibiotics Development, Schering Corporation, 86 Orange Street, Bloomfield, N.J. 07003 (U.S.A.)
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SUMMARY

A direct densitometric method for the assay of everninomicin D after thin-layer chromatography is described. The fluorescence quenching (transmittance) method and automatic integration of the spot area were used. The peak areas were linear with the amount spotted from 3 up to 40 μg. By direct evaluation of the thin-layer chromatograms, as little as 10 μg of everninomicin D can be determined with a relative standard deviation of 2.3%. The densitometric method was used to follow the course of the fermentation of everninomicin D on a laboratory scale.

INTRODUCTION

In situ analysis by densitometry offers speed, sensitivity and makes the detection of a few micrograms of a substance possible. Preliminary treatment of the sample in most instances is minimal. The technique has been applied widely to the analysis of antibiotics, of which tetracycline[1], erythromycin[2] and gentamicin[3] have recently been reported.

The present study is a further application of the densitometric method to the *in situ* analysis of everninomicin D. Everninomicin D is an oligosaccharide antibiotic[4] having ultraviolet absorption which allows the use of fluorescence quenching in the densitometric analysis without further procedures to render the substance visible.

The present densitometric method for assaying everninomicin D uses the fluorescence quenching method in the transmittance mode. The method is sensitive, specific and rapid.

EXPERIMENTAL

Materials

Solutions of everninomicin D in analytical-reagent grade organic solvents were prepared at a concentration of 1–10 mg/ml. Pre-coated TLC plates (E. Merck, Elmsford, N.Y.) with silica gel F_{254} with a 0.25-mm layer thickness were used without any pretreatment.

A Schoeffel SD 300 double-beam densitometer was equipped with an optical density computer, Model SDC 300 (Schoeffel Instrument Corp., Westwood, N. J.).

The output was recorded on a Honeywell 10-in. strip recorder (Honeywell, Fort Washington, Pa.) provided with a disc integrator Model 252 A (Disc Instruments, Inc., Santa Ana, Calif.).

Method

The TLC plates were divided into lanes of 1 cm width with a Schoeffel scoring device so as to avoid any sideways movements of the spots during the development of the chromatograms. A 17-cm termination line was scored at right-angles to direction of development. A 10-μl Kimax 51 micropipet (Kimble, a division of Owens Illinois, Toledo, Ohio) was used for spotting by the bulb expulsion technique 2 cm from the edge of the plate in the center of alternate lanes. The pipet was connected to the spotter with flexible rubber tubing. After filling the pipet to the mark, its tip was made to touch the TLC layer gently so as to avoid damaging the layer, and the liquid was squeezed out by applying pressure to the rubber tubing. The spots were dried under a stream of nitrogen. Because of the double-beam operating principle of the densitometer, only alternate lanes were loaded. The blank zones were used as references.

Four identical aliquots of the sample were alternately spotted with four identical amounts of the reference standard on a single plate.

Fresh ethyl acetate–acetone (1:1) gave a satisfactory separation. The plates were placed in chromatographic chambers, lined with filter-paper, and saturated with the solvent vapor for about 10 min. After the solvent had reached the 17-cm mark, the plates were removed, dried and placed in the densitometric scanning stage.

Scanning and recording took place in the direction of the chromatography at a speed of 4 in./min. The exciting wavelength in the fluorescence quenching transmittance mode was 265 nm, and the monochromator and exit slits were 1.5 and 1 mm, respectively. The operational settings of the density computer function were ratio and log. A full-scale range of 0.1 or 0.2 optical density unit was used in the course of the experiments. Integration of the peak areas was carried out by the disc integrator.

To follow the course of the fermentation of everninomicin D, 15 ml of the broth were extracted with 1 ml of dichloromethane, and 10 μl of the dichloromethane phase were spotted as outlined above.

RESULTS AND DISCUSSION

Peak areas of everninomicin D *versus* amount of sample spotted (3.0–96.6 μg) are plotted in Fig. 1. The response is linear only from 3 to 40 μg.

