CHROM. 5488

A THIN-LAYER CHROMATOGRAPHIC METHOD FOR THE DETECTION OF TYLOSIN IN BIOLOGICAL MATERIALS AND FEEDS

M. DEBACKERE AND K. BAETEN

Department of Pharmacology and Toxicology, Faculty of Veterinary Medicine, University of Ghent (Belgium)

(Received April 23rd, 1971)

SUMMARY

The detection of the antibiotic tylosin by means of a thin-layer chromatographic technique is described. Identification is made under UV light and by spraying the chromatoplates consecutively with acid iodoplatinate, Dragendorff's reagent and a saturated solution of silver sulphate in 10 % sulphuric acid. The sensitivity for the three consecutive sprays lies between 2 and 4 μg of tylosin base. The best extraction was obtained with a mixture of chloroform–ethyl acetate (2:1).

These extraction and chromatographic techniques were applied to the isolation and detection of tylosin from biological materials. Because of the disturbance by the proteins present in blood plasma, tissue homogenates and milk, deproteinization must be performed before extraction. For feeds, urine and tissue homogenates, two-dimensional chromatography was preferred. Tylosin could be detected with accuracy at concentrations of 2–4 p.p.m. in these different materials.

This method was also applied to the detection of tylosin in biological fluids such as blood plasma, urine and milk after injection in healthy cows and passage through the body.

INTRODUCTION

Tylosin, a fermentation product of a strain of the actinomycete *Streptomyces fradiae*, is an antibiotic discovered and developed by the Eli Lily & Company Research Laboratories, Indianapolis, Ind., U.S.A.

The particular strain of actinomycete was isolated from a soil sample collected by a missionary from a Thailand rice field and submitted in 1955; hence the derivation of the non-proprietary name "tylosin"[1].

Certain of the properties of tylosin indicate that it belongs to the "macrolide" class of antibiotics. It is a weak base that is very soluble in most common organic solvents and reacts readily with mineral acids and a number of organic acids to form water-soluble crystalline salts, *e.g.* tylosin tartrate (600 mg/ml). Aqueous solutions of tylosin exhibit a high degree of stability over the pH range 5.5 to 7.5 at 25° for periods as long as three months. However, below pH 4 or above pH 9, degradation was observed. In solution at pH $<$ 4.5, tylosin is converted by acid degradation to desmycosin, and in more severe conditions to the neutral sugars mycarose and myc-

aminose[2]. An important feature of tylosin is that the degradation product desmycosin exhibits physicochemical and antimicrobial properties that are very similar to those of tylosin.

Because of the effectiveness of tylosin against a number of poultry and livestock diseases and because of the ability of tylosin to improve liveweight gain and feed conversion efficiency of growing pigs and chickens, tylosin was developed specifically for agricultural uses. That means that tylosin can be used as a therapeutically active antibiotic, especially against infections caused by pleuropneumonia-like organisms (PPLO) and *Vibrio* in poultry and pigs or as a feed-additive probiotic factor for growth stimulation, mainly in pigs. In certain countries, however, *e.g.* Belgium, the latter use is not allowed.

The purpose of this paper is to describe a thin-layer chromatographic (TLC) method for the detection of tylosin in animal feeds and in biological materials of treated animals.

SEPARATION, IDENTIFICATION AND EXTRACTION OF TYLOSIN FROM PURE AQUEOUS SOLUTIONS

Separation

The separation of tylosin, as already mentioned, was carried out by TLC. As urine and feeds can contain many other substances that can interfere with the tylosin spot on the chromatoplates, we preferred to use two-dimensional TLC. Extracts from blood and milk, however, can be submitted to one-dimensional chromatography.

The tylosin base used to test out the best solvent and spray was a standard (Ref. 508-B/3-9/66) obtained from the Dista Laboratories (Speke, Liverpool, Great Britain) with a potency of 975 μg/mg and containing principally tylosin A (*ca.* 92 %) with small amounts of D and C and traces of B, E1 and E2. For the extraction procedure, we used the water-soluble crystalline salt tylosin tartrate.

Method

We used 20 × 20 cm precoated TLC plates, Silica Gel 254 (Merck), with a layer thickness of 0.25 mm. Silica Gel GF 254 plates were preferred because tylosin gives an absorbing spot in UV light at 254 nm so that it could be detected before spraying. Precoated plates were preferred to self-made chromatoplates because the perfect uniform layer gives better results in two-dimensional chromatography.

