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PHARMACOPOEIAL PROCEDURE FOR THE DETERMINATION OF TYLOSIN FACTORS BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

BRUCE J. FISH* and G. P. R. CARR*
British Pharmacopoeia Commission, Government Buildings, Honeypot Lane, Stanmore, Middlesex (U.K.)

SUMMARY

A method is described for the determination of the factors in tylosin base and tylosin tartrate as raw materials and in dosage forms. The reversed-phase chromatographic system is compared with other similar systems in terms of selectivity towards the major tylosin factors and an aldol condensation degradation product observed in tylosin injection. Experimental conditions affecting the separation of the components are discussed, together with procedures to demonstrate system validity. It is considered that the methods developed provide appropriate procedures for inclusion in pharmacopoeial monographs for tylosin, tylosin tartrate, tylosin premix, tylosin soluble powder, tylosin tablets and tylosin injection.

INTRODUCTION

Tylosin is produced by the fermentation of *Streptomyces fradiae* and is used as a broad-spectrum antibiotic in pigs and poultry and as a feed additive[1]. It is administered as either the base or tartrate salt in a range of dosage forms. Monographs for raw materials and dosage forms have been prepared for a new edition of the *British Pharmacopoeia (Veterinary)*[2]. Apart from the major component (tylosin A), several other related macrolide structures are produced in the fermentation process, and therefore the monographs required tests that were capable of determining tylosin A content and the levels of these other factors. The structures of the major tylosin factors are shown in Fig. 1.

Tylosin A activity was originally determined by descending paper chromatography followed by auto-biographic detection[3]. Such a method was considered unsatisfactory for publication in *British Pharmacopoeia* monographs for the following reasons: (a) it was time-consuming and imprecise, (b) the factors had different relative responses and some components may not even possess microbiological activity and, hence, would be undetected. The determination of the factors by a chemical method

* Present address: Wyeth Research (U.K.), Huntercombe Lane South, Taplow, Maidenhead, Berkshire, U.K.

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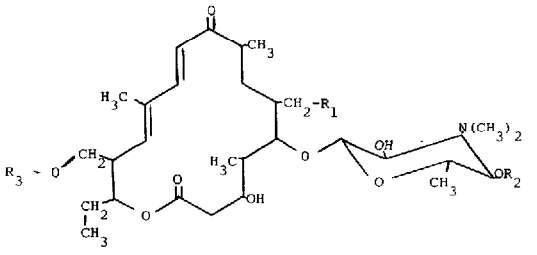
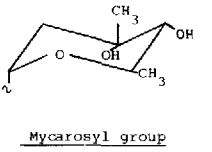
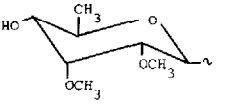




Mycarosyl group



Mycinosyl group

	R_1	R_2	R_3
Tylosin A (tylosin)	- CHO	mycarosyl	mycinosyl
" B (desmycosin)	- CHO	- H	mycinosyl
" C (macrocin)	- CHO	mycarosyl	3-O-desmethylmycinosyl
" D (relomycin)	- CH_2OH	mycarosyl	mycinosyl
DMT (desmycinosyl tylosin)	- CHO	mycarosyl	-H

Fig. 1. Structural formulae of major tylosin factors.

was therefore preferable. In the light of these observations, methods based on high-performance liquid chromatography (HPLC) seemed to offer a suitable approach. An HPLC method involving ultra-violet (UV) detection would overcome the aforementioned disadvantages and would be quicker, more precise, more robust and, hence, more suitable for inclusion in a pharmacopoeial monograph. The use of UV detection also has the advantage that similar responses from each of the components would be expected, because the chromophore is almost the same in each case.

Previously published methods were first examined. A system described by Kennedy[4] comprised a 30-cm μBondapak C_{18} column with a mobile phase consisting of 40% (v/v) acetonitrile and 2% (v/v) monoethanolamine in water. A UV detector, set at 290 nm, was used to monitor the eluent, and tylosin samples were dissolved in aqueous acetonitrile and then injected into the chromatographic system. When this method was tried, an identical elution order of the four main factors was observed to that quoted. However, the separation of the components from each other was poor and, also, the pH of the mobile phase was 11.7, which was considered too basic for continual use with reversed-phase octadecasilyl (ODS) columns. According to the author, lowering the pH was found to produce even poorer resolution.

More recently, Kennedy[5] described an improved method in which nine related tylosins were separated, but this procedure was unable to separate a significant degradation product of tylosin injection which was first observed in our method.

