Development of a Monoclonal-Based Enzyme-Linked Immunosorbent Assay for the Coccidiostat Salinomycin

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The polyether ionophoric coccidiostats are an important class of antimicrobials because of their extensive global use in modern agriculture. Salinomycin, the most widely used ionophoric anticoccidial in the U.S. broiler industry, has been marketed in all of the poultry-producing countries of the world. Established methods of analysis of salinomycin have disadvantages limiting their utility. We report here the development of 16 monoclonal anti-salinomycin antibodies. These monoclonal antibodies were used in competition enzyme-linked immunosorbent assays to differentially recognize salinomycin and the structurally similar narasin in the 10 ppb range. A preliminary immunoassay method has been developed, using one anti-salinomycin monoclonal antibody (SAL05), for quantization of salinomycin in poultry liver. The immunoassay gives a linear response with a good correlation between the spiked and observed values.

INTRODUCTION

Salinomycin (SAL) is a polyether, monocarboxylic acid antibiotic which possesses ionophoric properties (Pressman and Fahim, 1982). It is a major feed additive which is registered in the United States for use as a coccidiostat in broiler feeds (Shepard, 1992). Salinomycin is added to poultry feed either as the free acid or as the sodium salt at levels ranging from 44 to 66 ppm. Although this ionophore can be beneficial in broiler production when used properly, concerns have been raised about its toxic and sometimes lethal effects. Salinomycin toxicity can occur when it is present in feed not intended for a specific species or if it is fed at higher than recommended levels (AOAC, 1984). For these reasons, an accurate, precise, and rapid method to analyze SAL is required.

Analysis of SAL in feeds or tissues has utilized bioassays (AOAC, 1984), thin-layer chromatography (Vanderkop and MacNeil, 1990), and normal- or reversed-phase HPLC using postcolumn derivatization with vanillin (Blanchflower et al., 1985; Martinez and Shimoda, 1986; Lapointe and Cohen, 1988). The tissue bioassay is nonspecific and requires long incubation periods. The thin-layer chromatography and reversed-phase HPLC methods are labor intensive, have multistep sample cleanup, require expensive analytical equipment and technical help (HPLC), and use costly and environmentally negative extraction procedures.

Immunoassays are rapidly gaining acceptance as screening and quantitative methods for analysis of agricultural chemicals (Hammock et al., 1980; Greirson et al., 1991; Shelby et al., 1992). Many immunoassays have been developed to measure small organic molecules (haptens) which are not capable of stimulating the immune system to produce antibodies. The covalent conjugation of the hapten to an immunogenic carrier molecule, usually a protein, must be constructed so that the immunogen formed is capable of stimulating the immune system of an animal to produce specific antibodies that are capable of recognizing the unconjugated hapten. This recognition of unique sites on the hapten by the antibody is paramount to the specificity of the assay.

We describe the development of several anti-SAL monoclonal antibodies. These monoclonal antibodies (Mabs) were used to develop a competition enzyme-linked immunosorbent assay (c-ELISA) to detect low levels of SAL in buffers and in fortified liver samples.

MATERIALS AND METHODS

Chemicals and Supplies. The following chemicals and supplies were purchased: sodium salt of salinomycin (Calbiochem, La Jolla, CA; 563080); narasin (Elanco Products Co., Indianapolis, IN; 91059); lasalocid (Aldrich Chemical Co., Milwaukee, WI; 21,-111-7); monensin (Calbiochem, La Jolla, CA; 475896); HPTLC pre-coated plates (E. Merck, Darmstadt, Germany; 5715); Omnisolv grade *N,N*-dimethylformamide (DMF) and chloroform (EM Science, Gibbstown, NJ); methanol (Fischer, Fair Lawn, NJ); 1200–1400 mwco dialysis membrane tubing (Scientific Products, McGaw Park, IL; D1615-2); bovine serum albumin (BSA; A-7030), keyhole limpet hemocyanin (KLH; H-2133), Dowex 50-W cation-exchange resin (50x4-200), peroxidase-conjugated goat anti-mouse IgG (whole molecule) antiserum (A5278), peroxidase substrate 2,2′-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS; A1888), hydrogen peroxide (H1009) (Sigma Chemical Co., St. Louis, MO); *N*-hydroxysulfosuccinimide (Sulfo-NHS; 2451) and *N,N′*-dicyclohexylcarbodiimide (DCC; 20320) (Pierce Chemical Co., Rockford, IL), BALB/c mice (Harlan Sprague Dawley Inc., Indianapolis, IN), RIBI adjuvant (RIBI Immunochem Research Inc., Hamilton, MO); 96-well flat-bottom microtiter plates (Falcon 3072, Becton Dickinson and Co., Lincoln Park, NJ); food dehydrator (Excalibur, Sacramento, CA); isotype determinations (SBA Clonotyping, Fisher, Pittsburgh, PA); tissue homogenizer (Tekmar, Cincinnati, OH).

