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Analysis of penicillin residues in milk using high performance liquid chromatography

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Abstract
In the present study, High Performance Liquid Chromatography with Ultra-Violet detector (HPLC-UV) technique was standardized and validated for the detection and quantitation of penicillin antimicrobial residues viz. amoxicillin and cloxacillin from milk. The standardization procedure showed that the values for the system precision (% RSD) for both the analytes was <9% for area and <0.6% for retention time), linearity (r^2>0.99), specificity and accuracy (70-110%) and precision (<10%) were within accepted range and demonstrated system suitability for analysis of milk samples. The standardized and validated method was applied for the detection of penicillin residues from 100 milk samples randomly collected from local market of Hisar City (Haryana). Mean concentrations of amoxicillin and cloxacillin antimicrobial residues in market milk samples were 26.15 and 1.14 µg/kg, respectively. A total of 14 samples were found to be containing penicillin antimicrobial residues. Comparison of antimicrobial concentration in each positive sample of milk with international MRLs showed that, cloxacillin was responsible for maximum violations (3%). It was concluded that milk is significant source of antimicrobial residues.

Keywords: HPLC, Milk, Antimicrobial residues, MRL

1. Introduction
Milk is an excellent source of nutrients and considered as a natures perfect food since the antiquity (Enb *et al.*, 2009) [2]. It contains the optimal balance of proteins, fats, carbohydrates, vitamins and minerals providing a range of benefits for growth, immunity and development for the calves and also to human. More than 6 billion people around the world consume milk and milk products; the majority of them are from developing countries. India is on the first rank in milk production in the world. However, in contrast to health benefits, milk is, however, also a potential source of disease agents and chemical contaminants.

Antimicrobial residue is one of such contaminant. Veterinary antibiotics are chemical substances, which are extensively used in livestock during the animal breeding for both therapeutic and prophylactic purposes (Marchetti *et al*, 2001) [10]. They are also be used at sub-therapeutic levels to increase feed efficiency, promote growth and prevent diseases (Ronquillo and Harnandez, 2016) [12]. The most likely cause of violative drug residues is the failure to observe prescribed withdrawal times (Van Dresser and Wilcke, 1989) [17]. However, the extra label use of antibiotics, mainly dosages deviating from recommendations of the drug manufacturer fall under the main reason for occurrence of antibiotic residues in milk after the end of the withholding period in cows in India (Nisha, 2008) [11]. The inappropriate and abusive use of veterinary drugs and negligence regarding withholding periods of milk can lead to the presence of residues of these compounds or their metabolites.

Nowadays, beta-lactams (penicillin G, ampicillin, amoxicillin etc), aminoglycosides (streptomycin, neomycin etc) and tetracycline (tetracycline, oxytetracycline etc) antibiotics are the most frequently used antimicrobials for treatment of mastitis in dairy cows and consequently, the most commonly found residues in milk (Gustavsson *et al.*, 2004) [6]. Penicillins belong to a β-lactam class of antibiotics (Yang *et al*., 2008) [9]. Penicillin (benzylpenicillin) is the oldest antibiotic discovered by Sir Alexander Fleming in 1928 (Samanidou *et al*., 2006) [13]. The systematic use of penicillins may result in the presence of residues in edible animal products including meat and milk. The residues may cause the undesirable effects on consumer health such as allergic reactions and drugs-resistant strains in some sensitive people (Samanidou *et al*., 2009) [14]. Widespread use of antimicrobials has created potential residue problems in milk and milk products making it an important public health hazard. In India especially Haryana, there is a paucity of reports related to occurrence of antimicrobial residues in milk. Therefore, the present investigation was planned with the

objective to standardize the high performance liquid chromatography (HPLC) technique for detection and quantification of penicillin antimicrobial residues.

2. Material and method

2.1 Collection of samples: Milk samples were collected from Hisar city and were sourced to local vendors, mini dairies and pasteurized milk were collected from different places and stored at -20ºC till analysis**.**

2.2 Experimental

2.2.1 Chemicals and reagents

The analytical standards of antimicrobials viz. amoxicillin and cloxacillin having purity more than 98% were procured from Sigma-Aldrich. Supelclean™ LC-18 SPE Tube having bed wt. 500 mg and volume 3 mL were also procured from Sigma-Aldrich. HPLC grade solvents namely methanol and acetonitrile were procured from Fisher Scientific whereas anhydrous sodium sulphate was procured from Qualigens. HPLC grade water was prepared in the laboratory using Millipore (Bedford, MA, USA) Milli-Q system to give a resistivity of at least 18.2 M Ω cm.

