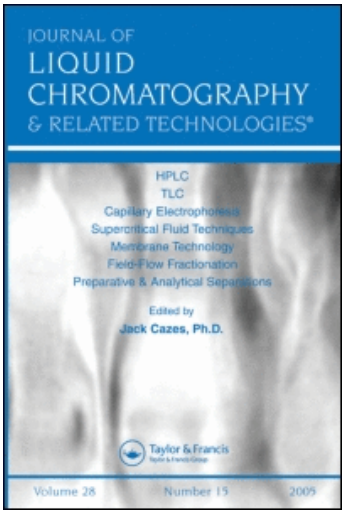

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Collaborative Study of the Analysis of Tylosin by Liquid Chromatography on Wide-Pore Poly(Styrene-Divinylbenzene)

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COLLABORATIVE STUDY OF THE ANALYSIS OF TYLOSIN BY LIQUID CHROMATOGRAPHY ON WIDE-PORE POLY(STYRENE-DIVINYLBENZENE)

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ABSTRACT

A previously developed method for analysis of tylosin A and related substances by liquid chromatography using a wide-pore poly(styrene-divinylbenzene) stationary phase was examined in a multicentre study involving 7 laboratories and 3 samples. The main component and the impurities were determined. An analysis of variance showed absence of consistent laboratory bias and significant laboratory-sample interaction at the 1 % level. Estimates for the repeatability and reproducibility of the method, expressed as standard deviation (SD) of the result of the determination of tylosin A, were calculated to be 1.3 % and 1.6 % respectively.

INTRODUCTION

Tylosin is a mixture of 16-membered ring macrolide antibiotics produced by fermentation of *Streptomyces* species. Tylosin A (TA) is the main component in this mixture. During fermentation several related substances can be formed: desmycosin or tylosin B (TB), macrocin or tylosin C (TC), relomycin or tylosin D (TD), demycinosyltylosin A (DMT) and 5-O-mycarosyltylonolide A (OMT) [1]. In neutral and alkaline medium tylosin A aldol (TAD) is formed [2,3]. Under the influence of light tylosin A in solution is partially converted into isotylosin (isoTA) [2]. Structures of these compounds were shown elsewhere [2,4].

A liquid chromatographic (LC) method for analysis of tylosin A and related substances on poly(styrene-divinylbenzene) (1000 Å) has been described previously [5]. The suitability of this method for general application was examined in this study, which was carried out in seven laboratories, using three samples.

EXPERIMENTAL

Apparatus and Columns

The protocol prescribed the use of a pump for isocratic delivery of the mobile phase at a flow rate of 1.0 ml/min. The equipment further consisted of a fixed loop injector with a loop of 20 µl, a column heating device, allowing continuous heating of the column at 60 °C (a water bath was preferred), a UV detector set at 280 nm and an integrator allowing peak area measurements. The participating laboratories were asked to use 25 x 0.46 cm i.d. columns, packed with poly(styrene-divinylbenzene) (PSDVB), 5 to 10 µm, 1000 Å. Laboratory packed columns were packed with PLRP-S, 8 µm,

1000 Å from Polymer Laboratories, Church Stretton, Shropshire, UK. Prepacked columns were purchased from different distributors of Polymer Laboratories.

Mobile phase

Liquid chromatographic grade or distilled tetrahydrofuran (200 ml) was added to a mixture of 50 ml of 0.2 M potassium phosphate buffer pH 9.0 and 750 ml of distilled water. The mobile phase was degassed by ultrasonication or by another suitable method. The collaborators were asked to adjust, if necessary, the tetrahydrofuran content in the mobile phase in order to reach the requirements for resolution.

Samples, Chemicals and Solvents

The reference sample (T-R) used was a house standard, containing 90.3 % m/m tylosin A, calculated on the substance "as is", and 9 % m/m water. A reference substance of TD was available, which was used to determine the resolution between TD and TA. The resolution between TB and TA was determined using a solution containing TB and TA. TB was formed in situ from TA in a solution of T-R in 0.05 M phosphoric acid solution (1.0 mg/ml) by heating for 5 min in a water bath at 60 °C. After cooling, the pH was adjusted to 7.0 and the solution was diluted to 100 ml using 0.05 M potassium phosphate buffer pH 7.0. Reference samples for the related substances were not used. The content of related impurities was expressed in terms of TA and calculated with reference to a 1 % dilution of the T-R reference solution. The samples to be examined (T-S1, T-S2, T-S3) were of commercial origin. Chemicals complied with Ph. Eur. requirements [6].

