Detection and Quantitation of Residual Antibiotics and Antibacterial Agents in Foods

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ABSTRACT—To detect and quantitate residual antibiotics and antibacterial agents in meats, we performed a biological assay employing the three microorganisms Bacillus subtilis ATCC 6633, Micrococcus luteus ATCC 9341, and Bacillus cereus var. mycoides ATCC 11778 for the screening purpose and developed a Gas Chromatography-Mass Spectrometry (GC/MS) analysis for the confirmation and quantitation.

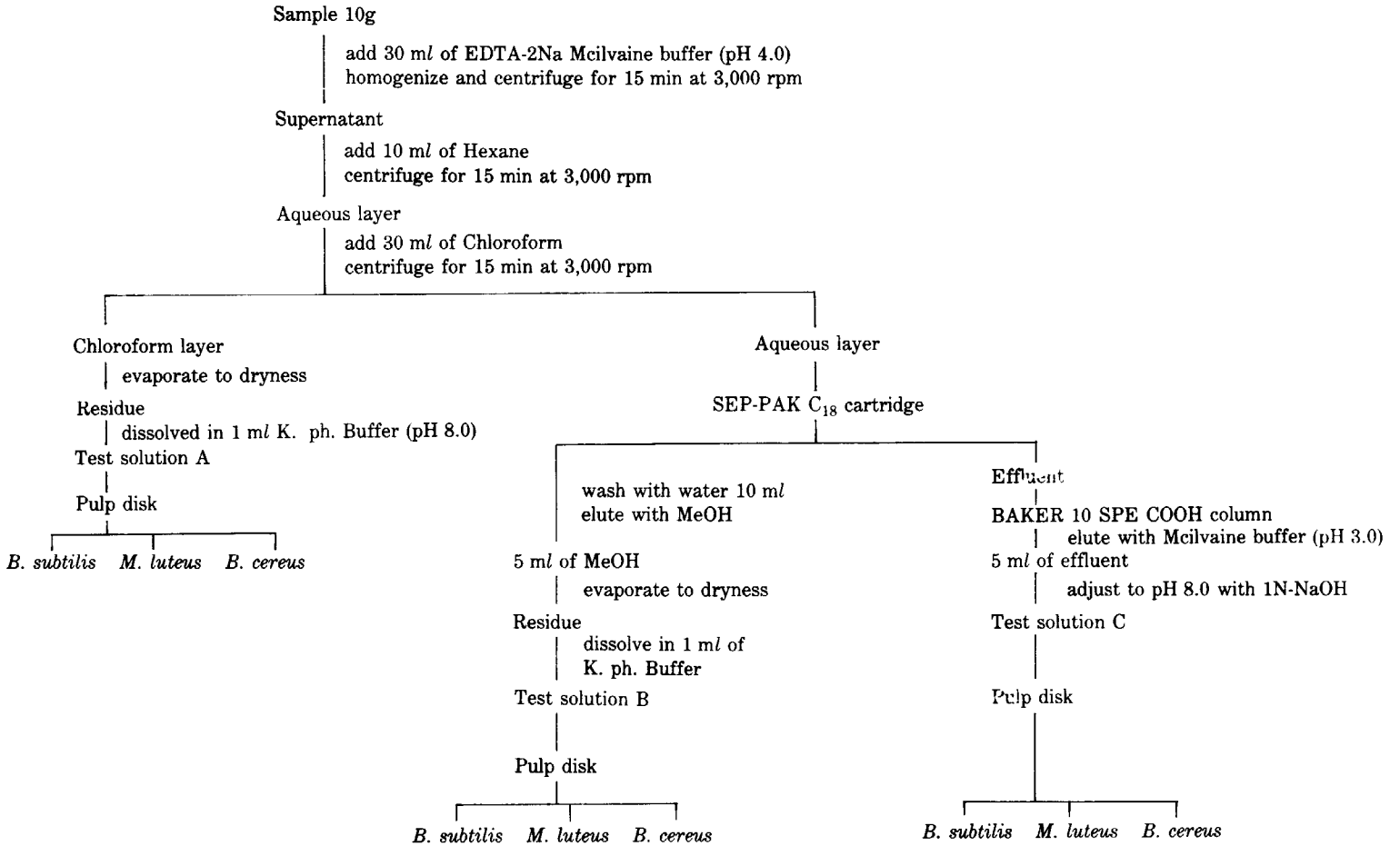
In the biological assay (paper disk method), three test solutions are used depending on the character of the residual antibiotics and antibacterial agents, follow by a simple clean up procedure which includes homogenization with Mcilvaine buffer, defatting with includes homogenization with Mcilvaine buffer, defatting with hexane, extraction with chloroform, and clean-up by Sep-Pak C_{18} and Bakerbond SPE carboxylic acid column. The chloroform layer is used for the analysis of sulfa agents, macrolides antibiotics and antibacterial agents. Adsorbed materials in the Sep-pak C_{18} were also employed for the analysis of penicillins and tetracyclines. Effluents from the Sep-Pak C_{18} were cleaned-up once more by Bakerbond 10 SPE COOH column and employed for the analysis of aminoglycosides.

