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Analysis of aminoglycoside residues in milk by high performance liquid chromatography

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Abstract
In the present study, High Performance Liquid Chromatography with Florescence detector (HPLC-FL) was standardized and validated for the detection and quantitation of aminoglycoside residues in milk. The validation studies showed that linearity (R^2>0.99), accuracy (70-110%) and precision (<10%) were within accepted range with good specificity. Aminoglycoside residues found to be positive in 15 samples out of 100 samples. Comparison of antimicrobial concentration in each positive sample of milk was done with international MRLs. The risk assessment based on per capita availability of milk in Haryana and India by calculating hazard quotient for each analyte under study revealed no risk due to dietary exposure of aminoglycoside residues through milk.

Keywords: Aminoglycoside, HPLC, milk, risk assessment

Introduction
Veterinary drugs particularly, antimicrobial agents are used in farm animals for treatment and prevention of various diseases as well as to improve feed efficiency and milk production. These are also used as growth promoters. Wide spread use of antimicrobials in agricultural and allied sectors led to their persistent presence in the environment at residual quantity. Due to their varying toxicity and development of drug resistance among the microorganisms, antimicrobial residues are considered as a major food safety and public health issue. In 2010, global consumption of antimicrobials in food animal production was estimated as 63,151±1,560 tons and India with share of 3% ranked 4th position (Van Boeckel *et al.*, 2015) [13]. Therefore, animal products such as meat and milk become a potential source for antimicrobial residues. Aminoglycosides are among the most commonly used antibacterials in veterinary medicine other being beta-lactams, macrolides, quinolones and sulfonamides.
Aminoglycosides defined as a group of compounds, having a broad spectrum activity against Gram's positive and negative bacteria and have been added to feeds for prophylaxis and to promote growth. Aminoglycosides are not metabolized to a small extent; they are bound to plasma proteins and are excreted almost entirely unchanged by the kidney. Chronic exposure to aminoglycosides can cause nephrotoxicity leading to renal failure, neuromuscular blockade and irreversible ototoxicity in human beings (Stead, 2000) [12].
In order to tackle this problem most of the industrialized nations have developed food safety and residue monitoring system and established maximum residue limits for chemical contaminants in foods. In India, Food Safety and Standards Authority of India (FSSAI) have been shouldered the responsibility of formulation and execution of food standards. Though FSSAI have established MRLs for pesticide residues and other harmful substances in milk, there are no MRL values for antimicrobial drug residues (FSSAI, 2006) [6]. The information on occurrence of antibiotic drug residues in India is available in the form of very limited academic research papers. Therefore, with due consideration to the facts and figures mentioned above, the present study was conducted with the objectives of detection of aminoglycoside residues in milk available in the local market and risk assessment due to dietary exposure of aminoglycoside residues.

Materials and Methods
Collection of samples: Milk samples were collected from Hisar. Different type of samples i.e. vendor milk, Dairy milk and pasteurized milk were collected from different places and stored at -20°C till analysis.

Chemicals and Reagents: Antibiotic reference standards with purity more than 98.0% of kanamycin (KM) and gentamicin (GM) and HPLC grade solvents and chemicals *viz.* methanol, 9-Fluorenylmethyl chloroformate (FMOC-Cl) and acetonitrile were procured from, Sigma Aldrich, U.S.A. Anhydrous sodium sulphate was procured from M/s Sisco Research Laboratories.

Preparation of standards reagent solutions: The primary standard solution of each antimicrobial was prepared by dissolving neat standards of AGs in borate buffer by using class A glassware (Final volume 25 ml) so that effective concentration remained more than 100 µg/mL. Standard solutions of AGs were stored at 4°C. For preparation of individual secondary standard solutions, the maximum residue limits (MRLs) prescribed by European Union Commission (EU, 2010) and Codex Alimentarius Commission of WHO (Codex, 2015) [2] for all antibiotics were considered. Based on these MRL values, a linearity range (50, 100, 150, 200, 250 µg/kg) was selected to cover the lowest MRLs for all the analyte molecules. Then, appropriate quantity of primary standard solution(s) was diluted to the required volume with same solvent to prepare individual secondary standard solution as well as standard mix.
Mobile phase used for the instrumental analysis of AGs was acetonitrile: water at 85:15 v/v. Derivatization agent in the form of a solution of 2.5 mm 9-Fluorenylmethyl chloroformate (FMOC-Cl) was prepared in acetonitrile. Borate buffer was prepared by adjusting the pH of boric acid solution with sodium hydroxide solution.