The error associated with scanning of various sample sizes is shown in Table I. The mean relative standard deviation was found to be 1.2% and was independent of sample size. The reproducibility of spotting on a plate was examined by spotting 10, 20 and 30 μg on their own respective plates 8–10 times. The mean relative standard deviation (Table II) was found to be 2.7%, which includes both the scanning error and the spotting error.

The reproducibility of identical amounts spotted on different plates is shown in Table III. The mean relative standard deviation was found to be 5.8%, which includes the scanning error, the spotting error and plate-to-plate variation.

The linearity of response was determined in the linear region (3–40 μg) for 10

plates (Table IV). The result for a given plate is given in the form of $y=a+bx$, where y is the peak area in terms of integrator counts, a is the y intercept, b is the slope of the line and x is the amount of everninomicin D in the spot.

The reproducibility of the above procedure was tested using three levels of samples with the corresponding identical levels of reference standard for plates 1–3

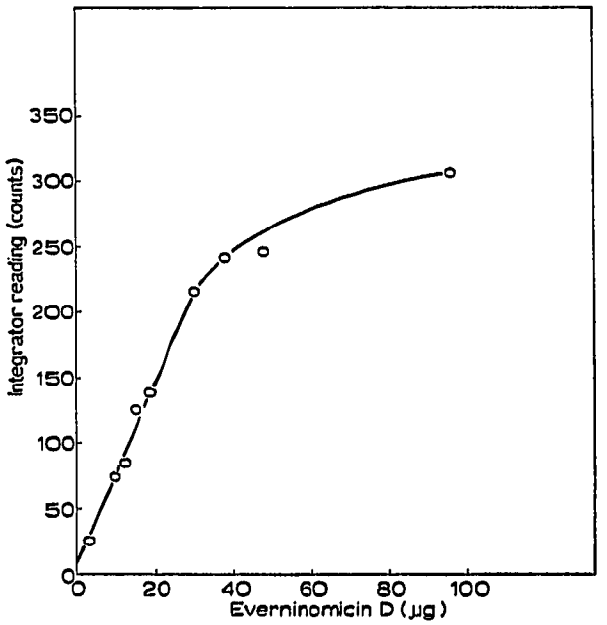

Fig. 1. Calibration curve for the determination of everninomicin D by the fluorescence quenching (transmittance) mode on silica gel F_{254} (E. Merck). Peak areas are evaluated by disc integrator. O.D. = 0.1.

TABLE I

REPRODUCIBILITY AS A FUNCTION OF SCANNING

Single TLC plate spotted once and scanned 10 times.

Amount spotted (µg)	*Mean area response*	*Standard deviation*	*Relative standard deviation (%)*
3.0	24.7	0.34	1.4
9.5	73.9	0.53	0.7
12.5	84.1	1.06	1.3
15.0	126.6	0.85	0.7
19.0	140.3	1.25	0.9
30.0	218.2	2.15	1.0
38.0	242.1	1.79	0.7
48.3	246.8	6.05	2.5
96.6	307.0	4.03	1.3
		Mean	1.2

TABLE II

REPRODUCIBILITY AS A FUNCTION OF SPOTTING

Single TLC plate spotted *m* times at one level and scanned once.

Plate No.	*Amount spotted (μg)*	*Number of spots*	*Mean area response*	*Standard deviation*	*Relative standard deviation (%)*
1	9.7	8	73.6	2.26	3.1
2	19.8	10	109.8	2.90	2.6
3	29.0	10	192.4	4.79	2.5
				Mean	2.7

TABLE III

REPRODUCIBILITY AS A FUNCTION OF TLC PLATES

Each of *m* plates spotted once at six identical levels and scanned once.