After trying different solvent systems, the most satisfactory results were obtained with a mixture of chloroform–acetone (60:40) for the first direction and a mixture of ethyl acetate–methanol (85:15) for the second direction. The plates were spotted and developed in the same manner as already described for alkaloids[3]. In the case of one-dimensional chromatography, only the second solvent was used. The total development for two-dimensional chromatography took about 3 h.

Identification

After development, the plates were dried with cold air and examined under UV light at 254 nm. Identification was possible at first by means of the R_F values under UV light before spraying. In the first solvent, tylosin had an R_F value of about 0.12 and in the second solvent this value amounted to about 0.60.

After examination under UV light, the sheets were sprayed consecutively with the following reagents for further identification:

(a) Iodoplatinate. 1 g of $PtCl_4 \cdot 2HCl \cdot 6H_2O$ and 20 g of KI are dissolved in 8 ml of concentrated HCl and diluted to 400 ml with distilled water. The chromatograms are sprayed until they are uniformly pink with a faint brown colour for tylosin.

(b) Dragendorff's reagent modified by MEUNIER AND MACHEBOEUF[4]. Solution A: 0.85 g of basic bismuth nitrate, 10 ml of acetic acid (96 %) and 40 ml of distilled water. Solution B: 20 g of KI dissolved in 50 ml of water. Both solutions were mixed and kept in a dark brown bottle. 10 ml of acetic acid and 35 ml of water are added to 5 ml of this mixture just before spraying. This reagent makes the spots for tylosin more pronounced.

(c) A saturated solution of silver sulphate in 10 % sulphuric acid. After this spray, the spots for tylosin become orange-brown against a dark background produced by the silver sulphate–acid iodoplatinate combination, especially after a few minutes exposure to light.

Sensitivity of the detection method used

Different plates were spotted with increasing concentrations of the pure tylosin base, and developed by one-dimensional chromatography. It was found that under UV light, quantities of 2 μg of tylosin could be seen as a faint absorbing spot, whereas 4 μg gave a definite spot. The sensitivity for the three consecutive sprays also lays between 2 and 4 μg of tylosin base.

Extraction procedure

Six different extraction solvents were tested, *viz.* chloroform, ether, dichloroethane, benzene, ethyl acetate and a mixture of chloroform–ethyl acetate (2:1). Before the extraction, a solution of tylosin tartrate (25 μg base/ml) in 25 ml of distilled water was made alkaline to pH 9–10 by the addition of 50 % NaOH. This solution was extracted three times with 10 ml of each solvent for 15 min by rotating the glass-stoppered tubes in a rotating apparatus. The respective solvent phases were collected, dried over anhydrous sodium sulphate and evaporated under vacuum in a rotary-film evaporator. The extracts obtained were redissolved in methanol and tested semi-quantitatively on chromatoplates against known amounts of tylosin base. From these results, it was found that the chloroform–ethyl acetate mixture gave the best results with a recovery of 80–100 %. Even in 25 ml solutions with a concentration of 1 μg/ml, the tylosin could be detected by the described procedure.

EXTRACTION, DETECTION AND IDENTIFICATION AFTER ADDING TYLOSIN TO BIOLOGICAL MATERIALS

These extraction and detection methods were further tested on biological materials such as blood plasma, urine, milk and liver homogenates. For these purposes, a known amount of tylosin tartrate was added to a certain volume (25 ml) or weight (20 g) of these materials. In view of the different composition of these biological media with regard to the pure aqueous solutions, it was necessary to adapt the extraction method described. The detection and identification, however, were based on the same TLC techniques as described for the pure aqueous solutions except that, as already mentioned for urine and tissues, two-dimensional chromatography was applied.

Blood plasma

After several extractions, it was found that the plasma proteins greatly disturbed the extraction procedure. It was therefore necessary to deproteinize the blood plasma. Because of the degradation of tylosin to desmycosin at pH less than 4.5 and the water insolubility of tylosin base at pH greater than 8, the deproteinizing agent must be nearly neutral. Therefore equivalent quantities of a saturated solution of $Ba(OH)_2$ of approximately 0.3 *N* and a 5 % solution of $ZnSO_4 \cdot 7H_2O$ were used. These solutions were previously titrated against each other so that a fixed volume of 5 % $ZnSO_4$ is neutralised by the same volume of $Ba(OH)_2$. For each ml of plasma, 1 ml of $Ba(OH)_2$ and 1 ml of 5 % $ZnSO_4$ were used. The mixture of these solutions produced a precipitate that had a great absorption capacity for the tylosin. To prevent this precipitation of tylosin, several millilitres of a saturated $(NH_4)_2SO_4$ solution were added previously to the plasma sample.