Analytical Procedures
Sample extraction and cleanup: HPLC with florescence detector (HPLC-FL) method used for detection of AGs from milk were standardized. Derivatization protocol required for detection of AGs was adopted as proposed by Stead and Richards (1996) [11] with slight modifications. Residue in flask was reconstituted with 2 ml borate buffer and subjected to derivatization with FMOC-Cl and chromatographically analyzed for AGs.

Chromatogramic Analysis
A Shimadzu prominence UFLC system equipped with DGU-20A5R degasser, SIL-20A HT autosampler and LC-20AD pump connected to C_8 column (Enable 4.6 mm x 250 mm porosity 5 um) housed in CTO-10AS column oven with FL detector was used throughout the experiment. The injection volume was 40 µL. For the analysis of AGs were determined on HPLC-FL at excitation wavelength of 260nm and emission wavelength of 315nm. The total run time was 30 min for AGs.

Results and Discussion
Standardization and validation studies
i) System Precision: The system precision was evaluated by studying the reproducibility of the instrumental response with respect to retention time and area of an analyte. The percent relative standard deviation (RSD) for analyte was found to be in range of 0.28-0.48 for retention time of AGs. The percent relative standard deviation (RSD) for analyte was found to be in range of 1.36-2.59 for area of AGs respect. The percent Relative Standard Deviation (%RSD) for all analyte was found to be less than 0.07 percent for area and 0.02 percent for retention time.

ii) Specificity: It was evaluated by visual observation of chromatograms of blank sample matrix and sample matrix spiked with standard mixture. For milk, chromatogramic signals at the retention times of AGs were absent in blank sample matrix.

iii) Linearity: The standard calibration curves of the analyzed AGs standards presented a good regression line (r^2>0.99) in the range of explored concentrations i.e. from 50 to 250µg/kg.

iv) Limit of detection (LOD) and limit of quantitation (LOQ): LOD and LOQ were determined by measuring the magnitude of the background response was analyzed by 10 blank samples and calculated by standard deviation of this response. Table 1 summaries the LOD and LOQ obtained for each analytes of AGs group.

Table 1: Limit of detection (LOD) and limit of quantification (LOQ) for AGs

Group of antimicrobials	Analytes	LOD (µg/kg)	LOQ (µg/kg)
Aminoglycosides	Kanamycin	4.4	9.7
	Gentamicin	80	134

v) Accuracy: The accuracy in terms of percent recovery of each analytes of AGs group at five different fortification levels (50, 100, 150, 200 and 250 µg/kg) was evaluated for milk and the results are presented in Table 2 and found satisfactory results. Recoveries for all analyte-matrix combinations ranged between 71- 110% in milk.

Table 2: Accuracy of method for detection of AGs residues in milk

Analytes	Accuracy (% Average Recovery ± SD) at various spiked Concentrations(µg/kg)				
	50	100	150	200	250
Kanamycin	96 ± 8.16	93.7 ± 4.29	92.45 ± 3.92	97 ± 8.10	100.3 ± 4.31
Gentamicin	110±10.99	107.5± 5.41	108.17± 6.29	110 ± 3.10	102.3 ± 7.7

vi) Precision: The precision was assessed, at five concentration levels (50, 100, 150, 200 and 250 µg/kg) by the recovery studies. Repeatability and intermediate precision values, expressed as relative standard deviation (CV percent) were found less than 10.51 for all analytes of AGs (Table 3).

Table 3: Precision of method for detection of AGs residues in milk

Analytes	Precision (% Relative Standard Deviation) at various spiked Concentrations(µg/kg)				
	50	100	150	200	250
Kanamycin	8.5	4.58	4.24	8.35	4.28
Gentamicin	9.98	5.02	5.81	2.8	7.48

Overall the multiresidue method followed for multiresidue detection and quantification of AGs antibiotic residues in milk was subjected to rigorous validation parameters. The system precision values indicated a good consistency in response by the HPLC instrument used during present study. A good linearity was noted for standards and spiked milk samples. Absence of interfering peaks in blank samples indicates good specificity of extraction and clean up method. Accuracy and precision of the method were in accepted range in comparison with international guidelines. These results of validation studies clearly demonstrated that the present method is suited for routine analysis of AGs in milk.

Determination of residues of AGs in milk

After successful standardization and validation of techniques for detection of AGs residues the extraction, detection and quantification was carried out on 100 samples of milk collected from Hisar city. The overall occurrence of AGs residues is presented in Table 4.

Mean concentration for each analyte is provided in Table 4 in milk samples. The results revealed that absolute mean concentrations of aminoglycosides were 67.33 µg/kg in which the residual concentration of kanamycin and gentamicin was 2.27 and 65.06 µg/kg respectively.