Other previously published HPLC methods were also reviewed. Omura *et al.*[6] described a reversed-phase system for separating a range of macrolide antibiotics including tylosin on a Jasco Pack SV-02-500 column. The separation medium has since been superseded by more efficient chromatographic packings, and this was one reason why this method was not examined here. The chromatogram which showed the separation of eight leucomycin factors had broad peaks, which meant that the

whole system was operating at a fraction of the efficiency of which current HPLC systems are capable.

Bhuwapathanapun and Gray[7] described both normal and reversed-phase HPLC methods for the estimation of four main tylosin factors. The reversed-phase system comprised a mobile phase of methanol–water (70:30) flowing at 1 ml/min, a column of Du Pont ODS Permaphase, and a UV detection wavelength of 254 nm. This system was unsatisfactory for the following reasons: (a) there was minimal retention of the least-retained factor which was tylosin A, (b) tylosins A and B were poorly resolved, (c) tylosin A and two other factors (tylosins C and D) were eluted as one band.

The normal-phase system comprised a mobile phase of 2-propanol–chloroform (35:65) at 1 ml/min, a column of Du Pont Zorbax SIL and a UV detector, set at 254 nm. It too was unsuitable because: (a) the analysis time was very long; tylosin B was eluted as a broad peak after nearly 1 h, (b) the system was not efficient because tylosin B was eluted as a broad peak over a 25-min interval, (c) tylosin A was not sufficiently well separated from tylosin D, (d) tylosin D and tylosin C were eluted as one peak. Altering the 2-propanol content of the mobile phase caused tylosin D to be better separated from tylosin C, which was now eluted together with tylosin A.

In the light of these observations, it was considered that a better HPLC system should be developed of a standard that is suitable for a modern pharmacopoeial monograph.

EXPERIMENTAL

Chemicals

Acetonitrile, methanol, and tetrahydrofuran (THF) were HPLC-grade material from Rathburns, Walkerburn, U.K. Hydrochloric acid, formic acid, acetic acid, 2-propanol, sodium perchlorate, and sodium pentanesulphonate were AnalaR-grade materials from BDH, Poole, U.K. Single-distilled, de-ionised water was used throughout. Samples of tylosin base, tylosin tartrate, dosage forms, and the major factors, which are: tylosin A (tylosin), tylosin B (desmycosin), tylosin C (macrocin), tylosin D (relomycin), were provided by Lilly Industries, Speke, U.K. Another factor produced by the fermentation process is desmycinosyl tylsoin (DMT). A pure sample was not available, but one sample of tylosin tartrate, provided by Lilly Industries, was known to contain this component. The following dosage forms were examined: tylosin premix, containing tylosin tartrate, tylosin soluble powder, containing 1 g tylosin (as tartrate) and 3 g sodium sulphathiazole; tylosin tablets, containing 200 mg tylosin base per tablet; and tylosin injection, containing 50 mg tylosin base per ml in 50% propylene glycol.

Instrumentation

All mobile phases were degassed by ultrasonic mixing for 15 min. The HPLC equipment comprised a Model 6000A pump (Waters Assoc., Harrow, U.K.), a U6K injector (Waters Assoc.) or Rheodyne 7125 valve injector, fitted with a 20-μl loop (Phase Sep, Queensferry, U.K.), an LC75 spectrophotometric detector (Perkin-Elmer, Beaconsfield, U.K.), a 308 integrator (LDC, Stafford, U.K.), and a 56 chart recorder (Perkin-Elmer), operating at 5 mm/min.

TABLE I

DETAILS OF CHROMATOGRAPHIC SYSTEMS

Details of systems 2, 3 and 4 were provided by Lilly Industries.

	System 1	*System 2*	*System 3*	*System 4*
Column	Nucleosil ODS 5 μm 20 cm × 0.46 cm I.D.	Zorbax C_8 pre-packed by Du Pont 15 cm × 0.46 cm I.D.	Hypersil C_8 5-μm pre-packed by Jones 15 cm × 0.45 cm I.D.	Zorbax C_8 20 cm × 0.46 cm I.D.
Mobile phase	40% acetonitrile in 0.85 *M* aq. sodium perchlorate (adjusted to pH 2.5 with 1 *M* hydrochloric acid)	acetonitrile–THF–0.0014 *M* sodium pentanesulphonate in 1% (v/v) acetic acid (1:1:3)	acetonitrile–THF–0.015 *M* sodium pentanesulphonate in 3% (v/v) formic acid (3:3:19)	acetonitrile–THF–0.0014 *M* sodium pentanesulphonate in 1% (v/v) acetic acid (23:19:58)
Flow-rate	1.0 ml/min	1.5 ml/min	1.5 ml/min	2.0 ml/min
Temperature	ambient	ambient	ambient	50°C
Injection volume	20 μl	15 μl	20 μl	20 μl
Detection wavelength	290 nm	280 nm	280 nm	290 nm
Sensitivity	0.08 a.u.f.s.	0.08 a.u.f.s.	0.08 a.u.f.s.	0.08 a.u.f.s.
Mobile phase pH	2.5	3.2	2.1	3.3

The extinction coefficients of the four tylosin factors, A, B, C and D were found by measuring the absorbance values of 0.004% solutions of each in mobile phase at the maximum of 290 nm, using 1-cm pathlength cells and a Perkin-Elmer 552 UV–VIS spectrophotometer. The relative responses were also measured by chromatographing individual factors and measuring the areas of the resulting peaks at 290 nm.

