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THE OVERVIEW OF FEED ADDITIVES AND VETERINARY DRUGS USED IN JAPAN AND THEIR RESIDUAL ANALYSIS IN LIVESTOCK PRODUCTS

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ABSTRACT: *The residue of drug in foods of animal origin has increasingly become of interest to the entire livestock industry as growing consumer health concerns. The current overview of feed additives and veterinary drugs used in Japan and their residual analysis has been reviewed. High performance liquid chromatographic technique(HPLC) with various detectors can be expected to be successfully applied for the routine analysis of residual feed additives and veterinary drugs including anabolic agents in livestock products.*

Key words: *feed additives, veterinary drugs, residual analysis, antibiotics, synthetic antibacterials, anabolic agents*

INTRODUCTION

Japanese expectancy of life are increased every year, so far both male and female has longest life span in the world as shown in Fig. 1. Several reasons for this increase might be considered and pointed out, among other things, the improvement of food habit might be highly contributed to the growing. Particularly young people, prefer Western style meal, which is mainly composed of meat, to representative Japanese style, live-stock industry has so grown prosperous. The breeding scale in the live-stock industry has been enlarged and become intensive year by year in order to reduce running expenses. As shown in Fig. 2, the future of broilers or livestock industry has taken the large scale breeding. In the case of chicken farm, no sunshine, just continous feeding without any excise are performed with conveyor system.

As shwon in Fig. 3, in proportion to the increase of production amount of livestock, the production of veterinary drugs such as antibiotics are also increased.

Although the Japanese are getting to take a meat than used to be, however, the consumption of fish in Japanese are still high compared with the other countries as shown in Fig. 4. The breeding style of fisheries in the countries has

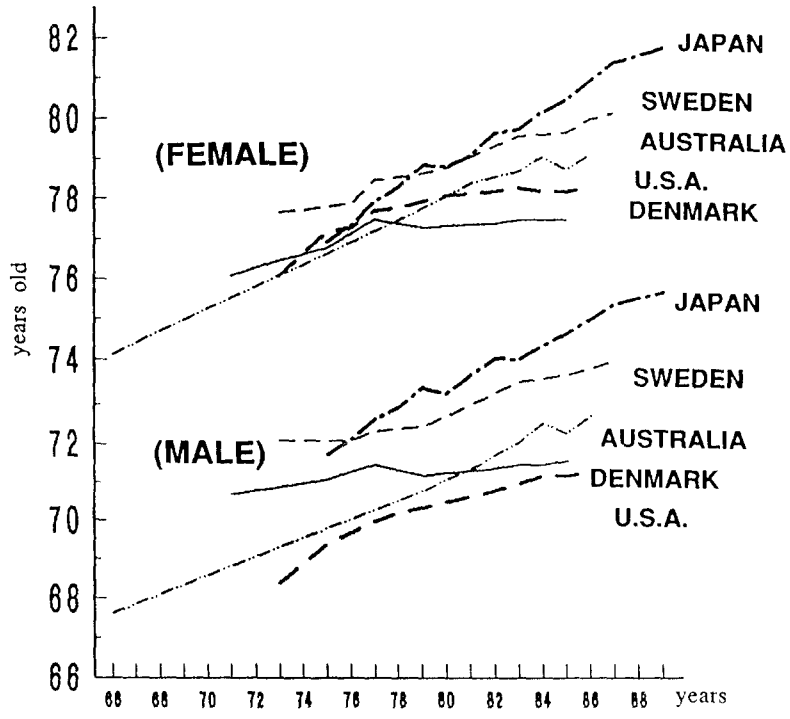


Figure 1. Expectancy of life.

