ELSEVIER

Journal of Chromatography A, 699 (1995) 93–97

JOURNAL OF CHROMATOGRAPHY A

Liquid chromatography of tylosin A and related substances on poly(styrene–divinylbenzene)

J. Paesen, P. Claeys, W. Cypers, E. Roets, J. Hoogmartens*

Laboratorium voor Farmaceutische Chemie en Analyse van Geneesmiddelen, Faculteit Farmaceutische Wetenschappen, Katholieke Universiteit Leuven, Van Evenstraat 4, B-3000 Leuven, Belgium

First received 13 June 1994; revised manuscript received 19 December 1994; accepted 20 December 1994

Abstract

A liquid chromatographic method for analysis of tylosin A and related substances was developed using wide-pore poly(styrene–divinylbenzene) as the stationary phase. As a mobile phase, a mixture of tetrahydrofuran–0.2 *M* potassium phosphate buffer pH 9.0–water (20:5:75) was used at a flow-rate of 1.0 ml/min. The column was kept at 60°C. Detection was performed by UV at 280 nm. Tylosin A is separated from all its known related substances that are potentially present as impurities in commercial samples of tylosin base and tylosin tartrate. The method shows good linearity and repeatability and is stability indicating. Results for a number of commercial samples analysed using the newly developed method are presented.

1. Introduction

A liquid chromatographic (LC) method for analysis of the 16-membered macrolide antibiotic tylosin using C_8 or C_{18} silica-based reversed phases was recently described [1]. In the same paper a review was given of the reversed-phase LC methods for tylosin. Major contributions were made by Kennedy [2,3] and Fish and Carr [4]. The major advantages of the latest method described by Roets et al. [1] over the previous were a better reproducibility on different stationary phases, a better selectivity and the less corrosive chromatographic conditions. However, there still was difference in retention behaviour between different silica-based reversed phases, which made it necessary to adapt the percentage of organic modifier to obtain satisfactory separation. Commercial samples of tylosin and tylosin tartrate were shown to contain different related substances in addition to the main component tylosin A (TA). A good LC method must be able to separate these related substances from TA and from each other. The impurities most frequently detected in commercial samples [1] are: desmycosin or tylosin B (TB) [5], macrocin or tylosin C (TC) [6], relomycin or tylosin D (TD) [7] and demycinosyltylosin (DMT) [8]. Structures of these compounds have been shown previously [1]. Other related substances have been isolated and their structures determined, e.g. 5-O-mycaminosyltylonolide (OMT) [9] and lactenocin (LACT) [6]. Other impurities, which may occur in solutions of tylosin, are tylosin A aldol (TAD) and isotylosin A (isoTA). Their isolation and structure elucidation is described in the companion paper [10].

Unlike the silica-based reversed phases used in

* Corresponding author.

0021-9673/95/$09.50
SSDI 0021-9673(94)01268-7

the latest described LC method for tylosin [1], poly(styrene–divinylbenzene) (PS–DVB) stationary phases can be used with alkaline mobile phases [11]. Wide-pore PS–DVB (PLRP-S, 8 μm, 1000 Å) showed good selectivity for the separation of the 14-membered ring macrolide erythromycin A and related substances, when used with a mobile phase of pH 9.0 [12,13]. In this paper a method for the analysis of tylosin is described which was derived from that for erythromycin and which is able to separate tylosin A from all its potential impurities. The method has been used to analyse a number of commercial samples.

2. Experimental

2.1. Solvents, reagents and samples

Acetonitrile (LC grade S) and tetrahydrofuran (THF) (LC grade), were from Rathburn (Walkerburn, UK). THF did not contain a stabilizer and was tested for absence of peroxides. Methanol (Roland, Brussels, Belgium) and 2-methyl-2-propanol 99.5% (Janssen Chimica, Beerse, Belgium) were distilled in glass apparatus before use. Water was distilled twice. Dipotassium hydrogenphosphate, potassium dihydrogenphosphate (Janssen Chimica) and tripotassium phosphate (Merck–Belgolabo, Overijse, Belgium) were of analytical-reagent quality.