The following materials were examined for use as suitable separation media: Nucleosil ODS, 5 μm packing (Camlab, Cambridge, U.K.); Zorbax C_8 packing (Du Pont, U.K.); Hypersil C_8 5 μm packing (Shandon, U.K.); and a prepacked Zorbax C_8 column. Nucleosil ODS was packed into a 20 cm × 0.46 cm I.D. stainless-steel column by upward packing, using 2-propanol–methanol (1:1) and was conditioned with 50% aq. methanol. Zorbax C_8 was packed into a 20 cm × 0.46 cm I.D. stainless-steel column by upward packing, using 2-propanol, and was conditioned with 50% aq. methanol. A 15 cm × 0.45 cm I.D. column, containing Hypersil C_8 5-μm packing was obtained already packed from Jones Chromatography, Glamorgan, U.K. A 15 cm × 0.46 cm I.D. column, containing Zorbax C_8, was obtained already packed from Du Pont.

Procedures for sample preparation

Tylosin and tylosin tartrate were dissolved in 50% aq. acetonitrile to produce *ca.* 0.02% tylosin solutions. Tylosin premix and soluble powder were shaken with sufficient 50% aq. acetonitrile to produce 0.02% solutions of tylosin. For tylosin tablets, a quantity of tablet powder equivalent to 200 mg tylosin was shaken with 50 ml methanol. This was filtered, and 5 ml of filtrate was diluted to 100 ml with 50% aq. acetonitrile. For tylosin injection, 1 ml of solution was diluted to 250 ml with 50% aq. acetonitrile.

HPLC methods

The chromatographic method developed for inclusion in the pharmacopoeia is based on the nucleosil ODS column (system 1, Table I). This table also describes the other methods that were examined for tylosin analysis.

RESULTS AND DISCUSSION

In choosing a new method, initial considerations suggested that a reversed-phase system was preferable to a normal-phase system, because reversed-phase is more applicable to the analysis of dosage forms. This is because the type of excipients that are likely to be encountered are generally polar and will be rapidly eluted on a reversed-phase system, whereas they will become strongly adsorbed to silica and eventually lead to a loss of column efficiency. The tylosin molecule contains a tertiary amino grouping which, due to adsorption on residual silanol sites on the column packing, can exhibit broad or tailing peaks in reversed-phase systems. These effects can be overcome by including an anionic ion-pairing agent in the mobile phase. This also necessitates using an acidic mobile phase to ensure that the amino group is fully protonated and effective ion-pairing is achieved with the counter-anion. In our experience, the perchlorate anion is a convenient counter-ion and, therefore, mobile phases containing aqueous sodium perchlorate were tried. A mobile phase, consisting