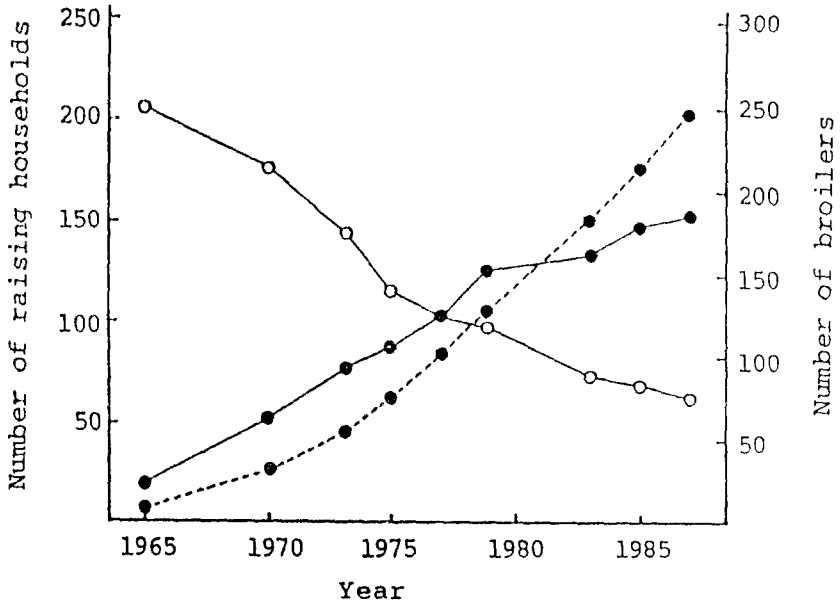


Figure 2. Comparison of annual population of broilers and numbers of raising households. ○–○, Number of raising households (×100); ●–●, Total number of broilers (×1,000,000); ●--●, Number of broilers per one farm (×100).

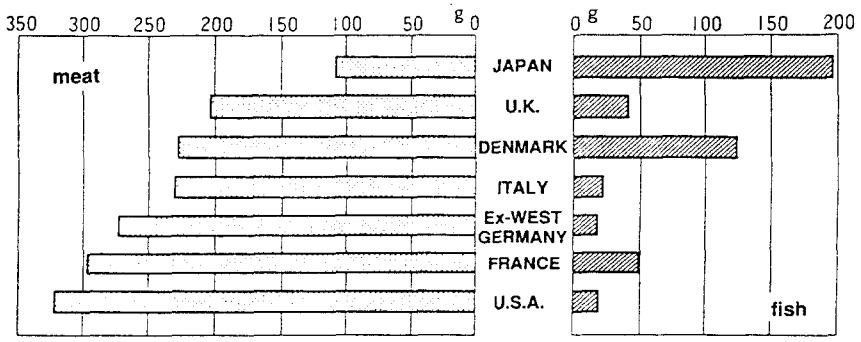


Figure 3. The amount of consumption of meat and fish of principal countries (g/day/person).

been also changed. The production of marine culture industry has increased high last 200 years.

In these industry for livestock and fisheries with a large population for breeding in small area, suffering disease causes a heavy economical loss. In order to decrease economically the cost of production, to improve the quality of product of livestock and fisheries and to raise the productivity, various compounds as feed additives, and to veterinary drugs are used as an effective means.

THE OUTLINE OF FEED ADDITIVES AND VETERINARY DRUGS

Feed additives used in Japan are divided into three categories owing to their purposes as shown in Table 1. First, antioxdants and antifungal agents have been used for prevention of quality deterioration of feed. The second group, which is 7 amino acids, 28 vitamins and 32 minerals, have been used as dietary supplements. As improvement of efficiency of food utilization as feed additives and nutritional ingredients, 9 synthetic antibacterials such as amprolium and sulfa drugs and also 20 antibitotics such as avoparcin, tetracycline etc. have been used. These substances are specified in the Law concerning Safety Assurance and Quality Improvement of Feed (Government Ordinance No. 68 of 1976).

Table 1. Feed additives used in Japan

Use	Classification	Designate feed additives
Prevention of quality deterioration	antioxidants (3)	BHA, BHT, ethoxyquin
	antifungal agents (3)	propionic acid and its Na and Ca salt etc.
	emulsifiers (4)	propyleneglycol etc.
Dietary supplements	amino acids (7)	DL-alanine, L-tryptophane etc.
	vitamins (28)	L-ascorbic acid, thiamine hydrochloride etc.
	minerals (32)	zinc sulfate, iron sulfate etc.
Promotion of effective utilization of nutritional ingredients in feed	synthetic antibacterials (9)	amprolium, ethopabate, sulfaquinoxaline etc.
	antibiotics (20)	avoparcin, tylosin, tetracycline etc.

*Safety assurance and quality improvement of feed.

