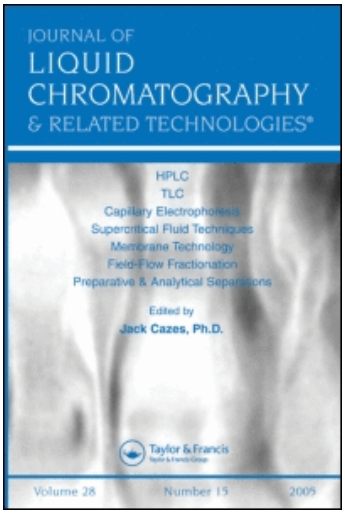

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RAPID DETERMINATION OF AVILAMYCIN IN FERMENTATION BROTHS

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INTRODUCTION

Avilamycin is a member of the orthosomycin family of antibiotics which is produced by *Streptomyces viridochromogenes*. Avilamycin is commonly used as a feed additive for a wide variety of domestic animals. To date, the only published assay procedure for the determination of avilamycin was performed by gas chromatography.[1] Several articles have detailed the separation of avilamycin factors on a preparative scale[2-4], however, no method has been published for the determination of avilamycin in fermentation broth. In this work, a rapid isocratic HPLC assay is described for the determination of avilamycin in fermentation broth.

EXPERIMENTAL

Chemicals

Avilamycin was obtained from Lilly Research Laboratories (Indianapolis, IN). Standards were prepared by dissolving the appropriate amount of material in 4:1 mixture of HPLC grade methanol and 0.1 M phosphate buffer, pH 7.0. Unless otherwise specified, all solvents were of HPLC grade and were purchased from Fisher Scientific (Springfield, NJ). All other chemicals were of reagent grade.

Apparatus

A Varian Vista 5500 liquid chromatograph (Varian Associates, Palo Alto, CA) was used. The guard column was a BrownLee Newguard RP-18 (dimensions: 15X3.2 mm, 7 µm particle diameter) and the analytical column was a Waters Nova-Pak C_{18} column (dimensions: 100X4.6 mm, 4 µm particle diameter). The chromatograph was fitted with 0.010" diameter stainless steel tubing. Unless otherwise specified, the detector wavelength was set at 290 nm. The detector range was set to suit a given application.

Chromatographic conditions

The mobile phase consisted of 45% methanol and 55% 15 mM ammonium phosphate adjusted to a pH value of 3.0 with concnetrated phosphoric acid. The flow rate was set at 3 mL/min, resulting in a typical back-pressure of about 250 atm. Unless otherwise specified, the injection volume was 10 µL.

Extraction of avilamycin

A 7 mL portion of fermentation broth was extracted with 45 mL of reagent grade methanol. The suspension was homogenized and allowed to settle for about an hour. A portion of the supernatent was withdrawn and filtered to 0.45 µm with a Lid/X filter (Genex, Gaithersburg, MD). The filtered solution was injected directly into the chromatograph.

Biological assay

The biological activity of avilamycin was measured against *Streptococcus faecium* (American Type Culture Collection, Rockville, MD). The assay broth was prepared by dissolving 5 g peptone, 1.5 g yeast extract, 1.0 g glucose, 3.0 g monobasic potassium phosphate, 1.5 g dibasic potasssium phosphate, and 10 mL of a Tween 80 solution in 1.0 L deionized water. Approximately 18 hours prior to analysis, a culture of *S. faecium* was transferred to a 500 mL erlenmeyer flask containing 250 mL brain heart infusion broth. The culture was then incubated overnight at room temperature. The culture was then chilled to retard growth and refrigerated until ready for use. Avilamycin broth samples were diluted with acetone to fall within a concentration range of 0.75-2.0 µg/mL. The diluted broth samples were then inoculated with *S. faecium*. The samples were placed in a 30°C water bath for a period of 4 hours and the turbidity of the solutions was measured with an Autoturb II reader (Elanco, Indianapolis, IN).

