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Note

High-performance liquid chromatographic analysis of erythromycins A and B from fermentation broths

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Preparations of erythromycin contain erythromycins A, B and C, erythromycin A being the major component. The activity spectra of erythromycins B and C are similar to that of erythromycin A[1]. Several paper chromatographic[2,3] and thin-layer chromatographic methods[4,5] are used for the separation of erythromycins, but these are not suitable for quantitative determinations. A gas–liquid chromatographic method[6] is available for the separation and quantitation of erythromycins and their derivatives and degradation compounds, but this requires a long time for derivatization.

Two high-performance liquid chromatographic (HPLC) methods[7,8] have been reported for the analysis of erythromycins; the separation of erythromycins A and B was described, but these reports failed to present quantitation data and did not demonstrate any separation of the numerous erythromycin epimers and their degradation products.

Two HPLC methods[9,10] have been reported for erythromycin analysis; one[9] is capable of quantifying selectively erythromycins A, B and C and at least nine other erythromycin epimers and degradation compounds; the other[10] is utilized as a rapid means of monitoring erythromycin fermentation processes.

A preparative HPLC method has been described[11] for the separation of erythromycin F.

We now report a method for HPLC determination of erythromycins A and B in fermentation broths of *Streptomyces erythreus*, after extraction in isoamyl acetate.

EXPERIMENTAL

Instruments

A high-performance liquid chromatograph (Pye Unicam, Cambridge, U.K.) equipped with a high-pressure mini-pump (PU 4010, Pye Unicam) and a variable-wavelength detector (LC3 UV detector, Pye Unicam) at 215 nm was used. The instrument was connected to a single-pen recorder (PM 8251, Philips, Eindhoven, The Netherlands) and to a computing integrator (DP 88, Pye Unicam).

The attenuation of the detector was 0.32 a.u.f.s. A sample was injected through a Rheodyne injector (Model 7120, Berkeley, CA, U.S.A.) with a 20-μl fixed loop. A reversed-phase column (Hibar RP 18, 10 μm, E. Merck, Darmstadt, F.R.G.) (250

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× 4 mm I.D.) with a 40 × 4 mm I.D. stainless-steel pre-column packed with LiChroprep RP-8, 5–20 μm (E. Merck), was used. The mobile phase was pumped at a flow-rate of *ca.* 1.5 ml/min (1300 p.s.i.) and the column was operated at room temperature. The column had to be conditioned for at least 24 h with the eluent before using.

Mobile phase

The mobile phase consisted of methanol–water–ammonia 28 Bé (80:19.9:0.1). The pH of the mixture was such that the packing material was not hydrolysed; it is possible to use the same column for 1000 analyses. After wetting the filter with methanol, the mobile phase was filtered through a 5.0-μm LS-type filter (Millipore, Bedford, MA, U.S.A.) and degassed under vacuum prior to use. At the end of each working day, the column was rinsed with deaerated water for *ca.* 1 h, followed by acetonitrile for a further *ca.* 1 h.

Reference standard solutions

Erythromycin A base. Approximately 50 mg of the powder were accurately weighed into a 10-ml volumetric flask. Just prior to the analysis, isoamyl acetate was added and the mixture was sonicated to facilitate dissolution.

Erythromycin B base. The standard solution was prepared as above.

Sample preparation

An appropriate quantity of the fermentation broth was accurately weighed into a 200-ml beaker. Isoamyl acetate was added in such a quantity to extract erythromycins in the solvent at 5–10 mg/ml; the pH was adjusted to 9.8 with ammonia 28 Bé. The mixture was stirred for *ca.* 5 min with a magnetic stirrer then centrifuged at *ca.* 20,000 r.p.m. to separate the solvent. The isoamyl acetate extract was filtered through a 0.5-μm FH-type filter (Millipore).

Calculations

For the analysis of erythromycins A and B in the isoamyl acetate extract, the peak areas were compared with those of the solutions of the two reference standards. No internal standard was used; using simple calculations it was possible to determine the quantities of erythromycins A and B contained in the fermentation broth.

RESULTS AND DISCUSSION

A typical HPLC chromatogram of erythromycins A and B is shown in Fig. 1.

Quantitation of erythromycins A and B

The precision of our HPLC method for the quantitation of erythromycins A and B was determined by analysing six individually weighed and individually prepared standard erythromycin reference powders. The relative standard deviations of the assay method were 2.62% for erythromycin A and 2.74% for erythromycin B (Table I).