Table 2. Synthetic antibacterials as veterinary drugs and/or feed additives

Synthetic antibacterials		Veterinary drugs[a]	Feed additives[b]
Sulfonamides	Sulfadiazine	*	
	Sulfadimethoxine	*	
	Sulfadimidine	*	
	Sulfadoxine	*	
	Sulfaisomidine	*	
	Sulfisozole	*	
	Sulfamethoxazole	*	
	Sulfamonomethoxine	*	
	Sulfaquinixaline	*	*
	Sulfathiazole	*	
	Sulfachlorpyridazine	*	
	Sulfamoyldapsone	*	
Furan derivatives	Difurazon	*	
	Furazolidone	*	
	Nitrofurazone	*	
	Nifurstyrenic acid	*	
Antiprotozoan agents	Amprolium		*
	Clopidol		*
	Decoquinate		*
	Ethopabate		*
	Nicarbazin		*
	Pyrimethamine	*	
Others	Carbadox	*	
	Morantel citrate		*
	Nalidixic acid	*	
	Olaquindox		*
	Ormetoprim	*	
	Oxolinic acid	*	
	Piromidic acid	*	
	Thiamphenicol	*	
	Trimethoprim	*	
	Calcium halofuginone polystyrenesulfonate		*

[a] Veterinary drugs : regulated by the Pharmaceutical Affairs Law

[b] Feed additives : regulated as feed additives in the law concerning Safety Assurance and Quality Improvement of Feed.

On the other hand, synthetic antibacterials and antibiotics are also used as veterinary drugs for the prevention and treatment of infectious diseases with relatively large dosage for short prescription period. The principal legal regulation related with feed additives is Law No. 145 established in 1960, so called the Pharmaceutical Affairs Law. In the article 83-2, it is defined that the veterinary drugs are intended for exclusive use with animals. Current regulated synthetic antibacterials as feed additives and veterinary drugs by the Pharmaceutical Affairs Law and the law concerning Safety Assurance and Quality Improvement of Feed in animal husbandry and aquaculture are summarized in the Table 2. An also summarized list of antibiotics as veterinary drugs and feed additives is given in

Table 3. Antibiotics as veterinary drugs and/or feed addivies

Antibiotics		Veterinary drugs[a]	Feed additives[b]
β-lactams	Amoxicillin	*	
	Ampicillin	*	
	Cloxacillin	*	
	Dicloxacillin	*	
	Mecillinam	*	
	Nafcillin	*	
	Penicillin G	*	
	Cepharonium	*	
Aminoglycosides	Apramycin	*	
	Destomycin A	*	*
	Dihydrostreptomycin	*	
	Fradiomycin	*	
	Gentamycin	*	
	Hygromycin B	*	*
	Kanamycin	*	*
	Kasugamycin	*	
	Spectinomycin	*	
	Streptomycin	*	
Tetracyclines	Chlortetracycline	*	*
	Doxycycline	*	
	Oxytetracycline	*	*
Macrolides	Carbomycin	*	
	Erythromycin	*	
	Josamycin	*	
	Kitsamycin	*	*
	Oleandmycin	*	*
	Sedecamycin	*	
	Spiramycin	*	*
	Tylosin	*	*
Polypeptides	Avoparcin		*
	Bacitracin	*	*
	Colistin	*	*
	Enramycin	*	*
	Mikamycin	*	
	Polymixin B	*	
	Thiopeptin	*	*
	Virginiamycin	*	*
Polysaccharides	Flavophospholipol	*	*
	Macarbomycin	*	
Polyethers	Lasalosid		*
	Monensin	*	*
	Salinomycin	*	*
Others	Bicozamycin	*	*
	Chloramphenicol	*	
	Fosfomycin	*	
	Lincomycin	*	
	Noshiheptide		*
	Nystatin	*	
	Novobiocin	*	
	Tiamulin	*	

[a] Veterinary drugs : regulated by the Pharmaceutical Affairs Law.

[b] Feed additives : regulated as feed additives in the law concerning Safety Assurance and Quality Improvement of Feed.

Table 4. Comparison of feed additives and veterinary drugs

	Feed additives	Vererinary drugs
Application	growth promotion	prevention and treatment of infectious diseases
Period	long (2-3 month)	short
Dose	trace	relatively large amount
Regulation	SAQIF*	Pharmaceutical Affairs Law
Synthetic Antibacterials	sulfaquinoxaline, clopidol, nicarbazin, olaquindox etc. (9)	sulfadimidine, carbadox, oxolinic acid etc. (25)
Antibiotics	chlortetracycline, lasalosid etc. (20)	ampicillin, kanamycin etc. (50)

*Safety Assurance and Quality Improvement of Feed.

