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QUANTITATIVE THIN-LAYER SPECTRODENSITOMETRIC DETERMINATION OF THE COMPONENTS OF POLYENE MACROLIDE ANTIBIOTIC COMPLEXES

JUAN F. MARTIN* and LLOYD E. McDANIEL

Institute of Microbiology, Rutgers, The State University, New Brunswick, N.J. 08903 (U.S.A.)

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SUMMARY

A direct spectrodensitometric method for quantitating the components of polyene macrolide complexes after separation by thin-layer chromatography is described. Resolution of the components of the candidin and candihexin complexes was good up to 2.5 and 10 μg/spot, respectively. The peak areas were linear with the amount spotted up to the same levels. Maximum peak areas for the components of the candidin and candihexin complexes were obtained using light wavelengths of 360 and 340 nm, respectively. Spotting errors rather than instrumental parameters were responsible for the variance of repeated determinations. Minimal relative standard deviation values were found at intermediate concentration levels in the linear range.

INTRODUCTION

The thin-layer spectrodensitometric (TLS) technique, involving the measurement of transmitted or reflected light, is a rapid, sensitive and convenient method for the *in situ* quantitative determination of substances separated by thin-layer chromatography (TLC)[1]. Studies of the parameters affecting the accuracy and reproducibility of densitometric determinations have been published[2–4]. This technique has been applied recently to the determination of the antibiotics tetracycline[5], erythromycin[6], gentamicin[7], everninomicin D[8] and penicillin G[9]. TLS is now used for routine analysis of pharmaceutical preparations[10] and is a promising technique for quantitation of many other antibiotics because of the widespread use of TLC for separation of antibiotics[11].

Polyene macrolide antibiotics have a strong UV–visible light absorption due to the presence in their macrolactone rings of conjugated systems of three to seven double bonds[12]. This property can be used to quantitate the amount of polyene macrolide present in TLC without previous treatment to render the substance visible. Polyene macrolide antibiotics are produced by Streptomycetes as mixtures of chemically related components[13]. Spectrophotometry of polyenes in solution is limited to the determination of the polyene complexes as mixtures of components but can not be used to quantitate each individual constituent. In most cases resolution of the

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*Present address: Department of Nutrition and Food Science, 56–126, M.I.T., Cambridge, Mass. 02739, U.S.A.

components of polyene complexes can be achieved by adequate selection of the ad-sorbent, solvent system and chromatographic conditions. As individual components of polyene complexes have different biological activities[14] and their proportions in the complex change according to the fermentation conditions and purification methods[15], the knowledge of the relative abundance of the individual components in the complex, and therefore in formulations of these drugs, is of supreme importance. We report here a TLS method that makes it possible to quantitate the components of the candidin (heptaene) and candihexin (hexaene) complexes with a study of the precision of the method as affected by instrumental and chromatographic parameters.

EXPERIMENTAL

Polyene complexes

The heptaene candidin complex and the hexaene candihexin complex having $E_{1\ cm}^{1\%}$ values of 1385 and 875, respectively, were obtained by fermentation and purifi-cation as described previously[13,16]. Solutions were prepared at different concentration levels (0.5 to 25 $\mu g/\mu l$) in N,N-dimethylformamide.

Thin-layer chromatography

Pre-coated 250-μm silica gel G plates (Analtech, Newark, Del., U.S.A.) were scored in 1-cm-wide strips using the SDA-303 Schoeffel scoring device (Schoeffel Instruments, Westwood, N.J., U.S.A.). Plates were cleaned from UV-absorbing im-purities by pre-developing the plates in the solvent system. After purification, the plates were dried in a ventilated oven and kept in a vacuum desiccator until used. One-microliter samples of the dimethyl formamide solution were applied to alternate lanes using 1-μl Microcaps disposable micropipettes. The blank lanes were used as references for the densitometric determinations. The plates were developed in the lower phase of the solvent system chloroform–methanol–20% ammonium hydroxide (2:2:1) in filter paper-lined chromatographic chambers. Resolution in activated (110°, 1 h) and non-activated plates as well as the effect of saturation of the plate with the solvent vapor prior to immersion in the solvent system, were studied. All plates were developed until the solvent front rose to 2 cm from the top. The solvent was evapo-rated at room temperature in the dark and the plates were scanned after 30 min on a definite time schedule to minimize any time factor involved due to degradation of the polyene.