Hapten Synthesis. Hapten synthesis is outlined in Figure 1. Salinomycin sodium salt (0.66 mmol) was dissolved in 3 mL of DMF and eluted with DMF through a 10-cm HCl-equilibrated Dowex 50-W cation-exchange column which had previously been washed with 2 volumes of 1 N HCl, 3 volumes of double distilled water, and 2 volumes of DMF. The SAL was eluted with DMF (pH 9) in 14.2 mL and divided into two fractions (2.4 and 11.9 mL) for conjugation. Thin-layer chromatography of the eluate was developed on concentration zone silica half-plates with chloroform/methanol (95:5). The SAL band had an R_f = 0.97, which was equal to that of the acid form of SAL.

Salinomycin–KLH Conjugation. Sulfo-NHS (0.117 mmol), dissolved in DMF (pH 9), was added to 2.4 mL of the SAL–DMF eluate. To SAL–Sulfo-NHS was added 0.1183 mmol of DCC in 1 mL of DMF (pH 9), and the mixture was stirred at 23 °C for 12 h. Fifty milligrams of KLH in 10 mL of water (pH 9) was added dropwise to the SAL–Sulfo-NHS–DCC.

Salinomycin–BSA Conjugation. Sulfo-NHS (0.59 mmol), dissolved in DMF (pH 9), was added to 11.8 mL of the SAL–DMF eluate. To SAL–Sulfo-NHS was slowly added 0.5836 mmol

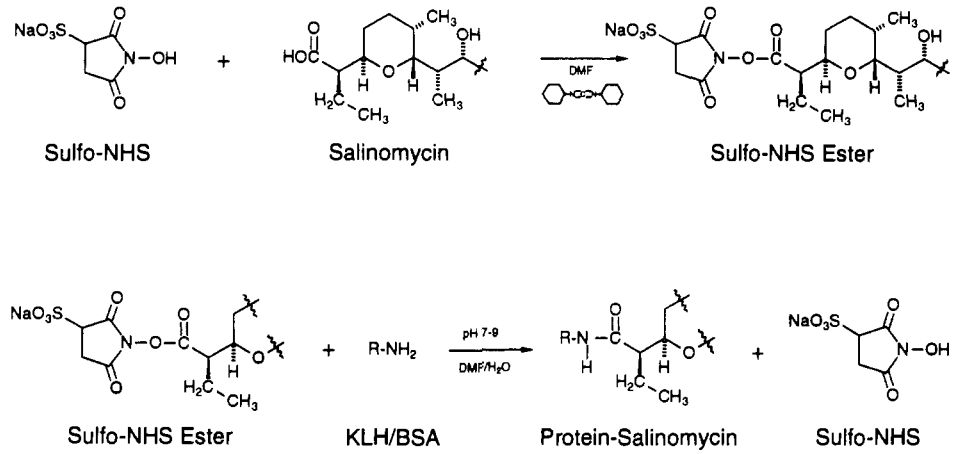

Figure 1. Synthetic pathway for production of the immunogen (SAL–KLH) or the bound plating antigen (SAL–BSA). Briefly, SAL and Sulfo-NHS in the presence of DCC were reacted in DMF (pH 9) to form the SAL–Sulfo-NHS ester. The ester was reacted with KLH or BSA to form the SAL–protein conjugate.

of DCC in 1 mL of DMF (pH 9), and the mixture was stirred at 23 °C for 12 h. Two hundred fifty milligrams of KLH in 10 mL of water (pH 9) was added dropwise to SAL–Sulfo-NHS–DCC.