Table 5. Application and dose of drugs used in the treatment of fish diseases

Fish	Disease	Active drugs	Application and dose	Day*
Yellowtail	Streptococcisis	Spiramycin	40 mg/kg of fish weight per day in feed	30
Red sea bream	Vibriosis	Oxytetracycline hydrochloride	50 mg/kg of fish weight per day in feed	3
Kuruma shrimp	Vibriosis	Oxytetracycline hydrochloride	50 mg/kg of fish weight per day in feed	25
Eel	Edwardsiellosis	Piromidic acid	10-20 mg/kg of fish weight per day in feed for 5-7 days	20
Rainbow trout	Vibriosis Furunculosis	Sulfa-monomethxine	150 mg/kg of fish weight per day in feed	30

*Period required by law for stopping treatments before harvesting for human consumption.

Table 3. Brief comparison of feed additives and veterinary drugs shown in Table 4 indicates that feed additives are used for a relatively long period at trace levels for the growth promotion. On the other hands, veterinary drugs are just shortly used for prevention and treatment of infectious diseases with relatively large dosage.

LEGAL RESTRICTIONS OF DRUG RESIDUE IN LIVESTOCK PRODUCTS

Table 5 indicates that one of the example of application and dose of veterinary drugs used in the treatment of fish disease. In the case of spiramycin, the drug is used as 4 mg/kg of fish weight per day in feed to treat streptococcisis for yellowtail. The period which is shown in Table 5 means how many days required by the Law for stopping treatments before harvesting for human consumption. In recent years, public concern over the presence of drug residues in meat products has rapidly grown in Japan.

To prevent the residues of veterinary drugs, the Law prescribes that animals should not be slaughtered shortly after the drugs are administered and while the

concentration of the drugs remains at therapeutically effective levels. However, the illegal use or overdosed use of these drugs is occasionally found. Another essential law is the Japanese Food Sanitation Law (Law No. 233) established in 1947, which describes standard and criteria of food.

RESIDUAL ANALYSIS OF FEED ADDITIVES AND VETERINARY DRUGS IN LIVESTOCK PRODUCTS

According to the Japanese Food Sanitation Law (Law No. 233), no food should contain antibiotics and in addition, meat, poultry eggs, fish and shellfish should not contain any synthetic antibacterial substances. Based on these legal regulations, it was required to analyze synthetic antibacterials and antibiotics in food used as feed additives or veterinary drug. For this purpose, official methods which provided by the Japanese Government are published as two volumes. In Vol. No. 1, biological assay using the disk method for residual antibiotics are described as shown in Table 6. The Scheme 1 just briefly tells how to prepare

Table 6. Microbiological assay for residual antibiotics in livestock products in Japan[a]

Test organisms	Antibiotics
○ Individual methods	
Bacillus stearothermophilus var. calidolactis C-953	Ampicillin, Cloxacillin, Lasalosid, Dicloxacillin, Salinomycin
Bacillus cereus var. mycoides ATCC 11778	Oxytetracycline, Chlortetracycline
Micrococcus flavus ATCC 10240	Bacitracin
Micrococcus luteus ATCC 9341	Tylosin, Spiramycin, Kitasamycin, Oleandmycin, Erythromycin, Penicillin G
Bacillus subtilis ATCC 6633	Kanamycin, Streptomycin, Enramycin, Quebemycin, Monensin
Bacillus cereus ATCC 19637	Flavophospholipol
Pseudomonas syringae×205	Hygromycin B
Corynebacterium xerosis NCTC 9755	Thiopeptin, Virginiamycin
Bordetella bronchiseptica ATCC 4617	Colistin
Bacillus brevis ATCC 8185	Macarbomycin, Destomycin A
Staphylococcus epidermidis ATCC 12228	Fradiomycin, Novobiocin
Escherichia coli NIHG	Chloramphenicol
Piricularia oryzae	Kasugamycin
○ Systematic methods	
Bacillus cereus var. mycoides ATCC 11778	Tetracyclines (Oxytetracycline, Chlortetracycline
Micrococcus luteus ATCC 9341	Macrolides (Tylosin, Spiramycin, Kitasamycin, Oleandmycin)
Bacillus subtilis ATCC 6633	Aminoglycosides (Streptomycin, Kanamycin, Fradiomycin, Destomycin A, Hygromycin B)