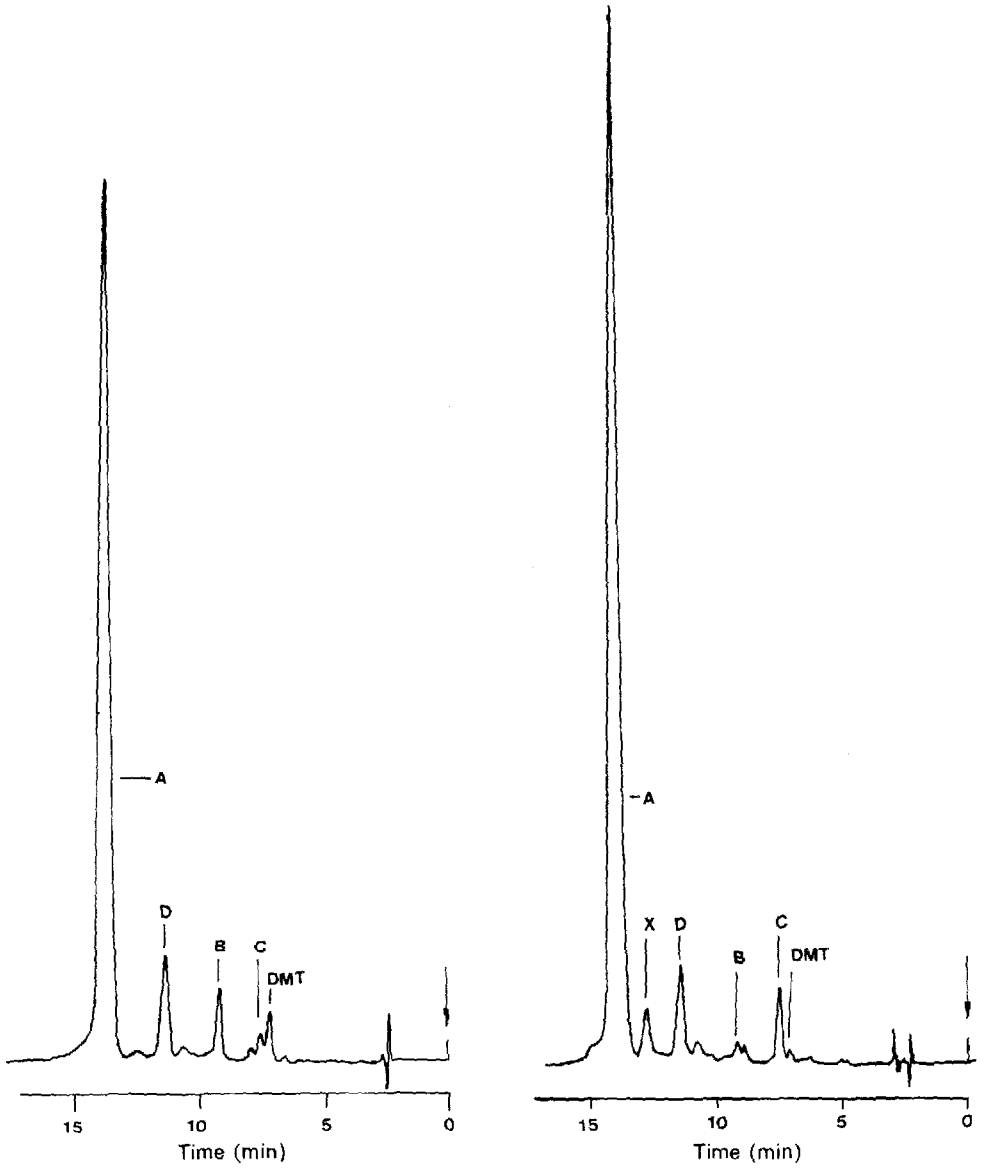


Fig. 2. Chromatogram of tylosin tartrate obtained with system 1. Twenty μl of 0.02% (w/v) tylosin tartrate in 50% (v/v) aqueous acetonitrile was injected. The mobile phase was 40% acetonitrile in 0.85 *M* sodium perchlorate (adjusted to pH 2.5) at a flow-rate of 1 ml/min. Column was Nucleosil ODS (5 μm), 20 cm × 0.46 cm I.D. Detector wavelength set at 290 nm.

Fig. 3. Chromatogram of tylosin injection with system 1 (Nucleosil ODS).

of 40% acetonitrile in 0.85 *M* sodium perchlorate (adjusted to pH 2.5 with 1 *M* hydrochloric acid), in combination with a 20 cm × 0.46 cm I.D. Nucleosil ODS (5 μm) column was found to be satisfactory for separating the tylosin factors of interest *i.e.* tylosins A, B, C, D and DMT. Peak assignments were made by injecting separate

