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Received for review November 25, 1985. Accepted May 5, 1986.

Determination of Sodium Salinomycin in Chicken Skin/Fat by High-Performance Liquid Chromatography Utilizing Column Switching and UV Detection

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A high-performance liquid chromatographic (HPLC) method has been developed for the quantitative determination of salinomycin in chicken skin/fat. For this procedure skin/fat homogenate (10 mL, equivalent to 2 g of tissue) was extracted with methanol. The extract was partitioned with carbon tetrachloride, applied to a silica gel column, and, in turn, run through a C_{18} column. The purified salinomycin solution was then oxidized with pyridinium dichromate and washed with sodium bicarbonate. The derivatized product was purified by running it through a silica gel column. The HPLC system utilized an automated on-line column-switching system with UV detection at 225 nm; the minimum limit of detection was 100 ppb. Peak height ratios of salinomycin to internal standard were used for quantitation.

INTRODUCTION

Sodium salinomycin (Figure 1) is a polyether antibiotic possessing anticocidial and growth-promoting activity (McClure et al., 1980). Its chemistry and biological activity have been described by Miyazaki et al. (1974).

Until now, a thin-layer, bioautographic technique (Heil et al., 1984) has generally been used for detecting salinomycin and similar compounds such as monensin (Donoho and Kline, 1967) in animal tissues. Although thin-layer bioautographic techniques are inherently selective and very sensitive, they lack precision and are time consuming. HPLC methods, on the other hand are generally precise, accurate, selective, sensitive, and amenable to automation and lend themselves to repetitive analyses. Goras and Lacourse (1984) and Blanchflower et al. (1985) have developed HPLC methods for salinomycin in feeds that utilize postcolumn reaction with vanillin and show potential for detecting low levels of salinomycin in tissue.

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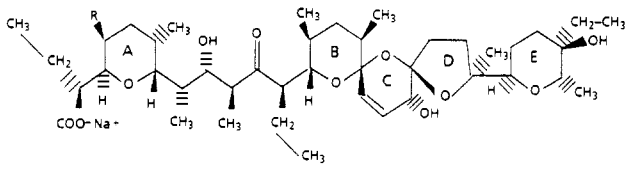

Figure 1. Structure of sodium salinomycin (R = H) and sodium narasin (R = CH_3).

To date, quantifying salinomycin in animal tissue has posed a twofold problem. The first problem concerns derivatizing the drug. Because salinomycin has no UV, fluorescent, or electrochemical properties, it must be derivatized either pre- or postcolumn for detection by HPLC. Derivatization of the carboxyl group is difficult because of the interference by the chelated sodium ion. Derivatization of the keto group is difficult because the group is sterically hindered. The allylic hydroxyl group on the C-ring can be derivatized by a number of reagents with UV adsorption; however, these reactions either degrade salinomycin or produce too many byproducts.

Recently Dohl and Greibrokk (1983) described an HPLC method for the UV detection of prostaglandins that were oxidized by pyridinium dichromate, a reagent that has the advantage of being soluble in aqueous solutions and hence can be removed from the reaction mixture. By use of this method the allylic hydroxyl group of salinomycin can be oxidized to form an α,β-unsaturated ketone that possesses strong UV absorption at 225 nm.

The second problem faced when trying to quantify salinomycin concerns the nature of complex biological matrices (such as animal tissues), which often present a problem for analysis by HPLC because of interference from endogenous tissue components. Automated, column-switching techniques (Cox and Pullen, 1984; Harvey and Sterns, 1984; Smets and Vanderwalle, 1984) have been used, however, for purifying samples on-column to improve resolution.

This paper describes an HPLC method for the quantitation of salinomycin in chicken skin/fat tissues that makes use of precolumn oxidation by pyridinium dichromate and an on-line, automated, column-switching technique.

