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Determination of Antibiotic Residues in Milk Marketed in SaudiArabia and Their Dietary Risk Assessment

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Abstract

Antibiotics are substances used in food-producing animals for therapeutic, preventive, or diagnostic purposes. Overuse can lead to antibiotic residues in food products that may have negative influence on human health. Milk, a crucial animal product, is likely to be contaminated with antibiotic residues, potentially fostering antibiotic-resistant bacteria. Long-term exposure to trace levels of antibiotic residues can have detrimental effects, including allergic reactions, gut flora disturbance, carcinogenic effects, and neurotoxicity. Sulfonamides take a long time to break down and remain in the body, leaving excess residues in poultry and cattle. Consuming animal products high in sulfonamide residues over an extended period may lead to the accumulation of these residues in the human body, potentially causing cancer, allergies, and hematopoietic system disorders. This study investigates the occurrence of sulfonamide residues in milk marketed in Saudi Arabia. The milk samples were analysed by LC-MS/MS and the Hazard Quotient (HQ) model was employed to evaluate the potential risk associated with the ingestion of milk. Fourteen sulfonamide antibiotics were identified in milk samples, and the risk exposure of sulfonamide was only measured for sulfamethazine in this study as it is the only one detected in the samples. The HQ values estimated in this research agreed with most published data from different countries and food groups indicating there is no significant health impact due to ingestion milk products based on the level of sulfonamide residues.

Keywords: Sulfonamide; LC-MS/MS; Milk; Risk Assessment; Hazard Quotient (HQ)

Introduction

Antibiotics are substances administered to food-producing animals, such as those yielding meat or milk, for therapeutic, preventive, or diagnostic purposes [1]. There are six types of antibiotics, which include antimicrobials, antiparasitic drugs, anti-inflammatory drugs, tranquilizers, drugs that promote growth, and other [2]. Despite that the overuse of antibiotics can lead to the presence of antibiotic residues in food products derived from animals, which can negatively impact human health [3,4]. Which raised focus on numerous research studies have demonstrated that consuming animal-based foods in our diet is a significant factor contributing to the exposure of antibiotics in human [5-8]. antibiotics residues refer to the remnants of drugs, their metabolites or degradation products that persist in food products obtained from animals [9].

Considering numerous health advantages, milk is one of the most essential animal products that can be found in different diets [10]. subsequently is also one

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of the products that is probable contaminated with antibiotic residue. Additionally, a significant concern associated with the existence of antibiotic residues in milk is the potential for fostering antibiotic-resistant bacteria. This resistance may transfer to humans, resulting in severe and life-threatening infections [11,12]. On other hand, long-term exposure to trace levels of antibiotic residues can have a many of detrimental effects, including allergic reactions, disturbance of gut flora, carcinogenic effects, and neurotoxicity [13,14]. In addition, antibiotic exposure has been linked to a higher incidence of metabolic disorders, including diabetes, obesity, asthma, and cardiovascular disease, according to epidemiological research [15-19]. For these reasons, it is crucial to systematically monitor the presence of antibiotic residues in animal-derived food products to ensure compliance with maximum residue limits and mitigate potential adverse effects on the quality of milk and meat [20].

For example, one of the most important and commonly used antibiotics is sulfonamide, a broad class of synthetic antibiotics commonly used as veterinary drugs for preventive and therapeutic reasons on fish farms and farm animal feed [21]. Sulfonamides have played an important role in the progress of livestock breeding industries since their widespread use and have many noteworthy features such as affordable cost, ease of application, large-scale antimicrobial activity and stable properties. Furthermore, when combined with synergists, improved bacterial effects and enhanced activity against microbes appear [22]. However, unfortunately sulfonamides take a long time to break down and remain in the body, leaving excess residues in poultry and cattle. Consuming animal products high in sulfonamide residues over an extended period of time may lead to the accumulation of these residues in the human body, which can have a number of harmful effects, including cancer, allergies, and disorders of the hematopoietic system [23]. In addition, the detection level of sulfonamide residues in food products intended for human consumption is a matter of significant concern due to their potential to induce carcinogenicity, provoke antibiotic resistance and elicit allergic reactions in humans. therefore, the EU has established an MRL value of sulfonamide about 100 g /1 kg for the total of all sulfonamides present in milk, tissues, and muscle obtained from animals that are being raised for food production [20]. The objectives of this study are to identify and detect the levels of sulfonamides in milk samples obtained from Saudi markets, involving the collection of 20 samples, using solid phase extraction method, followed by analysis through liquid chromatography-tandem mass spectrometry (LC-MS/MS). Furthermore, this study will assess the risk of residual exposure to sulfonamides in milk