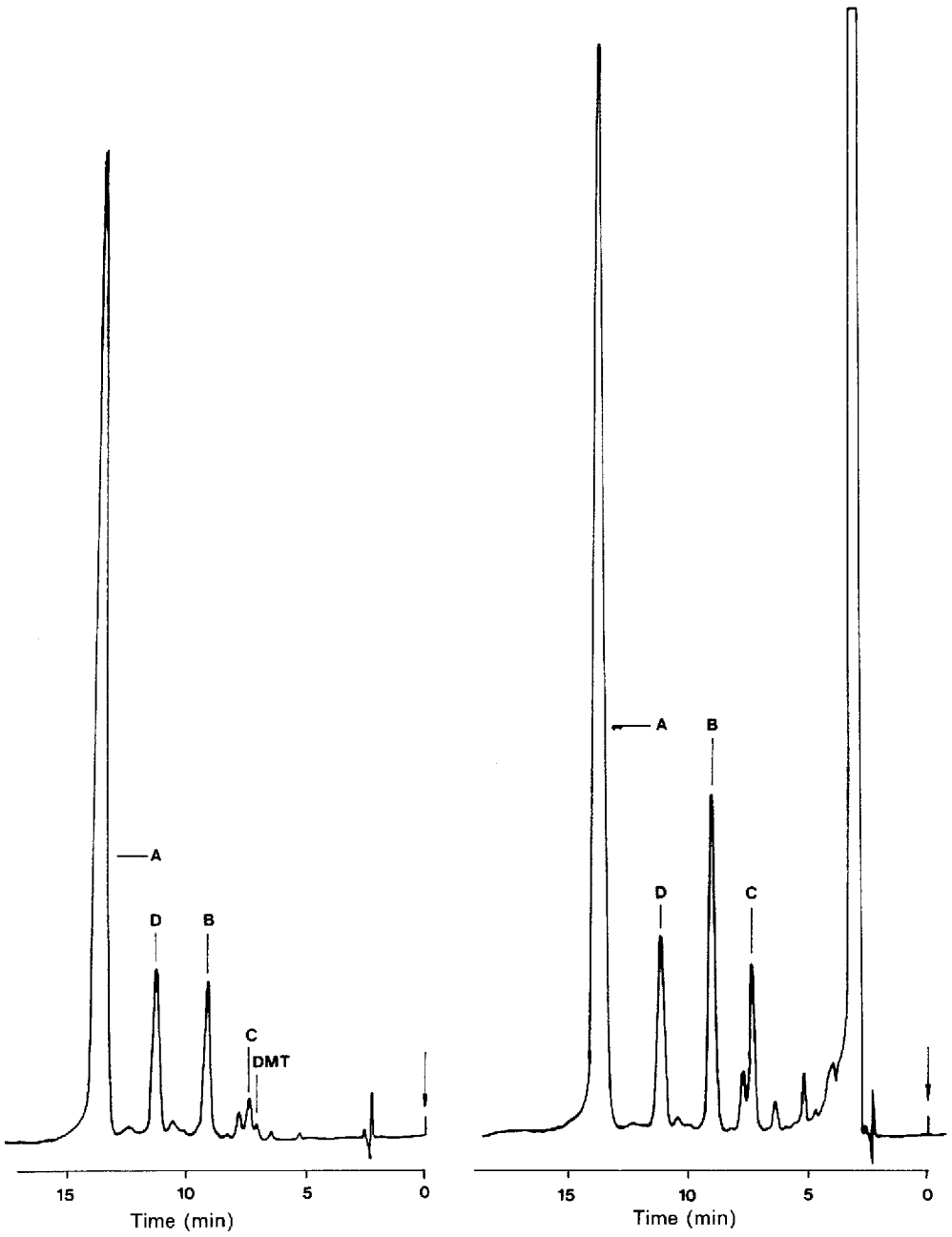


Fig. 4. Chromatogram of tylosin premix with system 1 (Nucleosil ODS).

Fig. 5. Chromatogram of tylosin soluble powder with system 1 (Nucleosil ODS).

