*Journal of Chromatography*, 281 (1983) 288–292
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROM. 16,229

## Note

# Influence of column temperature on the liquid chromatographic separation of tylosin and related macrolides

J. H. KENNEDY
*Eli Lilly and Company, P.O. Box 685, Lafayette, IN 7902 (U.S.A.)*
(Received August 16th, 1983)

Tylosin is a fermentation product primarily used to control animal diseases or to improve feed efficiency. Several process-related macrolides may be present with tylosin[1]. Previous publications from these laboratories reported separation of four macrolides in this series[2,3]. In this work nine macrolides, including tylosin, have been separated using ion-pair chromatography with elevated column temperature. The chemical structure of these macrolides can be found in Fig. 1. This report will describe the influence of temperature on the retention of the macrolides and the improvement in column selectivity at elevated temperatures. The retention order as related to structure will also be discussed.

## EXPERIMENTAL

### *Instrumentation*

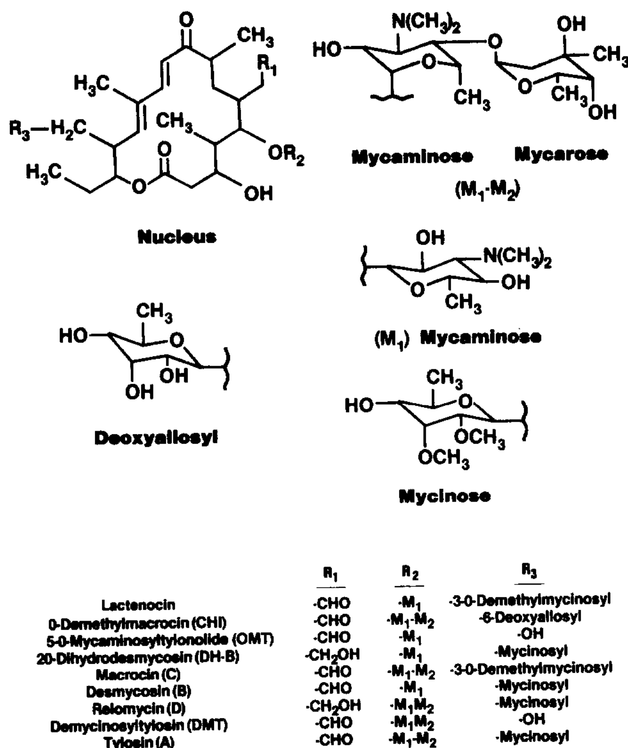
The liquid chromatography system used was a Spectra-Physics Model SP8000 equipped with an oven, a 280 nm UV detector (Spectra-Physics Model 8310) and a computing integrator (Spectra-Physics Model 4000).

Separations were carried out on the following columns: 150 × 4.6 mm I.D. Spherisorb ODS (Regis Chemical, No. 731318) and Zorbax $C_8$ (DuPont Instrument, No. 883952-711).

### *Materials and reagents*

Acetonitrile and tetrahydrofuran were purchased from Burdick and Jackson Lab. (Muskegon, MI, U.S.A.). Both solvents were UV grade obtained by distillation in glass. Water was treated with a Milli-Q water purification system (Millipore). Sodium salt of 1-pentane sulfonic acid was obtained from Eastman Kodak, and was used without further purification. Samples of the various macrolides were prepared in the Lilly Development Labs.

The eluent consisted of tetrahydrofuran–acetonitrile–ion-pair solution (24:29:47). The ion-pair solution was prepared by dissolving 250 mg of sodium 1-pentane sulfonate in one l of water containing 1% acetic acid. The pH of the eluent was 3.5. A flow-rate of 1.5 ml/min was used for all separations and 10 μl of sample solution were injected by a loop injector. Sample concentrations were typically 0.20 mg/ml of tylosin and 0.02 mg/ml of other macrolides in acetonitrile.