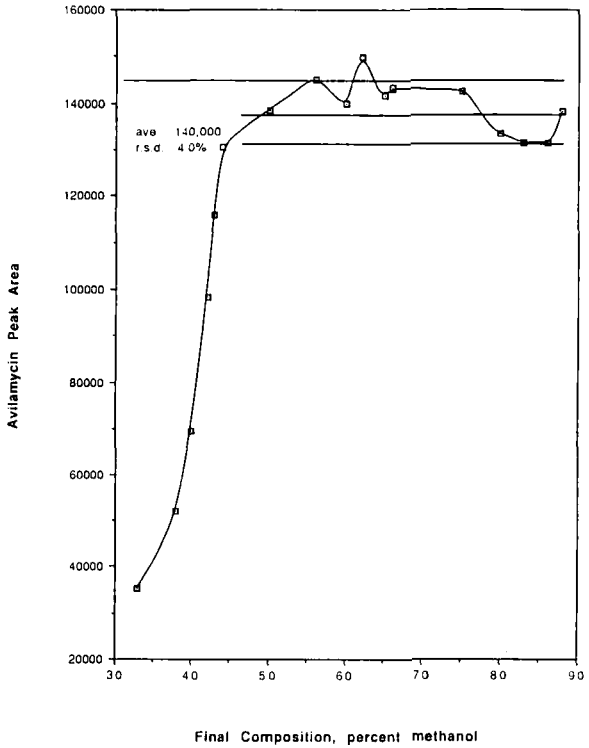

Fig. 1. Normalized plot of mass of avilamycin extracted versus ratio of methanol to broth. Dashed lines represent one standard deviation unit for data obtained with a methanol to broth ratio of 1.0 or greater. Relative standard deviation is 4.0%.

RESULTS AND DISCUSSION

Figure 1 depicts the relationship of extractant volume to avilamycin concentration. Incomplete extraction was observed with methanol:broth ratios less than 1. At final methanol concentrations above 50%, the extraction was quantitative, with a relative standard deviation of about 4%. The extraction procedure used throughout this work results in a final methanol concentration of 87%. The reproducibility of the extraction procedure was evaluated by extracting a broth sample five times. The coefficient of variation was 1.6%. In comparison, the coefficient of variation of multiple (N=5) injections of a single sample was 0.5%. The stability of avilamycin in the broth was investigated by alternating injections of standard and sample

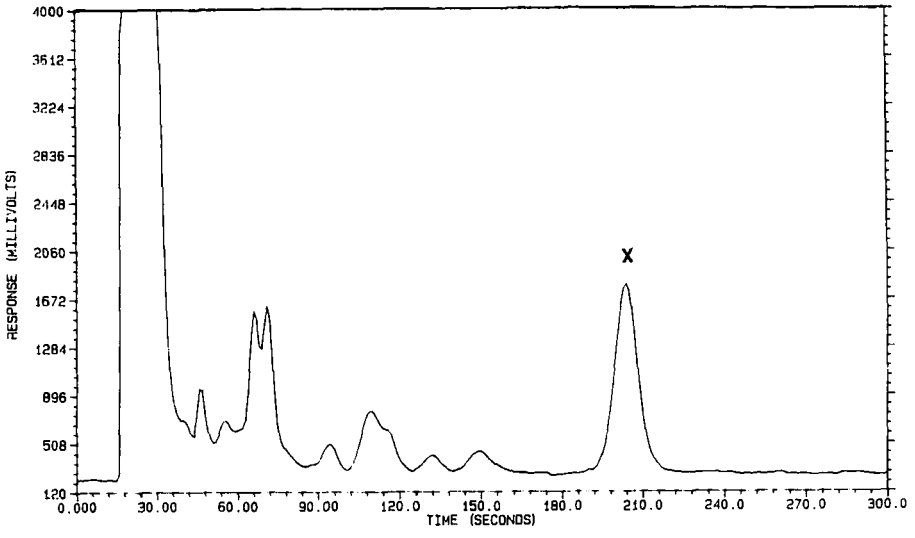

Fig. 2. Chromatogram of fermentation extract containing 0.350 mg/mL avilamycin factor A. Avilamycin factor A peak is labelled "X".

