Microbiological and Thin-Layer Chromatographic Identification of Aminoglycoside Antibiotics in Animal Body

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Abstract. Dihydrostreptomycin, kanamycin and fradiomycin were administered intramuscularly or orally to pigs and calves. An attempt was made to identify these aminoglycoside antibiotics qualitatively in tissues and urine collected from the animals after slaughter. The kidney, liver and muscle were homogenized and used to determine the diameters of inhibition zones to be produced on *Micrococcus luteus* ATCC 9341 and *Bacillus subtilis* ATCC 6633 plates at pH 6.0 and 8.0. The urine was used undiluted and after diluted serially. Thin-layer chromatographic identification was carried out only on kidney and urine samples characterized by capacity to produce larger inhibition zones on the *B. subtilis* plate at pH 8.0 than on any other plate. Next, the ultrafiltrate obtained from each tissue homogenate or urine was spotted on two silica gel plates. One of the plates was developed in the upper layer of chloroform-methanol-17% ammonia (2:1:1), and the other in n-propanol-pyridine-acetic acid-water (15:10:3:12). The R_f values obtained were a little higher than those with reference antibiotic solutions in some cases. It was possible, however, to distinguish the three antibiotics contained in a kidney or urine sample from one another by thin-layer chromatography with two solvents. Thus, the methods mentioned above were considered to be available for the identification of aminoglycoside residues in slaughtered animals. When the kidney is suspected to contain some aminoglycosides, it is recommended to carry out chromatography on the extract from the renal cortex for the identification of these substances.

The aminoglycoside antibiotics have been widely used to treat infectious diseases caused by diverse bacteria and to stimulate weight gains in animals. Dihydrostreptomycin (DSM), streptomycin (SM) and kanamycin (KM) are administered parenterally or orally, but fradiomycin (FM) is applied restrictedly by the oral and intramammary route because of its nephrotoxicity.

Much information has been presented on the absorption, distribution and residue of these antibiotics in domestic animals. Mercer et al. [10], and Andreini and Pagnattelli [2] reported that when given intramuscularly or subcutaneously, DSM and KM remained in the renal tissues for a long

period, although they disappeared sooner or later from any other tissue. FM was reported to be slightly absorbed from the intestinal tract in pigs and cattle following oral administration [4, 16]. On the other hand, destomycin A and hygromycin B having anthelmintic activities were shown to bring about no residues in pigs and chickens when given as feed additives [1, 7, 9, 22].

The residues of the aminoglycoside antibiotics have also become an object of discussion in slaughter animals [13, 18, 20]. In the present investigation, an attempt was made to identify DSM, KM and FM in tissues and urine of animals by the aid of

Table 1. Test protocol

Animal No.	Species and sex	Approx. body weight in kg	Antibiotic administered	Dose in mg	Time interval in hours between last adm. and slaughter
19	pig (♀)	50	Dihydro-streptomycin ¹⁾	750	24
20	pig (♂)	50		750	48
29	calf (♂)	250		2,500*	48
10	pig (♂)	70	Kanamycin ²⁾	750	6
8	pig (♂)	70		750	24
21	pig (♀)	50		750	48
27	calf (♂)	250		1,500*	48
22	pig (♂)	50	Fradiomycin ³⁾	3,000	6
23	pig (♀)	50		3,000*	6
24	pig (♀)	50		3,000	24
13	pig (♂)	50	control		
14	pig (♀)	50			

¹⁾ Dihydrostreptomycin sulfate solution 250 (Meiji) for veterinary use containing 250 mg/ml of dihydrostreptomycin.

²⁾ Kanamycin sulfate solution 250 (Meiji) for veterinary use containing 250 mg/ml of kanamycin.

³⁾ Dextromycin (Takeda) containing 662 mg/g of fradiomycin sulfate.

* The same dose of the antibiotic was administered ones 24 hours in advance.

microbiological and thin-layer chromatographic procedures.

Materials and Methods

1. Animals

Ten Yorkshire pigs weighing about 50 or 70 kg and two Holstein calves weighing about 250 kg were used. Of them, two pigs (Nos. 19 and 20) and one calf (No. 29) were given an aqueous suspension of DSM intramuscularly, and three pigs (Nos. 8, 10 and 21) and another calf (No. 27) an aqueous suspension of KM. Three pigs (Nos. 22, 23 and 24) were given FM sulfate dissolved in drinking water by the oral route with a catheter. To two calves (Nos. 29 and 27) and one pig (No. 23) were re-administered with the same dose of the antibiotic 24 hours after the first administration. Following the administration of the antibiotic, the animals were slaughtered at various times outlined in the test protocol (Table 1). The remaining two pigs (Nos. 13 and 14) served as controls.