In the instrumental analysis by using the GC/MSD, residual antibiotics and antibacterial agents were quantitated by selected ion monitoring (SIM) mode after derivatization. A simultaneous analysis of six residual antibiotics and antibacterial agents such as oxytetracycline, penicillin, ampicillin, chloramphenicol and thiamphenicol was developed with simple clean-up procedures revealing good recovery and reproducibility. Also, simultaneous detection of macrolides antibiotics such as erythromycin, spiramycin, and oleandomycin was developed after acid hydrolysis due to their large molecular structures.

Because of the high reproducibility and selectivity of these two methods, it is very desirable that the combination of the two methods be used in the bioassay for the screening of residual antibiotics and antibacterial agents and that GC/MSD analysis be used for the confirmation and quantitation.

Antibiotic and antibacterial drugs are extensively used in modern medicine. Also, an especially large number of these drugs have been employed for chemotherapeutical use or as food additives in the veterinary field and fish breeding because of their broad antimicrobial spectrum against both Gram-positive and Gram-negative pathogens. The extensive use of antibiotics and antibacterial agents for theapy of livestock and fish diseases may lead to problems with residues in medicated animals and in the environment. Thus adequate methods for detection and quantitation methods of these drugs are needed.

The conventional microbiological procedure provides a good sensivity, however it cannot identify the antibiotics. Although numerous analytical methods of antibiotics such as tetracyclines[1-4], penicillin[5], ampicillin[6-7], neomycin[8], streptomycin[9], novobiocin[10], oleandomycin[11], salinomycin and monensin[12-14], spiramycin[15], tylosin[16, 17], bacitracin[18, 19], chloramphenicol[20-22], erythromycin[23], and extensive review[24, 25] have been reported, simultaneous analysis by using the GC/MSD is very rare because of their differences



Scheme 1. Clean-up procedure for the bio-assay of antibiotics and antibacterial agents in meats.

in chemical and physical characters. Thus we will introduce a biological assay for the simultaneous screening of many antibiotics and antibacterial agents in meats. Also, simultaneous quantitation and confirmation of several antibiotics developed by using GC/MSD in our laboratory will be presented.

MATERIALS AND METHODS

A. Biological assay

1. Materials: Bacillus subtilis ATCC 6633 (B. sub.), Micrococcus luteus ATCC 9341 (M. lut.), Bacillus cereus var. mycoides ATCC 11778 (B. cer.) were purchased from Institute for Fermentation, Osaka, Japan. Antibiotic medium 5 for B. sub. and M. lut., and antiboitic medium 8 for B. cer. were purchased from Difco Lab., Michigan, U.S.A. Sensitivity test broth (code 05534) and sensitivity disk Agar-N (code 05530) were purchased from Nissui carboxylic acid column were also purchased from Waters Associates, Massachusetts, U.S.A. and J.T. Baker Inc., New Jersey, U.S.A., respectively. Other reagents and solvents were used as G.R. Grade.

2. Methods: The clean up procedure is summarized in Scheme 1. As you can see, three test solutions were prepared from meat samples. 10g of meat was homogenized with 30 m*l* of 0.01 M EDTA -2Na Mcilvaine buffer (pH 4.0), and centrifuged at 3,000 rpm for 15 min. The supernatant was deffated with 10 m*l* of hexane and extracted with chloroform. After centrifugation (3000 rpm, 15 min.), chloroform layer was filtered with Whatman 1PS phase separator (silicone treated, Cat. No. 2200125). After evaporation of the chloroform layer, the residue was dissolved in 1 m*l* of K-ph. buffer (pH 8.0). This is test solution A which is used for the detection of sulfa drugs (SAS), antibacterial agents and macrolide antibiotics. Test solution B for the detection of tetracyclines and penicillin antibiotics and test solution C for the aminoglycoside antibiotics were prepared by using above aqueous layer.