Solutions for analysis, containing 1.0 mg of tylosin/ml , were prepared in 0.05 M potassium phosphate buffer pH 7.0. Sample solutions had to be stored protected from light.

RESULTS AND DISCUSSION

Equipment and Method Performance

Table 1 includes information regarding columns, conditions used and results of performance checks carried out by each laboratory. The numbers assigned to the

TABLE 1
General Information on Equipment and Method Performance

Laboratory	Column	% THF in mobile phase	Retention time TA (n = 5) (RSD)	Sensitivity (S/N ratio)	S TA	Rs TD-TA	Rs TB-TA	Repeatability (n=5) Peak area RSD % TA	TC	TD	Linearity r TA
1	PP	19	36.4 (0.5)	23.7	1.0	4.6	8.7	0.4	2.1	0.7	0.9991
2	HP	20	31.3 (2.4)	10.0	1.1	5.5	9.8	0.9	2.0	1.1	0.9999
3	PP	24	28.1 (0.8)	16.8	1.1	6.1	9.7	0.8	4.2	1.8	0.9969
4	HP	21	25.5 (0.1)	42.0	1.4	4.9	8.3	0.9	1.6	1.0	0.9991
5	PP	18	28.4 (1.3)	10.0	1.0	5.2	7.7	0.6	2.0	1.8	0.9994
6	PP	25	26.6 (16)	3.2	1.1	4.5	ND	1.3	8.3	11.8	0.9990
7	HP	20	28.4 (0.2)	13.0	1.2	4.2	7.5	0.4	0.5	0.8	0.9997

PP = prepacked; HP = home-packed; THF = tetrahydrofuran; RSD = relative standard deviation; S = symmetry factor; Rs = resolution; r = coefficient of correlation; ND = not determined.

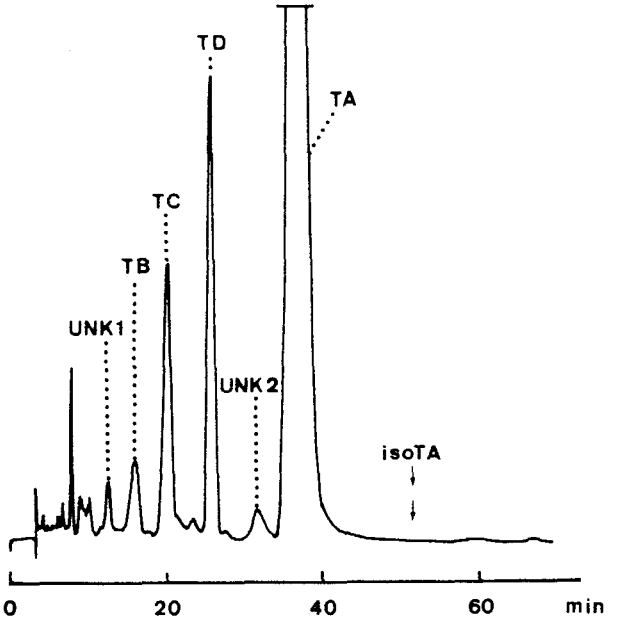

FIGURE 1: Typical chromatogram of sample T-S2, obtained in laboratory 1.
TA = tylosin A, TB = tylosin B, TC = tylosin C, TD = tylosin D,
isoTA = isotylosin A, UNK = substance of unknown identity.

laboratories do not correspond to the numbers assigned to the authors. A typical chromatogram is shown in Fig. 1. Three laboratories used home-packed columns, the other four used commercially purchased columns. The amount of tetrahydrofuran (THF) in the mobile phase varied between 18 % and 25 % v/v. The relative standard deviation (RSD) on the retention time of TA is not only a measure for the quality of the pumping system but also for the composition of hte mobile phase containing the volatile THF. In a first instance, laboratory 5 reported an increase of retention times during the day, due to loss of THF through evaporation. After advice was given to keep the mobile phase container well closed and not to purge continuously with helium, this problem was solved. Laboratory 6 was probably facing the same problem, but did not contact the organizing laboratory. The sensitivity of the detection system was also checked. In this test the signal-to-noise ratio was calculated of the TA peak obtained on injection of 50 ng of TA, which corresponds to 0.25 % of the amount used in sample

analysis. All laboratories, except laboratory 6, reached a ratio of at least 10. In laboratory 6 an older type of UV detector was used. However, the small amounts of impurities present in the samples were detected without any problem. Laboratory 5 used an injection volume of 10 µl instead of the prescribed 20 µl, because of overloading problems with the diode array detector used. Nevertheless, the required sensitivity was achieved.