[a] *Official Analytical Methods for Residual Substances in Livestock Products*, Vol. 1, Veterinary Sanitation Division, Environmental Health Bureau, Ministry of Health and Welfare, Japan.

the sample solution. In the analytical procedure, antibiotics are extracted with 50% EtOH and clean-up with liquid-liquid partition and column chromatography. By using this procedure, fraction No. 1, 2 and 3 obtained, which are macrolides, tetracyclines and aminoglycosides, respectively. As the bioassay for detection of the presence of drugs, microbial inhibition tests organized by Veterinary Sanitation Division, the Ministry of Health and Welfare, use antibiotic-sensitive strains of bacteria as the test organisms shown in Table 6. Monitoring samples obtained at abattoirs or collected by food inspectors are analyzed by the laboratories of government authorities.

The purpose of a screening test is to give a quick result whether the analyte is either not present in the target sample or is below the level of concern. The development of analytical methods have made it possible to detect the residual drugs at trace levels. The detection limit of each drug in livestock products should be defined by the evaluation of safety on the toxicological aspects. So far, the levels of 50 ppb has been considered as provisional detection limit for analysis of most of drugs.

For the assay of residual synthetic antibacterials, various chemical techniques have been developed using spectrophotometric procedures, thin layer chromatography, enzyme immunoassay, gas chromatography(GC), HPLC and

Table 7. Chemical assay for residual synthetic antibacterials in livestock products in Japan[a]

Antibacterials	Extraction and/or deprotenization	Measurement	Detection limit (ppm)
Sulfonamides	Acetonitrile	ECD-GLC (5% OV-17)	0.01-0.05
Furazolidone	Ethyl acetate	ECD-GLC (5% EGSS-X)	0.03
Difurazon	Ethyl acetate	TLC-densitometry (420 nm)	0.1
Nifulpirinol	Acetone	UV-HPLC (Gel[b], 360 nm)	0.2-0.4
Nifurstyrenic acid	Methanol	TLC-densitometry (400/520 nm)	0.05
Pyrimethamine	Isobuthanol-benzene	ECD-GLC (1.5% OV-17)	0.05
Robenidine	Ethyl acetate	ECD-GLC (3% OV-17)	0.05
Dinitolumid	Acetonitrile	ECD-GLC (1.5% OV-17)	0.01
Amprolium	Trichloroacetic acid	TLC-densitometry (400/460 nm)	0.02
Decoquinate	Methanol-chloroform	Fluorimetry (270/380 nm)	0.1
Clopidol	Methanol	ECD-GLC (10% DC-200)	0.05-0.1
Nicarbazin	Acetonitrile	UV-HPLC (Gel[b], 340 nm)	0.03
Ethopabate	Acetonitrile	UV-HPLC (ODS, 270 nm)	0.02
Carbadox	Acetonitrile	UV-HPLC (ODS, 380 nm)	0.05
Olaquindox	Acetonitrile	UV-HPLC (ODS, 380 nm)	0.05
Thiamphenicol	Acetone	FPD-GLC (2% OV-17)	0.5
Ormetoprim	Ethyl acetate	UV-HPLC (ODS, 230 nm)	0.05
Trimethoprim	Ethyl acetate	UV-HPLC (ODS, 320 nm)	0.05
Morantel citrate	Dichloromethane	UV-HPLC (ODS, 320 nm)	0.05
Oxolinic acid	Dichloromethane	UV-HPLC (ODS, 254 nm)	0.05
Nalidixic acid	Dichloromethane	UV-HPLC (ODS, 254 nm)	0.05
Piromidic acid	Methanol-chloroform	UV-HPLC (ODS, 280 nm)	0.05

[a] *Official Analytical Methods for Residual Substances in Livestock Products*, Vol. 2, Veterinary Sanitation Division, Environmental Health Bureau, Ministry of Health and Welfare, Japan.
[b] Styrene-divinylbenzene copolymer.

mass spectrometry(MS). In order to measure the synthetic antibacterials, official method Vol. 2 has achieved by using TLC, GC and spectrophotometry as listed in Table 7. By the technique which are listed in Table 7, some of the compounds such as thiamphenicol and clopidol are not good enough sensitivity to measure the trace amount. So, recently, these techniques were replaced by HPLC methods (Nakazawa *et al.*, 1990a; Nakazawa *et al.*, 1992b, 1992c). Several organic solvents are used for extraction and deprotenization. These most methods can only measure each antibiotics individually, the government has proposed the multiresidue analytical method with photodiode array detector, so far.

