Thin-Layer Bioautographic Assay for the Detection of Salinomycin Sodium in Rabbit Tissues

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A thin-layer bioautographic method for the semiquantitative determination of salinomycin sodium residues in edible tissues of different animal species was developed. The present paper describes the details of the method and the results of an exemplary study with rabbit tissues. The assay organism for the bioautographic detection was *Bacillus stearothermophilus*. The application of an agar diffusion assay as a pretest using a 9 cm diameter Petri dish containing a section of thin-layer sheet prespotted with salinomycin standards ensured successful evaluations using the bioautographic method. Results of the investigation of spiked tissue samples indicated a recovery of 50–100%. Test sensitivity was about 10 ng of salinomycin sodium/g of tissue.

Salinomycin sodium is a new polyether antibiotic (Kinashi et al., 1973) with anticoccidial (Miyazaki et al., 1974) and growth-promoting (McClure et al., 1980) activity. Its chemistry and biological activity have been described (Miyazaki et al., 1974).

For the potency determination of salinomycin sodium in premixtures and animal feedstuffs, colorimetric vanillin methods and microbiological agar diffusion and turbidimetric assay methods are available. Thin-layer chromatographic methods with vanillin or bioautographic detection have been developed for the identification of the antibiotic (Heil et al., 1982).

For the detection of the related compounds monensin and lasalocid in animal tissues, thin-layer bioautographic techniques have been described (Donoho and Kline, 1967; MacDonald, 1978; MacDonald et al., 1979). These techniques have been recognized as simple, selective, and sensitive. The present paper describes a thin-layer bioautographic method for the semiquantitative detection of salinomycin sodium in rabbit tissues.

MATERIALS AND METHODS

The standard substance was salinomycin sodium reference standard no. 1, potency 970 μg/mg. Solvents were reagent grade. Thin-layer chromatographic plates were precoated plastic sheets, 20 × 20 cm, Polygram Sil G UV 254, obtainable from Macherey & Nagel, D-5160 Düren, West Germany.

For tissue blending, three homogenizers were used: (a) Moulinette, obtained from Moulinex Marketing, D-5000 Köln, West Germany; (b) Ultra Turrax type 18/10, obtained from Janke & Kunkel, D-7813 Staufen, West Germany; (c) Universal Laboratory Aid, type 309, obtained from Mechanika Precyzyjne, Poland.

The test organism was *Bacillus stearothermophilus var. calidolactis* C 953, obtained from Netherlands Institute of Dairy Research.

The stock culture medium consisted of 1 g of beef extract (Difco), 2 g of yeast extract (Difco), 5 g of peptone (Difco), 5 g of NaCl, and 15 g of agar dissolved in 1 L of deionized water. The pH after autoclaving was 7.4. The working culture medium was 10 g of yeast extract (Difco), 20 g of casein peptone (Difco), and 0.5 g of glucose dissolved in 1 L of deionized water. The pH after autoclaving was 8. The assay medium was 2.5 g of yeast extract (Difco), 5 g of casein peptone (Difco), 1 g of glucose, and 12 g of agar dissolved in 1 L of deionized water. The pH after autoclaving was 8. Tetrazolium chloride solution consisted of 1 g of triphenyltetrazolium chloride dissolved in 100 mL of deionized water.

Preparation of Standard Solutions. Solutions of salinomycin sodium reference standard were prepared in methanol with the following concentrations: 0.031, 0.0625, 0.125, 0.25, 0.5, and 1.0 μg/mL. these solutions were assayed on each bioautographic plate.

Preparation of Working Culture. Stock cultures of the test organisms were grown on slants with stock culture medium at 60 °C for 2 days. The working culture was prepared by inoculation of 200 mL of working culture medium with the stock culture and incubation under magnetic stirring at 60 °C for 24 h.

Demonstration of Optimal Growth Properties of the Inoculum Suspension. Before preparation of the bioautographic assay with the actual thin-layer sheets, a pretest was carried out to indicate that the test organisms were active enough to give large clear inhibition zones. A 5 × 5 cm section of a thin-layer sheet was fixed on the bottom of a 9 cm diameter plastic Petri dish.

On the sheet was spotted 0.01 mL each of the lowest standard concentrations representing 0.31, 0.625, 1.25, and 2.5 ng of salinomycin sodium/spot. The plate was air-dried. A stock of such prepared plates can be stored for some weeks. In order to perform the pretest, to 8-mL portions of assay medium were added 0.03 mL tetrazolium chloride solution and different quantities (3–4 mL) of the working culture. The mixtures were shaken and poured into pretest plates. After 1–2 h of incubation at 60 °C, inhibition zones with diameters of approximately 8–14 mm for the four standards demonstrate optimal growth conditions of the test bacteria and the required quantity of working culture for inoculation of the assay medium.