A house standard for tylosin A was available in the laboratory. The purity of this standard was 90.3% (w/w), expressed on the substance as is. The base content of this house standard was determined by non-aqueous titration with 0.1 *M* perchloric acid using glacial acetic acid as the solvent. The water content was determined by Karl Fischer titration and the percentage of related substances by LC. The total mass explained by titration and water determination was 99.8%. Subtracting the water content and the content of related substances from 100 gave a purity for this house standard of 90.3% (w/w). Small amounts of TB, TC, TD, LACT, OMT and DMT were available as reference substances. TAD and isoTA were isolated and purified as described in the companion paper. Bulk samples of tylosin and tylosin tartrate were of known origin (Bulgaria, USA).

2.2. LC apparatus and operating conditions

Isocratic elution was used throughout the study. The equipment consisted of a Merck–Hitachi L-6200 intelligent pump (Darmstadt, Germany) set at a flow-rate of 1.0 ml min^{-1}, a Valco injector Model CV-6-UHPa-N60 (Houston, TX, USA) equipped with a 20-μl loop, a 250 × 4.6 mm I.D. column, packed with PLRP-S, 8 μm, 1000 Å (Polymer Labs., Church Stretton, UK), a Waters Model 440 UV detector (Milford, MA, USA) or a Merck–Hitachi L-4000 variable-wavelength UV detector set at 280 nm and an integrator HP 3393 A or HP 3390 (Hewlett-Packard, Avondale, PA, USA). The column was immersed in a water-bath, heated by a Julabo EM thermostat (Julabo, Seelbach, Germany). Other PLRP-S stationary phases (PLRP-S, 8 μm, 100 and 300 Å) were used to investigate their selectivity.

2.3. Buffers, sample preparation and mobile phase

Potassium phosphate buffers (0.2 *M*) used in the study were prepared by mixing suitable amounts of 0.2 *M* solutions of potassium dihydrogenphosphate, dipotassium hydrogenphosphate or tripotassium phosphate. All samples for qualitative work were dissolved in water. For quantitative work samples were dissolved in 0.04 *M* dipotassium hydrogenphosphate. The mobile phase finally used was prepared by mixing 750 ml of water, 50 ml of 0.2 *M* potassium phosphate buffer pH 9.0 and 200 ml of THF. The mixture was degassed by ultrasonication before use.

3. Results and discussion

3.1. Development of the chromatographic method

As wide-pore PS–DVB PLRP-S, 8 μm, 1000 Å showed excellent selectivity in the separation

of the macrolide antibiotic erythromycin [12], the suitability of this stationary phase was evaluated for the analysis of tylosin.

Several organic modifiers (x) were investigated, using mobile phases consisting of organic modifier–0.2 M phosphate buffer pH 9.0–water (x:5:95 − x) and a column temperature of 60°C: methanol, acetonitrile, 2-methyl-2-propanol and THF. Table 1 gives the resolution between the pairs TD–TA and TB–TC, the number of theoretical plates and the symmetry factor, calculated for TA using instructions of the European Pharmacopoeia [14]. Using methanol, very low efficiency and poor peak shapes were obtained, analogous to previous observations during the analysis of tetracycline [15], erythromycin [12] and cefalexin [16] using PS–DVB stationary phases. Using acetonitrile, TB and TC were coeluted. Using 2-methyl-2-propanol or THF, TA, TB, TC, TD and TAD were separated. There was no baseline separation between DMT and TB. THF was preferred over 2-methyl-2-propanol as the organic modifier for further chromatography, because of a much better efficiency and a better resolution between TD and TA. A mobile phase containing THF–0.2 M phosphate buffer pH 9.0–water was further evaluated by systematic examination of its components.

Variation of the pH in the range 7.0 to 11.0 revealed optimal separation at pH 9.0. Capacity factors decreased rapidly with increasing percentage of THF; 20% (v/v) THF in the mobile phase was adopted because of good selectivity and acceptable total analysis time (40 min). Varying the buffer concentration in the mobile phase in the range 2.5–10% had no influence on the selectivity. Using a column temperature of 50°C, the efficiency was lower than at 60°C. At 70°C the resolution between TB and TC decreased. Therefore, 60°C was finally chosen. Using the developed mobile phase, the selectivity obtained on PLRP-S, 8 μm, 1000 Å was better than that on PLRP-S having smaller pore sizes of 100 and 300 Å. Similar differences in selectivity were observed in the analysis of erythromycin [12].

A typical chromatogram of a commercial sample of tylosin tartrate, obtained with the selected chromatographic conditions, is shown in Fig. 1. The main component, TA, is well separated from all its potential impurities. Even a few components of unknown identity were separated. The latter were not detected using a previously reported method for tylosin using silica-based reversed phase [1].