As clearly realized in reviews (Nakazawa *et al.*, 1990a; Nakazawa *et al.*, 1992b, 1992c), HPLC method using a UV detector has recently been applied for the routine residual analysis. It is noted that GC techniques have been gradually replaceing HPLC ones for the analysis of residual drugs due to their ease of manipulation. However, when the peak of target substance appears on the HPLC chromatogram, the conventional HPLC method lacks qualitative informations. It is necessary to make sure the identification of observed peak for taking part of regulatory action from the standpoint of food hygiene. Although a few GC-MS methods have been developed for confirmation of target compounds, these are complicated and time consuming to prepare the suitable volatile derivatives for the large scale screening tests. For this purpose, the other instrumental analysis using photodiode-array detector (Horie *et al.*, 1990a) of mass spectrometry (Horie *et al.*, 1990b) are powerful ways to identify the compounds in comparison with authentic compound. Fig. 4 shows typical chromatogram of commercial pork samples in which sulfadimidine, widely used in the rearing of food-producing

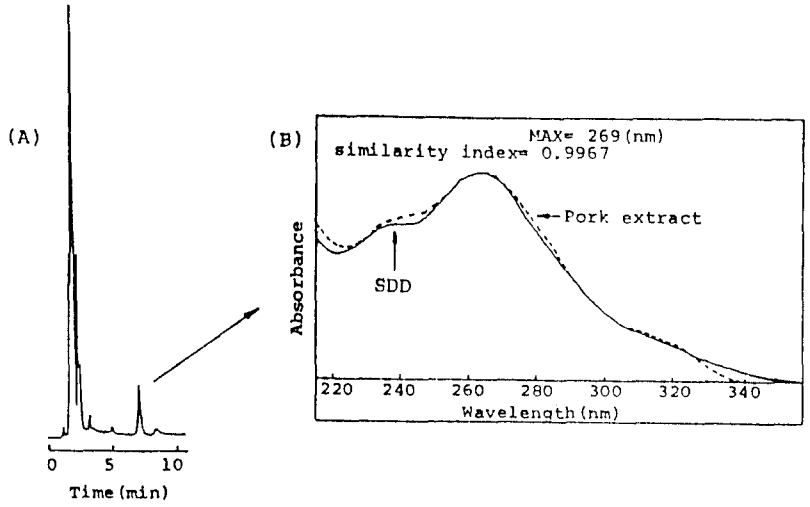
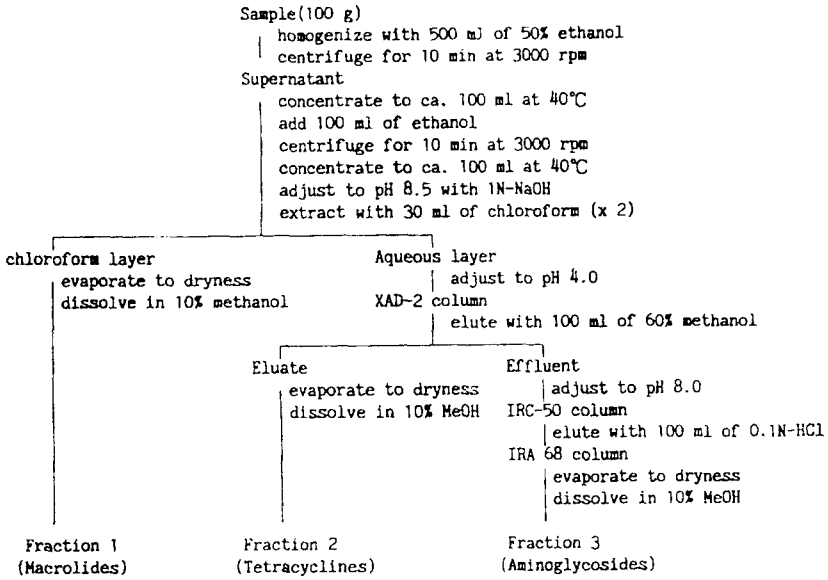


Figure 4. HPLC chromatogram obtained from pork sample.
(A) Chromatogram of pork sample in which sulfadimidine was detected at 0.1 μg/g, plotted at 275 nm.
(B) Normalized spectra of the peak (7.1 min) obtained from pork extract (dashed line) and standard sulfadimidine (solid line. LC conditions : column, TSK-gel ODS 80T_M (150×4.6 mm i.d.); mobile phase, 0.05 M sodium dihydrogenphosphate-acetonitrile (2 : 1); flow rate, 0.5 ml/min; detector, Shimadzu SPD-M6A.