The kidney, liver, muscle and urine were collected from each animal and frozen immediately.

2. Preparation of *Micrococcus luteus* and *Bacillus subtilis* plates

M. luteus ATCC 9341 and *B. subtilis* ATCC 6633 were seeded on to agar plates. On each plate the diameter of the inhibition zone was determined for

the tissue and urine samples handled in the same manner as described previously [21]. By using such plates, it was possible to screen tissue and urine samples containing more than 2 and 0.5 µg/g of DSM, 2 and 0.5 µg/g of KM, or 4 and 1 µg/g of FM.

3. Thin-layer chromatographic identification

A tissue sample homogenized with two volumes of distilled water was ultrafiltered through a pellicone membrane (PSED) in the 25-mm clinical ultrafiltration system (Millipore). The ultrafiltrate was concentrated in vacuo to about one-fifth. Aliquots of 50 µl of this ultrafiltrate were spotted on 2 silica gel plates (spot film for thin-layer chromatography (TLC), 20×20 cm, Tokyokasei), together with the same volumes of the reference antibiotics dissolved in distilled water to a concentration of 10 µg/ml of DSM, 10 µg/ml of KM, or 20 µg/ml of FM. One plate was developed in the upper layer of chloroform-methanol-17% ammonia (2:1:1, solvent A), and the other in n-propanol-pyridine-acetic acid-water (15:10:3:12, solvent B). When the two plates were dried, Rf values were determined by bioautography, as described previously [21], with antibiotic medium No. 1 (Difco) at pH 8.0 inoculated with *B. subtilis*. The urine sample was ultrafiltered intact and subjected to TLC.

4. Qualitative assay of antibacterial activities in tissues and urine

The detection of antibacterial activities was car-

Table 2. Diameter of inhibition zones on *M. luteus* and *B. subtilis* plates, and Rf values in chromatography by kidney samples

Animal No.	Inhibition zone (in mm)				Rf	
	<i>M. luteus</i>		<i>B. subtilis</i>		Solvent	
	pH 6.0	pH 8.0	pH 6.0	pH 8.0	A	B
19	NZ	17.6	12.5	22.3	0.14	0.38
20	NZ	16.3	NZ	21.9	0.14	0.36
29	NZ	17.0	13.0	21.8	0.11	0.40
10	NZ	NZ	NZ	17.3	0.32	0.12
8	NZ	NZ	NZ	17.3	0.32	0.11
21	NZ	NZ	NZ	17.3	no	0.14
27	NZ	NZ	14.5	22.5	0.32	0.11
22	NZ	10.5	NZ	16.3	no	no
23	10.0	12.0	NZ	16.8	no	no
24	NZ	NZ	NZ	NZ	ND	

NZ: No zone. ND: Not done. no: No area of inhibited bacterial growth on the chromatogram.

Rf values by reference antibiotic solutions in solvents A and B were 0 and 0.39 for DSM, 0.27 and 0.08 for KM, and 0.06 and 0.02 for FM.

ried out qualitatively in the same manner as described previously [21], with *B. subtilis* as the test organism.

5. Antibiotic concentrations in renal tissues

The cortex and medulla of the kidney were separated from each other and homogenized with 0.1 M phosphate buffer (pH 8.0). Antibiotic concentrations were determined by the microbiological method described previously [21], with No. 3 medium (pH 8.0) prepared according to the Minimum Requirements for Antibiotics for Animal Use [11]. *B. subtilis* was used as the test organism.

Results

1. Identification of aminoglycoside antibiotics in tissues

The diameter was determined in inhibition zones produced on *M. luteus* and *B. subtilis* plates by the tissue homogenates. Neither of these test organisms was inhibited by any liver or muscle sample. Table 2 presents the diameters of inhibition zones produced on these plates by kidney samples, along with Rf values in TLC. The largest inhibition zone was seen on the *B. subtilis* plate at pH 8.0, regardless of the type of antibiotics administered.

In animals given DSM, the Rf values were 0.11–0.14 and 0.36–0.40 when developed in solvents A and B, respectively. Conversely, they were higher with solvent A than with solvent B in animals given KM. These values obtained by kidney samples were a little higher than those with reference antibiotics in some cases. In animals given FM, no area of inhibited bacterial growth was recognized on the chromatogram.