Dialysis. Both SAL–KLH and SAL–BSA conjugates were dialized against DMF/water (80:20, pH 9) for 24 h. The concentration of DMF in the dialysis solution was decreased by 20% every 24 h until only water (pH 9) was present. The conjugates were separated into 0.5-mL aliquots and frozen at −70 °C until used.

Monoclonal Antibody Production. One-month-old BALB/c mice were injected intraperitoneally (ip) with 100 μg of SAL–KLH in 0.2 mL of isotonic saline with RIBI adjuvant. Mice received a single ip injection every other week for a total of three injections. Four days prior to fusion, the mouse was give an ip injection of 100 μg of SAL–KLH in 0.2 mL of saline with RIBI adjuvant. The spleen was removed, and splenocytes were fused with SP2/O myeloma cells and cultured under conditions described by Stanker et al. (1986).

A modification of the direct-binding ELISA (db-ELISA), described by Stanker et al. (1989), was used to screen tissue culture fluid from growing hybridomas for antibodies to SAL. Briefly, 96-well flat-bottom microtiter plates were coated with 100 μL of SAL–BSA (250 ng/well) in water and dried for 24 h at 37 °C in a food dehydrator. Nonreactive sites in each well on the microtiter plates were blocked for 1 h at 23 °C with 200 μL of a 3% solution of ovalbumin and incubated for 1 h at 37 °C with 100 μL of hybridoma supernatant containing the anti-SAL antibody. The plates were exhaustively washed with a solution of 0.05% Tween 20 in distilled water, and peroxidase-conjugated goat anti-mouse IgG (whole molecule) antiserum diluted 1/1000 in Tris-HCl assay buffer (pH 7.2) was added to each well. Following a second 1-h incubation at 37 °C, the microtiter plates were exhaustively washed again with Tween 20/water, and 100 μL of the peroxidase substrate (ABTS) with hydrogen peroxide in citrate buffer was added to each well. Absorbance measurements at 405 nm were taken after a 30-min incubation at room temperature, and the resulting data were analyzed.

Hybridomas from wells having a positive response in the c-ELISA screen were expanded and subcloned twice by limiting dilution to guarantee their monoclonal origin. Isotype determinations were done by ELISA using mouse heavy- and light-chain-specific antiserum.

Competition Enzyme-Linked Immunosorbent Assay. A c-ELISA was developed to quantify the amount of SAL in solution and to evaluate the ability of the antibody to distinguish between other ionophoric coccidiostats. The wells of a 96-well microtiter plate were coated with 250 ng of SAL–BSA and blocked with ovalbumin as previously described. Two hundred microliters of a competitor (20–100 ng/0.1 μL) in assay buffer was added to column 2 (rows A–H) of an antigen-coated well. The remaining wells in each column received 100 μL of assay buffer. A 100-μL aliquot of competitor was withdrawn from column 2 and serially diluted across the microtiter plate into wells of columns 3–10. Wells of columns 11 and 12 received no competitor. Next, 100 μL of a diluted monoclonal antibody that resulted in 50% of the plateau activity in a db-ELISA was added to each well of columns 2–12. Plates were incubated for 1 h at 37 °C and processed as described above. Percent inhibition was calculated by the following method: $(1 - B/B_0) \times 100$, where B is the OD of a well with a competitor and B_0 is the mean ODs of the wells without competitor (i.e., wells 11 and 12).

Extraction of Salinomycin from Liver. Liver was taken from 26, 21 day-old male broiler chicks (Hubbard × Hubbard) which had been fed *ad libitum* with feed containing no coccidiostats. One gram of sample from each liver was mixed with 10 mL of assay buffer and homogenized for 1 min. The resulting slurries were left as is or spiked with a SAL standard, homogenized again for 1 min, and refrigerated at 4 °C for 24 h. The homogenate was centrifuged at 1000*g* for 10 min at 4 °C. The liquid phase was recovered and used in a c-ELISA described above.

RESULTS

Hapten Synthesis and Hybridoma Production. Salinomycin sodium salt is an organic molecule with a molecular weight of 751. Because of its small size, it was conjugated to the carrier protein KLH to make it an immunogen. This SAL–KLH conjugate was used to immunize BALB/c mice.

