International Food Research Journal 17: 885-892 (2010)

Sulfonamides determination in chicken meat products from Malaysia

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Abstract: Sulfonamides (SAs), synthetic antibiotics, are commonly used by veterinarians in chicken for therapeutic, prophylactic or as growth promoter and halt the growth of bacteria in animal production. Four common SAs, Sulfadiazine (SDZ), Sulfamethazine (SMZ), Sulfamethoxazole (SMX) and Sulfaquinoxaline (SQX), were determined in chicken breast and liver samples using reverse phase HPLC using UV detector at 266nm. The concentration of SAs detected in samples from 11 states in Peninsular Malaysia ranged from 0.006-0.062 µg/g in breast meat samples and 0.08-0.193 µg/g in liver samples. Except for sample from Johor, concentration of SAs in all the samples were lower than MRLs established by Malaysia (0.1 µg/g). Exposure of sulfonamides in Malaysian consumers ranged from 0.002-0.088 µg/kg body wt. /day. The highest value of sulfonamides exposure was found in Johor with an estimated daily intake (EDA) of Sulfamethoxazole (SMX) in Johor.

Keywords: sulfonamides, HPLC-UV, chicken breast meat and liver, Malaysia

Introduction

Antibiotics are chemical substances that can inhibit the growth of, and even destroy, harmful microorganisms. Sulfonamides (SAs) are synthetic antibiotics with a wide spectrum against most gram-positive and many gram-negative organisms. They are regularly used by veterinarians in chickens for therapeutic, prophylactic, or growth-promoting purposes and halt the growth of bacteria in animal production. They are also used to treat many kinds of infections caused by bacteria and certain other microorganisms such as infectious diseases of digestive and respiratory tracts (Forth et al., 1987). Sulfonamides (SAs) inhibit multiplication of bacteria by acting as competitive inhibitors of p-aminobenzoic acid in the folic acid metabolism cycle (Hela et al., 2003).

The extensive use of SAs as a result of their low cost has resulted in the increase of many sulfonamide-resistant strains of bacteria. Use of SAs in chickens may result in SAs residues being present in the marketed tissues if inadequate withdrawal times for the chickens have not been observed or if these drugs have been indecently administered (Kishida, 2007). As a consequence of their extensive usage, considerable attention has been paid to the potential human health risk due to their carcinogenic potency and possible antibiotic resistance (Shao et al., 2005). Therefore, to ensure the safety of the food to the consumers, SAs are set at 0.1 µg g-1 of food producing animals for the maximum residue limit (MRL) by the European Union Regulation (1990) and Malaysian Food Regulation 1985 (2006).

The aim of this study is to determine level of four SAs, Sulfadiazine (SDZ), Sulfamethazine (SMZ), Sulfamethoxazole (SMX) and Sulfaquinoxaline (SQX,) in chicken muscle and liver samples marketed in Malaysia.

Materials and Methods

Chemicals and standard solutions

Acetonitrile and methanol (HPLC grade), were obtained from fisher scientific (Fisher Scientific UK Limited), whereas acetone, methylene chloride, acetic acid, ammonium acetate and n-hexane were purchases from Merck (Darmstadt, Germany). Deionized water was obtained through a Millipore-Q50 Ultrapure water system (Sartorius). The four sulfonamides, sulfadiazine (SDZ), sulfamethoxazole (SMX), sulfaquinoxaline (SQX) and sulfamethazine (SMZ), were obtained from Sigma (St. Louis, MO, USA). The stock solutions (c = 1mg/ml) were prepared by dissolving 0.01 g of each SA standard with 10 mL of 90% acetonitrile (n-hexane saturated).

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Working individual standard and mixed standard solution of these four SAs were prepared by diluting the stock solutions with 50% methanol in 0.01 M ammonium acetate (pH4.6). The stock standard was stored at 4 °C.