Both Sep-pak C_{18} activities with 5 m*l* MeOH, 5 m*l* of H_2O and 5 m*l* of saturated EDTA-2Na solution, and Bakerbond 10 SPE COOH column preactivated with 5 m*l* of hexane, 5 m*l* of MeOH, 5 m*l* of H_2O and 5 m*l* of Mcilvaine buffer (pH 1.0) were connected. Above aqueous layer was charged into these column, and Sep-Pak C_{18} was used for the preparation of test solution B and Bakerbond column for the test solution C. Sep-Pak C_{18} was washed with 10 m*l* of H_2O and eluted with 5 m*l* of methanol. After evaporation of MeOH fraction, the residue was dissolved in 1 m*l* of K-ph buffer (pH 4.5). This is test solution B. Test solution C was prepared from Bakerbond column which was eluted with 5 m*l* of Mcilvaine buffer (pH 3.0) and adjusted to pH 8.0.

3. Sample application: The pulp disks (i.d. = 10 mm) dipped into each test solution were applied to each medium plate, and incubated at 35°C for the sulfa medium, and at 30°C for the other mediums for an 18 hour overnight culture.

B. Instrumental analysis

1. Materials: Bond-Elute C_{18} column and Sep-Pak Silica column were purchased from International Analytichem, U.S.A. and Waters Associates, U.S.A., respectively. MSHFB (N-methyl-N-trimethylsilyl-heptafluorobutyramide, Macherey & Nagel Co., West Germany), TMS-Cl (trimethyl chlorosilane, Sigma Co., U.S.A.), and TMS-imidazole (N-trimethylsilyl imidazole, Sigma Co., U.S.A.) were purchased.

2. Instrumental conditions: Hewlett Packard 5890-5970A model of GC/MSD was operated at 70 eV. and the HP fused silica capillary column cross-linked phenylsilicone (SE-54) (length: 17 m, i.d.: 0.2 mm, film thickness: 0.33 μm) was used. Injection temperature was 280°C, and injection mode was splitless. Transfer line temperature and ion source temperature was 300°C and 200°C respectively. Helium was used as the carrier gas at a flow-rate of 0.7 m*l*/min. For the simultaneous analysis of oxytetracycline (OTC), chlortetracycline (CTC), penicillin (PC), ampicillin (Amp), chloramphenicol (CM) and thiamphenicol (TAP), the oven temperature was programmed that the initial temperature was set at 150°C and increased to 270°C at a rate of 20°C/min. After 3 minutes at 270°C, the tem-

perature further increased to 300°C at a rate of 5°C/min. This temperature further increased to 300°C at a rate of 5°C/min. This temperature was maintained for 6 minutes. Also for the analysis of macrolide antibiotics such as erythromycin (EM), spiramycin (SPM) and oleandomycin (OM), the oven temeprature program was set at 200°C as initial temperature and increased to 300°C at a rate of 10°C/min. This was maintained for 3 minutes at 300°C.

3. Extraction and clean-up procedure.

A) For the simultaneous analysis of tetracyclines (TCs), penicilins (PCs) and chlormphenicol (CM) groups.

A meat sample (10g) was blended three times with 20, 20 and 10 m*l* of 0.01 M Na_2 EDTA-Mcilvaine buffer (pH 4.0) using a stomacher and filtered with Whatman No. 2 filter paper. After centrifugation of filtrates at 4,000 rpm for 10 min., the supernatant was applied on a Bond-Elute C_{18} catridge activated with 10 m*l* of methanol, and 20 m*l* of water. The TCs, PCs and CM groups were eluted with 6 m*l* of 0.01 M methanolic oxalic acid solution. After evaporation and dry-up, it was derivatized with 50 μl of MSHFB:TMS-imidazole (100:5:2) at 80°C for 15 min. for the GC/MSD analysis.

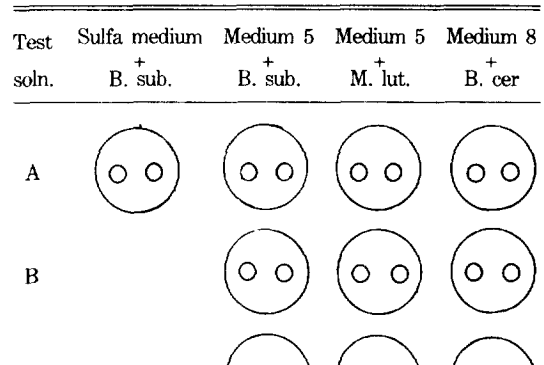
B) For the analysis of macrolide antibiotics (MLs)

