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Monitoring of Trimethoprim Antibiotic Residue in Livestock and Marine Products Commercialized in Korea

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Abstract A survey for trimethoprim (TMP) was performed using 369 livestock and marine products distributed in Korea. Product samples included beef, pork, milk, egg, chicken, flatfish, jacopever, common eel, and shrimp. TMP residues were analyzed using ultra performance LC with electrospray ionization tandem mass spectrometry (UPLC-ESI-MS/MS). The analytical method was validated according to FDA (U.S. Food and Drug Administration) guidelines and all results fully complied with FDA recommendations. TMP was detected in 7 product samples (1.9% incidence), including 5 jacopever, 1 flatfish, and 1 common eel. Residue levels were 1.17 to 16.43 μg/kg in jacopever, 40.0 μg/kg in flatfish, and 13.3 μg/kg in common eel. All positive product samples were below the legal residue limit of 20-50 μg/kg in the Korean food code.

Keywords: trimethoprim, LC-MS/MS, antibiotic, monitoring

Introduction

Antibiotics are administered to animals for treatment of diseases and infection and are also widely used at sub-therapeutic levels for promotion of growth in livestock and marine products. The Global consumption of antibiotics has been estimated between 100,000 and 200,000 tons/year (1). Trimethoprim (TMP) is an anti-infective agent that is commonly used for treatment of a wide variety of bacteria associated with infections of the middle ear, and the urine, respiratory, and intestinal tracts (2) and is also a potentiator when administered with sulfonamides (3). TMP, a dihydrofolate reductase inhibitor that differs structurally from fluoroquinolones and sulfonamides, is commonly prescribed in combination with sulfamethoxazole (as co-trimoxazole, which contains sulfamethoxazole: TMP in a 5:1 ratio), sulfadiazine (4), or separately (5,6).

The presence of antibiotic residues can increase proliferation of antibiotic-resistant pathogens and threats to public health. In order to protect consumer health, the European Union (EU) set maximum residue limits (MRL) of 100 and 50 μg/kg for TMP for members of family Equidae and all other food producing animals that supply milk, muscle, fat, liver, and kidney (7). The Positive List System of Japan contains an MRL of 20-100 μg/kg in animal and marine products (8). In Korea, TMP was licensed for treatment of urinary tract infections and the MRL was established by the Ministry of Food and Drug Safety (MFDS) at 50 μg/kg in cows, pigs, sheep, and chickens for muscle, liver, fat, kidney, and milk products, at 50 μg/kg for fish and crustaceans, at 20 μg/kg for eggs, and at 100 μg/kg in horses for muscle, liver, fat, and kidney products (9). Determination of TMP residues at MRL levels requires sensitive analytical methods to comply with current regulations.

HPLC is a commonly used analytical method (10,11). Most previous work has involved only one matrix, for instance, blood, plasma, serum, or urine with HPLC (12). Several analytical methods have been described for TMP analysis in combination with sulfonamides in different biological fluids (10,13). Current study for TMP analysis with LC-MS/MS is underway for some species (3,12,14); however, no study has been performed regarding TMP analysis in livestock and marine products using LC-MS/MS.

One of the main problems of TMP analysis in livestock and marine products is the complexity of extraction steps and a need for clean-up procedures before instrumental analysis. For this purpose, liquid-liquid extraction (LLE), solid-phase extraction (SPE), pressurized liquid extraction (PLE), and matrix solid-phase dispersion (MSPD) have been used for extraction of TMP from biological fluids (11), wastewater (5,14), and plasma (15).

In this study, a method is proposed involving easy preparation, rapid extraction, and simplified solid phase extraction (SPE) steps. Moreover, ultra performance LC with electrospray ionization tandem mass spectrometry (UPLC-ESI-MS/MS) was used for TMP analysis. The purpose of this study was to monitor TMP residues in livestock

and marine products commercialized in Korea using UPLC-ESI-MS/MS. Koreans enjoy eating flatfish and jacopever sashimi, roasted eel, and Korean style raw beef. Therefore, a survey of TMP residues in livestock and marine products is important in Korea.