The extraction procedure was performed as follows: to 25 ml of blood plasma, 3 ml of a saturated $(NH_4)_2SO_4$ solution, 25 ml of a 5 % $ZnSO_4$ solution and 25 ml of a saturated $Ba(OH)_2$ solution were added consecutively, with thorough shaking after each addition. After centrifugation for 15 min at 1500 rev/min, the supernatant was introduced into a 100-ml glass-stoppered extraction tube, made alkaline to pH 9 and extracted three times with 20 ml of chloroform–ethyl acetate (2:1), as already described. It was found that even at concentrations of 1 μg/ml, tylosin could be detected, and that the extraction rate varied between 60 and 70 %.

Milk

The same extraction procedure was followed as for blood plasma. Here also, concentrations of 1 μg/ml of milk could be detected with an extraction rate of 50–60 %.

Liver homogenates

To 20 g of liver in a 100-ml glass-stoppered centrifuge tube 15 ml of a 56.4 % Na_2HPO_4 (0.2 *M*, 28.392 g/l) + 43.6 % citric acid monohydrate (0.1 *M*, 21.015 g/l) buffer of pH 5.5 were added. To prevent precipitation of the tylosin with the tissue proteins, 3 ml of a saturated $(NH_4)_2SO_4$ solution were added before homogenization. After homogenization, the tube was centrifuged at 1500 r.p.m. for 15 min. The supernatant was transferred to another glass-stoppered centrifuge tube, deproteinized with 25 ml of 5 % $ZnSO_4$ and 25 ml of saturated $Ba(OH)_2$ solution, and centrifuged at 1500 r.p.m. for 15 min. The supernatant was then made alkaline to pH 9 with 50 % NaOH, and extracted three times with 30 ml of extraction solvent over 15 min, as already described.

In some cases, however, it was found that some interfering substances masked the spots for tylosin on the chromatoplates. After evaporation, the residue therefore was redissolved in several millilitres of dilute HCl (pH 5–6) and extracted three times with 5 ml of ether. The ether layers were then discarded and the aqueous phase was made alkaline and re-extracted three times with 5 ml of chloroform–ethyl acetate (2:1). This extract was dried with anhydrous sodium sulphate, evaporated and spotted on the chromatoplates for two-dimensional chromatography. Concentrations of 2 p.p.m. of tylosin added to liver tissues could be detected with an extraction rate of 60–70 %.

Urine

25 ml of urine were extracted three times after alkalinization of the urine to pH 9 with 20 ml of the extraction solvent. As for the liver extract residue, a clean-up by prior acid extraction with ether is necessary to eliminate interfering substances. After two-dimensional chromatography, concentrations of 1 μg/ml could be detected with an extraction rate of 50–60 %.

EXTRACTION, DETECTION AND IDENTIFICATION OF TYLOSIN FROM BIOLOGICAL FLUIDS OF LACTATING COWS AFTER PASSAGE THROUGH THE BODY

Materials

Eight healthy, lactating cows with a body-weight of 450–560 kg were injected intramuscular (i.m.) with a dose of 10 mg tylosin/kg (Tylan, Eli Lily & Co.).

Just before the injection, the animals were placed in the experimental box and control samples of milk, blood and urine were taken. Milk was taken from the four quarters and these were totally milked. This was also the case after each milk sampling so that no milk remained in the udder.

The blood was collected by puncture of the jugular vein and collected in a receptacle containing some heparin.

Urine was collected continiously during 12 h by fixing a catheter in the urethra and draining the urine by means of a plastic tube in a measuring glass[5].

Samples of blood, urine and milk were taken again in the same manner 1, 3, 6, 9, 12 and 24 h after the injection. For blood, a sample was also taken after 0.5 h.

Extraction, detection and identification

Immediately after collection, the blood samples were centrifuged, and the plasma and urine samples were stored in a freezer until the moment of extraction. Milk samples were extracted immediately for tylosin detection.