Densitometry

A Schoeffel SD 3000 double-beam densitometer equipped with a SDC 300 density computer-recorder was used in the transmission mode. Dried plates were scanned in the direction of the development at 2 in./min with a chart speed of 4 in./min. Scanning was done at a wavelength of 340 nm for candihexin and 360 nm for candidin using a monochromator slit bandwidth of 1.5 mm and an exit beam slit width of 0.5 mm with a length of 3.5 mm. Settings in the density computer were posi-tive, ratio and log (for optical density output). Full-scale density ranges of 0.2, 0.4 or 1.0 O.D. were used. Integration of the peak area was done by multiplying peak height by peak width at mid-height.

RESULTS AND DISCUSSION

Separation of the components of polyene macrolide complexes

TLC of candidin and candihexin complexes on the upper phase of neutral or basic solvent systems formed by chloroform, a primary alcohol and water (or 20% ammonium hydroxide) resulted in spots with higher R_F values than those obtained in the lower phase of the same solvent systems. Diffusion of the spots in the upper phase was considerable and poor resolution of the components was obtained. No separation and considerable tailing, as revealed by the densitometric determinations, were obtained in acid solvent systems. Basic solvent systems produced small, dense spots which gave high and well-resolved peaks in the densitometer. The best separation of components was achieved in the lower phase of the solvent system chloroform–methanol–20% ammonium hydroxide, (2:2:1). Six components have been identified in the candihexin complex. Five of them, having R_F values of 0.26, 0.29, 0.36, 0.39 and 0.43, were separated in the solvent system used (Fig. 1). Traces of another component
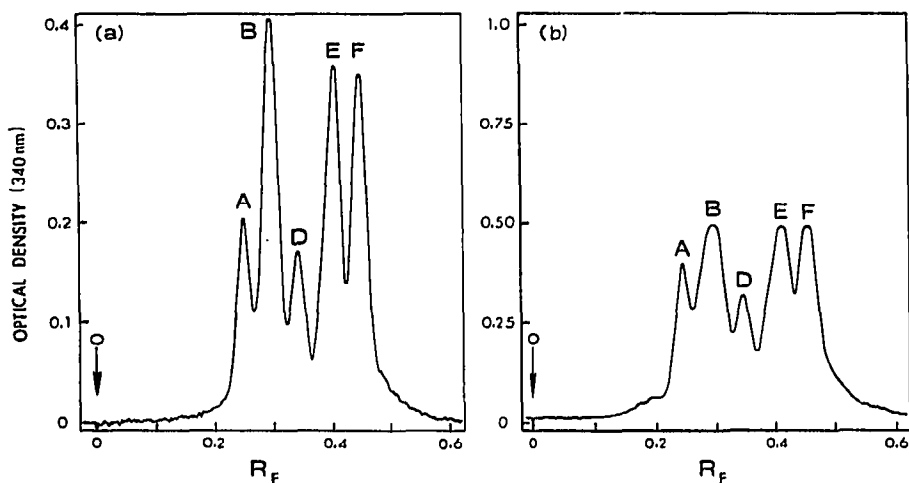

Fig. 1. TLC resolution of the components of the candihexin complex (A–F). (a), 10 μg/spot; chart width O.D. range = 0.4. (b), 25 μg/spot; chart width O.D. range = 1.0.

were detected by countercurrent distribution and column chromatography[15]. Candidin, as reported by Borowski *et al.*[17], has been resolved into two major components having R_F values of 0.27 and 0.32 and traces of a third one with an R_F value of 0.36. This minor component of the candidin complex which forms about 5% of crude candidin preparations was not evident in the purified candidin used for these experiments. Substitution of methanol by ethanol, propanol or butanol gave poorer resolution of the complex. Separation of the components of both polyenes was better in non-activated than in activated plates. No significant difference in resolution was obtained by saturating the plate with the solvent system before starting the development.

Peak area of the individual components as a function of the amount spotted

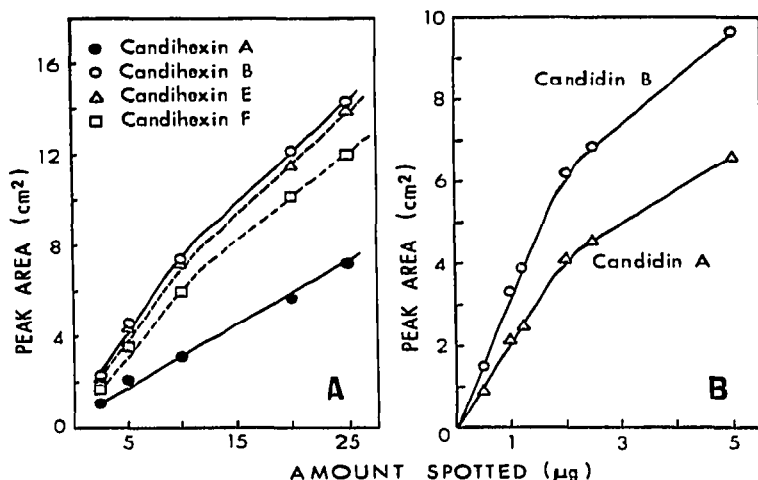The peak areas of the four main components of the candihexin complex *versus*