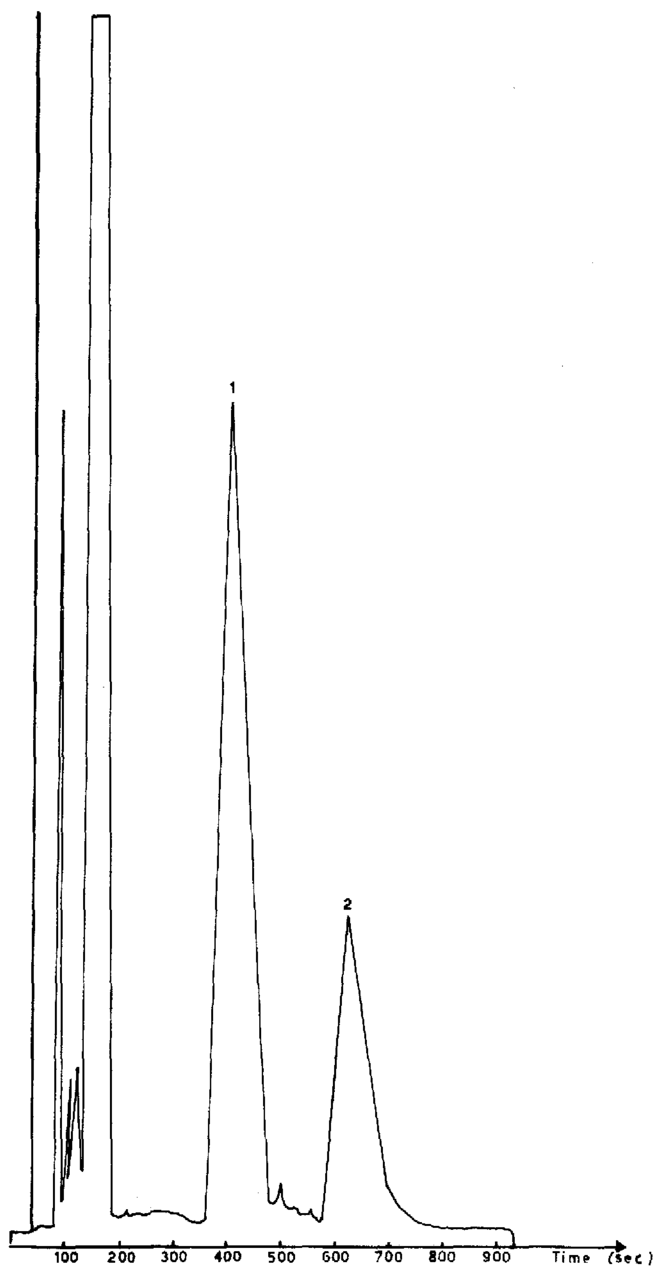

Fig. 1. HPLC chromatogram of erythromycin A (1) and B (2).

Comparison of various methods

We have compared the determinations of erythromycins A and B during a fermentation process utilizing the HPLC method of Tsuji and Goetz[10] and the HPLC method developed by us. The results, reported in Table II, show that our results are the higher of the two methods. It is possible that the procedure of Tsuji and Goetz

TABLE I

PRECISION OF THE ERYTHROMYCIN A AND B HPLC ASSAY METHOD

Weight of erythromycin injected (μg)	*Peak area of erythromycin*	*Peak area/weight ratio*
Erythromycin A		
100.6	1150322	11434.61
100.9	1201877	11911.56
103.4	1202622	11630.77
101.5	1150622	11336.18
100.5	1148892	11431.76
99.7	1097892	11011.95
Erythromycin B		
100.3	1142322	11389.05
99.7	1098782	11020.88
102.7	1158620	11281.60
100.2	1166720	11643.91
101.2	1192622	11784.80
99.8	1101722	11039.30

for the purification of the fermentation broth, before the injection into the HPLC apparatus, causes a loss of erythromycins. In fact, under our experimental conditions, we recovered using the method of Tsuji and Goetz[10] *ca.* 75–80% of the erythromycins A and B.

We also compared the determinations of erythromycins during a fermentation process by utilizing a chemical method[12], a microbiological method[13] and the HPLC method developed by us. The results are reported in Fig. 2.

The highest values were obtained using the chemical assay because the colorimetric method with sulphuric acid is not selective and assays also other fermentation products such as erythronolides A and B and traces of erythromycins C–F.

The microbiological method gives slightly higher values than our HPLC method. It is possible that the test organism (*S. lutea* ATCC 9341) is partially inhibited by other substances different from erythromycins A and B, such as erythromycins C–F.