The extraction, detection and identification were carried out as already described for the respective biological materials.

Results

The results of these eight experiments are summarized in Table I. The different crosses in Table I have a comparative value with respect to each other only for the same kind of sample within a given experiment.

EXTRACTION, SEPARATION AND IDENTIFICATION OF TYLOSIN FROM FEEDS AND PREMIX

Feed samples with concentrations of tylosin varying from 2 to 20 p.p.m. were examined. To 25 g of these samples, 50 ml of distilled water were added and the tubes were thoroughly shaken for 20 min. After centrifugation, the aqueous phase was decanted into another extraction tube and the procedure was repeated. The collected aqueous phases were made alkaline and extracted three times with 40 ml of chloroform–ethyl acetate (2:1). The collected extracts were dried with anhydrous sodium sulphate and evaporated to dryness under vacuum in a rotary-film evaporator. As for the urine and liver tissue extracts, it was necessary to clean up the residue and to apply two-dimensional chromatography. A definite spot was obtained for the

J. Chromatogr., 61 (1971) 125–132

TABLE I

RESULTS AFTER THE INJECTION OF TYLOSIN INTO LACTATING COWS

Cow No.	*Plasma*							*Milk*						*Urine*					
	0.5 h	*1 h*	*3 h*	*6 h*	*9 h*	*12 h*	*24 h*	*1 h*	*3 h*	*6 h*	*9 h*	*12 h*	*24 h*	*1 h*	*3 h*	*6 h*	*9 h*	*12 h*	*24 h*
1	−	?	+++	++	++	+	−	−	++++	+++	++	+	−	−	++++	++	++	+	−
2	−	−	−	−	−	−	−	−	++++	+++	++	+	−	++	+++	++	+	−	−
3	+	++	+++	+++	++	+	−	−	+	++	++	+++	−	++	+++	++++	++	+	+
4	−	++	+	−	−	−	−	−	++	+++	++	+	−	+	++++	+++	++	++	+
5	−	−	−	−	−	−	−	−	+++	++++	++	+	−	++	++++	+++	++	++	+
6	−	+	++	−	−	−	−	−	+	++	+	+	−	+	++	+++	++	+	−
7	−	+	++	−	−	−	−	−	+	++	++	+	−	+	+++	++++	+++	++	+
8	+	++	+	−	−	−	−	−	−	+	+++	++	−	++	++++	+++	++	+	−

5 p.p.m. concentration level whereas for the 2 p.p.m. concentration level a faint spot was seen in some cases so that we can say that the sensitivity of the method used was situated between 2 and 5 p.p.m.

In some cases, however, a gel was formed in the organic solvent layer, presumably by the starch present in the feed, so that it was impossible to separate this layer even after centrifugation. The upper aqueous layer was then transferred as quantitatively as possible to a new extraction tube for further extraction. Some more extraction solvent was added to the organic layer and the gel was precipitated by the addition of a sufficient amount of anhydrous sodium sulphate to dry the extract.

Even for tylosin premix, this method could be applied notwithstanding the presence of tylosin in a gelatinised form and of paraffin as an antidust agent.

DISCUSSION

Until now, only a few papers concerning the identification of tylosin have been published[2,6–10]. They involve mostly microbiological techniques and are based on the growth inhibition of tylosin against sensitive organisms such as *Sarcina lutea* on agar plates or on a turbidimetric test against *Staphylococcus aureus*. Although the sensitivity of these methods is very high and even concentrations of 0.2 p.p.m. can be detected in tissues, it is clear that all these methods are not specific enough to be used for the detection of tylosin alone in samples of feeds or biological materials. Other antibiotics or chemotherapeutics used as feed additives or as anti-infectious agents can also give growth-inhibition when they are isolated from feeds or biological materials. To eliminate this interference by other similarly acting agents, it is necessary to have more specific detection and identification methods that are not based on pharmacological but on chemical and physical properties. Therefore a chemical method such as chromatography is much more reliable.

In this paper, we have proved that tylosin can be revealed and identified very easily by TLC and that the detection method used is very sensitive since tylosin is readily detectable in quantities of 5 μg and even 2 μg. Moreover, the two-dimensional chromatography technique offers greater specificity. The extraction method also is of great importance for this kind of work. It must have such a high yield that the extracts contain sufficient quantities for them to be detected chromatographically. From our results, it is seen that for biological fluids such as urine, milk and blood plasma, concentrations of 1 μg/ml, and for feeds and tissues, concentrations of 2 p.p.m., could be detected with accuracy.