Spleen cells from the mice immunized with SAL–KLH were fused with SP2/O myeloma cells, and the resulting hybridomas were cultured in 22 96-well microtiter plates. One or more growing hybridomas were observed in greater than 95% of the wells at 10 days. The supernatant from each of the 2112 wells was screened for antibodies against SAL in a db-ELISA using microtiter plates coated with 250 ng of SAL–BSA/well. Over 500 wells (approximately 25%) gave positive signals and were observed to have 1 or more hybridoma clones which were secreting antibodies that recognized some epitope on the SAL–BSA conjugate. The cells from those wells showing the strongest response (125 wells) were expanded and tested again in a c-ELISA for their ability to recognize unconjugated (free) SAL. Forty of the hybridomas produced antibodies that recognized unconjugated SAL. The most active of these hybridomas were subcloned twice by limiting dilution to guarantee their monoclonal origin. This resulted in 16 clones. These 16 monoclonal antibodies (Mabs) were named SAL02–SAL13 and SAL22–SAL25. Using an isotype-specific ELISA, all antibodies were determined to be IgG2α antibodies with kappa light chains. Culture fluid from the 16 Mabs was used in all subsequent experiments.

Assay Development and Antibody Characteristics. Direct binding ELISA experiments were performed on

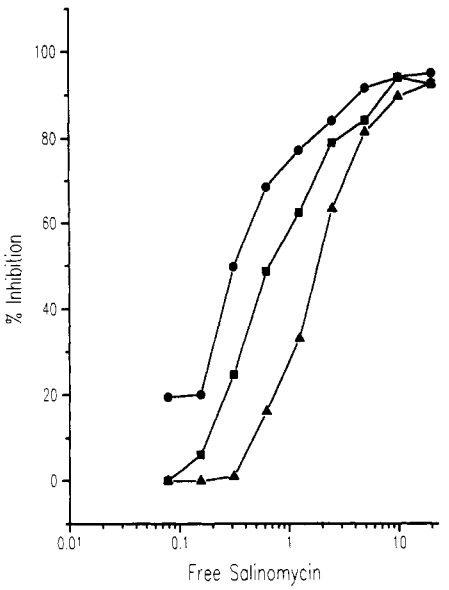

Figure 2. Competitive ELISA for three typical monoclonal antibodies using unconjugated (free) SAL as the competitor. Each point represents the mean of 10 assays. Of the 16 monoclonal found, SAL05 had the lowest IC_{50} (0.33 ± 0.10 ng/well) and SAL10 the highest (1.76 ± 0.29 ng/well). Nanograms per well can be converted to parts per billion (ppb) by multiplying the IC_{50} value by 10.

culture fluid from the 16 Mabs using SAL–BSA as the coating antigen (250 ng/well). The concentration of a monoclonal antibody that resulted in 50% of the plateau activity in the db-ELISA was used in all c-ELISAs.

Competitive ELISA results for four typical SAL monoclonal antibodies using free SAL as a competitor are shown in Figure 2. The concentrations of SAL that resulted in a 50% inhibition (IC_{50}) of the control activity (i.e., wells 11 and 12 with no competitor present) ranged from 0.33 (SAL05) to 1.76 ng (SAL10).

The antibodies were further characterized by their ability to recognize other commonly used coccidiostats that are ionophores (Figure 3). Representative inhibition curves for the monoclonal antibody SAL05, with the ionophores SAL, narasin (NAR), lasalocid, (LAS), and monensin (MON) as competitors, are shown in Figure 4. These data illustrate the relative ability of SAL05 to distinguish between these different ionophore coccidiostats. The IC_{50} values for 13 of the 16 monoclonal antibodies were not significantly different in their abilities to distinguish between SAL and NAR. Values from the c-ELISA for all 16 antibodies for the competitors SAL, NAR, LAS, and MON are summarized in Table I. None of the 16 monoclonal antibodies recognized either LAS or MON at or below 1000 ng/well. The means are based on 10 independent assays run on 10 different days during a 2-week period. The ability of the 16 monoclonal antibodies to detect SAL and NAR ranged from 0.33 to 1.76 ng/well (0.796 ± 0.374) for SAL and from 0.34 to 1.17 ng/well (0.779 ± 0.274) for NAR.

Immunoassay of Salinomycin in Liver. An immunoassay capable of detecting SAL in broiler chick liver was developed with SAL05. Liver samples spiked with 1.25, 2.5, and 5.0 ppm of SAL were determined to have 1.06, 2.49, and 3.97 ppm of SAL, respectively (Figure 5). The correlation beween the spiked and observed levels was 0.9838. The means for the observed levels were 87% of the spiked levels.