Fig. 2. Relationship between peak areas and amount spotted for the components of (A) the candihexin complex and (B) the candidin complex. All samples were applied in 1 μl. Chart width O.D. range = 1.0.

the amount of sample (candihexin complex) spotted are plotted in Fig. 2A. A linear response was obtained up to 10 μg/spot, but partial overlapping of the peaks occurred above the 20 μg/spot level as illustrated in Fig. 1. The determination of the components of the candidin complex was more sensitive than that of the components of the candihexin complex. The linear response range was obtained at lower levels of candidin (0.62 to 2.5 μg/spot) (Fig. 2B). Resolution of candidins A and B was good up to 2.5 μg/spot, but no separation took place at the 5 μg/spot level (Fig. 3).

The calibration curves for the components of both polyene complexes, using the mean area response vs. concentration, were straight lines only for low concentra-
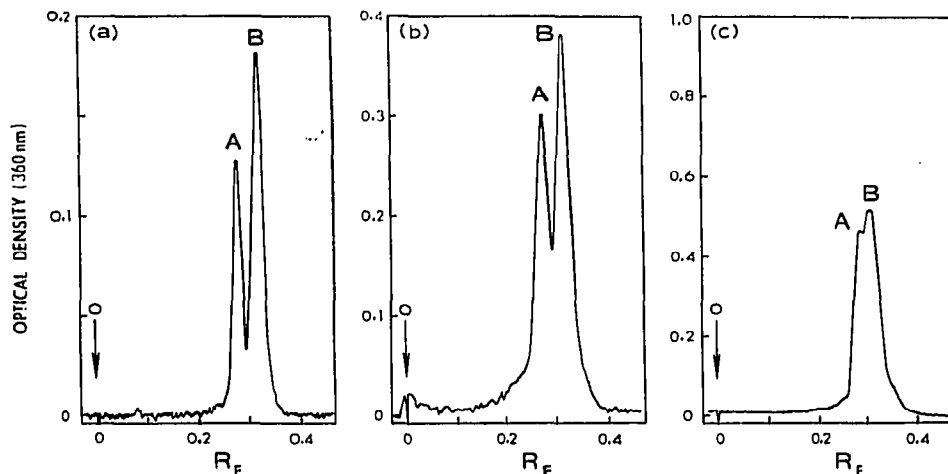

Fig. 3. TLC resolution of the components of the candidin complex. (a), 1 μg/spot; (b), 2 μg/spot; (c), 5 μg/spot. Chart width O.D. ranges are 0.2, 0.4 and 1.0, respectively.
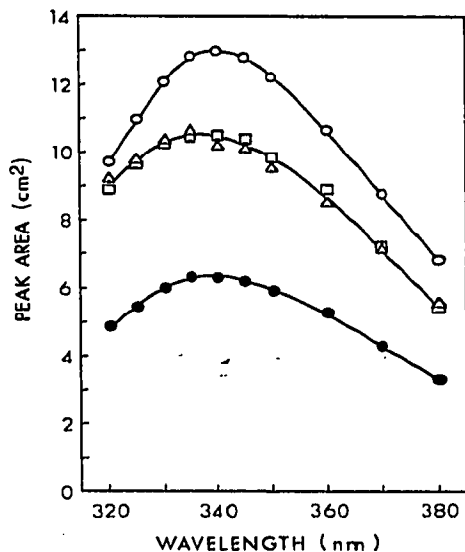
Fig. 4. Peak area responses of the components of the candihexin complex at different wavelengths. 5 μg/spot. Chart width O.D. range = 0.4. ●—●, Candihexin A; ○—○, candihexin B; △—△, candihexin E; □—□, candihexin F.