| | $R_1$ | $R_2$ | $R_3$ |
|---|---|---|---|
| Lactenocin | -CHO | $-M_1$ | -3-0-Demethylmycinosyl |
| 0-Demethylmacrocin (CHI) | -CHO | $-M_1-M_2$ | -6-Deoxyallosyl |
| 5-0-Mycaminosyltylonolide (OMT) | -CHO | $-M_1$ | -OH |
| 20-Dihydrodesmycosin (DH-B) | $-CH_2OH$ | $-M_1$ | -Mycinosyl |
| Macrocin (C) | -CHO | $-M_1-M_2$ | -3-0-Demethylmycinosyl |
| Desmycosin (B) | -CHO | $-M_1$ | -Mycinosyl |
| Relomycin (D) | $-CH_2OH$ | $-M_1M_2$ | -Mycinosyl |
| Demycinosyltylosin (DMT) | -CHO | $-M_1M_2$ | -OH |
| Tylosin (A) | -CHO | $-M_1-M_2$ | -Mycinosyl |

Fig. 1. Chemical structures of macrolides.

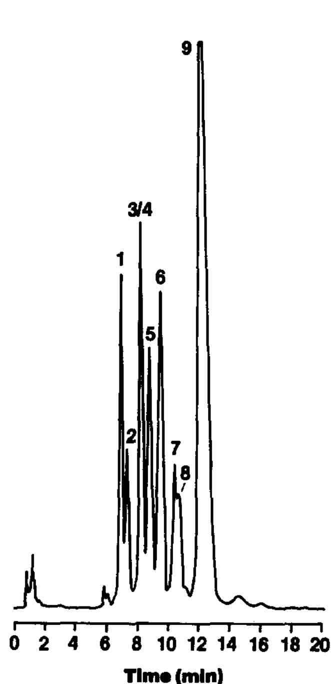
## RESULTS AND DISCUSSION

A separation of eight of the compounds of interest was obtained at room temperature by the optimization procedure described by Glajch and Kirkland[4] using acetonitrile, tetrahydrofuran, and ion-pair solution. A typical separation of the Spherisorb ODS column, is presented in Fig. 2. Since temperature effects were of interest, further modification of solvent strength or pH to improve separation were not examined.

A chromatogram illustrating the separation of all compounds at 55°C on a Spherisorb ODS column is presented in Fig. 3. The same effect of improved selectivity at higher than ambient temperature was observed on a Zorbax $C_8$ column. However, the total analysis times were shortened due to decreased aliphatic chain length in the stationary phase. A typical chromatogram on the Zorbax $C_8$ column at 55°C is presented in Fig. 4.

Column temperature was increased in five degree increments from 25 to 70°C and the capacity ratio, $k'$, of each compound was measured at each temperature. The dependence of $k'$ on temperature is given by:

$$\ln k' = \frac{\Delta H}{RT} - \frac{\Delta S^0}{R} + \Phi$$



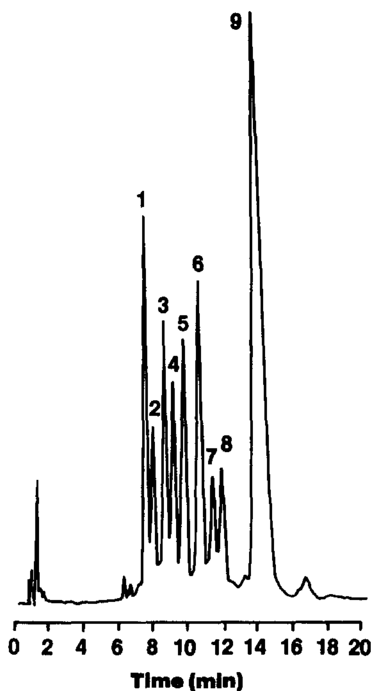




Fig. 2. Effect of column temperature on performance at 25°C on Spherisorb ODS. Peak identity: 1 = lactenocin, 2 = *o*-demethylmacrocin, 3 = 5-O-mycaminosyltylonolide, 4 = 20-dihydrodesmycosin, 5 = macrocin, 6 = desmycosin, 7 = relomycin, 8 = demycinosyltylosin, 9 = tylosin.