MATERIALS AND METHODS

Apparatus for Extraction and Oxidation Procedure. A tissue homogenizer (Polytron, Model PT-35; Brinkmann Instruments, Inc., Westbury, NY) with a probe generator (Model PT20ST, Brinkmann) was used in this experiment. The following extraction columns (Bond-Elut, Analytichem International) were used: a 3-mL column containing 0.2 g of silica gel, a 3-mL column containing 0.5 g of silica gel, and a 3-mL column containing 0.2 g of C_{18}. A vacuum manifold (Vac-Elut, Analytichem International) was used to force solvent through the extraction columns. A Rotavapor-R (Brinkmann) and a 24-position nitrogen evaporator (Organomation, South Berlin, MA) were used for solvent evaporation.

Materials. Skin/fat for the standard curves was taken from chicken breasts purchased at a local grocery store. Abdominal skin/fat was taken from chickens dosed with salinomycin at the A. H. Robins Research Farm. The tissues were obtained and frozen as soon as possible after the chickens were killed.

The standard substance used in this work was sodium salinomycin with a potency of 926 μg/mg. The internal standard (IS) used was sodium narasin, obtained from Hoechst AG, Frankfurt, West Germany. All solvents were of either nanograde or HPLC grade and were purchased from Mallinckrodt, Paris, KY. Water was deionized and distilled in glass.

Preparation of Standard Solutions. Sodium salinomycin was prepared in methanol at the concentrations of 0, 2.0, 6.0, 10.0, 14.0, and 20.0 μg/mL. The internal standard was dissolved in methanol at a concentration of 20.0 μg/mL. The standard solutions were stored at 4 °C and were warmed to room temperature before being used.

Synthesis of Pyridinium Dichromate. Pyridinium dichromate was synthesized according to the method of Corey and Schmidt (1979). Chromium(VI) oxide (10 g, 100 mmol) was added to 10 mL of water that was being stirred in an acetone–ice bath. Pyridine (8 mL, 98.9 mmol) was slowly added. Forty milliliters of acetone was then added, which produced a crop of orange crystals. The mixture was allowed to sit in the ice bath, after which the crystals were filtered over a Buchner funnel and washed with acetone. The crystals were dried under vacuum oven overnight. This procedure produces pyridinium dichromate that is stable for several months if kept in an amber bottle at room temperature and is purer than that which is commercially available. Because of its purity cleaner chromatography is made possible.

Extraction and Oxidation. Skin/fat was cut into small pieces, weighed, homogenized in water (tissue/water, 20/80, w/v), frozen, and stored until analyzed. In preparation for analysis the homogenate was thawed in lukewarm water. While the homogenate was being stirred, 10-mL aliquots (equivalent to 2 g of tissue) were pipetted into 50-mL centrifuge tubes that had Teflon-lined screw-on caps. Duplicate aliquots were spiked with 100 μL of standard solutions. The resulting solutions contained salinomycin at concentrations of 0, 100, 300, 500, 700, or 1000 ppb and IS at a concentration of 1000 ppb. Homogenate samples containing unknown amounts of salinomycin were spiked with 100 μL of IS only.

The aliquots were extracted by being shaken with 40 mL of methanol on a reciprocal shaker for 15 min. The samples were then centrifuged at room temperature at 2000 rpm for 10 min. The resultant supernatant was partitioned with 30 mL of carbon tetrachloride two times in a 125-mL separating funnel. The carbon tetrachloride layers were collected in a round-bottom flask and concentrated to dryness on the rotovap. The carbon tetrachloride partitioning was done under a fume hood. The residue was reconstituted in 2.5 mL of methylene chloride. The resultant solution was then transferred onto a 0.5-g silica gel column that was attached to a Vac-Elut vacuum manifold. A water aspirator was used to create a vacuum. The flask was washed with 2.5 mL of methylene chloride, and the wash was added onto the silica column. The silica column was washed with an additional 7.5 mL of methylene chloride.