Scheme 1. Analytical procedure for antibiotics in livestock products.

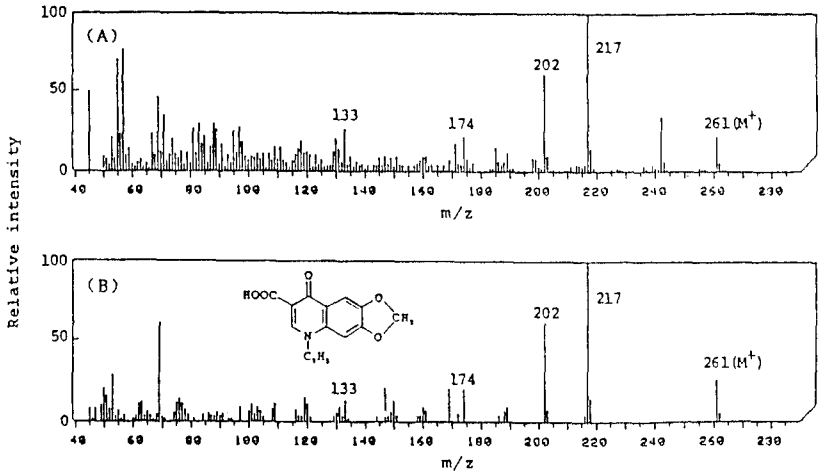
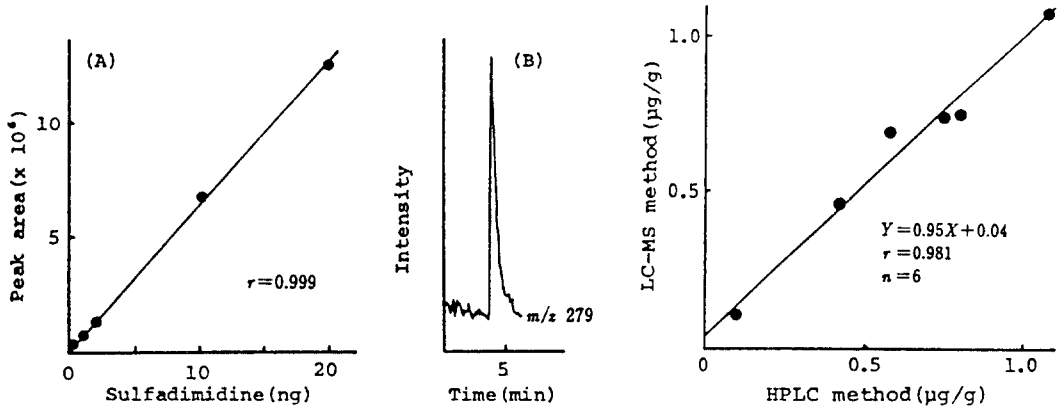


Figure 5. Mass spectra of (A) oxolinic acid isolated by HPLC from sweet fish, and (B) authentic drug.

animals to prevent and treat diseases and to promote their growth, was detected at 0.1 μg/g (Horie *et al.*, 1990c) using by photodiode-array detector. The peak component with a retention time of 7.1 min was compared with a standard sample of sulfadimidine. The high similarity index representing the similarity of the two spectra, indicates that the two spectra were almost identical, confirming the peak as sulfadimidine. The other confirmation approach of target compound found in sample is combination with mass spectrometry. Fig. 5 demonstrates the mass spectra of standard oxolinic acid ($C_{13}H_{21}NO_5$, mw 261), which is widely used to fish to treat variety of gram-negative organisms, and isolated sample from sweet



(A) Calibration Curve for Sulfadimidine and (B) SIM Chromatogram of 200 pg Sulfadimidine

Correlation between HPLC and Thermospray LC-MS Methods for Sulfadimidine in Pork

Figure 6. Thermospray LC-MS methods for determination of sulfadimidine in pork.

