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LIQUID CHROMATOGRAPHIC DETERMINATION OF LINCOMYCIN IN FERMENTATION BEERS

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SUMMARY

A reversed-phase ion-pairing liquid chromatographic method with UV detection at 214 nm is described for the determination of lincomycin A and lincomycin B in fermentation beers. The chromatographic system consists of a microparticulate octylsilica column and a mobile phase composed of 10 m*M* sodium dodecyl sulfate in ammonium phosphate-buffered aqueous acetonitrile (pH 6). Thermostating the column at 45°C improves the symmetry of the lincomycin peaks by eliminating fronting. The sample is diluted with phosphate buffer, centrifuged and the supernatant injected on the chromatographic column. The ion-pairing reagent causes lincomycin A and lincomycin B to be separated from each other and from all other substances present in raw fermentation beers. Precision of the assay for lincomycin A was 1.2% relative standard deviation. Recovery of spiked lincomycin A and lincomycin B from a fermentation beer sample was quantitative. A comparison of this high-performance liquid chromatographic (HPLC) method to a standard automated wet chemical method shows the HPLC method is more precise, specific and accurate, while being as simple to accomplish.

INTRODUCTION

Lincomycin is a medium spectrum antibiotic which is produced by fermentation as a mixture of the compounds lincomycin A and lincomycin B. The structures of these compounds are shown in Fig. 1. Lincomycin B shows only 25% the activity of lincomycin A, and typically represents less than 5% of the total lincomycin concentration in the bulk drug[1].

The lincomycin fermentation beer is a complex, heterogeneous mixture, whose composition changes as the fermentation progresses. The lincomycin fermentation is a directed fermentation in which the formation of side reaction products, such as lincomycin B, is suppressed. In order to monitor and direct the fermentation, the concentration of both lincomycin A and lincomycin B are monitored as the fermentation progresses.

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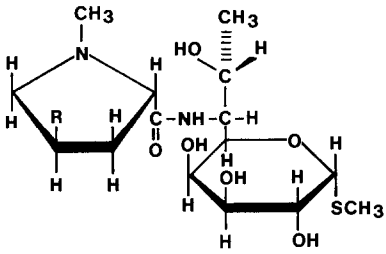

LINCOMYCIN A: R= $CH_2CH_2CH_3$

LINCOMYCIN B: R= CH_2CH_3

Fig. 1. Chemical structure of lincomycin A and lincomycin B.

A wet chemical method developed by Prescott[2] is the method most used for the determination of lincomycin in fermentation beers. The method is based on the reaction of 5,5′-dithiobis(2-nitrobenzoic acid) with the methanethiol generated by acid hydrolysis of the methylthioglycosido group of the antibiotic molecule. The colored product is then measured spectrophotometrically. Automation of the method with an AutoAnalyzer™ makes possible 150 assays per day, with an assay precision of 6% R.S.D. The AutoAnalyzer method is not specific for lincomycin A and measures all compounds having a hydrolyzable thio group, such as for example, lincomycin B. In addition, a positive bias is obtained from certain components, in the fermentation media[2]. Other assay methods which solve the AutoAnalyzer's methods specificity problem are a gas chromatographic procedure[3] and a gas chromatographic–mass spectrometric assay[4]. These methods, however, require derivatization of the lincomycin to form a volatile analyte, and are not rapid enough for routine monitoring of fermentation beers.

Our intention in developing a process assay for lincomycin was to replace the AutoAnalyzer method with one having a simple sample preparation, better specificity for lincomycin A and an analysis time comparable to the AutoAnalyzer method. The assay method developed and described below is an isocratic high-performance liquid chromatographic (HPLC) method using reversed-phase ion-pairing chromatography with UV detection at 214 nm. Sample preparation is very similar to the AutoAnalyzer method sample preparation, except in this chromatographic assay samples are diluted with pH 3 phosphate buffer solution instead of water. Chromatographic resolution and quantitation of both lincomycin A and lincomycin B within 15 min provides very useful information on the progress of the fermentation. The utility of the method was demonstrated by analyzing several samples of fermentation beers and comparing the analytical results obtained to the results from the AutoAnalyzer method.

EXPERIMENTAL

Chromatography

Experiments were conducted on a modular liquid chromatograph consisting of a Model 110A pump (Altex, Berkeley, CA, U.S.A.), an autosampler containing an injection valve with a 50-μl loop, (Valco, Austin, TX, U.S.A.), a RP-8 guard column

(Brownlee Labs., Santa Clara, CA, U.S.A.) and a Model 1203 detector with a zinc source (214 nm) (LDC, St. Petersbery, FL, U.S.A.) and a recorder (Sargent-Welch, Skokie, IL, U.S.A.). For quantitation, the data was collected and processed by a PDP 11 digital computer (Digital Equipment, Maynard, MA, U.S.A.).