It seemed likely that DSM and KM might be distinguishable from each other in tissues, although DSM-containing ultrafiltrates caused obvious tailing on the chromatogram when developed in solvent A (Fig. 1). When developed in solvent B, the ultrafiltrate produced an area of inhibited bacterial growth, which was round or oval in shape for any antibiotic, on the chromatogram.

2. Identification of aminoglycoside antibiotics in urine

Urine samples were used undiluted and after diluted serially, but few inhibition zones could be found on *M. luteus* and *B.*

Fig. 1. TLC pattern of inhibited bacterial growth on silica gel plate by kidney samples.

1 and 2: pig (No. 8) and calf (No. 27) given KM, 3 and 4: pigs (Nos. 19 and 20) given DSM, 5: pig (No. 23) given FM, 6: FM standard, 7: DSM standard, and 8: KM standard.

subtilis plates with any dilution of the urine. The diameters of inhibition zones produced on these plates by undiluted urine samples are indicated in Table 3, along with Rf values in TLC.

The largest inhibition zone was produced on the *B. subtilis* plate at pH 8.0 by all the

urine samples, except two (Nos. 8 and 21) collected from pigs given KM. These samples gave rise to inhibition zones which were conversely larger on the *B. subtilis* plate at pH 6.0 than on that at pH 8.0.

The Rf values by DSM were higher in solvent B than in solvent A, and those by KM vice versa. In the case of development in solvent A, tailing was recognized to be similar in appearance to that noticed in kidney samples. The Rf values by FM were less than 0.1 in both solvents. TLC revealed that when administered orally to pigs, this antibiotic was excreted in urine.

3. Qualitative assay

When collected from animals given the aminoglycosides, kidney and urine samples were all positive for this assay, but liver and muscle samples were not always positive. All the tissue and urine samples were negative for this assay when collected from control pigs (Table 4).

4. Antibiotic concentrations in kidney

The antibiotic concentrations in the renal cortex were several times as high as those in the renal medulla, in spite of the presence of the aminoglycoside antibiotics administered (Table 5).

Discussion

The aminoglycoside antibiotics were shown to be more highly sensitive to *B. subtilis* than to any other test organism for the detection of antibiotic residues, especially at pH 8.0 than at pH 6.0 [3, 8, 11, 12, 18]. On the other hand, Ikekawa et al. [5] and Ito et al. [6] stated that the aminoglycoside antibiotics could be distinguished from one another by TLC. In the present study, TLC was applied to the identification of DSM, KM and FM in tissue and urine samples previously screened by using agar plates at different pH values inoculated with *M. luteus* and *B. subtilis*.

Table 3. Diameter of inhibition zones on *M. luteus* and *B. subtilis* plates, and Rf values in chromatography by urine samples

Animal No.	Inhibition zone (in mm)				R f	
	<i>M. luteus</i>		<i>B. subtilis</i>		Solvent	
	pH 6.0	pH 8.0	pH 6.0	pH 8.0	A	B
19	11.5	17.0	15.8	20.5	0.03	0.46
20	NZ	9.5	13.0	17.5	no	no
29	16.0	25.5	23.0	28.8	0.11	0.45
10	10.1	15.5	22.8	27.5	0.30	0.11
8	NZ	NZ	19.5	15.0	no	no
21	NZ	13.1	19.0	16.0	no	no
27	13.8	21.0	20.0	26.8	0.29	0.11
22	ND		ND		ND	
23	17.0	24.0	21.5	27.0	0.08	0.05
24	NZ	15.5	13.3	16.0	no	0.02

See the footnote of Table 2.

Rf values by reference antibiotic solutions in solvents A and B were 0 and 0.43 for DSM, 0.27 and 0.08 for KM, and 0.09 and 0.02 for FM.

Table 4. Qualitative assay of antibacterial activities in tissues and urine by using *B. subtilis* plates at pH 6.0 and 8.0

Animal No.	Kidney		Liver		Muscle		Urine	
	pH 6.0	pH 8.0	pH 6.0	pH 8.0	pH 6.0	pH 8.0	pH 6.0	pH 8.0
10	+	+	—	—	—	—	+	+
20	+	+	—	—	—	—	—	+
29	+	+	—	—	—	—	+	+
10	+	+	—	—	—	—	+	+
8	+	+	—	—	—	—	+	+
21	+	+	—	—	—	—	+	+
27	+	+	—	—	—	—	+	+
22	+	+	—	—	—	—	ND	
23	+	+	—	—	—	—	+	+
24	+	+	—	—	—	—	+	+

ND: Not done.