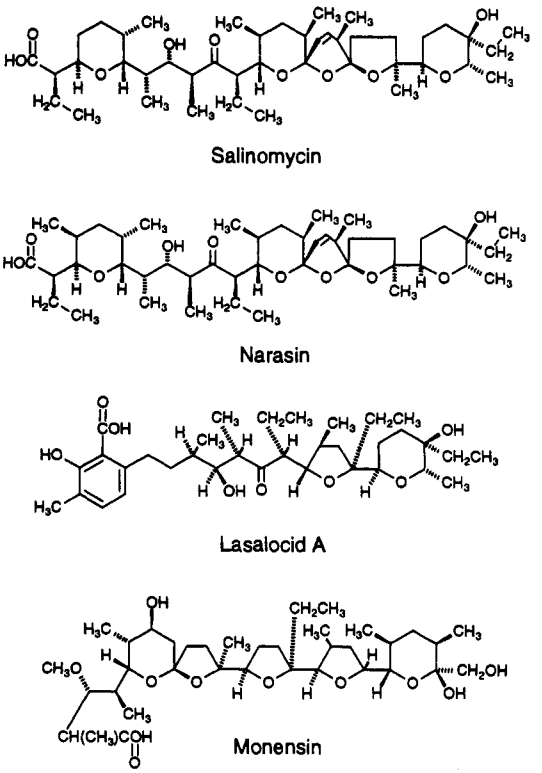

Figure 3. Structures of the four common ionophoric coccidiostats used in agriculture today. These four coccidiostats account for $73.5 of the $87.5 million (84%) spent in the poultry coccidiostat market in the United States in 1990. Salinomycin, narasin, lasalocid, and monensin were the competitors in c-ELISAs with the 16 anti-SAL monoclonal antibodies.

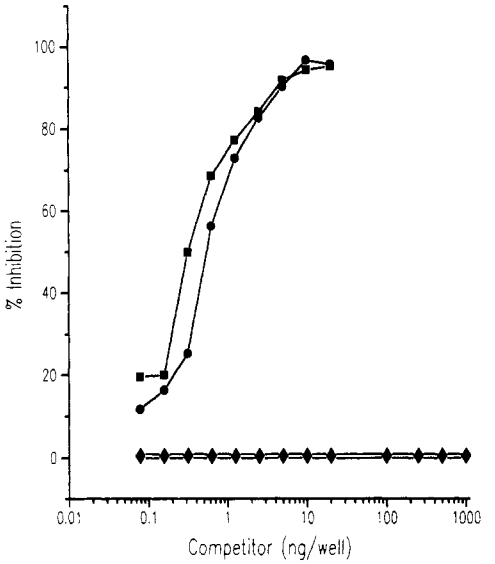

Figure 4. Typical c-ELISA showing the ability of SAL05 to react with salinomycin, narasin, lasalocid, or monensin. Each point represents the mean of 10 assays. The IC_{50}s for salinomycin and narasin were 0.33 ● 0.10 and 0.52 ± 0.62 ng/well, respectively. Lasalocid or Monensin was not recognized by SAL05 at or below 1000 ng/well.

DISCUSSION

The polyether ionophores are a major class of antimicrobials because of their extensive global use in agriculture. These compounds, which render cell membranes permeable to sodium and potassium ions, were found to have anticoccidial activity and improve feed utilization by ruminants (Pressman, 1976). Most of the commercial production depends on the large-scale fermentation of

Table I. Mean 50% Inhibition Values (IC_{50}) of Competitors for the 16 Anti-Salinomycin Monoclonal Antibodies[a]

antibody	competitors, ng/well	
	SAL[b]	NAR[c]
SAL02	0.62 ± 0.11[d]	0.97 ± 0.97[d]
SAL03	0.78 ± 0.72	0.82 ± 0.58
SAL04	0.35 ± 0.17	0.34 ± 0.15
SAL05	0.33 ± 0.10 b	0.52 ± 0.62 a
SAL06	0.78 ± 0.62	0.39 ± 0.15
SAL07	1.44 ± 1.28	0.93 ± 0.85
SAL08	0.60 ± 0.30 b	1.09 ± 0.15 a
SAL09	0.97 ± 0.28	1.17 ± 0.27
SAL10	1.76 ± 0.29 a	1.18 ± 0.49 b
SAL11	0.57 ± 0.25	0.69 ± 0.28
SAL12	0.49 ± 0.28	0.67 ± 0.19
SAL13	0.75 ± 0.49	0.55 ± 0.20
SAL22	0.56 ± 0.23	0.51 ± 0.09
SAL23	0.92 ± 0.15	0.99 ± 0.25
SAL24	1.15 ± 0.45	0.98 ± 0.46
SAL25	0.61 ± 0.18	0.68 ± 0.34