The analytical column, a Zorbax C_8 (25 cm × 4.6 mm) (DuPont, Wilmington, DE, U.S.A.) was operated at a flow-rate of 2 ml/min. The use of a guard column in this assay method is very important due to the large amount of particulate matter and very hydrophobic impurities in raw fermentation beers. Changing the guard column when poor peak shape is observed will, in most cases, restore the separation to initial conditions. Both columns were thermostated at 45°C. The mobile phase was prepared by dissolving 2.9 g of sodium dodecyl sulfate and 10 ml of concentrated (85%) phosphoric acid in 660 ml distilled water and then adding 330 ml of HPLC grade acetonitrile (Burdick & Jackson Labs., Muskegon, MI, U.S.A.). The solution is titrated to an apparent pH of 6.0 with concentrated ammonium hydroxide and then filtered. Using these chromatographic conditions, the retention times of lincomycin A and lincomycin B are approximately 11 and 6 min, respectively.

Procedure

Before sampling, the fermentation beer is thoroughly mixed by shaking and inverting the sample container to obtain a homogeneous mixture. An accurately weighed aliquot of the sample is diluted with a phosphate buffer solution to obtain a

Fig. 2. Chromatogram of lincomycin fermentation beer sample preparation. Conditions: mobile phase, 10 m*M* sodium dodecyl sulfate and 150 m*M* ammonium phosphate in 33% acetonitrile, pH 6.0; flow-rate, 2.0 ml/min; temperature, 45°C. Peaks: 1 = lincomycin B; 2 = lincomycin A.

lincomycin A concentration of 20–400 μg/ml. The phosphate buffer is prepared by dissolving 7 ml of concentrated phosphoric acid in 1000 ml of distilled water and adjusting to pH 3.0 with concentrated ammonium hydroxide. The sample is shaken for 1 min and an aliquot of the diluted sample centrifuged for at least 10 min at a minimum of 2000 *g*. A 25–100-μl volume of the clear supernatant is analyzed by LC. The concentration of lincomycin A and B in the sample preparations is then determined by a comparison to standard curves over the range of 20–400 μg/ml lincomycin A and 2–40 μg/ml lincomycin B.

RESULTS AND DISCUSSION

A typical chromatogram of a lincomycin fermentation beer sample is shown in Fig. 2. The lincomycin A and lincomycin B peaks are sharp and well resolved from each other and from the rest of the sample constituents. The only sample preparation is dilution of the beer with phosphate buffer and centrifugation. The addition of the dodecyl sulfate ion to the mobile phase enables the separation of the lincomycin peaks from the many other compounds in this very complex matrix without any sample "clean up". Without dodecyl sulfate as an ion-pairing reagent, these drug peaks are lost in a "forest" of constituent peaks.

Dodecyl sulfate was chosen as the ion-pairing reagent after several other alkyl sulfate and sulfonates were tried. In general, we found that the more hydrophobic the ion-pairing reagent, the greater was the retention of lincomycin and the greater the selecitivity. As the dodecyl sulfate concentration was increased retention was increased. Both of these observations agree with well known reversed-phase ion-pairing behavior. The use of dodecyl sulfate imparted enough selectivity to the system in order to separate lincomycin A and lincomycin B from the rest of the UV-absorbing constituents in this sample, while still being soluble in the mobile phase. The dodecyl sulfate concentration was arbitrarily set at 10 m*M* to allow fairly rapid loading on the column, so that stable retention times would be obtained within 2 h. With this concentration of dodecyl sulfate, the acetonitrile concentration of 33% gives a lincomycin A retention time of approximately 11 min. In addition to controlling selec-

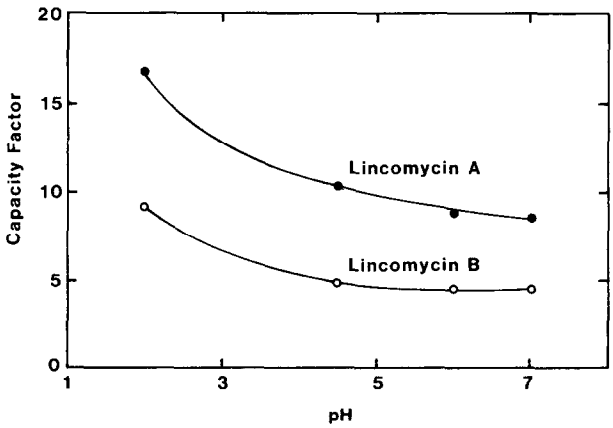

Fig. 3. Effect of mobile phase pH on retention. Conditions as in Fig. 2.

tivity, the sodium dodecyl sulfate helps reduce tailing of the lincomycin peak on the silica based reversed-phase packing material. Addition of ion-pairing reagents to the mobile phase has previously been shown, in some cases, to reduce tailing of nitrogen-containing compounds[5].