Materials and Methods

Sampling and Sample Preparations

A total of 20 samples of processed packaged milk were collected from different markets in Riyadh city. A volume of approximately 20 mL of milk was carefully placed into a falcon tube and labeled appropriately for each sample. The tube was then transported to the reference laboratory for food chemistry (RLFC) using a cool box. Ultimately, all samples were kept in a deep freezer at a temperature of -20 C until the day of analysis.

Reagents and Chemicals

All solvents used in the extraction and analysis were LC-MS/MS grade. Acetonitrile, Methanol and Formic Acid were purchased from Merck (Darmstadt, Germany). Water was also obtained from Merck (Darmstadt, Germany). Sulfacetamide, sulfamethazine, sulfachlorpyridazine, sulfathiazole, sulfadiazine, sulfapyridine, sulfamerazine, sulfamethizole, sulfamethoxypyridazine, sulfaclozine, sulfamethoxazole, sulfadimethoxine, sulfabenzamide and sulfaquinoxaline analytical standards were obtained from Sigma-Aldrich (St. Louis, Missouri, United States).

Analytical standard preparation

Standard solutions containing 1000 µg/mL of each antibiotic were prepared by accurately weighting and diluting stock standard in methanol and later stored in a freezer maintained at 4 °C. From the stock, working solutions were prepared.

Sample extraction and clean-up

Sample extraction methods and analysis were performed in accordance with the technique described by Waters Applications [24]. One milliliter of homogenized sample was weighted into 15 mL polypropylene tube. Then, 4 mL of 0.2% formic acid in acetonitrile was added, followed by shaking the mixture for one minute. Then, the sample was centrifuged for 5 minutes at 10000 rpm. The supernatant was passed through hydrophilic lipophilic balanced (HLB) cartridge and collected. The sample was evaporated to dryness and then reconstituted in 1 mL of 5% methanol in water. Finally, the extracted sample was filtered through 0.45µm nylon syringe filter and transferred into the analysis vial.

Liquid Chromatography with Mass Analysis

The LC-MS/MS utilized for antibiotic determination was a UHPLC Thermo system connected to the Alts Thermo Scientific TSQ triple quadrupole instrument. The compounds ionization was carried out using Electrospray Ionization (ESI) in positive ionization mode. The mass spectrometer parameters included a Spray Voltage of 4000 V, Seath Gas at 20 psi, Auxiliary Gas at 20 a.u., and Capillary Temperature set to 350 °C. A chromatographic column, the kinetex C18, with dimensions of 150 mm × 2.1 mm and particle size of 3 µm, manufactured by phenomenex, was employed for the separation of antimicrobial residues. The separation process was carried out at a consistent flow rate of 400 µL min^{-1}. Two mobile phases were prepared for the determination of

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sulfonamide residues. One consisted of water with 0.1% formic acid (solvent A), while the other consisted of methanol with 0.1% formic acid (solvent B). The gradient elution programs utilized are provided in table 1. The quantification ion is emphasized in table 2, which includes the multiple reaction monitoring (MRM) parameters and retention times. The data acquisition and instrument control were carried out using software, Version 2.3 (Thermo Fisher).

Table 1: The gradient elution programs of LC-MS/MS.