TABLE II

DETERMINATIONS OF ERYTHROMYCINS A AND B DURING A FERMENTATION PROCESS UTILIZING THE METHOD OF TSUJI AND GOETZ AND THE METHOD DEVELOPED BY US

Sample No.	*Our method (μg/ml)*		*Method of Tsuji and Goetz*[10] *(μg/ml)*	
	Erythromycin A	*Erythromycin B*	*Erythromycin A*	*Erythromycin B*
1	785	265	604	199
2	920	298	717	224
3	1480	460	1184	354
4	1512	452	1179	357

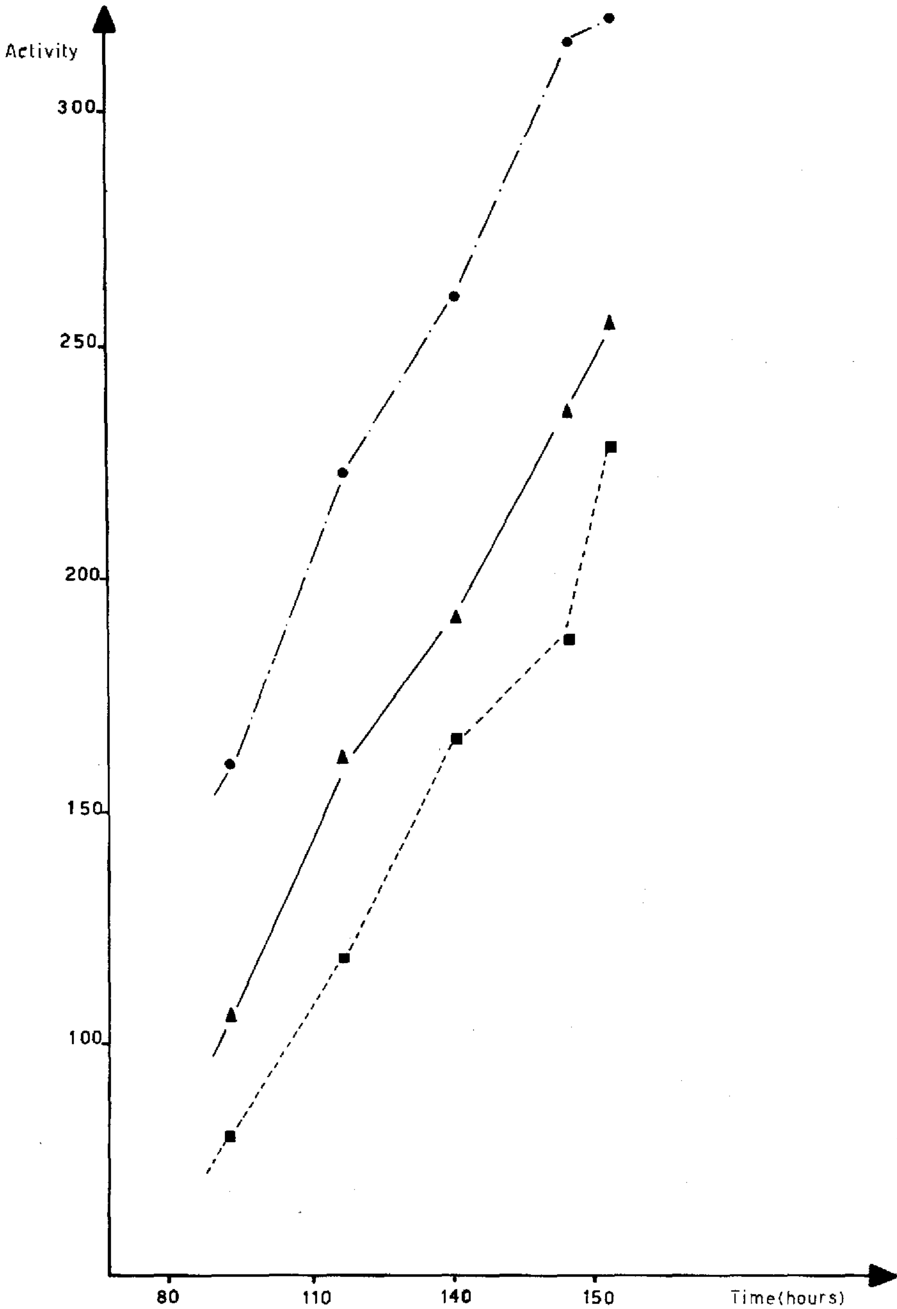

Fig. 2. Comparison of chemical, microbiological and our HPLC assay of erythromycin A + B during the fermentation process. ● = chemical; ▲ = microbiological; ■ = HPLC assay.