2.2.2 Preparation of standards reagent solutions

The primary standard solution of each antimicrobial was prepared by dissolving neat standards of Penicillins in methanol by using class A glassware (Final volume 25 ml) so that effective concentration remained more than 100 µg/mL. Standard solutions were stored at -18°C. For preparation of individual secondary standard solutions, the maximum residue limits (MRLs) prescribed by European Union (EU, 2010) and Codex Alimentarius Commission of WHO (Codex, 2015) for all antibiotics were considered. Based on these MRL values, a linearity range was selected to 50, 100, 200, 300, 400 ng/mL for cloxacillin and 500, 1000, 1500, 2000 and 2500 ng/mL for amoxicillin. Then, appropriate quantity of primary standard solution(s) was diluted with methanol 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 Pencillins was composed of solvent A (water: formic acid at 1000:1 v/v) and solvent B (water: acetonitrile: formic acid (at 100:900:1 v/v/v).In the present study, HPLC-UV method was standardized and validated for the determination of penicillins i.e. amoxicillin and cloxacillin based on the method reported by Stolker *et al*. (2008) [15] with slight modifications.

2.3 Analytical Procedures

2.3.1 Sample extraction and cleanup

10 ml of spiked milk sample was taken in centrifuge tube and mixed with 25-30 g sodium sulphate until slurry was formed. Twenty millilitre acetonitrile was added to it and centrifuged at 7000 rpm for 15 minutes. 15 mL of the supernatant was taken out in a beaker and 10 mL of acetonitrile was again added to the centrifuge tube and re-centrifuged (7000 rpm/15 minutes). Supernatant was collected in a 50 mL beaker. This procedure was repeated again and supernatant was added to previously collected extract in measuring cylinder.

For sample cleanup, solid phase C_{18} cartridge was attached to vacuum manifold and activated with 6 ml methanol followed by 6 ml water using vacuum manifold. Sample extract was loaded on the activated cartridge. Then cartridge was eluted using 15 mL methanol. The cleaned up extract as well as eluent was collected in pear shaped evaporating flask and evaporated to dryness at 55ºC using a rotary evaporator. Residue in flask were redissolved in 2 mL methanol and subjected for chromatographic analysis for penicillins.

2.4 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 µm) housed in CTO- 10AS column oven and SPD-20A UV-VIS detector was used throughout the experiment. The system was controlled by Lab Solution® Software. Operating conditions of the instrumental methods were as detailed Table 1.

Table 1: Specific HPLC conditions for each antibiotic

Parameters	Amoxicillin	Cloxacillin
Mobile-phase A:B	10:90	35:65
Detection wavelength	310 nm	280 nm
Flow rate	1 ml/min.	1 ml/min.
Oven temperature	30 ºC	30 ºC
Injection volume	40 µL	40 µL
Runtime	20 min.	15 in.

3. Results and Discussion

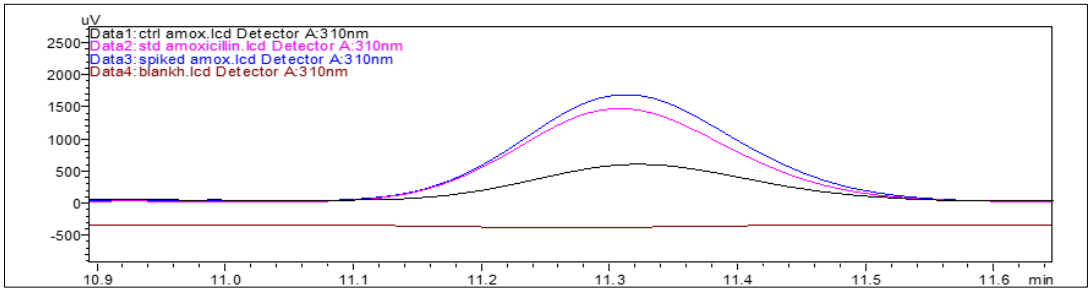
3.1 Standardization and validation studies