[a] For each monoclonal antibody, means followed by different letters are significantly different from each other at $p > 0.05$ (i.e., SAL05, SAL08, and SAL10). Neither lasalocid nor monensin inhibited the antigen–antibody binding at or below 1000 ng/well (10 ppm). [b] SAL, salinomycin. [c] NAR, narasin. [d] Mean ± SD, $n = 10$.

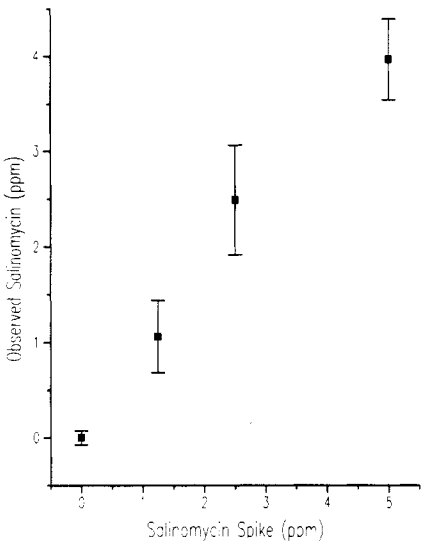

Figure 5. c-ELISA used to determine the level of salinomycin contamination in broiler liver samples spiked with 1.25, 2.50, and 5.00 ppm of salinomycin. Salinomycin levels were calculated from the competition curve as described in the text. The mean recovery of the observed levels was 87% of the spiked values. Each point represents the mean ± SD of 26 assays.

Streptomyces or *Actinomadura* rather than chemical manufacture (McDougald, 1990). The ionophores typically are coccidiocidal in action and have a narrow margin of safety when given to chickens or other animals.

Salinomycin, discovered in the early 1970s, has been marketed in all of the poultry-producing countries of the world (McDougald, 1990). This ionophore, the most widely used anticoccidial in agriculture in the United States, had sales accounting for $48.1 of the $87.5 million (55%) spent in the coccidiostat market for broilers in 1990 (SRI International, 1992). Salinomycin or its sodium salt is approved for use in broiler feed in the United States at levels of 44–66 ppm (Shepard, 1992). Because of its widespread use, and potential for misuse, convenient methods for monitoring of salinomycin in food and feeds are needed.

We have successfully produced a salinomycin-protein conjugate (SAL-KLH) and have used it to immunize BALB/c mice. Spleen cells from these mice were fused with SP2/O myeloma cells and produced hybridomas which secreted a variety of antibodies. Sixteen distinct hybridomas have been isolated that produce monoclonal antibodies which recognize free salinomycin. Using an isotype-specific ELISA, all antibodies isolated were determined to be IgG2α antibodies with kappa light chains. The abilities of these antibodies to detect free salinomycin in a c-ELISA ranged from 0.33 to 1.76 ng/well (3.3–17.6 ppb). Using the least and most sensitive antibodies, the detection limit for salinomycin was 28–150 times more sensitive than those previously reported (Miller et al., 1986). Three of the 16 antibodies produced significant differences in binding to salinomycin and the structurally similar ionophore narasin. Neither lasalocid or monensin influenced the c-ELISA, even at 1000 times the concentration of salinomycin or narasin needed to produced their IC_{50}. The data presented here, while preliminary, suggest that analysis of salinomycin by c-ELISA in broiler spiked liver homogenates will not involve extensive, costly, or environmentally negative extraction procedures. This ease of sample preparation in tandem with a sensitive c-ELISA for salinomycin will allow liver samples to be assayed quickly on a large-scale basis.

We have described the development of a preliminary immunoassay that can be useful as a rapid, inexpensive screening procedure and can be easily automated to test for salinomycin contamination in liver tissue following minimal sample preparation.

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