Instrumentation

The HPLC system consisted of a Waters 600, Controller HPLC pump and Waters 486 Tunable Absorbance detector was used to analyze SA residues in chicken breast meat and liver samples. The mobile phase used was 0.01 M ammonium acetate at pH 4.6 and acetonitrile. Chromatographic separation was performed with gradient elution on reverse phase TSKgel ODS-80TM, C18 (5 µm, 4.6 mm x 25.0 cm) analytical column following the methods described by Ismail-Fitry et al (2008) (Table 1). The flow rate was 1.0 ml/min and 20-µl volume of the sample was injected. The sulfonamides were detected at 266nm (Figure 1).

Other apparatus used were a rotary evaporator (N-1001S-W, EYELA, 1L, Tokyo, Japan), , equipped with an aspirator (A-1000S, Tokyo, Japan) and a digital water bath, , (SB-1000, Tokyo, Japan), a nitrogen-evaporating unit (Pierce, Reacti-Therm Heating Module, Rockford, IL, USA), bowl cutter/mixer (ADE SL-18, Hamburg, Germany), Homogenizer Ultra Turrax basic (IKA Labortechnik, Staufen, Germany), benchtop centrifuge (Clements 2000, Sydney, Australia) and Eppendorf microfuge (EBA 12, Hettich Zentrifugen, Germany).

Table 1. Gradient of mobile phases used in HPLC determination of SA residues in chicken breast meat and liver samples.

Time (min)	A (%)	B (%)
0	95.0	5.0
18	63.0	37.0
23	63.0	37.0
25	95.0	5.0
30	95.0	5.0
35	95.0	5.0

A: 0.01M ammonium acetate pH 4.6
B: Acetonitrile

Collection of samples

A total of 66 samples consisted of breast meat and liver were purchased from wholesale markets in eleven states at Peninsular Malaysia which included Perlis, Kedah, Penang, Terengganu, Pahang, Kelantan, Perak, Selangor, Melaka, Negeri Sembilan and Johor. The samples were immediately frozen to -20°C, placed in ice-box (-20°C) during transportation and immediately placed in the -20°C freezer upon arrival in the laboratory. Deep-frozen samples were kept at -20ºC until analysis.

Sulfonamide extraction

The sulfonamide extraction was carried out following the method described by Ismail-Fitry et al. (2008). Breast meat and liver samples were cut into small portion (dimension) and blended An accurately weighed 10g amount of sample was place in 200ml PTE centrifuge bottle with 30ml 90% acetonitrile (n-hexane saturated) is added. The sample is homogenized for 1 minute with a homogenizer (IKA Labortechnik, Staufen, Germany) and centrifuged at 3500 rpm for 10 min (Clements 2000, benchtop, Sydney, Australia). The supernatant was transferred into 250 ml round bottom flask, whereas the residue (sample) was extracted with 20 ml acetone after sonication (Ultrasonik 104X, Neytech, Bloomfield, CT, USA) for 5 minutes. Then the mixture was centrifugated at 3500 rpm for 10 minutes; the supernatant was poured into the pear-shaped flask and evaporated at 50 °C until near to dryness. Then, 5ml methylene chloride was added, mixed using vortex and transferred into test tube; this step was repeated 3 times. The methylene chloride was then dried under nitrogen at 50 °C. The solution was reconstituted with 1 ml 50 % methanol in acetate buffer and mixed using vortex (Stuart, Oregon, USA). Then 2ml n-hexane was added and the mixture was mixed again using vortex. The mixture was then filtered and 20-µl of filtrate was injected into the HPLC system.

Linearity, Recovery, Limit of Detection (LOD) and Limit of Quantification (LOQ)

The linearity, R2, of SDZ, SMZ, SMX and SQX were obtained from the calibration graphs, which composed of seven points by plotting peak areas of SA standards having concentrations from 0.025 to 1.0 µg g-1. The correlations of determination (R2) for all SAs were more than 0.99. The LOD were determined by measuring the peak height of the blank chicken samples. LOQ were measured based on a signal-to-noise ratio (S/N) 10:1. In order to determine the recovery, 10 g of blended samples were spiked with