Table 5. Antibiotic concentrations ($\mu\text{g/g}$) in renal cortex and medulla

Dihydrostreptomycin			Kanamycin			Fradiomycin		
Animal No.	Cortex	Medulla	Animal No.	Cortex	Medulla	Animal No.	Cortex	Medulla
19	21.25	5.25	10	17.75	3.50	22	8.76	0.98
20	13.50	4.80	8	13.00	2.80	23	10.25	2.45
21	19.50	12.00	21	12.75	2.85	24	1.85	0
			27	50.00	14.50			

In the present study, DSM, KM and FM contained in kidney and urine samples were shown to be distinguishable from one another by TLC with two solvents. In each animal, antibiotic residues were confirmed in kidney and urine, but not in muscle, by qualitative assay. In general, it is possible to assume the presence of antibiotic residues in meat by examining such excretory organs as the kidney and liver of the same subject, because there is a close correlation in antibiotic concentrations among the organs and tissues in various regions. The aminoglycoside antibiotics, however, cannot be assumed to be present in meat even when detected in the kidney of the same subject, because they are known to be sequestered in the kidney long after detectable concentrations have disappeared from any other organ or tissue [2, 4, 10, 18]. The residues of the aminoglycoside antibiotics can be anticipated to be in meat only when confirmed by qualitative assay, and in the kidney of the same subject only when identified by TLC.

FM excreted in urine was identified by TLC. Therefore, it was suggested that this antibiotic might have been absorbed a little from the alimentary tract when administered by the oral route, as described by Huber [4] and Schollenberger and Sobczyk [16]. Schothorst et al. [19, 20] reported that FM was hardly distinguishable from SM when its concentration was too low. In the present study, however, these two antibiotics were shown to be discriminated from each other by TLC.

Schmidt and Cremmling [15] and Terplan et al. [17] stated that non-specific inhibitory substances were occasionally detectable in the kidney and urine from slaughtered animals and eliminated by passing through the dialysis membrane. The antibacterial activities found in the urine from two of the

four animals given KM might be due to such non-specific inhibitory substances.

In contrast to penicillins [21], the aminoglycoside antibiotics showed much higher concentrations in the renal cortex than in the renal medulla. This result was in accord with the finding of Rollins et al. [14]. It strongly suggests the possibility that identification by TLC will be enhanced in accuracy by the use of the renal cortex, if some aminoglycosides are distributed in the kidney from slaughtered animals.

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要 約

動物体内におけるアミノグリコシッド系抗生物質の鑑別法: 吉村治郎・伊藤 治・米沢昭一(農林水産省動物医薬品検査所)——ジヒドロストレプトマイシン, カナマイシンおよびフラジオマイシンを豚または牛に投与し, 殺処分後に採取した腎, 肝, 筋および尿に含まれるこれら抗生物質の鑑別を試みた。まず, 蒸留水を用いて, 各検体の希釈乳剤を作成し, *Micrococcus luteus* ATCC 9341 と *Bacillus subtilis* ATCC 6633 に対する阻止径を, pH 6.0 と 8.0 に調整した寒天培地を用いて測定した。この方法により, *B. Subtilis* に対し pH 8.0 の培地で最も大きな阻止径を示した腎と尿だけを, 薄層クロマトグラフィーによる鑑別に供した。すなわち, 組織乳剤および尿の限外濾液を作成し, 2枚のシリカゲルプレートの原点にスポットした。1枚のプレートはクロロホルム-メタノール-17%アンモニア(2:1:1, 展開液A)の上層で, 残り1枚のプレートは *n*-プロパノール-ピリジン-酢酸-水(15:10:3:12, 展開液B)で展開し, *B. Subtilis* を試験菌とする bioautography により Rf 値を求めた。いずれの抗生物質においても, それぞれの供試検体の Rf 値は, 常用標準品のそれよりもやや高くなる傾向があった。また, 展開液Aを用いた場合には, しばしばテーリングがみられた。しかし, 薄層クロマトグラフィーを2枚のシリカゲルプレートを用いて行えば, 屠畜の腎や尿に残留するアミノグリコシッド系抗生物質の鑑別も可能と思われた。