Chromatographic characteristics were calculated according to the monograph "liquid chromatography" of the Ph. Eur. [7]. The symmetry factor S was calculated for the TA peak. The results varied between 1.0 an 1.4. Home-packed columns gave higher values. The resolution Rs was calculated first for the pair TD-TA (R_STD-TA). In the protocol a limit of 4.0 was put on this resolution. Collaborators were asked to adjust the THF content of the mobile phase in order to improve the resolution when necessary. The required resolution was reached by all participating laboratories. In a second test, the resolution between TB and TA (R_STB-TA) was determined using the conditions established for the first resolution test. Laboratory 6 did not report a result. The highest values for R_STB-TA were obtained by the collaborators who also achieved the best values for R_STD-TA. The intention was to check whether the resolution TB-TA could be used as a system suitability test instead of the resolution TD-TA. In contradistinction with TD, TB can easily be obtained by in situ decomposition of TA. The repeatability, expressed as the relative standard deviation (RSD, %) was calculated for five consecutive injections of the same solution of T-S2. This sample contained relatively high amounts (4 to 5 %) of the impurities TC and TD. The required RSD value for the peak area of TA was < 1 %. Laboratory 6 reported a slightly higher value, which might be due to the higher variation on the retention times mentioned above. This laboratory also reported the highest RSD values on the area of the impurities TC and TD.

The linearity is a measure for the quality of the detection system. The correlation coefficient r was calculated for a regression line determined over the range 16-24 µg of T-R injected, corresponding to 80 % - 120 % of the prescribed amount to be analysed. In a first instance, laboratory 4 reported a very high value for the intercept, which was probably due to overloading of the detector. The problem was solved by replacement of the detector.

Analysis of samples

Samples were analysed four times, using four separately prepared solutions. Individual results for the main compound, expressed as % m/m TA, and calculated

relative to the content of T-R, are mentioned in Table 2. Means and RSD values for TA are given in Table 3.

The protocol prescribed to calculate the mean results using the mean value of all the analyses of the reference. In a few cases this prescription was not followed. Laboratory 3 analysed the samples over a time period of four days, because an autosampler was not available. Results obtained on day 4 were systematically higher than those on the other days. Therefore, results were calculated with reference to the T-R samples analysed on that day. Laboratory 6 obtained a large variation of the results, which may be explained by the above mentioned variation of the retention times. Results for this laboratory were calculated using the nearest results obtained with the reference. The variation between replicates (RSD) was mostly less than 2 %, except in laboratories 3 and 4 (samples T-S1 and T-S2) and in laboratory 2 (sample T-S3). Laboratory 3 also obtained higher RSD values in the repeatability test. However, using Dixon's test for outlying individual results [8], no value had to be eliminated. Overall, RSD values obtained in the analysis of samples were higher than those in the repeatability test (Table 1). The means of means have RSD values lower than 2 %, which indicates that the method is robust enough to be generally applicable.

Table 4 reports means of mean values for the related substances. The content of impurities is expressed in terms of TA. Laboratory 6 did not properly integrate the peaks for UNK 1 and TB in all the samples. These results were not included in the mean values for these impurities. Laboratory 3 had a problem with the assignment of UNK 1. The reason for this is not clear, since the sensitivity test requirement (see Table 1) was met by this laboratory. Laboratory 2 reported formation of isoTA upon storage of the samples. The samples were probably not protected from light during analysis. This formation of isoTA explains the high RSD value for isoTA in sample T-S1. Although the content of impurities is rather low, the RSD values are quite small, indicating a good reproducibility due to good selectivity of the method towards the different related substances.