Salinomycin was eluted from the column with 6 mL of a solution of methylene chloride and methanol (9/1). The eluate was then evaporated under a stream of nitrogen. The resultant residue was reconstituted in 2 mL of water and 2–3 drops of concentrated ammonium hydroxide. The solution was added onto an activated 0.2-g C_{18} column. The column was washed first with 4 mL of water and then with 3 mL of a solution of methanol and water (50/50). Salinomycin was eluted from the column with 3 mL of methanol. The eluate was dried over anhydrous, powdered sodium sulfate.

The purified extract was evaporated to complete dryness under a stream of nitrogen, reconstituted in 1 mL of methylene chloride, and transferred to a culture tube (16 × 100 mm) that had a Teflon-lined, screw-on cap. The

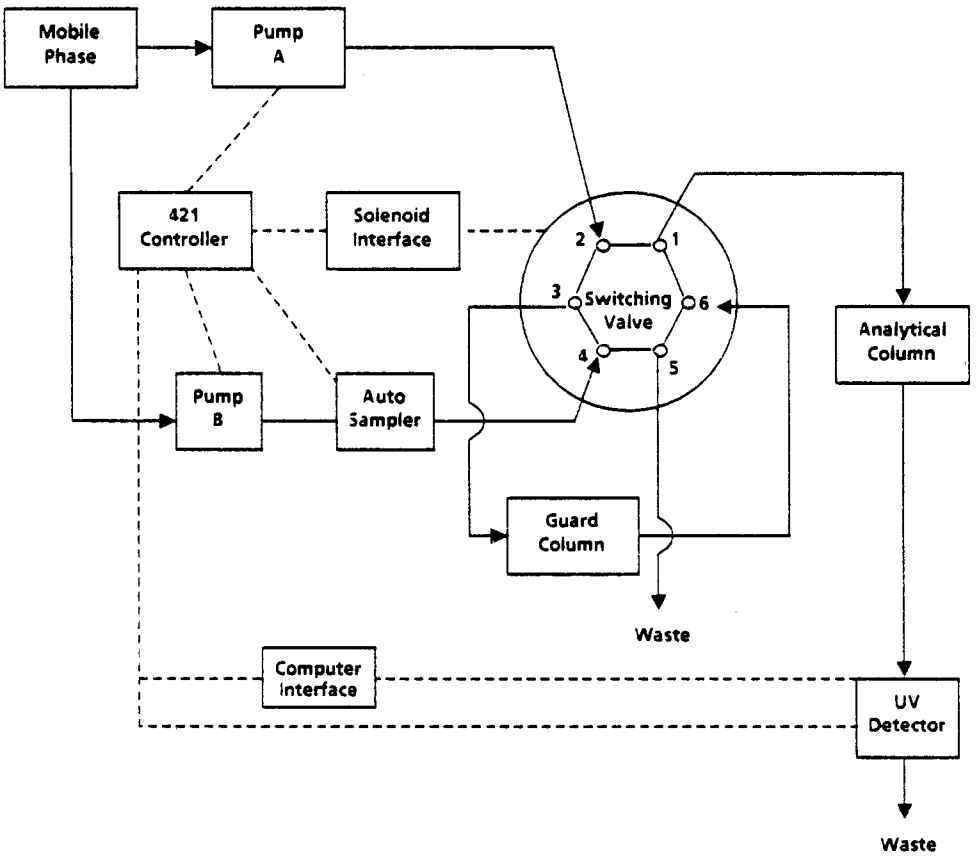

Figure 2. Schematic representation of the HPLC column switching apparatus. Solid lines represent tubing; dashed lines represent electrical connections.

tube contained 10 mg of pyridinium dichromate. Each tube was shaken for 30 min at room temperature. One milliliter of 5% sodium bicarbonate was added to each tube, and the solution was mixed on a vortex mixer. One milliliter of methylene chloride was then added, and the solution was mixed again on a vortex mixer. The aqueous layer of each mixture was removed, and the methylene chloride layer was washed an additional four times with 1-mL portions of 5% sodium bicarbonate and once with 1 mL of water. The methylene chloride was then dried over sodium sulfate and put onto a 0.2-g silica gel column. The column was washed with 12 mL of methylene chloride and eluted with 3 mL of a solution of methylene chloride and methanol (9/1). The wash was evaporated to dryness under a stream of nitrogen and reconstituted in 400 μL of acetonitrile.