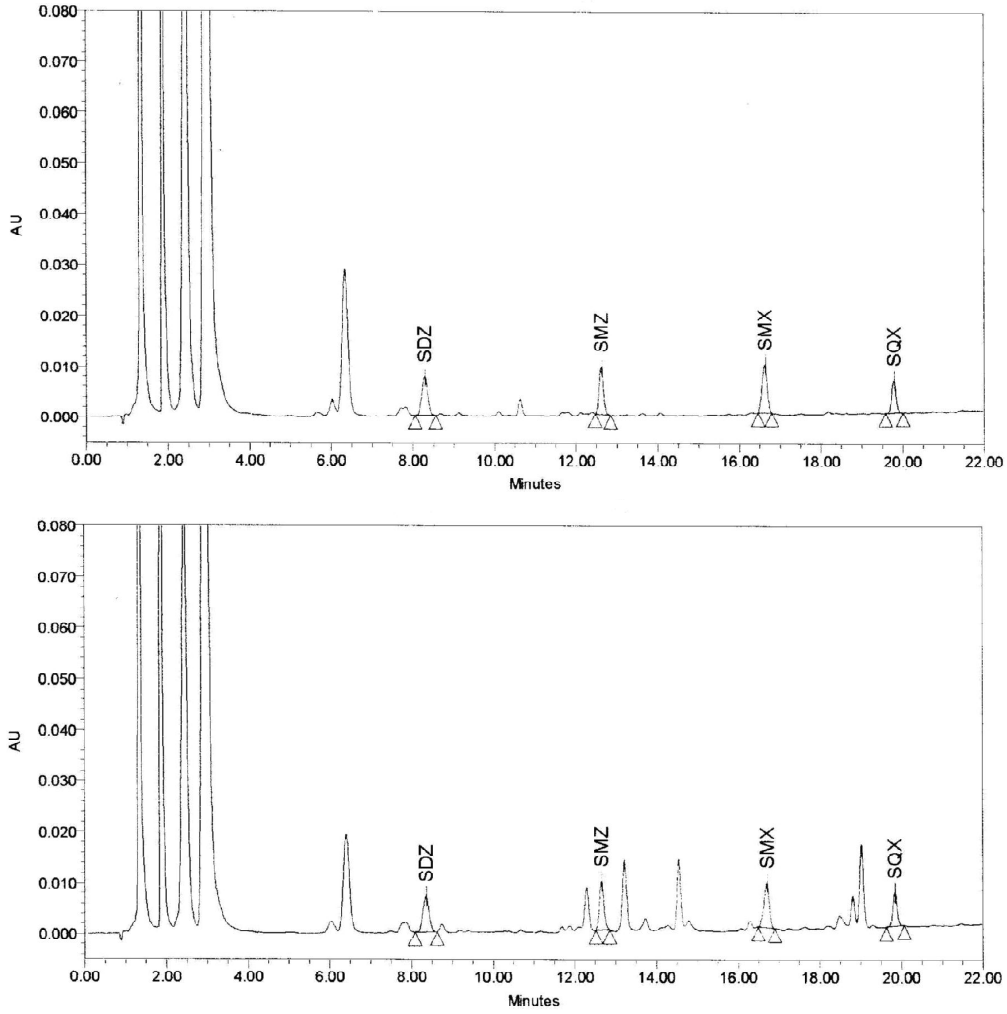

Figure 1. HPLC chromatogram of A: SAs standards (0.1µg g^{-1} SAs); B: SAs in chicken breast meat sample.

0.1 µg g-1 of standards SAs to the samples followed by homogenization for 1 min.

Exposure assessment

The estimated daily intake (EDI) values for different Sulfonamides (µg/kg body wt. /day) from chicken consumption in eleven states of Peninsular Malaysia were calculated using the averages chicken consumption reported by Ministry of Health Malaysia in each state (2006). The average Sulfonamide values in samples (µg/g) from each state were multiplied by mean chicken consumption (g/day) and by the average weight of an individual (50 kg) (Hajeb et al., 2009).

Statistical analysis

The statistical analysis has been performed using using SPSS (Version 11.5, SPSS Inc., Chicago, IL, USA). A two-way analysis of variance (ANOVA) was employed to determine the variation in SA concentrations among samples and locations. A $p < 0.05$ was considered to indicate statistical significance.