Analysis of variance

In order to further analyse the results obtained for the main component, a number of statistical calculations were performed following described procedures [8,9]. The results were first examined for outliers. The means were ranked for outlying laboratories [8]. The ranked mean values were also examined for outlying mean values by using Dixon's criterion [8]. No laboratory was excluded and no outlying

TABLE 2
Individual Results (% m/m) for Tylosin A

Laboratory	Sample											
	T-S1				T-S2				T-S3			
1	80.22	79.80	81.33	80.99	74.64	74.45	74.74	76.99	74.83	74.98	75.40	76.03
2	80.49	80.19	82.23	78.59	76.24	74.22	77.49	77.36	76.00	73.81	72.21	71.39
3	80.23	77.58	78.44	82.21	74.72	79.60	77.08	76.71	75.77	76.06	75.54	75.33
4	78.93	77.14	78.46	81.38	73.99	72.46	78.15	77.72	73.40	74.96	74.17	74.08
5	80.20	80.14	79.76	80.45	77.84	80.10	77.72	78.50	77.17	78.66	77.36	76.02
6	81.80	82.09	81.04	79.79	76.52	78.69	78.60	76.77	75.30	76.38	76.14	74.39
7	80.00	80.87	80.99	80.35	75.49	76.16	74.45	75.87	74.28	74.79	75.45	75.65

TABLE 3

Mean values (% m/m) for Tylosin A

Laboratory	Sample T-S1	Sample T-S2	Sample T-S3
1	80.6 (0.9)	75.2 (1.6)	75.3 (0.7)
2	80.4 (1.8)	76.3 (2.0)	73.4 (2.8)
3	79.6 (2.6)	77.0 (2.6)	75.7 (0.4)
4	79.0 (2.2)	75.6 (3.7)	74.1 (0.9)
5	80.1 (0.4)	78.5 (1.4)	77.4 (1.4)
6	81.2 (1.3)	77.6 (1.5)	75.6 (1.2)
7	80.6 (0.6)	75.5 (0.8)	75.0 (0.6)
Mean of means	80.2 (0.9)	76.5 (1.6)	75.2 (1.7)

Relative standard deviations (RSD, %) are mentioned in parentheses.

TABLE 4

Mean of mean values (%) for related substances

Sample	UNK 1[a]	TB[a]	TC	TD	UNK 2	isoTA
T-R	0.24 (16)	0.44 (15)	0.59 (17)	1.01 (15)	0.35 (29)	ND
T-S1	0.60 (15)	1.09 (14)	0.39 (23)	4.25 (12)	0.79 (26)	0.88 (68)[b]
T-S2	0.63 (15)	1.35 (13)	4.47 (15)	5.78 (14)	0.68 (19)	ND
T-S3	0.98 (19)	1.31 (14)	3.21 (14)	2.19 (14)	0.77 (22)	ND

ND = not detected; RSD (%) are given in parentheses. (a) Results for lab 6 not included. (b) Lab 2 reported formation of isoTA upon storage of the sample solution.

TABLE 5
Analysis of variance

Source of variation	Sum of squares	Degrees of freedom	Mean square	Variance ratio
Between labs (L)	50.43	6	8.41	L/LS = 2.77 F_{99} (6,12) = 4.82
Laboratory - sample interaction (LS)	36.47	12	3.04	LS/S = 1.71 F_{99} (12,63) < 2.50 > 2.34
Between replicates (S)	112.09	63	1.78	

mean value was found. The data were examined in order to determine whether the within-sample and within-laboratory variances may be considered as sufficiently homogeneous [9]. For both variances the limit was not exceeded.

An analysis of variance was carried out to search for consistent laboratory bias or significant laboratory sample interaction [9]. The results which are shown in Table 5 reveal that there is no significant between laboratory variance at the 1 % level, so no consistent laboratory bias exists. The laboratory-sample interaction is also not significant at the 1 % level. Estimates of the repeatability of the analytical method (intra-laboratory variance) and of the reproducibility (inter-laboratory variance) were calculated. The standard deviations thus obtained were 1.3 % and 1.6 % respectively. The method will show greater variation when carried out by different laboratories than within one laboratory, but evidently no consistent laboratory bias exists (the between-laboratory variance is not significant).

CONCLUSION

It can be concluded that the LC method shows a reproducible selectivity on PLRP-S columns of different origin and that the method is suitable for purity control and assay of tylosin.

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