HPLC Mobile Phase. The mobile phase was prepared fresh daily by adding 60 mL of water, 40 mL of tetrahydrofuran, and 0.1 mL of concentrated phosphoric acid to 900 mL of acetonitrile. The solution was mixed and then filtered through a 0.45-μm membrane filter. To compensate for intercolumn variation and column aging, the ratio of tetrahydrofuran to water can be modified to obtain optimum resolution.

Apparatus for HPLC. The HPLC apparatus consisted of a pump A (Beckman, Model 112), a pump B (Altex, Model 110A), an autosampler (WISP, Model 720B; Water Associates, Inc.) set to inject 50 μL with a run time of 15 min, a UV detector (Hitachi, Model 638-0412) set at 225 nm and 0.005 AUFS, a system controller (Altex, Model 421), a solenoid interface (Autochrom, Model 201), and a six-port switching valve (Autochrom, Model 401). The analytical column was an Altex Ultrasphere ODS column (25 cm × 4.6 mm, i.d.), and the guard column was a Brownlee MPLC C_{18} column (3 cm × 4.6 mm, i.d.). Packing materials in both columns were spherical particles, 5 μm in diameter. The flow rates through the analytical column and guard column were 2.0 and 1.3 mL/min, respectively.

A schematic representation of the HPLC is shown in Figure 2. The switching valve was pneumatically actuated, and its positions were controlled by the system controller. The system controller was interfaced to the switching valve by a solenoid interface. An electrical connection was made between the autosampler and the remote-input response flag on the system controller; thus, sample injection initiated a file in the system controller. Subsequently, the system controller started the computer interface to allow for data acquisition and automatically set the detector to zero.

The switching valve positions used during the analysis are shown in Figure 3. The times for controlling the switching valve were established by determining the retention times of oxidized salinomycin and narasin on the guard column. The time required to elute all of the endogenous peaks off the guard column was established, and the run was accordingly set on the autosampler.

Quantitation. The analogue output signal generated by the UV detector was sent by way of an interface (Mark III, analogue to digital converter) to a Computer Automated Laboratory System (CALS) on a Hewlett-Packard 1000 computer. An equation for the least-squares regression line was calculated by comparing the peak height ratio of salinomycin to internal standard with known concentrations of salinomycin. This equation was used to determine the concentration of salinomycin in unknown samples.

RESULTS AND DISCUSSION

Comparative Chromatograms. Figure 4 shows a chromatogram of a skin/fat sample, purified according to the method and containing 700 ppb salinomycin, that was