Materials and Methods

Product sample collection Livestock and marine products (*n*=369) were purchased from different markets in Seoul, Busan, Incheon, Daegu, Daejeon, Gwangju, Ulsan, and Jeju, Korea in 2012. Product samples were classified into 9 product categories of beef (*n*=75), pork (*n*=63), milk (*n*=77), egg (*n*=36), chicken (*n*=21), flatfish (*n*=23), jacopever (*n*=27), common eel (*n*=22), and shrimp (*n*=25). All product samples were finely ground with blender and stored in a freezer at-20 until use.

Chemicals and materials Trimethoprim (TMP) and formic acid were obtained from Sigma-Aldrich (St. Louis, MO, USA). Acetonitrile and methanol of HPLC grade were supplied by Burdick & Jackson (Ulsan, Korea). Acetic acid (99.9%, HPLC grade) was purchased from J.T. Baker (Phillipsburg, NJ, USA). Oasis HLB, MCX, and WAX solid-phase extraction (SPE) cartridges were obtained from Waters (Milford, MA, USA).

Standard preparation A stock solution of TMP (100 μg/mL) was dissolved in methanol (Burdick & Jackson) and stored at 4°C in the dark. A new stock solution was prepared monthly. Standard working solution (1 μg/mL) was prepared daily and diluted in 0.5 formic acid with 20% methanol. For quantification, a calibration curve for TMP was prepared at 6 concentrations between 0-50 ng/mL (0, 1, 2, 5, 10, and 50 ng/mL).

Preparation and clean-up procedures One g of blended sample of beef, pork, milk, egg, chicken, flatfish, jacopever, common eel, and shrimp was placed into a 50 mL polypropylene tube and subjected to extraction using 10 mL of acetonitrile (Burdick & Jackson). The mixture was homogenized (HeidolphReax top; Heidolph, Schwabach, Germany) for 5 min, sonicated (Power sonic 520; Hwashin, Seoul, Korea), for 10 min, and centrifuged (Allegra X-22R; Beckman Coulter, Brea, CA, USA) at 1,000×*g* for 10 min. The supernatant was filtered through a syringe filter (PVDF membrane, pore size 0.45 μm; Whatman®, Little Chalfont, England) and evaporated under a gentle stream of nitrogen at 45°C (GM-2200; EYELA, Tokyo, Japan). The residue was dissolved in 2 mL of 20% methanol.

An Oasis HLB cartridge was conditioned using 5 mL of methanol followed by 5 mL of distilled water. The entire volume of extract was applied to the SPE cartridge, which was subsequently washed with 2 mL of 5% ammonium hydroxide in 10% methanol. After drying the cartridge for 3 min, the residue was eluted using 2 mL of 2% formic acid in 80% methanol. The elution fraction obtained from SPE was diluted using deionized water (1:3) and filtered through a syringe filter (PVDF membrane, pore size 0.2 μm; Whatman®), after which the diluted eluate was injected into a UPLC-ESI-MS/MS apparatus. Every product sample was analyzed 3x and results were expressed as a mean value of the total residue concentration±standard deviation (SD).

LC-MS/MSanalysis of trimethoprim LC analysis was performed using an Acquity Ultra Performance (Waters) and ESI-MS/MS measurements were performed using a Quattro premier XE (Waters). Data were recorded using MassLynx 4.1 software on a personal computer. Trimethoprim was separated on an Acquity UPLC BEH C_{18} column (2.1×100 mm, 1.7 μm particle size) (Waters).

The binary mobile phase was composed of A: 0.1% formic acid in deionized water and B: 0.1% formic acid in acetonitrile. The gradient conditions started at 5% B and increased to 80% B within 3 min, followed by holding at 80% B until 4 min. At 4.1 min, the gradient was programmed to re-equilibrate the column for 1.9 min under initial conditions. The total time of analysis was 6.0 min. The flow rate was 0.3 mL/min and the injection volume was 20 μL in full-loop mode. MS determination was performed in electrospray ionization (ESI) positive ion mode combined with monitoring of the most abundant MS/MS (precursorproduct) ion transitions with a dwell time of 0.04s.The MS parameters were a capillary voltage of 3.5 kV, a source temperature of 150, a desolvation temperature of 350, cone gas flow of 50 L/h, and desolvation gas flow of 800 L/h.