Results and Discussion

The limit of detection (LOD) of SDZ, SMZ, SMX, and SQX in the samples was 0.008, 0.007, 0.008, and 0.005µg g-1, respectively. Meanwhile, the limit of quantification (LOQ) was 0.023, 0.022, 0.025, 0.021 µg g-1 for SDZ, SMZ, SMX and SQX, respectively, which was lower than the MRL specified by Malaysia and EU countries of 0.1 µg g-1. The recoveries range from 82.0 to 98.9 % and RSDs from 0.7 to 7.6 %; these values are still within the criteria of the Codex for residue analysis (recovery of 70-110 % and RSD of <20 %).

Samples of chicken breast meat and liver samples

Table 2. Sulfonamides (SAs) concentrations in chicken breast meat and liver samples from different states of Peninsular Malaysia.

Location	Samples	Sulfonamides (mean ± S.D) (µg/g)			
		Sulfadiazine (SDZ)	Sulfamethazine (SMZ)	Sulfamethoxazole (SMX)	Sulfaquinoxaline (SQX)
Kedah	Breast meat	0.013±0.003	0.020± 0.009	-	-
	liver	0.019±0.003	0.013± 0.003	-	-
Perlis	Breast meat	-	0.039± 0.002	0.008± 0.003	0.005± 0.003
	liver	0.027±0.009	0.014± 0,734	0.015± 0.001	-
Penang	Breast meat	0.016±0.011	-	-	-
	liver	0.021±0.001	0.014± 0.005	0.013± 0.009	-
Terengganu	Breast meat	0.011±0.005	0.012± 3,631	-	-
	liver	0.007±0.001	0.007± 0.001	0.008± 0.004	-
Pahang	Breast meat	0.049±0.013	-	-	-
	liver	0.012±0.005	0.007± 0.005	0.014± 0.002	-
Kelantan	Breast meat	-	0.011± 0.004	-	-
	liver	-	0.019± 0.002	0.037± 0.007	-
Perak	Breast meat	0.030±0.003	0.029± 0.002	-	-
	liver	-	-	0.009± 0.004	-
Johor	Breast meat	0.008±0.003	-	-	-
	liver	0.010±0.003	0.008± 0.002	0.152± 0.072	-
Malacca	Breast meat	0.012±0.004	0.007± 0.003	-	-
	liver	-	0.004± 0.019	0.015± 0.001	-
Selangor	Breast meat	-	0.009± 0.002	-	-
	liver	0.010±0.003	0.009± 0.004	0.006± 0.004	-
N.Sembilan	Breast meat	0.009±0.001	0.010± 0.002	-	-
	liver	-	0.012± 0.004	-	-

Table 3. Estimated daily intake of Sulfonamides (µg/kg body wt. /day) from chicken consumption in different states of Peninsular Malaysia.

Location	Chicken consumption (g/day)	Estimated daily intake of Sulfonamides (µg/kg body wt./day)			
		Sulfadiazine (SDZ)	Sulfamethazine (SMZ)	Sulfamethoxazole (SMX)	Sulfaquinoxaline (SQX)
Kedah	24.77	0.008	0.008	-	-
Perlis	24.77	0.013	0.013	0.006	0.002
Penang	24.77	0.009	0.007	0.006	-
Terengganu	31.17	0.006	0.006	0.005	-
Pahang	31.17	0.019	0.004	0.009	-
Kelantan	31.17	-	0.009	0.023	-
Perak	32.77	0.020	0.019	0.006	-
Johor	28.81	0.005	0.005	0.088	-
Malacca	28.81	0.007	0.003	0.009	-
Selangor	32.77	0.007	0.006	0.004	-
N.Sembilan	28.81	0.005	0.006	-	-

purchased from the eleven states in Peninsular Malaysia were analyzed for the four SAs. Table 2 shows the average concentration and standard deviation of each SAs compound in breast meat and liver samples from the states. The SAs concentrations detected in the marketed samples were considered acceptable if they did not reach maximum residues limits (MRLs) of 0.1 µg/g adopted by Malaysia (Malaysian Food Regulation, 2006).