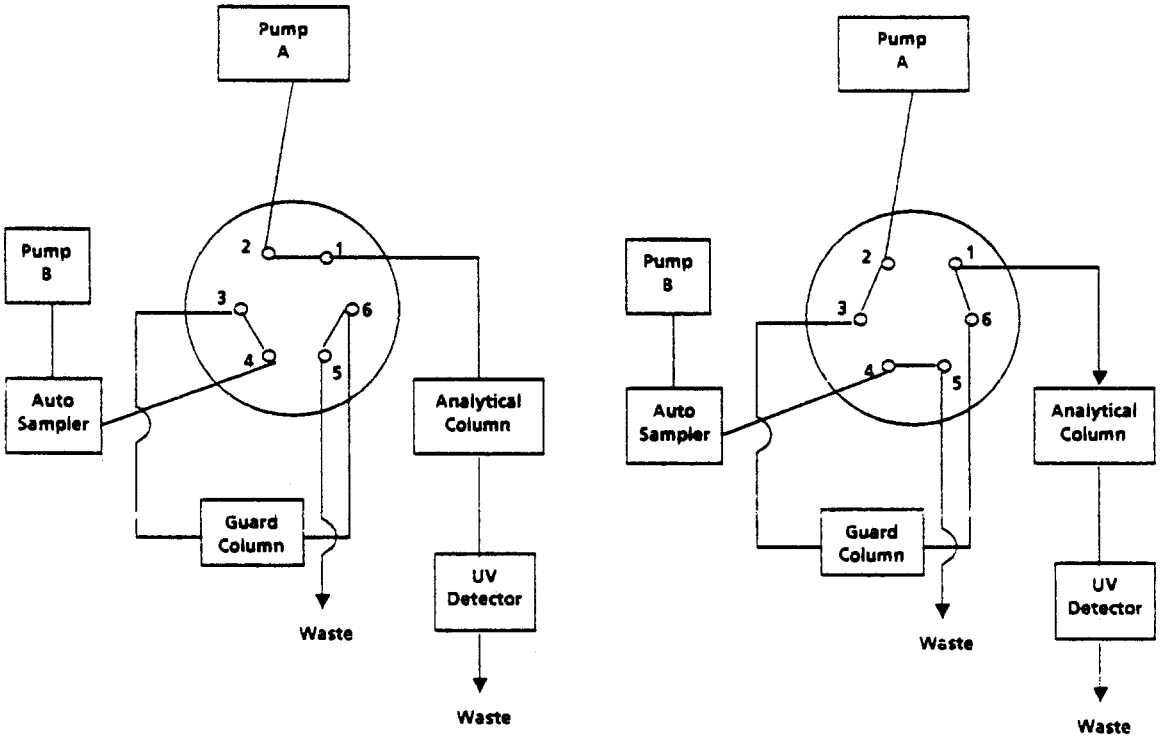

Figure 3. Schematic representation of the switching valve positions during analysis. Position A injects the sample onto the guard column that is vented to waste. Position B injects the guard column eluate onto the analytical column. System controller program times: position A, 0–0.8 min; position B, 0.8–2.2 min; return to position A at 2.2 min.

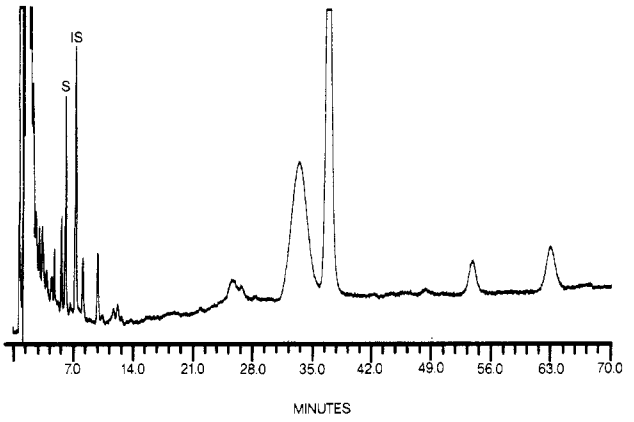

Figure 4. Chromatogram of a control skin/fat sample spiked with 700 ppb salinomycin and 1000 ppb IS injected on the analytical column only (S = salinomycin, IS = internal standard).

Table I. Calibration Curve Analysis of Sodium Salinomycin Concentration vs. Salinomycin/Internal Standard Peak Height Ratios

salinomycin, ppb	salinomycin/IS peak ht ratios		
	mean	SD	% CV
0	0.01	0.007	70.0
100	0.12	0.020	16.7
300	0.32	0.024	7.5
500	0.54	0.030	5.6
700	0.74	0.036	4.9
1000	1.05	0.079	7.5

injected directly onto the analytical column. This sample contained many endogenous components that showed UV absorption at 225 nm, which would, consequently, make quantitation of salinomycin difficult and the analysis time prohibitively long. This was to be expected because of the nature of the tissue and the wavelength used (225 nm is a relatively nonspecific wavelength for UV absorption). When a tissue sample was injected onto a guard column only, all of the endogenous components eluted off the column within 16.0 min (Figure 5). Salinomycin and the IS started to elute off the guard column at 1.2 min and were totally eluted off the guard column by 2.2 min. When the fraction of the eluate from the guard column containing salinomycin and IS was diverted to the analytical column, resolution was greatly enhanced and analysis time was significantly decreased (Figure 6). The retention times of salinomycin and IS were 8.0 and 9.7 min, respectively.