Results and Discussion

TMP was analyzed in 369 livestock and marine products including 75 beef, 63 pork, 77 milk, 36 egg, 21 chicken, 23 flatfish, 27 jacopever, 22 common eel, and 25 shrimp. TMP levels were determined using UPLC-ESI-MS/MS in all product samples to avoid false positive errors due to matrix interference. Comparison with background noise levels in 9 matrices showed no interference peaks detected at the expected retention time of TMP. For confirmation, 2 characteristic fragmentations of the protonated molecular ion $[M+H]^+$ were monitored. The most abundant fragment was used for quantification, while the other was used as a qualifier. Multiple Reaction Monitoring (MRM) mode was used to increase the sensitivity and selectivity of the determination. The UPLC system showed advantages with respect to speed, sensitivity, and resolution as an attractive option for analysis of TMP in animal origin products (16). A UPLC-MS/MS chromatogram is shown in Fig. 1. The most abundant ion was at m/z=229.77.

The method was validated following FDA guidelines (17) for quantitative methods. Recovery of the analyte was replicated 6x at levels of 0.5 MRL, MRL, and 2 MRL in accordance with the Food Code in Korea (9). Recovery results were 76.84-80.52% for beef, 81.13-90.81% for pork, 81.50-85.34% for milk, 83.68-87.04% for eggs, 87.92-95.55% for chicken, 72.18-81.07% for flatfish, 85.64-93.13%

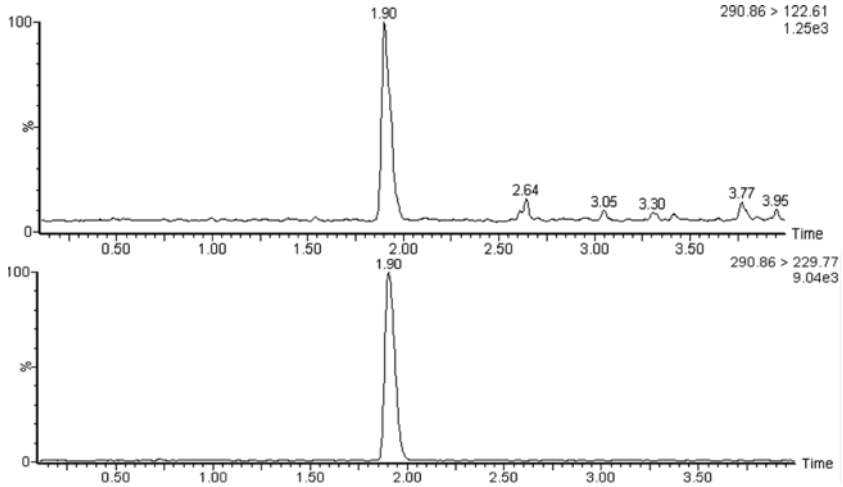

Fig. 1. UPLC-ESI-MS/MS chromatograms obtained in positive ion mode for trimethoprim at 200 μg/kg in bovine muscle.

for jacopever, 75.05-81.99% for common eel, and 70.08-80.03% for shrimp. Relative standard deviation (RSD) values for results did not exceed 9.60%, indicating good reproducibility and high precision (Table 1). The limit of detection (LOD), limit of quantification (LOQ), and linearity of TMP measurements are shown in Table 2. The LOD is the lowest concentration of analyte that an analytical process can reliably differentiate from background levels, while the LOQ is the lowest concentration of analyte that can be quantified. LOD and LOQ values were calculated based on signal-to-noise ratio (S/N) values of 3 and 10. The LOD ranged from 0.15 to 0.30 μg/kg and the LOQ ranged between 0.5 and 1.0 μg/kg. The linearity was calibrated from 0 to 50 ng/mL (6 points) and the squared correlation coefficient (R^2) was 0.999. The method was acceptable for determination of MRL values in animal origin products.