Fig. 3. Effect of column temperature on performance at 55°C on Spherisorb ODS. Peak identity: 1 = lactenocin, 2 = *o*-demethylmacrocin, 3 = 5-O-mycaminosyltylonolide, 4 = 20-dihydrodesmycosin, 5 = macrocin, 6 = desmycosin, 7 = demycinosyltylosin, 8 = relomycin, 9 = tylosin.

where $\Delta H$ is the enthalpy of transfer from stationary to mobile phase, $\Delta S^0$ is the associated change in standard entropy, and $\Phi$ is the log of the phase ratio in the column which remains constant[5]. In most bonded phase chromatographic systems, retention decreases in a linear fashion with increased temperature[6,7]. The plot of log $k'$ versus $1/T$ presented in Fig. 5 clearly indicates an increase in $k'$ with temperature.

Snyder[8] alluded to several mechanisms and/or structural changes which could favor increased retention at higher temperatures. Reasons for this so-called "irregular" behavior include: (i) combined chemical and Van der Waals interactions found in ion-pair systems, (ii) increased branching of alkyl substituents, or (iii) increased cyclization of the molecule, whether alicyclic or aromatic rings. Several of these effects can be used to explain the temperature behavior of the macrolides.

All of these macrolides will form ion-pairs and ion-pairing can cause "irregular" temperature behavior. Retention in ion-pair chromatography is based on distribution of the ion and ion-pairs in both stationary and mobile phases. The $k'$ values are proportional to distribution coeeficients between the mobile and stationary phase which are in turn a function of two main processes —dissociation of the ion-pair and ion exchange mechanisms between the mobile phase and the stationary phase. Depending on the enthalpy change of the dominating process, the $K_{eq}$ of ion-pair for-

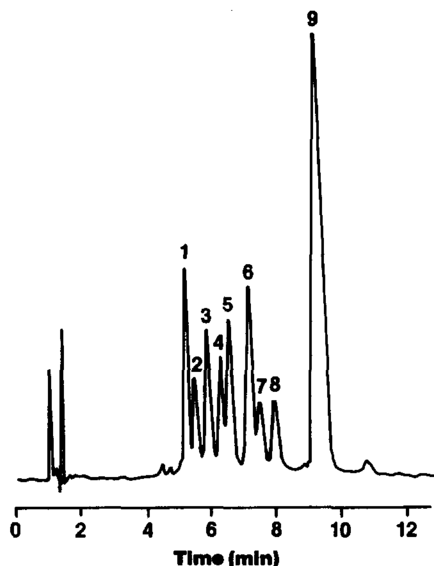



Fig. 4. Effect of column temperature on performance at 55°C on Zorbax $C_8$. Peak identity: 1 = lactenocin, 2 = *o*-demethylmacrocin, 3 = 5-O-mycaminosyltylonolide, 4 = 20-dihydrodesmycosin, 5 = macrocin, 6 = desmycosin, 7 = demycinosyltylosin, 8 = relomycin, 9 = tylosin.

mation will increase or decrease with temperature, and therefore, the distribution coefficient will increase or decrease with temperature[9]. Although the mechanism of ion-pairing is not clear, the distribution coefficients at higher temperature are such that increased solubility in the stationary phase is the favored mechanism for these macrolides.

Two compounds, 5-O-mycaminosyltylonolide (OMT) and demycinosyltylosin (DMT), behave in a more "regular" manner relative to the other macrolides. The $k'$ of these compounds remain relatively constant with temperature. This could be due