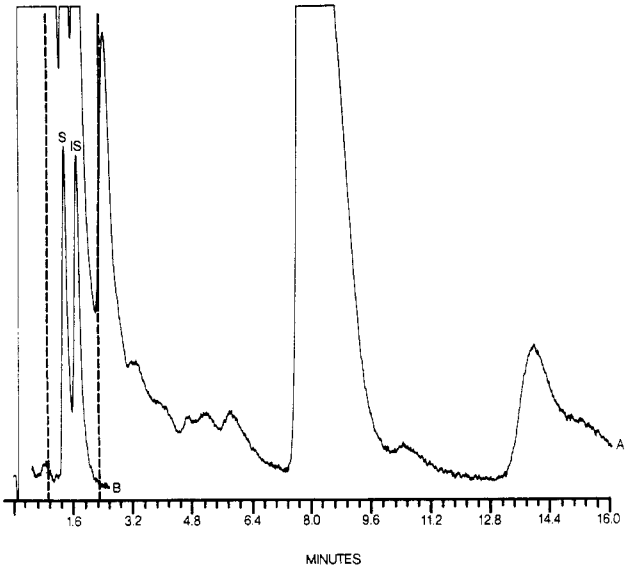

Figure 5. Multiple chromatograms of (A) a skin/fat sample spiked with 700 ppb salinomycin and 1000 ppb IS and (B) oxidized salinomycin and IS injected on the guard column only. Dotted lines represent the fraction that is subsequently injected onto the analytical column (S = salinomycin, IS = internal standard).

Table II. Concentration of Salinomycin in Skin/Fat Tissues of Chickens Fed Salinomycin, Roxarsone, and Lincomycin[a]

chicken no.	salinomycin, ppb	chicken no.	salinomycin, ppb
4069	BQL[b]	8076	149.5
4073	BQL	8064	155.3
8073	120.4	4074	291.9

[a] No salinomycin was found in the control samples. [b] BQL = below quantifiable limits (<100 ppb).

Standard Curve. The results of standards (run on 11 successive days) for salinomycin concentration vs. salinomycin/IS peak height ratios are shown in Table I. A linear response was obtained for concentrations of salinomycin of 100 to at least 1000 ppb. The mean slope, intercept, and correlation were 0.0010, 0.0102, and 0.9988, respectively; the percent coefficients of variation were 7.0, 125.5, and 0.2, respectively. The quantifiable limit of detection for salinomycin was 100 ppb. This limit could probably have been lowered because the peak height ratio at 100 ppb was 1 order of magnitude greater than that at 0 ppb. However, this limit of detection provided sufficient sensitively, and based on the results of a residue study conducted earlier at the Robin's facilities, the levels of salinomycin sodium detected are well below the finite tolerance level (680 ppb) for chicken skin/fat (Table II).

Accuracy and Precision of Method. The accuracy and precision of the method were determined by assaying aliquots of skin/fat homogenate that were spiked with various concentrations of salinomycin (Table III). The concentrations of salinomycin in the samples were unknown to the analyst at the time the samples were run. The concentrations of salinomycin found in the samples was nearly identical with the concentration that was added. The accuracy in predicting unknown concentrations of salinomycin and the low degree of variation of the slopes