A 10g meat sample was homogenized with 20 m*l* of methanol. This was centrifuged at 2,000 rpm for 10 min. The supernatant was filtered with Whatman No. 1 filter paper, and shake with 20 m*l* of n-hexane to remove the lipids. Add 2 m*l* of 1 N-NaOH and 30 m*l* of $CHCl_3$ to methanol phase and mix by swirling. In addition, add 30 m*l* of 1% Na_2HPO_4 solution and shake vigorously 1 min. Set aside to separate and drain lower chloroform phase. After evaporation, the residue was dissolved into 5 m*l* of $CHCl_3$, and charged into Sep-Pak Silica catridge activated with 30 m*l* of $CHCl_3$. The MLs was eluted with 30 m*l* of $CHCl_3$:MeOH = 2:1 solution, and evaporated. To hydrolyse in acidic condition, 15 m*l* of 0.3 N-HCl was added, and heated at 50°C for 1 hour. To extract in above pH 9 condition, 20 m*l* of $CHCl_3$ and 1.3 m*l* of 5 N-NaOH were added, and shake for 10 min. After evaporation of $CHCl_3$ layer, it was derivatized with 50 μl of MSHFB:TMSCl:TMS-imidazole (100:5:2) at 80°C for 15 min. for the GC/MSD analysis.

RESULTS AND DISCUSSION

The microbiological assay using paper disk have well been studied by Matsumoto *et al.*[26]. Also, to improve the bio-assay system, K. Jibo group used Micrococcus luteus mutants, as test microorganism[27], and reported the basic studies on biological detection of residual antibiotics in foods[28]. The Jinbo group practically applied the biological assay to detect the residual antibiotics and antibacterial agents in cultured fish[29] and pork[30]. The biological method in this text was further improved[31] and was practically used in the Tokyo metropolitan Research Laboratory of Public Health. This bio-assay was characterized by using three test microorganism and three test solutions.

As shown in Schem 2, 4 plates were made by test-solution A because of the preparation of one extra plate for the detection of sulfa drugs. The sensivtivity of this bio-assay preformed by Dr. Jinbo[31] was very high, for example, EM, OTC, CTC, PCG, AMP, SM, sulfadimethoxine (SDM) and sulfamonomethox-



Scheme 2. Simple illustration of bio-assay.

Table 1. Judgement of Antibiotics and Antibacterial Agents in Bio-Assay.

Test Soln.	Test microorganism			Judgement
	B. sub.	M. lut.	B. cer.	
A	+	+++	−	MLs
	+	−	−	SAs
B	+	−	+++	TCs
	+	+++	−	PCs
C	+++	−	+	AGs

Table 2. Characteristic ions of antibiotics in GC/MSD analysis.

Drugs	M.W.	Retention time	Characteristics
OTC	480.44	4.768	410, 395, 292
CTC	478.88	18.602	468, 427
PC	334.38	5.735	232, 290
Amp	349.42	4.136	178, 217, 319
CM	356.23	6.536	330, 257
TAP	323.14	7.337	225, 208

ine (SMM) was 0.05, 0.05, 0.01, 0.0025, 0.0025, 1.0, 0.1 and 0.1 μg/g, respectively. The judgement of class of antibiotics in the plates is summarized in Table 1.

The strength of inhibition diameter is an excellent parameter to divide a class of antibiotics. Therefore, the microbiological method will be a good screening method of residual antibiotics and antibacterial agents.

To quantify and confirm the residual antibiotics, we also developed a GC/MSD analytical method with selected ion monitoring (SIM) mode having a simple clean up procedure. For the simultaneous analysis of TCs, PCs and CM groups, we selected characteristic ions for each drug, as summarized in Table 2.

The recoveries of OTC, CTC, PC, Amp, CM and TAP in this clena-up procedure were about 80, 80, 70, 60, 90 and 90%, respectively. The limit of detection in TCs was about 50 ppb., however, that of PCs and CM groups is below 1 ppb. Therefore, this clean up procedure and GC/MSD SIM analysis is very powerful method for the detection of residual antibiotics in meats.

Also, simultaneous detection and quantitation of macrolides antibiotics such as EM, SPM and OM was developed after acid hydrolysis due to its large molecular structures. We also selected characteristic ions summarized in Table 3.

Table 3. Characteristic ions of macrolide antibiotics after acid hydrolysis in GC/MSD analysis.

Drugs	M.W.	Retention time	Characteristic ions
EM	733.92	12.417	230, 123, 157, 436
OM	687.89	15.284	157, 230, 246
SPM		18.088	159, 318, 160

The recovery and the limit of detection of three macrolides antibiotics are 90% and below 1 ppb., respectively. Although Oka *et al.*[32] reported an application of C_{18} cartridge for the analysis of TC residue in animal livers and Takatsuki *et al.*[33] also reported GC/MSD determination of EM in beef and prok, they did not reported a simultaneous analysis of antibiotics.

Because of the high ability of broad screening in the microbiological assay, and the high reproducibility and selectivity of GC/MSD analysis, it is very desirable that the combination of the two methods be used in the bioassay for the screening of residual antibiotics and antibacterial agents and GC/MSD analysis for the quantitation and confirmation.

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