tion levels. However, a linear calibration curve over the entire range of concentrations was obtained when concentrations were plotted *versus* the area squared although the lines do not extend through the origin. A similar result has been reported by Frei[3]. The linearity of calibration curves using the peak area, peak area squared and peak height plotted as functions of the concentration has been studied[10]. The use of the peak area squared *versus* concentration as calibration function was recommended for
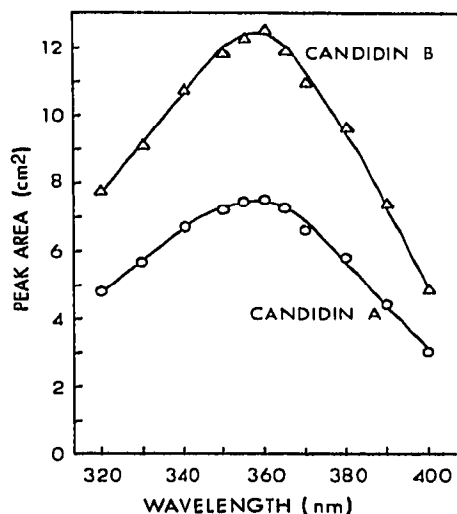

Fig. 5. Peak area responses of the components of the candidin complex at different wavelengths. 2.5 μg/spot. Chart width O.D. range = 0.4.

concentrations above 1 μg/spot while a better linearity was obtained using peak height *versus* concentration at lower concentrations.

Peak area as a function of the wavelength of the incident light

The mean area response of the four main candihexin components at different wavelengths is indicated in Fig. 4. A maximum area response was obtained at 340 to 345 nm. It dropped considerably at higher wavelengths although methanol solutions of candihexin show a first and highest absorption peak at 380 nm[13]. The maximum area response for the candidin components was obtained at 360 nm (Fig. 5) well below the highest peak of candidin in methanol solution which occurs at 405 nm.

Solutions of candidin and candihexin have typical absorption spectra with a main absorption band resolved into four sharp narrow peaks separated by about 20 nm. As the bandwidth, at the slit width of 1.5 mm used throughout the experiments, is in the range of 20 to 25 nm, it resulted in an overlapping of the absorption of the different peaks of the polyenes. It gave a non-resolved band as depicted in Figs. 4 and 5 instead of the multipeak band of the polyenes in solution.

The hypsochromic shift observed in the absorption maxima in TLS with respect to those obtained in solution is probably due to the scattering and reflecting effect of the silica gel that results in a different fraction of transmitted light being absorbed by the sample. A recent study on the effect of the adsorbent layer on the bathochromic shift of the absorption maxima[18] suggests that the shift is a function of the layer thickness and the particle size of the silica gel.

A small peak area was obtained for both antibiotics in the 320 to 330 region, which agrees with the lower end of the absorption band of the polyenes in solution. Under 320 nm the transmitted light was not enough for experimental determinations owing to a drastic absorption of UV light by the silica gel[19].

Reproducibility as a function of the scanning, spotting and amount of sample spotted

The variance due to the scanning (instrumental parameters) of the candidin components is given in Table I as the ratio of the standard deviation (S.D.) to the mean (relative standard deviation). Relative S.D.s were about 1.5 % for candidin A

TABLE I

REPRODUCIBILITY AS A FUNCTION OF SCANNING

Single TLC plate spotted once with 1.25 μg of candidin complex and scanned 10 times.

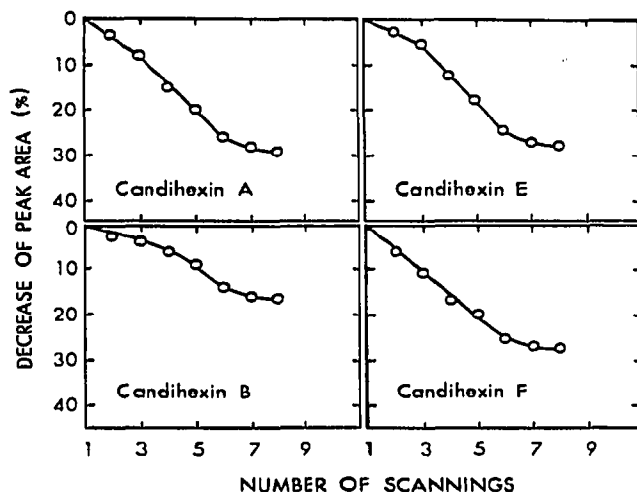| Experiment number	Mean area response (mm²)	S.D.	Relative S.D. (%)
Candidin A			
1	575	10.1	1.7
2	558	8.1	1.4
		Mean	1.55
Candidin B			
1	1039	12.4	1.2
2	1083	10.0	0.9
		Mean	1.06

Fig. 6. Decrease of the peak area response of the candihexin components upon repeated scannings. 5 μg/spot.

and 1 % for candidin B. It was found that the larger the proportion of the component, the smaller the relative S.D. Repeated scannings of the spots of the candidin complex produced no degradation of the polyene as judged by the area response. By contrast, a significant degradation of the candihexin components was found after repeated scannings (Fig. 6). These results reflect the different photostabilities of the components of the two polyene complexes to UV degradation. The lability of polyenes to UV light is a well-known phenomenon which has been correlated with *cis-trans* isomerization of the chromophore[20].