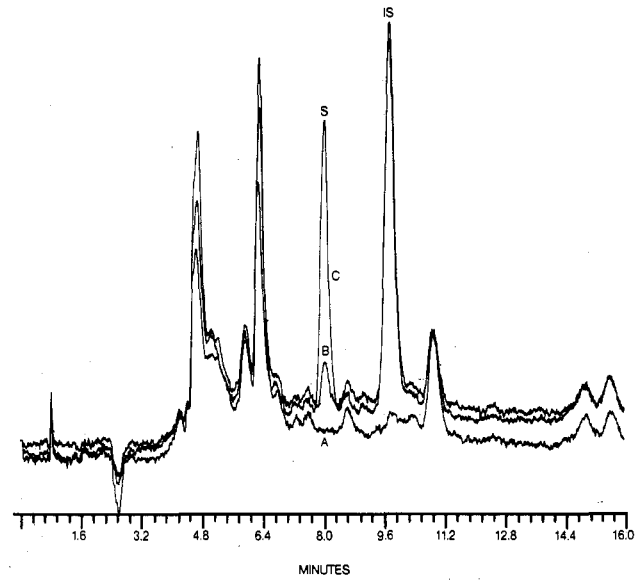

Figure 6. Multiple chromatograms of control skin/fat tissue spiked with (A) no drugs, (B) 100 ppb salinomycin and 1000 ppb IS, and (C) 700 ppb salinomycin and 1000 ppb IS (S = salinomycin, IS = internal standard).

of the standard curves demonstrate that the method has sufficient sensitivity to permit a differentiation of a violative sample from samples containing less than the tolerance level.

Interference. To test for possible interference of other drugs with the detection of salinomycin, control skin/fat homogenate was spiked with various combinations of approved drugs commonly used in chicken feed. Combinations of roxarsone (2000 ppb) and zinc bacitracin (500 ppb), roxarsone (2000 ppb) and lincomycin (200 ppb), roxarsone (2000 ppb) and oxytetracycline (1000 ppb), and roxarsone (2000 ppb) and virginiamycin (200 ppb) did not interfere

Table III. Determination of Unknown Amounts of Salinomycin Added to Skin/Fat Homogenate

	salinomycin found						
salinomycin added, ppb	no. 1	no. 2	mean	mean of set, ppb	SD of set	CV of set, %	% of salinomycin added
0	BQL[a]	BQL	BQL	BQL			
	BQL	BQL	BQL				
	BQL	BQL	BQL				
	BQL	BQL	BQL				
125[b]	100.8	100.8	100.8	118.3	17.50	14.8	94.6
	118.2	118.2	118.2				
	130.7	140.9	135.8				
275	277.8	267.8	272.8	278.6	8.66	3.1	101.3
	287.8	287.8	287.8				
	283.6	283.6	283.6				
	270.0	270.0	270.0				
450	481.8	430.3	456.1	459.4	14.78	3.2	102.1
	477.5	477.5	477.5				
	482.2	441.8	462.0				
	421.5	462.0	441.8				
600	656.8	595.0	625.9	605.5	11.53	1.9	100.9
	589.8	610.2	600.0				
	589.8	610.2	600.0				
	633.7	563.0	598.4				
	583.2	623.6	603.4				
750	749.5	780.3	764.9	763.3	39.05	5.1	101.8
	706.9	736.9	721.9				
	841.1	789.5	815.3				
	766.0	735.7	750.9				
925	916.5	896.5	906.5	930.4	19.92	2.1	100.6
	936.7	916.3	926.5				
	954.7	954.7	954.7				
	923.7	944.3	934.0				

[a] BQL = below quantifiable limit (<100 ppb). [b] Two samples at 125 ppb were not included because their duplicate values did not agree.

with the accurate detection of salinomycin (700 ppb). These drugs did not produce any peaks at the retention times of salinomycin or the IS. Monensin (700 ppb) and lasalocid (700 ppb) did not interfere with the detection of salinomycin (700 ppb) and did not show any peaks. In a similar experiment, radiolabeled metabolites of salinomycin were extracted and isolated by thin-layer chromatography from the livers of dogs dosed with radiolabeled salinomycin. The metabolites were oxidized and chromatographed under similar conditions reported here, and no metabolite eluted at the retention time of salinomycin.

ACKNOWLEDGMENT

I acknowledge Allen Miller for his editorial assistance in the preparation of the text of this article.