The pH of the mobile phase was used as a further means of altering the selectivity of the separation between lincomycin and interfering compounds. Fig. 3 illustrates the dependence of lincomycin retention on the pH of the mobile phase. With decreasing pH, the retention of lincomycin increases. This is attributed to greater protonation of the pyrrolidine nitrogen atom at lower pH, the pK_a of which is 7.6[6]. With an increase in the concentration of the positively charged lincomycin, more ion interaction occurs and therefore greater retention. The beer samples contain other compounds which are also more retained as the pH of the mobile phase is lowered. However, by raising the mobile phase pH to 6, the selectivity between the drug and other beer constituents is greatly enhanced so that adequate resolution is obtained.

Another problem encountered with the development of this separation was a "fronting" lincomycin peak when the assay was attempted at room temperature. Thermostating the analytical column and guard column at 45°C dramatically improves peak shape over that observed at room temperature by elimination of the peak front (Fig. 4). The reason for this improvement in peak shape has not been investigated in detail. However, the addition of the competing amine, N,N-dimethyloctylamine, to the mobile phase did not affect the fronting phenomenon. Since competing amines have been shown to reduce solute interaction with residual

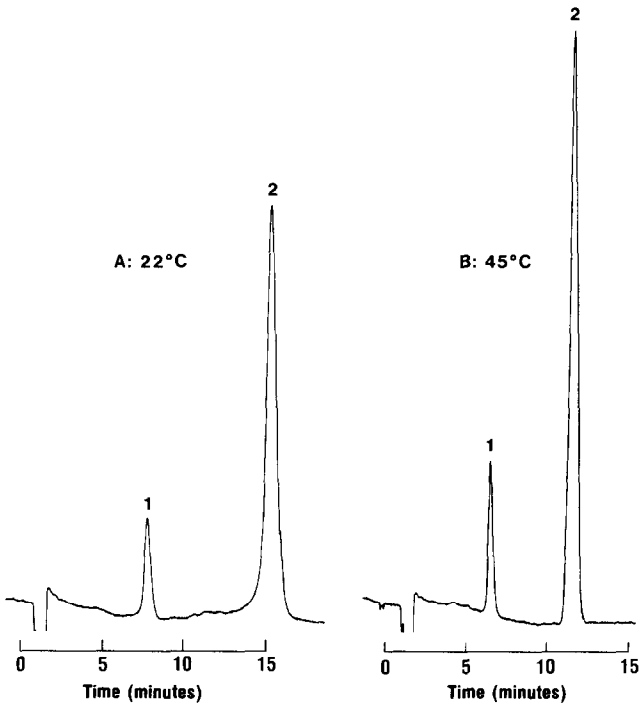

Fig. 4. Chromatograms of lincomycin standards: A, 22°C and B, 45°C. Conditions as in Fig. 2.

TABLE I

STANDARD ADDITION TO LINCOMYCIN BEER SAMPLE

	Lincomycin A (mg)		*Lincomycin B (mg)*	
	Added	*Found*	*Added*	*Found*
	0	0.86	0	0.05
	1.763	2.62	0.291	0.36
	3.525	4.58	0.582	0.63
	5.288	6.41	0.873	0.90
	8.813	9.76	1.455	1.53
Slope	1.016		1.007	
Intercept	0.904		0.050	
Corr. coeff.	0.9995		0.9995	
E.S.D.* slope	0.018		0.019	
E.S.D. intercept	0.087		0.016	

* Estimated standard deviation.

silanol groups[7], these results indicate that the disturbing effect is not an ion-exchange or adsorption retention mechanism.