Time (min.)	A%	B%
0	90	10
1	90	10
12	40	60
15	40	60
16	0	100
17	0	100
18	90	10
20	90	10

Quality control

Blank milk samples, spiked with 50 ug/kg of the targeted antibiotics, were analyzed with each batch of samples to guarantee the accuracy and precision of the results. The recovery of antibiotics fell within the range of 70–120%, and the permissible relative standard deviations (RSDs) for repeated measurements were established at less than 20%. Additional quality control methods employed include the utilization of reagent blank, matrix-matched calibration, and duplicate analysis. The calibration curves were required to have a minimum correlation coefficient (r2) of >0.99 at a minimum of three levels. The limit of detection (LOD) and the limit of quantification (LOQ) were calculated by multiplying the ratio of the slope and the standard error of regression of the calibration curve by 3 and 10, respectively (table 2).

Risk Assessment for dietary exposure to sulfonamides

Consumption Data: We explored two different scenarios for our study. In the first scenario, we utilized the sum of daily milk intake (g/day) from the GEMS Food platform, along with our own Saudi data study, estimating it at 161 g/day [27]. In another scenario, we based our analysis on the recommended serving size listed on milk labels, which approximates to 1 - 2 cups, or approximately 250 - 500 g/day.

Estimated Dietary Intake (EDI): EDI values were estimated using mean daily consumption (CONS) in g/day, body weight (BW) of Saudi adults in kg (70) which is adopted based on Almutairi et al, study [28], and mean contaminant content (C) in ug/kg. Equation 1 below represents the exposure of adults to toxic substance through milk products.

$$EDI\ (\mu g/kg\ bw/day) = (C\ (\mu g) \times CONS\ (g/day)) / 1000\ (g)) / BW\ (kg) \quad (1)$$

Hazard Characterisation: The Hazard Quotient (HQ) model was employed to evaluate the potential risk associated with the ingestion of milk residues. The HQ is calculated by dividing the estimated daily intake (EDI) of a substance by the Acceptable daily intake (ADI) equation 2 [25].

$$HQ = EDI / ADI \quad (2)$$

Table 2: LC-MS/MS retention time, MRM parameters, LOD and LOQ values.

Antibiotic compounds	LC-MS/MS-retention time (min)	Precursor ion	Product ions	LOD (µg/Kg)	LOQ (µg/Kg)
Sulfacetamide	2.74	215	107.967	2.15	6.52
Sulfamethazine	4.96	279.033	124.067	5.3	16.05
Sulfachlorpyridazine	5.96	284.967	91.95	5.04	15.27
Sulfathiazole	3.53	256.017	91.967	7.44	22.56
Sulfadiazine	3.64	250.3	91.967	3.62	10.96
Sulfapyridine	3.64	250.033	91.883	1.53	4.64
Sulfamerazine	3.99	265.117	91.967	8.04	24.35
Sulfamethizole	5.12	270.967	91.967	1.75	5.32
Sulfamethoxypyridazine	5.3	281.2	91.967	2.65	8.04
Sulfaclozine	5.97	285	91.967	5.19	15.72
Sulfamethoxazole	6.37	254.033	91.967	1.22	3.7
Sulfadimethoxine	6.52	311.051	107.967	7.88	23.88
Sulfabenzamide	7.4	277.05	107.967	1.8	5.45
Sulfaquinoxaline	8.17	301.067	155.967	7.39	22.4

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ADI is a calculated quantity of residue that is permitted to be consumed everyday over a person's lifetime without posing any significant health risks. According to Codex Alimentarius database. ADI value of sulfonamide antibiotic residue was estimated as 50 µg/kg. A hazard index of one or less indicates a low level of hazard, while a value larger than one suggests a risk of harm.

Here, in our study, Monte Carlo simulation was further used to take into consideration the uncertainty and variability of estimates [30]. Simulations were conducted as 10,000 iterations, and health risk calculation values were provided at the 95% percentile. Monte Carlo Simulation, and other data treatment in this study were performed using Microsoft Office Excel 2016.