Registry No. Salinomycin, 53003-10-4.

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Received for review December 11, 1985. Accepted April 29, 1986.

Occurrence of the Mutagens 2-Amino-3,8-dimethylimidazo[4,5-*f*]quinoxaline (MeIQx) and 2-Amino-3,4,8-trimethylimidazo[4,5-*f*]quinoxaline (4,8-Me_2IQx) in Some Japanese Smoked, Dried Fish Products

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Mutagenicity of a variety of Japanese smoked foodstuffs such as smoked salmon, herring, trout, chicken, ham, sausage, and smoked, dried mackerel products (Sababushi) was measured with *Salmonella typhimurium* TA98 in the presence of the microsome S9 system. Smoked, dried mackerel products showed significant mutagenicity, whereas other smoked products showed little or no mutagenicity. The mutagens in a certain brand of smoked, dried mackerel were purified, and the major mutagenic substance was suggested to be 2-amino-3,8-dimethylimidazo[4,5-*f*]quinoxaline (MeIQx) and the minor one 2-amino-3,4,8-trimethylimidazo[4,5-*f*]quinoxaline (4,8-Me_2IQx). This major mutagen in the smoked, dried mackerel was estimated at 0.8 ng/g. The activity of the minor mutagen was about one-tenth that of the major mutagen. These mutagens may be formed from the constituents of the fish meat by the heat supplied during smoking and drying. It is unlikely that the smoke supplied during the process contributes to the formation of the mutagenicity.

Smoking is a common practice for processing meat and fish. Smoking supplies not only flavor but also materials that exert antibacterial action. It has been demonstrated that the smoking is the cause for the mutagenic benzo[*a*]pyrene in the products (Shiraishi et al., 1973; Engst and Fritz, 1977). Recently, we found two mutagens, 2-amino-3,8-dimethylimidazo[4,5-*f*]quinoxaline (MeIQx) and 2-amino-3,4,8-trimethylimidazo[4,5-*f*]quinoxaline (4,8-Me_2IQx), in the smoked, dried bonito (Kikugawa et al., 1985; Kikugawa et al., 1986). MeIQx is carcinogenic in mice (Sugimura et al., 1986). Since there are several different ways to carry out smoking of foods (see Table I), it is important to know which of these procedures can generate MeIQx. Here we report the results of our survey for the MeIQx-type mutagens in various smoked meat and fish products. The results indicate that not only the smoking but also the heating at around 100 °C are necessary factors for the generation of this type of mutagen.

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MATERIALS AND METHODS

Materials. Japanese smoked foodstuffs assessed for mutagenic potential were obtained at a local market in Tokyo. They are listed in Table I and classified into three groups according to the conditions for smoking. Benzo[*a*]pyrene was the product of Wako Pure Chemical Industries, Ltd., Osaka, Japan. Authentic specimens of 3-amino-1,4-dimethyl-5*H*-pyrido[4,3-*b*]indole (Trp-P-1), 3-amino-1-methyl-5*H*-pyrido[4,3-*b*]indole (Trp-P-2), 2-amino-6-methyldipyrido[1,2-*a*:3′,2′-*d*]imidazole (Glu-P-1), 2-aminodipyrido[1,2-*a*:3′,2′-*d*]imidazole (Glu-P-2), 2-amino-9*H*-pyrido[2,3-*b*]indole (AαC), 2-amino-3-methyl-9*H*-pyrido[2,3-*b*]indole (MeAαC), 2-amino-3-methylimidazo[4,5-*f*]quinoline (IQ), 2-amino-3,4-dimethylimidazo[4,5-*f*]quinoline (MeIQ), MeIQx, 4,8-Me_2IQx, and 2-amino-3,7,8-trimethylimidazo[4,5-*f*]quinoxaline (7,8-Me_2IQx) were generous gifts of Dr. S. Sato and Dr. M.

0021-8561/86/1434-0810$01.50/0 © 1986 American Chemical Society