Linearity of the method was determined over the concentration range of 1.76–35.2 μg/ml and 0.29–5.82 μg/ml for lincomycin A and lincomycin B respectively. The resulting standard curve for lincomycin A is described by the following regression equation:

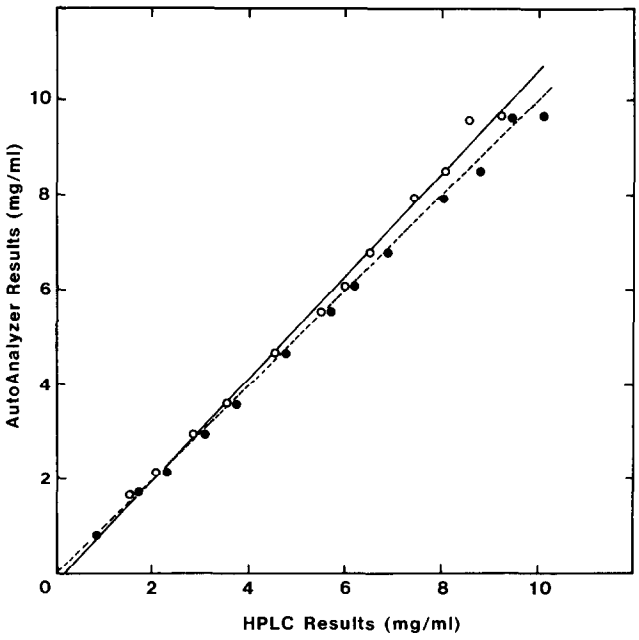

Fig. 5. Correlation of HPLC and AutoAnalyzer results. Regression equation for lincomycin A (solid line): $y = 1.093\ (\pm\ 0.001)x - 0.188\ (\pm\ 0.123)$; $r = 0.998$. Dashed line = theoretical curve.

peak area ratio = 0.343 × concentration (mg/ml) + 0.070

For lincomycin B the regression equation was:

peak area ratio = 0.344 × concentration (mg/ml) + 0.004

The correlation coefficients of the regression were 0.9998 and 0.9991 for lincomycin A and B, respectively.

Precision of the assay method was tested by preparing and analyzing a typical beer sample five times. The relative standard deviations obtained were 1.2% and 6.6% for lincomycin A and B, respectively.

A standard addition experiment was performed by spiking 1-ml aliquots of a young beer sample with various amounts of lincomycin and analyzing the resulting samples. The results of this study are shown in Table I. The linear regression equations obtained show that lincomycin is not being adsorbed by components in the beer sample. The slopes of the lines are not significantly different from the theoretical value of 1.000 ($p = 0.05$), and the correlation coefficients of the regression are greater than 0.999. The intercepts give a very accurate measurement of the lincomycin A and B concentrations in the original sample.

To compare the HPLC procedure for lincomycin with the AutoAnalyzer

TABLE II

COMPARISON OF LINCOMYCIN ASSAY RESULTS OBTAINED BY HPLC AND AUTOANALYZER METHODS (mg/ml)

Sample	*Time of sampling**	*AutoAnalyzer Total lincomycin*	*HPLC*		
			Lincomycin A	*Lincomycin B*	*Total*
A	0.25	0.74	0.81	<0.03	0.81
A	0.30	1.67	1.54	0.16	1.70
A	0.36	2.15	2.06	0.21	2.27
A	0.42	2.96	2.85	0.24	3.09
A	0.48	3.59	3.56	0.20	3.76
A	0.54	4.65	4.54	0.19	4.73
A	0.60	5.53	5.48	0.21	5.69
A	0.66	6.08	5.95	0.21	6.17
A	0.72	6.81	6.44	0.39	6.83
A	0.84	7.96	7.38	0.63	8.01
A	0.89	8.54	8.01	0.71	8.73
A	0.95	9.57	8.49	0.84	9.33
A	1.00	9.65	9.14	0.88	10.02
B	0.18	0.33	0.23	0.03	0.26
C	0.73	6.14	5.95	0.15	6.11
D	0.32	1.53	1.57	0.10	1.66
D	0.52	4.33	3.84	0.18	4.02
E	0.20	0.35	0.34	<0.03	0.34
F	0.37	2.21	2.21	0.18	2.39

* Normalized to the end of the fermentation.

method one lot of fermentation beer sampled at various times during the course of the fermentation, as well as different beer samples, were assayed by both methods (Table II). As shown in Fig. 5, close agreement between the methods is obtained when a comparison of the AutoAnalyzer result and the sum of the lincomycin A and B concentrations by HPLC is made. The regression equation for the line (not shown) is $y = 0.999\ (\pm\ 0.014)x - 0.089\ (\pm\ 0.084)$. However, when the lincomycin A concentration only by HPLC is plotted against the AutoAnalyzer results (solid line in Fig. 5), we see a statistically significant deviation ($p = 0.05$) between the two methods; with the AutoAnalyzer method giving the higher result. The reason for the deviation is clearly the lincomycin B concentration.

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