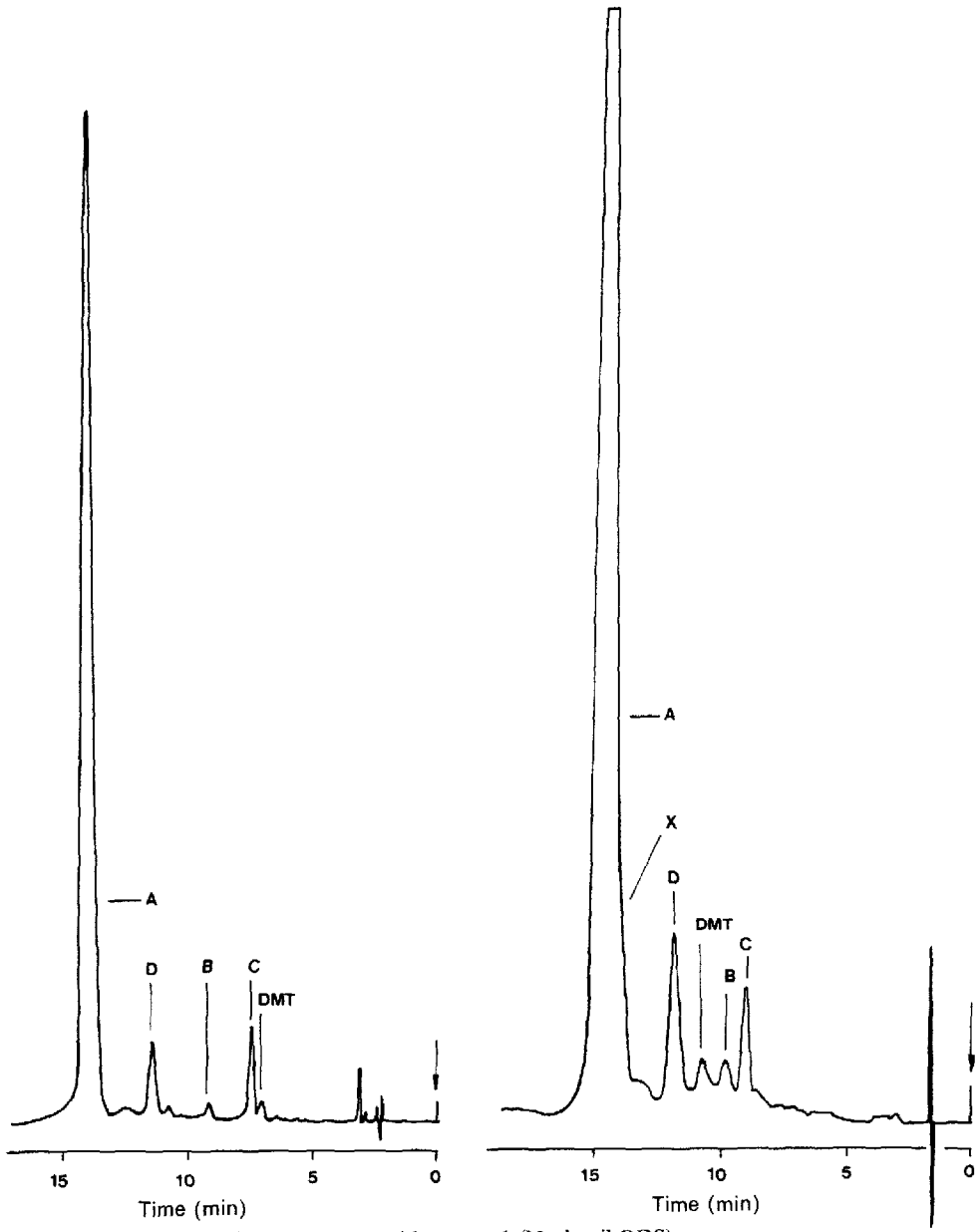


Fig. 6. Chromatogram of tylosin tablets with system 1 (Nucleosil ODS).

Fig. 7. Chromatogram of tylosin injection with system 2 (Zorbax C_8).

reference solutions of the factors. Fig. 2 shows a typical separation by this system. A pH of 2.5 was found to be the optimum value, as a higher pH of 3.5 was found to give broad peaks, showing unacceptable degrees of tailing. It was not desirable to use a lower pH because of the possibility of hydrolysing ODS from the silica matrix.