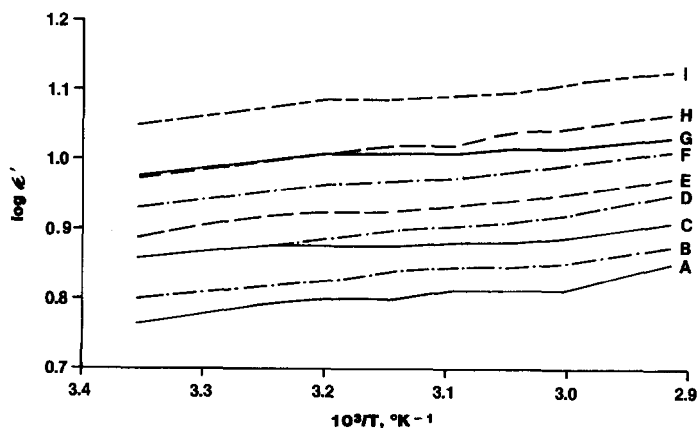



Fig. 5. Effect of temperature on $k'$. A = Lactenocin, B = *o*-demethylmacrocin, C = 5-O-mycaminosylthylonolide, D = 20-dihydrodesmycosin, E = macrocin, F = desmycosin, G = demycinosyltylosin, H = relomycin, I = tylosin.

to the differences in substitution at the $R_2$ and $R_3$ positions. OMT and DMT have –OH substitution in the $R_3$ position whereas the other macrolides have a branched mycinosyl type substitution. Branching in the molecule at the $R_3$ position appears to be the primary cause for the "irregular" temperature behavior of the macrolides.

These data indicate that functional groups such as –OH, –CHO, $-CH_2OH$ and $-OCH_3$, as well as the degree of branching, affect the elution order. The relationship between elution order and either the functional group or the degree of branching is as follows:

(I) Rate of elution is dependant on hydroxyl groups on $R_3$: *o*-demethylmacrocin > macrocin > tylosin.

(II) Decreased branching of $R_2$, where all other substituents are equal, will increase the rate of elution: lactenocin > macrocin.

(III) When branching at $R_2$ position is the same, $-CH_2OH$ substitution at $R_1$ position elutes faster than –CHO substitution; decreased branching increases the rate of elution: 20-dihydrodesmycosin > desmycosin > relomycin > tylosin.

Predicting the elution order for tylosin and related macrolides prior to chromatography would be speculative since the dominating effect of branching or functional groups present are unknown. OMT and DMT are different from the other macrolides in that the $R_3$ position has –OH substitution. Both compounds are aldehydes. However, OMT does not have the mycarose group therefore giving reduced branching and earlier elution than DMT.

## CONCLUSION

Improved resolution for macrolide separations were obtained at elevated column temperatures. Column selectivity improved and $k'$ increased. The macrolides studied provided a good group of compounds to examine the effects of branching and functional group substitution on elution behavior at varied temperature. The "irregular" temperature behavior was used to resolve nine previously unseparated macrolides, and their retention order was related to differences in chemical structure.

## REFERENCES

1 B. H. Baltz, E. T. Seno, J. Stonesifer, P. Matsushima, and G. M. Wild, *Trends in Antibiotic Research*, Japan Antibiotic Research Association, 1982, p. 65.
2 J. H. Kennedy, *J. Chromatogr. Sci.*, 16 (1977) 492.
3 J. H. Kennedy, *Ion-Pair Chromatographic Analysis of the Macrolide Antibiotic Tylosin, Analytical No. 46, ACS Conv.*, Washington, DC, Sept. 1979.
4 J. L. Glajch, J. J. Kirkland, K. M. Squire and J. M. Minor, *J. Chromatogr.*, 199 (1980) 57.
5 H. Colin, J. C. Diez-Masa, G. Guiochon, T. Czajkowska, and I. Miedziak, *J. Chromatogr.*, 167 (1978) 41.
6 J. J. Kirkland and L. R. Snyder, *Introduction to Modern Liquid Chromatography*, 1979, 2nd ed., p. 292.
7 G. Vigh and Z. Varga-Puchony, *J. Chromatogr.*, 196 (1980) 1.
8 L. R. Snyder, *J. Chromatogr.*, 179 (1979) 167.
9 J. C. Kraak and J. F. K-Huber, *J. Chromatogr.*, 102 (1974) 333.