The usefulness of the method described has further been shown by our results obtained after injection of tylosin in a group of cows. It can be seen from the results in Table I that tylosin was very easily detectable in milk and urine samples after a single injection of a 10 mg/kg dose. In nearly all the cows examined, a peak concentration was found between 3 and 6 h after the injection for both the urine and the milk. For the milk, however, it was not possible to detect tylosin within the first hour and 24 h after administration, whereas this could be achieved in the case of the urine. Nevertheless, the concentrations for the milk were much more lower than for the urine, and, although an accurate quantitative dosage was not possible by the TLC method used, it could be estimated from the sensitivity limit of our method that the peak concentrations in the milk were mostly higher than 4 μg/ml whereas the 12-h

samples still had concentrations above 1 μg/ml. For the urine, the peak concentrations were at least 10–12 times higher than the concentrations at 12 h, which were mostly lower than those for the 1-h samples.

It must be noted also that in many cases the spots for the urine extracts were tailed and consisted of two parts. As it is known that tylosin can be converted to desmycosin and that desmycosin exhibits physical and chemical properties that are very similar to those of tylosin, it could be that the spot on the chromatoplates consisted of tylosin and desmycosin. Further experiments will be carried out to examine this possibility.

It was much more difficult to detect the injected tylosin in the blood plasma and in two cases it was impossible to detect tylosin at all. Except for two cows (Nos. 1 and 3), the quantity of tylosin present in the positive samples at the peak concentrations was just beyond the sensitivity limit of our method, namely 1–2 μg/ml. This means that in most cases, the highest concentration of tylosin after a single dose of 10 mg/kg in cows probably varied from 2 to 4 μg/ml blood plasma.

The results for the extraction and detection of tylosin from feeds gave a sensitivity limit of 2–5 p.p.m. As the minimal concentration of tylosin used as feed additive ranges between 5 and 10 p.p.m., it is clear that the method described is sensitive enough to detect tylosin in these feeds. This can be very useful, especially in those countries where the use of tylosin as feed additive is not allowed.

In addition to the lower sensitivity of this chemical method for the detection of tylosin in blood plasma in comparison to the microbiological method, it is impossible to obtain an accurate dosage of the tylosin present because TLC methods permit only a semi-quantitative estimation. However, studies are now in progress to elute the tylosin spots from the chromatoplates and to determine the tylosin in the eluates by UV spectrophotometry.

ACKNOWLEDGEMENTS

We wish to thank the Dista Laboratories (Speke, Liverpool, Great Britain) for the supply of the tylosin base standards, and Mr. J. VINCKE for his technical assistance.

REFERENCES

1 *Elanco Technical Services Reports*, 23 Jan., 1967, Ref. X/E/4, *Tylan Premix – Fundamental Documentation*, Eli Lily International, Wimbledon.
2 R. L. HAMILL, M. E. HANCY, M. STAMPER AND P. F. WILEY, *Antibiot. Chemother.*, 11 (1961) 328.
3 M. DEBACKERE AND L. LARUELLE, *J. Chromatogr.*, 35 (1968) 234.
4 MEUNIER AND MACHEBOEUF, in E. STAHL, *Dünnschichtchromatographie*, Verlag Chemie, Weinheim, 1962, p. 829
5 G. PEETERS AND R. COUSSENS, *Arch. Int. Pharmacodyn.*, 84 (1950) 209.
6 DISTA LABORATORIES, *Laboratory Test Methods*, personal communication.
7 J. M. MCGUIRE, W. S. BONIECE, C. E. HIGGINS, M. M. HOEHN, W. M. STACK, J. WESTHEAD AND R. N. WOLFE, *Antibiot. Chemother.*, 11 (1961) 320.
8 F. KAVANAGH AND L. J. DENNIN, *Analytical Microbiology*, Academic Press, New York, 1963, p. 369.
9 R. M. KLINE, C. E. REDMANN, J. B. TEPE AND R. L. HAMILL, *Agric. Food Chem.*, 13 (1965) 305.
10 *Official Methods of Analysis of the Association of Official Analytical Chemists*, 11th Ed., George Bonta Company, Menasha, 1970, p. 762.