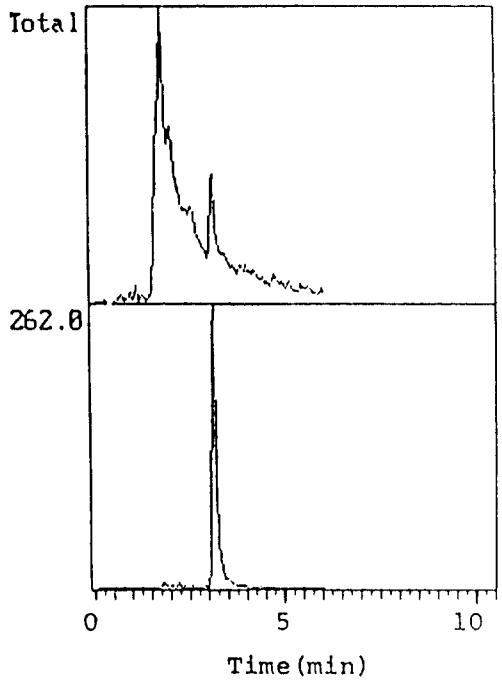


Figure 7. Thermospray LC-MS total ion chromatogram and mass chromatogram of sweet fish sample containing 1.50 μg/g incurred oxolinic acid residue. Conditions : column, Intetsil ODS-2 (150 ×4.6 mm i.d.; mobile phase, 0.05 M ammonium acetate (pH 4.5)-acetonitrile (7 : 3); flow rate, 0.8 ml/min; column temperature, 35°C; vapour temperature, 165°C; ion source temperature, 270°C.

fish. After the isolation of the oxolinic acid fraction from HPLC separation, the eluated was offered to the MS analysis. The retention of the sample peaks coincides with those of the standard oxolinic acid and with the mass spectrum

of oxolinic acid; a molecular ion peak at m/z 261 and a parent peak at m/z 217. Based on these results, the compound found in sweet fish was identified as oxolinic acid. By application of the method to the analysis of 115 commercial fishes including yellowtail, eel, common carp, rainbow trout and sweet fish, oxolinic acid was found in 24 samples of sweet fish at levels ranging from 0.01 to 1.90 μg/g (Horie *et al.*, 1987). Furthermore, a thermospray HPLC-MS method has been developed for the analysis of 12 sulfonamides including sulfadimidine and sulfadimethoxine (Horie *et al.*, 1990b). LC separations were carried out on C_{18} column by using 0.1 M ammonium acetate buffer and acetonitrile mixture as the mobile phase. The intensity of the protonated molecular MH^+ ions strongly depended on vaporizer temperatures. The mass spectra obtained from sulfonamides were very simple with base peaks corresponding to protonated molecular ions MH^+. An excellent correlation between the results of the thermospray HPLC-MS method and HPLC method was obtained ($r=.999$). Recently, by this thermospray HPLC-MS, oxolinic acid in sweet fish was successfully confirmed with total ion cromatogram and mass chromatogram as shown in Fig. 7 (unpublished data).

ANABOLIC AGENTS FOR GROWTH PROMOTION

In addition to the foregoing antibiotics and synthetic antibacterials, naturally occurring hormones such as progesterone and estradiol have been used for growth promotion and feed efficiency in heifers as anabolic agents. A vast assay of chemical techniques including radioimmunoassay, GC, GC-MS, and so on, have been developed for the determination of trace levels present (Nakazawa, 1989). Because of the trace amount of existing hormones, the tedious and troublesome sample preparation steps for extraction and cleanup are required for the analysis. With the aid of the advantage of chromatography over separation ability, HPLC techniques also have been applied (Miyazaki *et al.*, 1989; Watabe *et al.*, 1989). However, the actual levels present are usually very low, besides showing wide variation, depending on the physical conditions of animals. Since these compounds are naturally present, all tissues and biological samples have these hormones as a constituent. So far, it is really hard to estimate the residual amount of anabolic agents, which is administered.

CONCLUSION

According to the Japanese Food Sanitation Law, any food should not contain antibitotics and synthetic antibacterial substances. In order to monitor the drug residue levels in livestock products, a simple and reliable methods are required. In addition to the efforts of development of improved residual analysis, evaluation on residues of veterinary drugs should include the parent compounds and/or their metabolites in any edible portion of the animal product. The development of new veterinary drugs must be also evaluated based on their efficacy, safety to the intended animal species and safety to humans consuming products of animal origin.

Moreover, although residues of animal drugs in livestocks do not appear to be a problem, it should be required to survey the verious products by appropriate method in respect with food hygiene.

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