3.1.1 System Precision

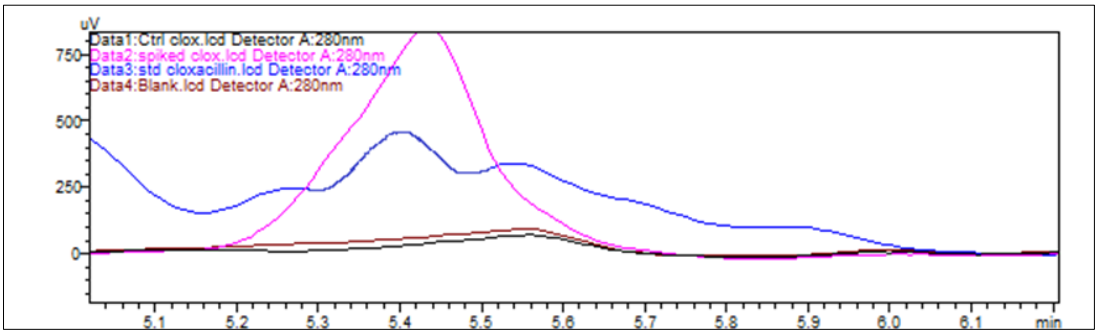
The system precision was evaluated by studying the reproducibility of the instrumental response with respect to retention time and area of an analyte Retention time of the analytes were 11.485 ± 0.062 and 5.495 ± 0.023 for amoxicillin and cloxacillin, respectively. Relative standard deviation (RSD) of retention time was in range of 0.41 – 0.53% for penicillins. Relative standard deviation (RSD) of the area under curve was in the range of 1.70–8.59 % for penicillins.

3.1.2 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 penicillins viz. amoxicillin and cloxacillin were absent in blank sample matrix.



(A)

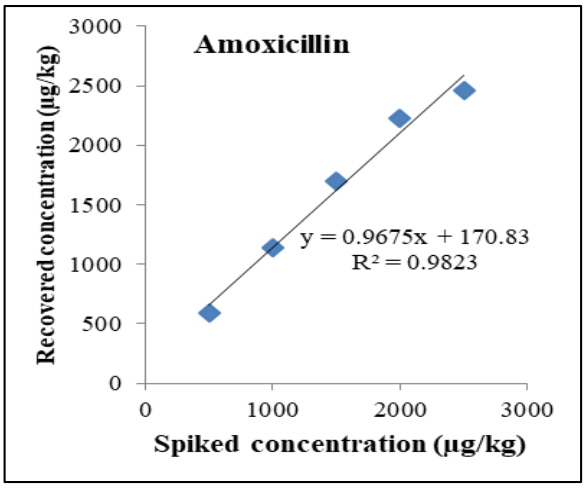


(B)

Fig 1: Comparison of chromatograms of blank and spiked milk samples demonstrating specificity Amoxicillin (A), Cloxacillin (B)

3.1.3 Linearity

The standard calibration curves of the analyzed Penicillins standards presented a good regression line (r2>0.98) in the range of explored concentrations i.e. 500 to 2500 µg/kg for amoxicillin and from 50 to 250µg/kg for cloxacillin.



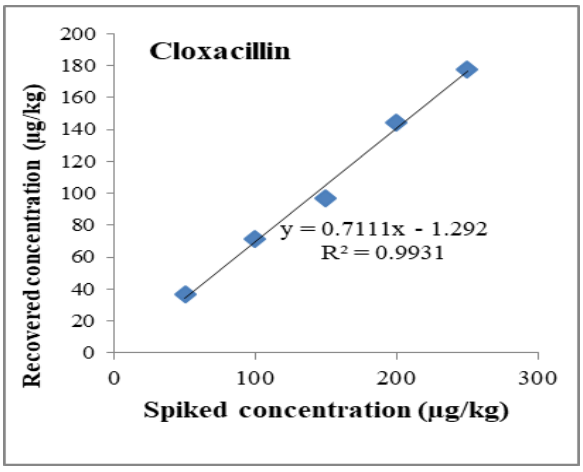


Fig 2: Linearity graphs of penicillins recovered from spiked milk samples

3.1.4 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. LOD was found to be 693.72µg/kg and 9.16 µg/kg for amoxicillin and cloxacillin, respectively. Whereas, LOQ values were 1777.47 µg/kg and 25.80 µg/kgfor amoxicillin and cloxacillin, respectively.

3.1.5 Accuracy

Accuracy was estimated on the basis of ability of the method to recover the known spiked quantity of penicillin antimicrobials in milk. It is expressed as percent average recovery and evaluated for each analyte of penicillin group at five different fortification levels i.e. 500 to 2500 µg/kg for amoxicillin and from 50 to 250µg/kg for cloxacillin. Table 2 shows the accuracy of method for detection of penicillins.

Table 2: Accuracy of penicillin antimicrobials spiked in milk

Analyte	Accuracy (%Average recovery ± SD)				
	50	**100**	**150**	**200**	**250**
Cloxacillin	72.74±3.92	71.43±1.97	70.80±2.44	72.08±3.96	71.11±6.06
	500	1000	1500	2000	2500
Amoxicillin	117.40±1.77	113.55±6.11	113.38±5.27	111.35±6.61	98.40±3.88

SD= Standard deviation, RSD = Relative Standard Deviation

3.1.6 Precision

The precision expressed as relative standard deviation and was assessed at five concentration levels i.e.500 to 2500 µg/kg for amoxicillin and from 50 to 250µg/kg for cloxacillin. Repeatability and intermediate precision values, (CV percent) were found less than 9 for all analytes of penicillins (Table 3).