Residue of SAs detected in breast meat samples ranged from 0.006-0.062 µg/kg. In comparing between states, breast meat samples from Pahang (0.049±0.013) showed the highest concentration of total SAs followed by Perlis (0.039± 0.002) and Perak (0.029± 0.002). Sulfamethazine (SMZ) was detected in samples from each state except for Penang, Pahang and Johor. Sulfamethazine (SMZ) which is a suspected carcinogen has been identified and determined in meat, fish, milk and cheese (Clark et al., 2005, Gehring et al., 2006, Pena et al., 2004 and Wen et al., 2005), and has been rendered as the major cause in approximately 95% of all violations involving sulfonamides in tissues (Zhenga et al., 2007). SMZ is more stable towards heat compare to other SAs which it need longer time to be destroied and detoxified (Rose et al. (1995). Sulfadiazine (SDZ) was detected in breast meat samples from each state except for Perlis, Kelantan and Selangor. While Sulfamethoxazole (SMX) and Sulfaquinoxaline (SQX) were only detected in samples from Perlis.

Concentration of SAs detected in chicken liver samples was from 0.008-0.193 µg/kg. The highest levels of SAs detected were for SMZ follow by SMX, SDZ and SQX. In comparing between states, Johor

showed the highest SAs residues detected follow by Kelantan and Malacca. SAs residue detected in liver sample from Johor was 0.152± 72.727 which exceed MRLs of 0.1 µg/g. This concentration may illustrate inadequate withdrawal period before the chicken was being slaughtered. SMZ was detected in liver samples from each state except for Perak. SMX was not detected in liver samples from Kedah and Selangor. While SDZ were detected in samples from all states except for Kelantan, Perak, Malacca and N. Sembilan. None of the liver samples showed SQX residue. The level of SAs residues in chicken liver was signifantly ($p<0.05$) higher compared to breast meat samples, except for SQX which wasn not detected in liver samples. Liver play a major role in body metabolism and has a number of functions in the body including glycogen storage, plasma protein synthesis and drug detoxification. Therefore, the levels of SAs compound in liver sample are usually higher than other parts of chicken.

In comparison to those reported in other countries, sulfonamides detected in chicken samples in Malaysia is considered low. For instance in USA, the contamination rates of sulfonamides was reported to over 4% (Dey et al., 2003), while in Italy it was lower (less than 1% violation). Samples of poultry meat surveyed in Italy showed contamination of sulfadiazine at 0.64- 21.0 µg/kg and sulfaquinoxaline at 0.98- 116.0 µg/kg (Weiss et al., 2007). Positive samples detected for sulfonamides in their study were always in the liver. Study on the occurrence of veterinary drug residues, including sulfonamides, in poultry products in Nigeria showed contamination of 1% in eggs and 33.1% in broilers, 23.6% in slaughter and 4.8% in local chickens (Kabir et al., 2004). However, there was no report on sulfonamides occurrence in Malaysia or other Asian countries for more realistic comparison.

Table 3 shows exposure of sulfonamides from chicken consumption in Malaysian consumers from the eleven states. It ranged from 0.002-0.088 (µg/kg body wt. /day). The highest value of sulfonamides exposure was found in Johor with an estimated daily intake (EDA) of Sulfamethoxazole (SMX) in Johor. However, there is no Allowed Daily Intake (ADI) recommended by regulatory agencies for exposure to sulfonamides. Furthermore, there is no previous study on sulfonamides exposure from other countries to be compared with.

Conclusions

The residue of sulfonamides detected in chicken liver was higher than breast meat samples. Study findings did not detect any evidence of misuse or abuse of the investigated drugs. Only two sample out of 66 (chicken liver samples from Johor) was in violation of the regulation due to the presence of Sulfamethoxazole (SMX). Residue control in Malaysia is primarily focused on consumers' protection and it should be based on risk analysis, therefore it might be useful to prepare guidelines for the uniform enforcement of residue control all over the country.

Acknowledgments

The authors wish to acknowledge the Ministry of Health, Malaysia for financial support.

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