Results and Discussion

Fourteen sulfonamide antibiotics, including sulfacetamide, sulfamethazine, sulfachlorpyridazine, sulfathiazole, sulfadiazine, sulfapyridine, sulfamerazine, sulfamethizole, sulfamethoxypyridazine, sulfaclozine, sulfamethoxazole, sulfadimethoxine, sulfabenzamide, and sulfaquinoxaline, were identified in milk samples (see table 3). For the experiment, 4 milk samples were chosen and examined to verify that there were no traces of sulfonamide antibiotics. These samples will be used later as a reference for spiking quality control materials. In the descriptive analysis, every compound with a value below the limit of quantification (LOQ) was assigned a zero value.

The research revealed that sulfonamide antibiotic residues in milk samples were below LOQ except on sample was detected. Sulfamethazine was the only sulfonamide residue found in 20 milk samples analyzed in this study. The mean concentrations of sulfamethazine were 0.85 µg/l, with a detection rate of 5%. Fortunately, the residue of sulfamethazine level in this study was within tolerable levels set by Saudi Food and Drug Authority (SFDA) regulation for veterinary drug residues in food [31]. SFDA regulation agrees with the Codex as the MRL of sulfamethazine in milk is 25µg/l. However, in the European Commission the MRL of sulfamethazine in bovine milk is 100µg/kg [32].

Based on the dietary exposure assessment results, the HQ was estimated to characterize the risk of exposure to sulfonamide. The risk exposure of sulfonamide was only measured for sulfamethazine in milk as based on our findings this antibiotic was the only one found in our samples tested. The average concentration was used, and the below LOQ values were treated in two scenarios, in the lower bound (LB) the below LOQ=0 and in the upper bound (UB) the below LOQ=LOQ value. The average results of the estimated daily intake (EDI) of sulfamethazine was between 1.96×10^{-4} µg/kg bw /day and 0.115 µg/kg bw /day. The 95% percentile of EDIs of sulfamethazine for adult in Saudi Arabia were from 2.99×10^{-3} µg/kg bw /day to 0.176 µg/kg bw /day for those drinking milk (table 4). The hazard quotient of sulfamethazine varied from 3.94×10^{-5} to 3.45×10^{-3}, which indicates that there is no health concern related to the residue of sulfamethazine in milk products (table 5).

Table 3: Mean concentration (µg/Kg) of 14 sulfonamide antibiotic residues in milk samples.

Antibiotics	Milk sample n=20
Sulfacetamide	<LOQ
Sulfamethazine	0.85
Sulfachlorpyridazine	<LOQ
Sulfathiazole	<LOQ
Sulfadiazine	<LOQ
Sulfapyridine	<LOQ
Sulfamerazine	<LOQ
Sulfamethizole	<LOQ
Sulfamethoxypyridazine	<LOQ
Sulfaclozine	<LOQ
Sulfamethoxazole	<LOQ
Sulfadimethoxine	<LOQ
Sulfabenzamide	<LOQ
Sulfaquinoxaline	<LOQ

n stands for the number of the samples

Table 4: Estimation of EDI in adult consumers due to milk ingestion.

Antibiotic		EDI of milk from GEMS Food platform		EDI of milk depends on lower serving size (ug/kg.b.w)		EDI of milk depends on upper serving size (ug/kg.b.w)	
		Mean	95%	Mean	95%	Mean	95%
Sulfamethazine	LB	0.0019694	0.0029974	0.003053	0.0046431	0.0061111	0.0092867
	UB	0.037189	0.0568601	0.0578221	0.0881694	0.1149736	0.1755868