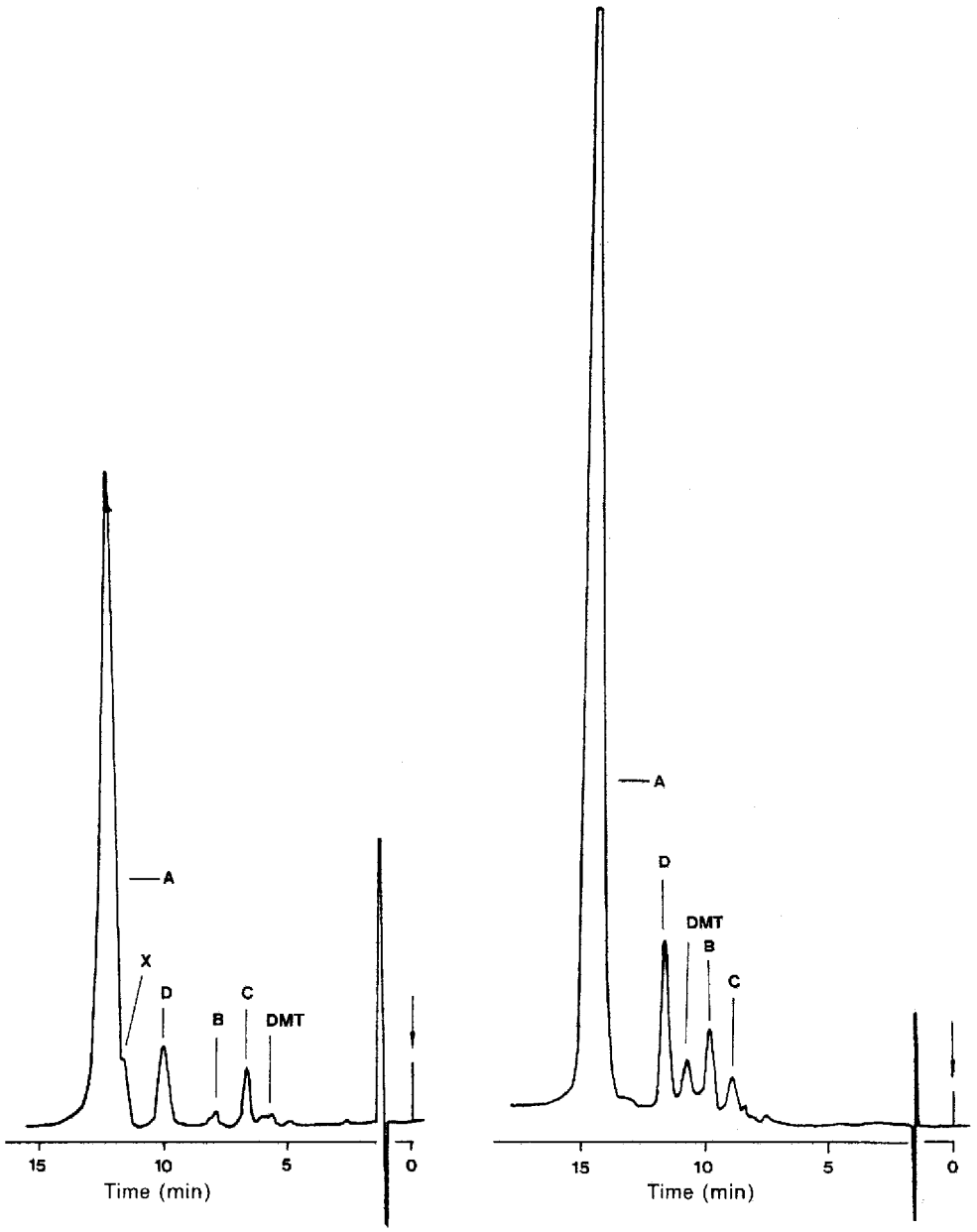


Fig. 8. Chromatogram of tylosin injection with system 3 (Hypersil C_8).

Fig. 9. Chromatogram of tylosin tartrate with system 2 (Zorbax C_8).

Most ODS materials have a working range of pH 2 to 8. The above method was applicable to both raw materials and to all of the dosage forms.

Typical chromatograms from each of the dosage forms are shown (Figs. 3–6). The chromatogram, obtained from tylosin soluble powder which contains sodium

sulphathiazole, showed that the sulphonamide was rapidly eluted with the solvent dead-volume and so did not interfere with the tylosin factors. No interference due to other ingredients were observed from any of the dosage forms.

The HPLC of tylosin injection was interesting in that a substance was observed to be eluted between tylosins A and D which was not observed in any of the other preparations or raw materials (see Fig. 3). This extra peak (X) was neither directly due to the benzyl alcohol preservative nor caused by leaving the solution to stand before commencing analysis.

When the sample of tylosin injection was chromatographed by the methods based on Zorbax C_8 and Hypersil C_8 columns and mobile phases containing pentanesulphonate as pairing-ion (Table I, systems 2 and 3), it was noticed that this peak (X) was very poorly separated from tylosin A, as shown in Figs. 7 and 8. These systems were evidently not as selective for X as the system based on Nucleosil ODS. This difference in selectivity was also reflected in the different elution order of DMT in the Zorbax C_8 system, as shown in Fig. 9, although the elution order of tylosins A, B, C and D was identical in all of the systems examined. Adjusting the amounts of acetonitrile and THF did not enable the unknown peak (X) to be separated. In fact the resolution of the other factors deteriorated so that it was clear that systems 2 and 3 were already being operated under optimum conditions. Using a slightly modified eluent with a Zorbax C_8 column maintained at 50°C (Table I, system 4) slightly improved the separation of X from tylosin A but the resolution was not as good as that achieved using the Nucleosil ODS system. Also, the resolution of DMT had decreased and was unsatisfactory. This system was very similar to the one published by Kennedy[5] in which nine related tylosins were separated, but this did not include X. The compositions of the organic modifiers in system 4 were adjusted in an effort to improve separation but none of the mobile phases examined could completely separate all tylosin factors and impurity X.

Using the Nucleosil ODS system, levels of X up to 4% of total peak area were found in samples of tylosin injection. In such samples, the amount of tylosin A was significantly less than expected, which implied that the formation of X was accompanied by a decrease in tylosin A content. This confirmed the importance of using a chromatographic system that was capable of separating X from tylosin A. X has recently been identified as being an aldol condensation product, formed by the condensation of the aldehyde group at R1 in Fig. 1. Aldol condensations are base-catalysed, and since the pH of the injected sample is about 9, favourable conditions prevail for this type of reaction. The λ_{max} of the aldol condensation product was 280 nm as compared to the λ_{max} of 290 nm of the other tylosin factors.