The error associated with the spotting is shown in Table II for the components of both candidin and candihexin complexes. Different relative S.D.s were obtained for each individual component as a result of (1) the specific light absorption and (2) the particular spot diffusion of each component. A higher relative S.D. was observed for

TABLE II

REPRODUCIBILITY AS A FUNCTION OF SPOTTING

Single TLC plate spotted *n* times at one level (10 μg candihexin/spot or 2 μg candidin/spot) and scanned once.

Component	Number of spots, n	Mean area response (mm²)	S.D.	Relative S.D. (%)
Candihexin				
A	9	772	25.2	3.2
B	7	1842	44.8	2.4
D	8	802	39.4	4.9
E	9	1815	50.6	2.7
F	8	1497	50.0	3.3
Candidin				
A	7	1101	46.2	4.2
B	7	1686	66.9	3.9

TABLE III

REPRODUCIBILITY AS A FUNCTION OF THE AMOUNT OF CANDIHEXIN APPLIED

Candihexin complex was spotted *n* times at levels 2.5, 5, 10 and 20 μg/spot and scanned once.

Amount spotted (µg)	Number of spots, n	Mean area response (mm²)	S.D.	Relative S.D. (%)
Candihexin A				
2.5*	7	507	21.4	4.2
5.0**	6	566	32.1	5.6
10.0**	9	751	25.6	3.4
20.0***	8	566	39.2	6.9
Candihexin B				
2.5	8	1174	32.9	2.8
5.0	6	1211	49.7	4.1
10.0	9	1783	48.4	2.7
20.0	8	1225	59.2	4.8
Candihexin E				
2.5	7	1109	52.9	4.9
5.0	6	1177	34.6	2.9
10.0	9	1764	45.1	2.6
20.0	8	1147	72.2	6.2
Candihexin F				
2.5	6	922	49.1	5.3
5.0	7	1046	39.7	3.8
10.0	9	1485	58.8	3.9
20.0	8	1027	62.5	6.0

   * Full-scale chart range = 0.2 O.D.
  ** Full-scale chart range = 0.4 O.D.
*** Full-scale chart range = 1.0 O.D.

the spotting than for the repeated scanning of one single spot, even though a uniform size of spots was used (1 μl applied at once in all cases). Spotting rather than instrumental parameters are thus responsible for the main error in quantitating thin-layer chromatograms of polyenes by densitometry. This conclusion agrees with the results of Frei[3] using reflectance spectroscopy, although Welch and Martin[21] presented evidence showing that the spotting error does not seem to be a significant source of variation on TLS analysis of sugars.

    The study of the relative S.D. as a function of the amount spotted is summarized in Table III for the components of the candihexin complex. Small relative S.D.s were obtained with samples of 5 and 10 μg/spot while either 2.5 μg/spot or 20 and 25 μg/spot samples gave higher variation. The high variation for small samples is due to their small peak areas and were also described in the densitometric determination of penicillin G[9]. The high relative S.D. values obtained for very large samples are a consequence of the poor resolution of the components under these conditions (Fig. 1). Results of the reproducibility of the area response of increasing amounts of candidin are shown in Table IV. The lowest level of sample gave the highest relative standard deviation. Minimal values of the relative standard deviation at intermediate levels in the linear range of samples were also reported by Frei[3].

TABLE IV

REPRODUCIBILITY AS A FUNCTION OF THE AMOUNT OF CANDIDIN APPLIED

Candidin complex was spotted n times at levels 0.62, 1.0, 1.25, 2.0, 2.5 and 5 μg/spot and scanned once.

Amount spotted (μg)	Number of spots	Mean area response (mm²)	S.D.	Relative S.D. (%)
Candidin A				
0.625*	9	490	32.1	6.5
1.00**	8	543	30.6	5.6
1.25**	7	604	33.9	5.6
2.00**	6	1108	49.9	4.5
2.50***	8	422	20.3	4.8
Candidin B				
0.625	8	759	53.4	7.0
1.00	8	835	35.4	4.2
1.25	7	965	63.5	6.5
2.00	6	1696	67.3	3.9
2.50	8	653	36.8	5.6

* Full-scale chart range = 0.2 O.D.
** Full-scale chart range = 0.4 O.D.
*** Full-scale chart range = 1.0 O.D.

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