Solvent extraction including centrifugation and syringe filtration was performed, which enabled deproteinization and defatting of product samples and also elimination of matrix interference. Furthermore, at this initial step of method development, extracts were directly injected into the UPLC-MS/MS apparatus without clean-up in order to confirm whether a clean-up step was required or whether adequate results could be acquired without sample manipulation. Consequently, use of SPE cartridges showed a crucial effect on recovery of the analyte. The SPE cartridge purification step was kept as simple as possible for minimization of ion suppression effects due to the complexity of the product sample matrix.

SPE clean-up procedures for analyte extracts in blank milk samples spiked with 20 μg/kg TMP was investigated using Oasis HLB, Oasis MCX, and Oasis WAX SPE cartridges. The HLB cartridge is a high-performance, water-wettable copolymer and is a hydrophilic-lipophilic balanced reversed-phase sorbent for acid, basic, and neutral compounds. The MCX cartridge is a mixed-mode cation exchange sorbent for bases, and the WAX cartridge is a mixed-mode weak anion exchange sorbent for strong acids. Mixed-mode sorbents provide both reversed-phase and ion exchange modes of retention, enabling greater cleanup selectivity and sensitivity for both acidic and basic compounds (18).

Table 1. Recovery (%) for the proposed method using UPLC-MS/MS

Samples	Spiked conc. (μg/kg)	Mean±SD[1)]	RSD[2)] (%)
Beef	25	76.84±2.74	3.52
	50	78.18±3.77	4.82
	100	80.52±7.73	9.60
Pork	25	81.13±4.06	5.00
	50	90.81±4.49	4.94
	100	86.15±3.68	4.27
Chicken	25	87.92±2.50	2.84
	50	94.44±5.52	5.85
	100	95.55±3.68	3.19
Milk	25	85.34±3.67	4.30
	50	81.50±2.87	3.53
	100	84.71±3.87	4.57
Egg	10	85.29±3.63	4.25
	20	83.68±3.67	4.39
	40	87.04±5.83	6.70
Flat fish	25	72.18±1.88	2.74
	50	77.80±2.26	2.91
	100	81.07±6.72	8.29
Jacopever	25	85.64±2.98	3.48
	50	86.24±3.82	4.70
	100	93.13±1.97	2.12
Common eel	25	75.05±1.89	2.52
	50	81.99±3.55	4.33
	100	77.10±1.71	2.22
Shrimp	25	70.08±4.32	6.16
	50	80.03±2.11	2.64
	100	75.58±1.46	1.93

[1)]standard deviation
[2)]relative standard deviation

Table 2. Validation parameters for detection of trimethoprim using UPLC-MS/MS

Sample	LOD (μg/kg)[1)]	LOQ (μg/kg)[2)]	Calibration curve[3)]	R^2
Beef	0.3	1.0	y=264.54x+69.95	0.9994
Pork	0.3	1.0	y=219.34x+59.21	0.9991
Chicken	0.15	1.0	y=256.33x+49.86	0.9991
Milk	0.3	0.5	y=269.08x+63.84	0.9991
Egg	0.3	1.0	y=226.14x+13.69	0.9995
Flatfish	0.3	1.0	y=274.78x+130.61	0.9999
Jacopever	0.3	1.0	y=288.73x+96.30	0.9993
Common eel	0.3	1.0	y=233.06x+33.75	0.9997
Shrimp	0.3	1.0	y=146.32x+104.75	0.9991

[1)]Limit of detection
[2)]Limit of quantification
[3)]x=concentration of trimethoprim (μg/kg), y=intensity

Table 3. Incidence and range of trimethoprim levels in 369 livestock and marine products