3.2. Quantitative aspects of the LC method

The sample load capacity of the column was found to be 100 μg. For quantitative analysis of tylosin an amount of 20 μg was used. For this quantity the limit of detection was 0.06%, determined at a signal-to-noise ratio of 3 [14]. The limit of quantitation was 0.12% [$n = 5$; relative standard deviation (R.S.D.) = 9.2%]. The repeatability was checked by analysing 10 times the same solution of tylosin tartrate (20 μg). The

Table 1
Resolution between TB and TC (R_s TB–TC) and between TD and TA (R_s TD–TA), numbers of theoretical plates (N_{TA}/m) and symmetry factors (S_{TA}) for TA, using different organic modifiers

Organic modifier		R_s TB–TC	R_s TD–TA	N_{TA}/m	S_{TA}
Name	% (v/v) (x)				
Methanol	55	< 1.0	1.5	700	3.0
Acetonitrile	30	< 1.0	4.4	6 560	1.5
2-Methyl-2-propanol	15	1.5	1.6	5 600	1.4
THF	20	1.5	5.1	12 320	1.3
THF + acetonitrile	15 + 6	1.3	3.6	7 740	1.2

Stationary phase: PLRP-S, 8 μm, 1000 Å (250 × 4.6 mm I.D.); mobile phase: organic modifier–0.2 M phosphate buffer pH 9.0–water (x:5:95 − x); flow-rate: 1.0 ml min^{-1}; column temperature: 60°C; detection: UV at 280 nm.

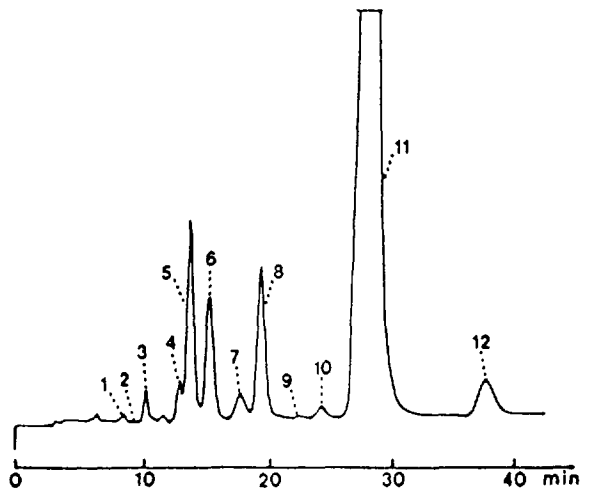

Fig. 1. Typical chromatogram of a commercial tylosin tartrate obtained with the described method. Chromatographic conditions: stationary phase: PLRP-S, 8 μm, 1000 Å (250 × 4.6 mm I.D.); mobile phase: THF–0.2 *M* phosphate buffer pH 9.0–water (20:5:75, v/v/v); flow-rate: 1.0 ml min $^{-1}$; column temperature: 60°C; amount injected: 20 μg; detection: UV at 280 nm. Peaks: 1 = OMT; 2 = LACT; 3 = unknown; 4 = DMT; 5 = TB; 6 = TC; 7 = unknown; 8 = TD; 9 = TAD; 10 = unknown: 11 = TA; 12 = isoTA.

R.S.D. on the area of the TA peak was 0.6%. When stored protected from light, solutions of tylosin and tylosin tartrate in water (1.0 mg ml^{-1}) are stable for at least 24 h. Nevertheless, it was preferred to dissolve the samples for quantitative analysis in 0.04 *M* dipotassium hydrogenphosphate in order to have a pH of solution similar to that of the mobile phase. These buffered solutions as well are stable for at least 24 h. The method showed good linearity in the range of injected mass examined (8–25 μg). The following relationship was found, where X = amount injected in μg and Y = peak area/1000: $Y = 5315X + 2334$, $S_{Y.X}$ (standard error of estimate) = 831, r (correlation coefficient) = 0.9997, n (number of measurements) = 19.