In the course of developing a method, it was noticed that not all Nucleosil ODS columns exhibited identical selectivity when the eluent containing 0.85 *M* sodium perchlorate was used. This variance in selectivity resulted in either loss of resolution between X and tylosin A or loss of resolution between DMT and tylosin C or both. It was possible to overcome this by adjusting the molarity of the sodium perchlorate whilst keeping the pH constant at 2.5, and so regain the desired separation. Increasing the sodium perchlorate molarity decreased the retention of DMT relative to tylosin C, thus causing an increase in resolution while the retention of X relative to tylosin A increased, thus causing a decrease in resolution between these two factors. For each column it was necessary to establish the composition which provided optimum resolution between these components.

Variations in performance among batches of stationary phases is quite a common feature of HPLC packings. To allow for such variations in pharmacopoeial monographs, it is necessary to introduce performance requirements into those tests where HPLC is used. This often includes a column efficiency test, and in this case a minimum theoretical plate number of 22 000 plates per metre was considered necessary. However, such a test does not allow for the possibility of selectivity variations among batches of packing. In this case, it is proposed to overcome this by establishing a reference substance which contains tylosins A, B, C, and D, as well as DMT and X at their respective expected levels. This reference material will be made available in support of the HPLC test, together with a reference chromatogram prepared from it. An analyst wishing to carry out the test will then be directed to use the reference material to establish the chromatographic conditions necessary for achieving the separation, as demonstrated in the reference chromatogram.

Quantification of tylosins A, B, C, D, DMT and X

By virtue of the fact that the method based on the Nucleosil column separated tylosins A, B, C, D, DMT and X, it was possible to determine the content of tylosin A in a sample of raw material or in any of the dosage forms. It was also possible to determine the levels of each of the other components. In the monographs for the raw materials and dosage forms, it is intended to include statements to the effect that the content of tylosin A and the content of tylosins A + B + C + D are not less than minimum percentage values. The method of calculating the contents of tylosins A, B, C, and D is total peak area normalisation of all peaks seen in the chromatogram. This is possible, because the responses of the tylosin factors to ultra-violet light at the 290 nm maximum are virtually identical (Table II). Peak area normalisation is necessary, as the alternative method of using an external reference standard is not possible. This is because reference standards of all the components will not be available.

A sample of tylosin tartrate was injected six times, and the area percent of each of the tylosin factors, together with the coefficient of variance of injection reproducibility was calculated. The results are shown in Table III. Table IV shows the content of the factors found in production batches of each of the dosage forms.

TABLE II

RESPONSES OF TYLOSIN FACTORS TO ULTRA-VIOLET LIGHT AT 290 nm

Tylosin	*Molecular weight*	*Theoretical response*	*Response**	*Response***
A	916.1	1.0***	1.0***	1.0***
B	771.9	1.2	1.1	1.0
C	902.1	1.0	1.0	1.0
D	918.1	1.0	1.0	0.9

* Responses found by measuring the absorbances of 1-cm layers of solutions of each factor in a spectrophotometer.

** Responses found by analysing solutions of each factor in the HPLC system 1 and measuring the area of the resultant peak.

*** Response is relative to tylosin A on a weight for weight basis.

TABLE III

CONTENT OF TYLOSIN FACTORS IN A SAMPLE OF TYLOSIN TARTRATE

Tylosin	*Mean content (by area percent)*	*Coefficient of variance* $\left(\frac{100 \times S.D.}{mean}\right)$*
A	83.4	0.9
B	4.6	1.3
C	1.6	2.6
D	7.1	1.3
DMT	2.5	1.3

* S.D. = standard deviation (n = 6).

TABLE IV

CONTENT OF TYLOSIN FACTORS IN DOSAGE FORMS (BY AREA PERCENT)

Tylosin factor	*Tylosin premix**	*Tylosin soluble powder**	*Tylosin tablets*	*Tylosin injection*
A	77.3	66.4	86.1	83.2
B	8.6	13.5	0.9	0.9
C	1.9	6.1	5.0	3.7
D	9.5	8.6	5.1	5.6
DMT	0.5	0.0	1.0	0.4
X	0.0	0.0	0.0	3.5

* These are old samples and show a low tylosin A content that is not representative of current production batches.

CONCLUSIONS

An HPLC method that is capable of separating and quantifying the factors present in tylosin raw material and dosage forms has been developed and has been shown to be superior to other available methods. The method is suitable for inclusion in a pharmacopoeia, and it is intended to include it in tylosin monographs due to be published in the next edition of the *British Pharmacopoeia (Veterinary)*[2].

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