In the present study, aminoglycosides were detected in 10% of samples. These were present in all categories of milk. However, vendor milk samples showed highest occurrence followed by samples from mini dairies and pasteurized milk samples. The mean concentration of aminoglycosides was 66.33 µg/kg which was mostly contributed by gentamicin (65.06 µg/kg). Mean concentration of kanamycin was found to be 1.27 µg/kg.

Table 4: Mean concentration of AGs residues in milk

Group of antimicrobials	Analytes	Mean concentration (µg/Kg)			
		Raw milk-Vendor (n=40)	Raw milk-Dairy (n=40)	Pasteurized Milk (n=20)	Total (n=100)
Aminoglycosides	Kanamycin	0.66	0.61	-	1.27
	Gentamicin	123.50	15.89	113.37	65.06

Reports on detection of aminoglycosides in milk are very scanty. Gradinaru *et al.* (2011) [7] studied antibiotic residues in milk samples collected from farms in the NE Romania (Moldavia). Out of 2785 total milk samples Gentamicin and neomycin were identified in 25% of samples, at an average concentration of 198.68 µg/kg for gentamicin and 2048.53 µg/kg neomycin. Haasnoot *et al.* (1999) [8] examined 776 randomly collected milk samples using enzyme linked immunosorbent assays (ELISA) for the presence of the four aminoglycosides but none of the sample was found to contain residues above MRL values. Bando *et al.* (2009) [1] studied the occurrence of antimicrobial residues in pasteurized milk commercialized in the state of Parana, Brazil and detected gentamicin residues in 4 out of 82 samples.

With respect to aminoglycosides, the EU MRLs are available for AGs but Codex MRL is available only for gentamicin. According to these MRLs, total 8% samples exceeded the tolerance limits for gentamicin antimicrobials and kanamycin was present in traces but not having the violative concentration (Table 5). Violative concentrations in milk samples were due to AGs (8%).

Based on frequency of detection and concentration of various analytes of AGs studied in the present work, it can be stated that, with exception of kanamycin, milk samples were laden with other all antibiotics included in the study. From the findings of the present study it can be concluded that, the antibiotic residues in milk is more it may be because of lack of awareness of farmers about the withdrawal period of milk during the treatment period. However, further monitoring studies are required to produce residue free milk for domestic consumers.

Table 5: Comparison of AGs residue levels in milk samples with the national and International MRLs

Analyte	International MRLs		Number of samples violating MRLs	
	EU (2010) (µg/kg)	Codex (2015)(µg/l)	EU	Codex
Kanamycin	150	NE	NE	0
Gentamicin	100	200	8	8

NE = Not established

Risk assessment due to dietary exposure to the exposure to the aminoglycoside residues

In the current study, the risk of dietary exposure to residual quantity of AGs via milk consumption was evaluated for the population of Haryana and India. Risk assessment is done by comparing the estimated daily intake of antimicrobial residues with their acceptable daily intake (ADI) values recommended by regulatory agencies. Table 6 and 7 shows the dietary intake of antimicrobial residues expressed as µg per kilogram of body weight per day (µg/kg BW/day) in comparison with acceptable daily intake (ADI) values recommended by JECFA i.e. 0-20 µg/kg body weight/day for gentamicin. The hazard quotient based on ADI values and dietary intake values was also calculated and presented in Table 6 and 7.

Table 6: Dietary exposure of aminoglycoside residues through Milk consumption by the population of India

Group of antimicrobials	Analytes	Dietary Intake (µg/kg b.wt/day)	ADI (µg/kg b.wt/day) (JECFA, 2002)	Hazard Quotient
Aminoglycosides	Kanamycin	0.06	NA	-
	Gentamicin	2.69	0-20	0.05

ADI = Acceptable daily intake; NA= not available

Table 7: Dietary exposure of aminoglycoside residues through Milk consumption by the population of Haryana

Group of antimicrobials	Analytes	Dietary Intake (µg/kg b.wt/day)	ADI (µg/kg b.wt/day) (JECFA, 2002)	Hazard Quotient
Aminoglycosides	Kanamycin	0.02	NA	-
	Gentamicin	1.09	0-20	0.13

ADI = Acceptable daily intake; NA= not available

Conclusions

In this study, Total 15% samples were found positive for AGs with high prevalence of gentamicin (10%) and kanamycin (5%). Kanamycin was present only in traces but gentamicin was found in 8% samples above the MRLs. Gentamicin was detected more in vendors milk sample as 10% and 7.5% in dairies milk samples. Gentamicin was also found in 15% pasteurized milk samples. Based on hazard quotient, results indicated that consumers are not at risk of dietary exposure of the antimicrobials included in the present study through milk.

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