3.3. Analysis of commercial samples

Table 2 shows the results obtained for one tylosin base and five tylosin tartrate bulk samples. The content of TA was calculated with reference to the tylosin house standard (90.3%, w/w, as is). As the specific absorbances of TA, TB, TC and TD at the wavelength of detection (280 nm) are similar, the content of the impurities was expressed in terms of tylosin A. The water content was determined by Karl Fischer titration. The R.S.D. on the TA content mostly did not exceed 1.0%. The total mass was not

Table 2
Composition of bulk samples of tylosin tartrate (samples 1–5) and tylosin (sample 6)

	Composition (%, w/w)					
	Sample 1	Sample 2	Sample 3	Sample 4	Sample 5	Sample 6
TA	79.4	78.5	78.8	77.5	78.1	81.9
	(1.0)	(0.8)	(0.6)	(0.4)	(0.5)	(1.0)
n	5	5	5	5	5	5
TB	2.2	2.5	2.1	2.8	2.7	0.8
TC	0.6	0.9	0.5	0.7	0.6	1.4
TD	2.4	2.7	2.0	2.9	2.2	2.7
DMT	0.2	0.3	0.1	0.5	0.3	1.7
TAD	<0.06	0.2	0.1	<0.06	<0.06	<0.06
isoTA	0.5	0.2	0.3	0.4	0.4	0.4
Other	1.5	1.8	1.5	1.6	1.7	4.0
Total base	86.8	87.1	85.4	86.4	86.0	92.9
Total tartrate	93.9	94.2	92.3	93.4	93.1	–
Water	3.5	3.9	4.0	4.0	4.0	2.6
Total mass explained	97.4	98.1	96.3	97.4	97.1	95.5

R.S.D. in parentheses; *n* = number of solutions analysed = number of analyses. Chromatographic conditions as in Fig. 1.

entirely explained by the sum of the content of TA, impurities and water. This could be explained by the fact that some of the compounds of unknown identity have a lower UV absorbance at 280 nm than TA or by the presence of salts.

4. Conclusions

The LC method using wide-pore (1000 Å) PS–DVB material enables the separation of TA from all its potential impurities. Compared to previously published methods using reversed-phase columns, the method is highly selective since all known potential impurities and even a few unidentified impurities are separated from each other. The method shows good repeatability, linearity and sensitivity. The robustness of the method will be further investigated in a collaborative study.

References

[1] E. Roets, P. Beirinckx, I. Quintens and J. Hoogmartens, *J. Chromatogr.*, 630 (1993) 159.
[2] J.H. Kennedy, *J. Chromatogr. Sci.*, 16 (1978) 492.
[3] J.H. Kennedy, *J. Chromatogr.*, 281 (1983) 288.
[4] B.J. Fish and G.P. Carr, *J. Chromatogr.*, 353 (1986) 39.
[5] R.L. Hamill, M.E. Haney, M. Stamper and P.F. Wiley, *Antibiot. Chemother.*, 11 (1961) 327.
[6] R.L. Hamill and W.M. Stark, *J. Antibiot.*, 17 (1964) 139.
[7] H.A. Whaley, E.L. Patterson, A.C. Dornbush, E.J. Backus and N. Bohonos, *Antibiot. Agents Chemother.*, (1963) 45.
[8] H.A. Kirst, G.H. Wild, R.H. Baltz, E.T. Seno, R.L. Hamill, J.W. Paschal and D.E. Dorman, *J. Antibiot.*, 36 (1983) 376.
[9] R.B. Morin and M. Gorman, *Tetrahedron Lett.*, 34 (1964) 2339.
[10] J. Paesen, W. Cypers, R. Busson, E. Roets and J. Hoogmartens, *J. Chromatogr.*, 699 (1995) 99.
[11] J.V. Dawkins, L.L. Lloyd and F.P. Warner, *J. Chromatogr.*, 352 (1986) 157.
[12] J. Paesen, E. Roets and J. Hoogmartens, *Chromatographia*, 32 (1991) 162.
[13] J. Paesen, D.H. Calam, J.H. McB. Miller, G. Raiola, A. Rozanski, B. Silver and J. Hoogmartens, *J. Liq. Chromatogr.*, 16 (1993) 1529.
[14] *European Pharmacopoeia*, Maisonneuve, Sainte-Ruffine, France, 2nd ed., 1987, V.6.20.4.
[15] N.H. Khan, P. Wera, E. Roets and J. Hoogmartens, *J. Liq. Chromatogr.*, 13 (1990) 1351.
[16] C. Hendrix, J. Thomas, L.-M. Yun, E. Roets and J. Hoogmartens, *J. Liq. Chromatogr.*, 16 (1993) 421.