Extraction. Ten to twenty grams of tissue (muscle, liver, kidney, fat) was macerated with the Moulinette homogenizer. Ten grams of cutted tissue was blended with the Ultra Turrax or Mechanika Precyzyjna homogenizers. If necessary, a small quantity of water was added. A quantity of the homogenate equivalent to 5 g of tissue was extracted with 50 mL of acetone by shaking on a reciprocal shaker for about 15 min at room temperature. The entire sample was filtered, and the tissue and filter were rinsed with 20 mL of acetone. The entire acetone fraction was evaporated to dryness by rotary vacuum evaporation. The residue was dissolved in 25 mL of 80% ethanol and 20 mL of petroleum ether. In a separatory funnel the ethanol fraction was drawn off. The petroleum ether phase was again extracted with 25 mL of 80% ethanol. The combined ethanol fractions were evaporated to dryness. The

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Table I. Salinomyin Recovery Study: Bioautographic Assay Results of Rabbit Tissue Samples Spiked with Various Quantities of Salinomycin Sodium (Average of Three Spiked Samples)

spiked with salinomycin, ng/g of tissue	salinomycin content of spiked samples recovered, % of spiked quantity			
	liver	kidney	muscle	fat
10	50	50	80	70
20	65	50	75	100
50	63	52	63	50
100	59	59	56	90
200	100	80	75	75

residue was dissolved in 1 mL of methanol. This solution was used for the thin-layer bioautography.

Control Samples. Together with the experimental samples control tissue samples known to be free of salinomycin were processed through the whole procedure: one tissue sample as a blank and another tissue sample spiked with salinomycin standard solution (10 ng/g of tissue).

Thin-Layer Chromatography. A total of 0.01 mL each of sample solution, standard solution, and control solution was applied to thin-layer sheets. The sheets were developed in a solvent system of *n*-hexane–ether–methanol–acetic acid (70:30:4:0.5) within 2 cm of the top. The sheet was dried in the air for 10 min and then kept for 1 h in an ammonia atmosphere over ammonia solution. The remaining ammonia on the sheet was air-evaporated. To demonstrate optimal assay conditions prior to the bioautography, 2 drops containing 0.31 and 5 ng of salinomycin sodium standard were spotted on the upper range of the thin-layer sheet.

Bioautography. The thin-layer sheet was fixed on the bottom of a quadratic bioassay dish (Nunc). Assay medium (110 mL) was mixed with the required amount of working culture determined in the pretest and 0.3 mL of tetrazolium chloride solution and carefully poured into the dish over the thin-layer sheet. After the agar solidified the plate was incubated at 60 °C for 1–2 h until inhibition zones developed.

Evaluation. A semiquantitative interpretation was given by comparison of the zone diameters of the various standard concentrations and the sample solutions. For permanent recording, the finished bioautographic plate was photographed with a Polaroid camera.

RESULTS

When standard solutions were applied to the thin-layer sheets, salinomycin concentrations of 0.31 ng/spot and greater showed defined inhibition zones in all experiments. In separate assays standard concentrations of 0.08 and 0.15 ng/spot gave positive responses but not sharply limited inhibition zones. The zones were of symmetrical oval shape, and the zone size increased with increasing concentrations of the standards.

For determination of the recovery rates, different tissue samples have been spiked with 10, 20, 50, 100, and 200 ng of salinomycin sodium/g of tissue. Three spiked samples of each concentration were assayed. The recovery varied between 50 and 100% of the spiked amount (Table I). Spiking of 10 ng/g of tissue resulted in inhibition zones that could be evaluated semiquantitatively. Lower spiking concentrations gave positive responses. Therefore, the sensitivity of the method was considered to be about 10 ng of salinomycin sodium/g of tissue with a precision of about ±30%.

Results of a Routine Residue Study with Rabbits (Table II). Rabbits were fed with a feed containing 50 ppm of salinomycin sodium for 45 days. The animals were sacrificed at withdrawal times of 0, 1, 2, 3, and 4 days. Samples of liver, kidney, and leg muscle of four individual animals were assayed. The samples of cardiac muscle and abdominal fat of four animals were pooled before assay. At a withdrawal time of 0 days all four of the liver samples contained salinomycin in concentrations of approximately 10 ng/g. The pooled samples of abdominal fat contained less than 10 ng/g. In the samples of kidney, leg muscle, and cardiac muscle as well as in all tissue samples after withdrawal times of 1 and more days, no detectable levels of salinomycin sodium were found.

Table II. Salinomycin Residue Study: Bioautographic Results from Tissues of Rabbits Fed with Feed Containing 50 ppm of Salinomycin Sodium

withdrawal time, days	salinomycin content, ng/g				
	liver	kidney	leg muscle	cardiac muscle	fat
0	10	–[a]	–	–	<10
1	–	–	–	–	–
2	–	–	–	–	–
3	–	–	–	–	–
4	–	–	–	–	–

[a] (–) Not detectable.

DISCUSSION

As for monensin (Donoho and Kline, 1967) and lasalocid (MacDonald et al., 1979), the thin-layer bioautographic technique is very useful for the assay of salinomycin sodium in animal tissues. The introduction of a pretest before the actual bioautography indicates optimal growth conditions of the assay organisms that are essential for the successful bioautographic detection of the antibiotic. So after completion of most of the analytical work, the last step of the procedure, i.e., the bioautography, can be evaluated accurately.

The results dealing with the recovery and sensitivity of the described method have been confirmed by results obtained from the investigation of tissues of various animal species: turkey, broiler, cattle, sheep, pig, and rat (Heil et al., 1981). The results agree with those of Turnbull and Thompson (1977) obtained from the assay of chicken tissues.

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