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The HQ value estimated in this study were in agreement with most published data from different country in different group of food. Zuo et al., investigate the types and quantities of sulfonamide compound residues in livestock and poultry products in Shijiazhuang and determine the risk level of the dietary intake of sulfonamides. The mean value of Index of Food Safety (IFS) calculated for sulfonamide residues in different age groups. The range of IFS was between 1.51×10^{-4} and 3.57×10^{-3} indicating that the sulfonamide residues in animal/poultry products available in Shijiazhuang market are safe [34]. Based on the study conducted by Al-Shaalan et al. in Saudi Arabia and Egypt, various of antibiotic residue groups including sulfonamide were analysed in 200 milk samples utilizing bacteriological test kit and HPLC-MS/MS. only 4 milk samples were positive for oxytetracycline residues with detection rate of 2%. The hazard quotient was measured, and it was found to have an insignificant effect on consumer health [35]. Sulfonamide residues and other antimicrobial residues were also determined in 119 raw milk sample in Croatia, the calculated EDIs were 20 to 1640 times lower than ADIs stated by European Medicines Agency and WHO showing that there is no health concern related to consuming milk products [36] (Figure 1).

Table 5: Estimation of HQ in adult consumers due to milk ingestion.

Product	Antibiotic	UB		LB	
		HQ		HQ	
		Mean	95%	Mean	95%
Milk from GEMS Food platform	Sulfamethazine	0.00074532	0.0011087	3.94×10^{-5}	5.85×10^{-5}
Milk depends on lower serving size		0.00114923	0.00171442	6.14×10^{-5}	9.12×10^{-5}
Milk depends on upper serving size		0.00231298	0.00344822	0.0001219	0.00018118

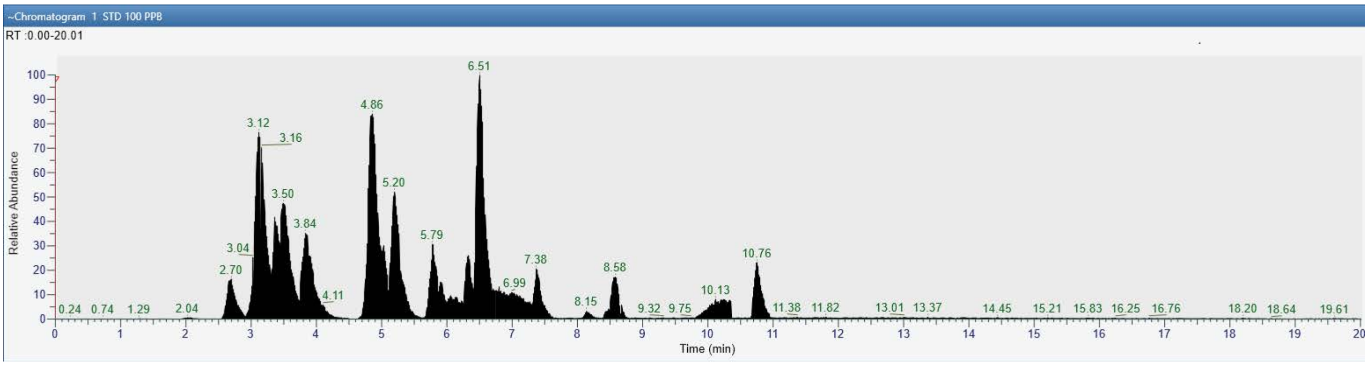

Figure 1: The chromatogram of separated sulfonamide antibiotics by LC-MS/MS.

Conclusions

This study investigates the presence of sulfonamide residues in milk marketed in Saudi Arabia. The milk samples were analyzed using LC-MS/MS and the Hazard Quotient (HQ) model to evaluate the potential risk associated with ingestion of milk. Fourteen sulfonamide antibiotics were identified in the milk samples, with the risk exposure of sulfamethazine being the only detected in the samples. The HQ values estimated in this research agreed with most published data from different countries and food groups, indicating no significant health impact due to ingestion milk products based on the level of sulfonamide residues. The study aims to identify and detect the levels of sulfonamide in milk samples obtained from Saudi markets.

Declaration of Interests

The authors declare that no competing interests exist in relation to this manuscript.

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Disclaimer

The views expressed in this paper are those of the author(s) and not do not necessarily reflect those of the SFDA or its

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stakeholders. Guaranteeing the accuracy and the validity of the data is a sole responsibility of the research team.

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