Sample category	Analyzed samples	Detected sample	Range of TMP level (μg/kg)
Beef	n=75	ND[1)]	-
Pork	n=63	ND	-
Chicken	n=21	ND	-
Milk	n=77	ND	-
Egg	n=36	ND	-
Flatfish	n=23	1	40.0
Common eel	n=22	1	13.1
Jacopever	n=27	5	1.17-16.43
Shrimp	n=25	ND	-

[1)]Non-detection

Both MCX and WAX cartridge recovery ranged from 44.0 to 49.1%. HLB cartridge recovery was 86.4%. Thus, the HLB cartridge was used. Besides, the HLB cartridge is commonly available on the market and the type of sorbent covers a large variety of polarities. Therefore, the HLB cartridge is useful for dealing with a wide range of analytes (19). Conditioning and equilibration of the SPE cartridge were performed with a 100% organic methanol solvent and a 100% distilled water aqueous solvent, respectively. Methanol was used for elimination of interference and for elution of TMP residues from cartridges. Each ratio of organic solvent was evaluated for washing and elution steps, and consequently both 10 and 80% methanol showed improvement in recovery values and, therefore, were used.

TMP residue levels detected in 369 livestock and marine products are listed in Table 3. TMP residues were detected in 7 marine product samples (1.9% incidence). Five jacopever out of 27 jacopever samples (18.5% incidence), 1 flatfish out of 23 flatfish samples (4.3% incidence), and 1 common eel out of 22 common eel samples (4.3% incidence) exhibited TMP residues. These product samples were probably intended for treatment or prevention of a particular type of infection/disease, or were contaminated during production. Detected concentrations were 1.17 to 16.43 μg/kg in jacopever, 40.0 μg/kg in flatfish, and 13.3 μg/kg in common eel. All positive product samples were below the legal residue limit of 20-50 μg/kg in Korea.

Generally, a combination of trimethoprim and sulfadiazine is sold under the name Aqua-Sulprim® made by the Korea Thumb Vet Company (Iksan, Korea). The combination of trimethoprim and sulfadiazine is often applied for treatment of vibriosis in flatfish, jacopever, common eel, and yellowtail in Korea (6). Furthermore, Won *et al*. (20) reported monitoring of trimethoprim levels in marine products due to use of the sulfadiazine and trimethoprim combination and reported that sulfadiazine was found in 1 flatfish (14 μg/kg) and 1 jacopever (26 μg/kg). The level of sulfadiazine residue in marine products exhibited considerable similarity with results reported herein.

In this study, the incidence of TMP residues was more common in jacopever. In Korea, the usual dose for fish is 6-30 mg/kg of body weight with a withdrawal period of 30 days (21). In Korea, intensive farming, which can lead to a high potential for spread of infectious disease, has been maintained to satisfy an increasing demand for marine products. Moreover, pharmacokinetics in aquaculture and stockbreeding are affected by species specificity, health conditions, age, size, water temperature, and salinity (22). Thus, treatments were carried out in accordance with proper usage of veterinary drugs and withdrawal periods were observed.

According to the Korean National Health & Nutrition Examination Survey intake frequency per week for livestock and marine products was 0.5 to 2.6 times for 12 years or older consumers (23). Besides, in Korea and other countries in Southeast Asia, many people traditionally consume a variety of animal origin byproducts, such as blood, guts, and legs as foods (24) and consistently consume jacopever and flatfish sashimi. Consequently, monitoring of TMP residues in animal origin products is important in Southeast Asia.

In this study, a simple and reliable method using a UPLC-MS/MS system for rapid analysis of TMP residues in livestock and marine products was proposed. Acquired validation parameters fully complied with FDA recommendations. The proposed method was useful for detection of TMP residues. Furthermore, a large number of products commercialized in Korea were analyzed and quantified using the method. Although TMP residues appeared to be at relatively safe levels in livestock and marine products, the possibility of antibiotic

misuse and incomplete observation of prescribed withdrawal times by stock breeders was evident. Therefore, monitoring of trimethoprim and sulfonamide combination antibiotics in farmed livestock and fish products should be expanded.

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Disclosure The authors declare no conflict of interest.

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