TABLE I

ANALYSIS OF AVILAMYCIN AT SEVERAL WAVELENGTH

Sample	Result (mg/mL)			
Wavelength:	220 nm	250 nm	290 nm	average (rsd)
1	0.35	0.32	0.35	0.34 (4.1%)
2	0.76	0.70	0.75	0.74 (4.4%)
3	1.12	1.06	1.09	1.09 (3.0%)
4	1.48	1.43	1.46	1.46 (1.7%)
5	1.82	1.77	1.81	1.80 (1.4%)

TABLE II

SUMMARY OF SPIKING EXPERIMENTS

Solution	Percent of Native Concentration	$Conc_{theory}$ (mg/mL)	$Conc_{found}$ (mg/mL)	Recovery
A	100	NA	0.423	NA
B	116	0.491	0.473	96.3%
C	132	0.557	0.567	101.8%
D	163	0.691	0.697	100.8%
E	195	0.824	0.841	102.1%
F	226	0.957	0.975	101.9%
G	258	1.091	1.096	100.5%

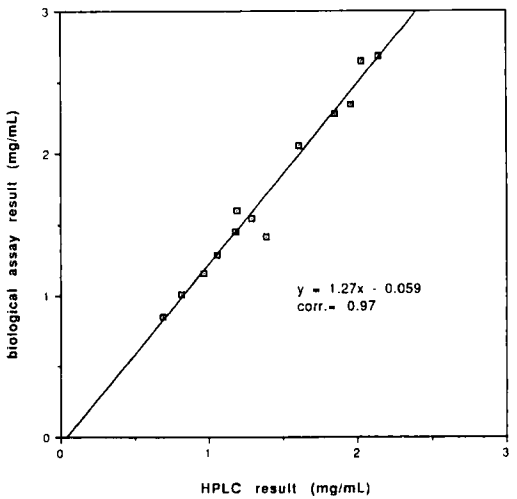

Fig. 3. Comparison of HPLC and microbiological assays. The samples were obtained by spiking avilamycin into broth extract. Line: bio = 1.27*HPLC - 0.059.

over a period of 12 hours. Both solutions were at room temperature. The relative standard deviations of the peak areas of the standard and sample were 1.1% and 1.4%, respectively (N=25). Data acquired over a longer time period indicated that the avilamycin in the extract was stable for at least five days at room temperature.

The peak height and area were linear with concentration over the domain 0.1 to 3.0 mg/mL. Typical calibration plots gave correlation coefficients of 0.999 or better with origin intercepts. At avilamycin concentrations in excess of 3.0 mg/mL, the calibration curve was non-linear.

Figure 2 depicts a typical chromatogram of a fermentation extract. Avilamycin exhibited a retention time of 206 seconds. The column efficiency was measured to be about 25,000 theoretical plates per meter. The peak symmetry was found to be 1.05. The analytical column typically lasted about 1000 injections before the theoretical plate count decreased by 20%.

The selectivity of the chromatography was investigated by performing the analysis on four broth extracts at several detection wavelengths, 220, 250, and 290 nm. The pertinent data is listed in Table I. Clearly, the assay results were independent of detection wavelength, suggestive of a single component peak. Table II lists the recovery of avilamycin spiked into the fermentation extract. Over the concentration domain studied, the recovery of avilamycin averaged 100.6%.

Avilamycin was spiked into a blank broth extract to produce a series of samples with varying avilamycin concentration. The samples were analyzed by HPLC and a microbiological assay. The correlation of the two assays is presented in figure 3. A linear relationship is evident, although the microbiological assay gives slightly inflated results. This effect is most likely related to the presence of substituted avilamycin isomers, which exhibit some antimicrobial activity. The HPLC assay, however, is specific for the major factor of avilamycin, factor A (75% of the factor distribution). The other factors elute prior to factor A.

CONCLUSIONS

A rapid HPLC assay has been developed for the determination of avilamycin in fermentation broth. The assay correlates well with microbiological activity and is sufficiently durable for applications involving high sample throughput.

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