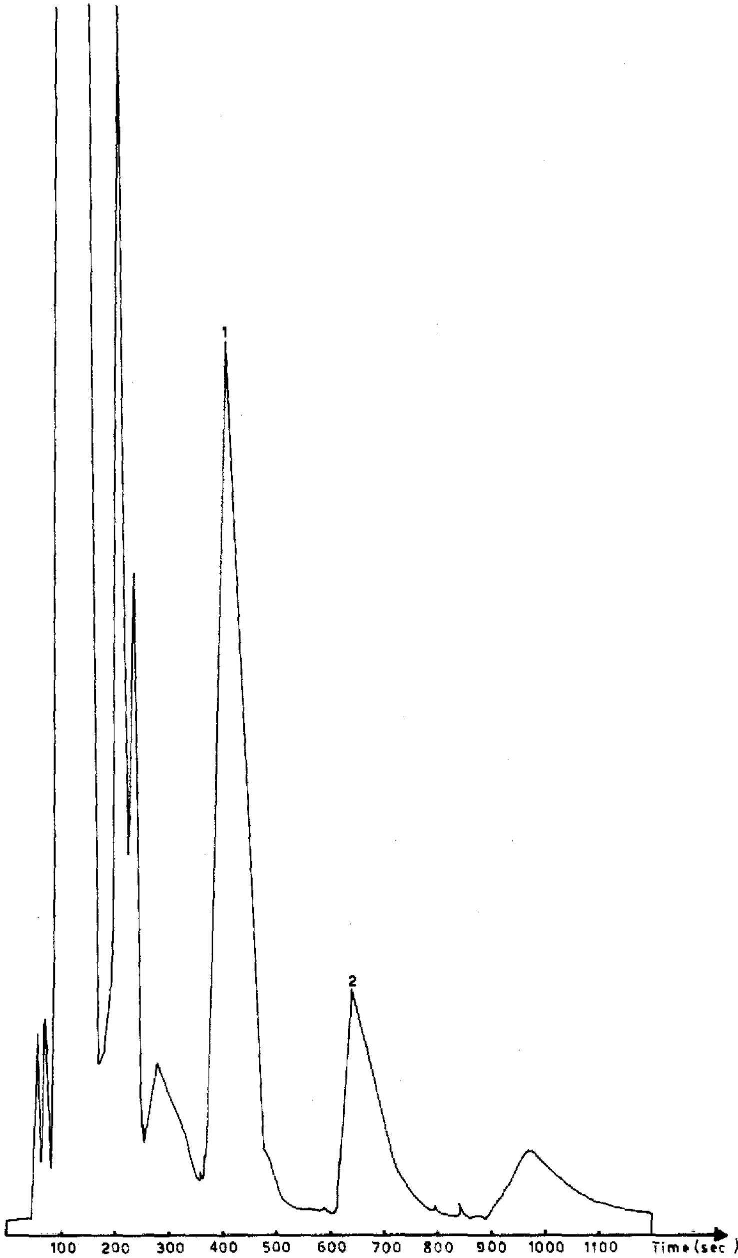

Fig. 3. HPLC chromatogram of erythromycin A (1) and B (2) in the fermentation broth.

Determination of erythromycins A and B in fermentation broths

The reliability of the method has been checked by placing an exact quantity of an erythromycin A or B reference standard in a sample of a fermentation broth. Erythromycin determinations were performed before and after addition of the antibiotic. The recoveries of the two erythromycins were very good, as is shown in Table

TABLE III

RECOVERY EXPERIMENTS CARRIED OUT ON A LABORATORY-PREPARED MIXTURE OF ERYTHROMYCINS A AND B IN A SAMPLE OF FERMENTATION BROTH

Results are means of the analysis of three samples, each of which was chromatographed six times.

Compound	*Weight (mg)*	*Found mean, $\bar{x}$ (mg)*	*Recovery, $\bar{x}$ (%)*
Erythromycin A	50.00	48.66	97.32
Erythromycin B	25.00	24.11	96.44

III. Fig. 3 shows a typical HPLC chromatogram obtained from our fermentation process.

This method was developed so as to drive our strain of *S. erythreus* to produce more erythromycin A and to reduce the production of other erythromycins (B–F) and erythronolides A and B. We believe that our technique can be applied to the other erythromycins (C–F); the results will be reported in a future publication.

In conclusion, the proposed method is suitable for a sensitive and reproducible quantitative evaluation of erythromycins A and B during the fermentation process.

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