Amount spotted (μg)	*Number of plates*	*Mean area response*	*Standard deviation*	*Relative standard deviation (%)*
2.9	7	25.6	1.68	6.6
10.3	10	74.5	4.03	5.4
14.5	10	98.2	6.42	6.5
20.6	10	130.8	8.11	6.2
29.0	9	168.9	9.20	5.4
41.2	9	225.6	10.19	4.5
			Mean	5.8

TABLE IV

LINEARITY OF PEAK AREA *VERSUS* AMOUNT SPOTTED

Each plate spotted once at six different levels and scanned once.

Plate No.	*Regression line*	*Correlation coefficient*	*Standard error*
1	$y=14.1+5.39\,x$	0.9962	5.1
2	$y=20.1+5.34\,x$	0.9867	9.5
3	$y=18.1+5.13\,x$	0.9847	9.8
4	$y=23.1+5.02\,x$	0.9790	11.3
5	$y=21.5+5.24\,x$	0.9935	6.5
6	$y=16.5+5.33\,x$	0.9956	5.5
7	$y=16.0+4.84\,x$	0.9398	18.9
8	$y=13.7+5.68\,x$	0.9679	11.9
9	$y=18.1+5.15\,x$	0.9828	10.5
10	$y=12.6+4.74\,x$	0.9785	10.8

TABLE V
RECOVERY OF EVERNINOMICIN D

Plate	*Sample*		*Standard*		*Recovery*		
No.	$\bar{Y}_u$	$S_{\bar{y}}^2$	$\bar{Y}_S$	$S_{\bar{y}}^2$	%	V_T^2	V(%)
1	74.0	1.38	73.3	0.80	101.0	4.01	2.0
2	109.0	2.25	111.0	0.75	98.2	2.50	1.6
3	196.0	6.00	190.0	1.00	103.2	1.84	1.4
4	83.7	1.41	124.7	5.63	101.4	5.63	2.4
5	85.3	5.85	122.7	1.19	105.1	8.83	3.0
6	87.0	6.00	130.0	0.89	101.1	8.45	2.9
				Average	101.7	5.21	2.3

(Table V), while for plates 4–6 the level of sample was held constant at two-thirds of the level of the reference standard. The results calculated by the method of Jones *et al.*[5] yielded an average recovery of 101.7% with a relative standard deviation (V) of 2.3%.

One of the useful applications of the densitometric procedure is in following the course of the fermentation of everninomicin D. The results are shown in Fig. 2.

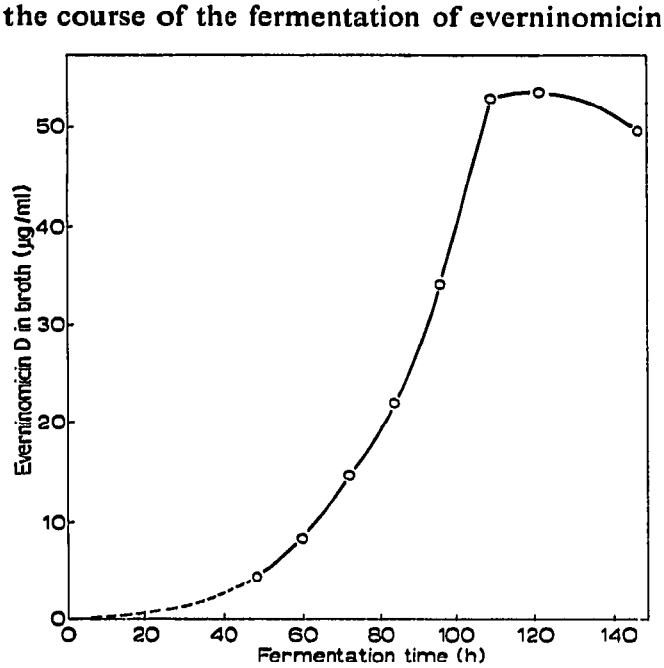

Fig. 2. The course of the fermentation of everninomicin D by the fluorescence quenching (transmittance) mode. O.D. = 0.1.

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