Table 3: Precision of penicillin antimicrobials spiked in milk

Analyte	Precision (% RSD)				
	500	**1000**	**1500**	**2000**	**2500**
Amoxicillin	1.51	5.38	4.65	5.93	3.95
	50	100	150	200	250
Cloxacillin	5.39	2.76	3.49	5.50	8.53

Overall the method followed for multi-residue detection and quantification of Penicillins 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 was indicating good specificity of extraction and cleans up method. In comparison with international guidelines the, accuracy and precision of the method were found to be in accepted range. These results of validation studies were evident that the present method is suited for routine analysis of penicillins in milk.

3.2 Determination of residues of penicillins in milk

After successful standardization and validation, the technique for detection of penicillins residues was implemented for on extraction, detection and quantification of 100 milk samples randomly collected from the local market of which 40

samples were obtained from vendors, 40 samples from mini dairies (private milk collection and selling counters), whereas, 20 samples of pasteurized milk were obtained from retail shops of Hisar city. The occurrence of penicillins residues with their mean concentration in milk samples is presented in Table 4. The results revealed that absolute mean concentrations of penicillins were 27.29 µg/kg in which the residual concentration of amoxicillin and cloxacillin was 26.15 and 1.14 µg/kg respectively.

In the present study, penicillins were detected in 14% 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 penicillins was 27.92 µg/kg which was mostly contributed by amoxicillin (26.15µg/kg). Mean concentration of cloxacillin was found to be 1.14 µg/kg.

Table 4: Mean concentrations of penicillins in milk samples

Analyte	Mean concentration (µg/kg)			
	Raw milk- Vendor (n=40)	Raw milk- Dairy (n=40)	Pasteurized milk (n=20)	Total (n=100)
Amoxicillin	54.31	-	-	26.15
Cloxacillin	3.52	-	-	1.14

Similar studies were conducted by other researchers for the detection of penicillins also showed considerable prevalence from various patches of world and India. Khaskheli *et al.* (2008) [8] analysed 137 milk samples for β-lactam in Pakistan and found a prevalence of 36.5 %. Also, 69% prevalence was recorded for penicillin antimicrobial residues in raw milk by Junza *et al.* (2010) [7] in Spain. Gaare *et al.* (2012) [4] conducted a study in Karnal (Haryana) and recorded 5 positive milk samples for penicillin antimicrobials in milk. During a study for the detection of antimicrobial residues in milk in Punjab, Gaurav *et al.* (2014) [5] recorded 7(2.2 %) positive for cloxacillin residues in milk. Similarly, Thapaliya *et al.* (2014) recorded penicillin antimicrobial residues in 18 (12%) out of 150 market milk samples analyzed.

The concentration of each of the antimicrobial under study in each of the milk samples (if detected) was compared with available MRLs set forth by the EU and Codex. Amongst the antimicrobials included in the present study, EU MRLs are available for both amoxicillin (4 µg/kg) and cloxacillin (30 µg/kg) in milk. Whereas, Codex MRLs is available only for amoxicillin (4 µg/kg). Accordingly, the results showing violation of MRLs are presented in Table 5.

Table 5: Comparison of antimicrobial residue levels with EU and Codex MRLs in various types of milk samples

Analytes	International MRLs		Number of samples with violative concentration			
	EU,2010 (µg/kg)	Codex,2015 (µg/kg)	Raw milk- Vendor (n=40)	Raw milk- Dairy (n=40)	Pasteurized milk (n=20)	Total (n=100)
Amoxicillin	4	4	1 (2.5%)	Nil	Nil	1 (1%)
Cloxacillin	30	NE	3 (7.5%)	Nil	Nil	3 (3%)

NE- Not established

Raw milk samples were found to have penicillins residues more than the established MRL. 3% of total milk samples were found with amoxicillin above MRLs and 1% of the total samples were having cloxacillin residues above the prescribed limits. Violations with respect to MRLs have also been reported by few workers. In a study reported by Khaskheli *et al.* (2008) [8] from Pakistan, amoxicillin residues level in market milk was 9.03 fold higher compared to values recommended by European Union. Bilandzic *et al.* (2011) [1] from Croatia reported violations in 5.4 % of milk samples for penicillins residues which is slightly higher than as compared to this study.

Based on the frequency of detection and concentration of analytes, the milk samples were found to be laden with antimicrobial residues of Penicillin group. On the basis of 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 consumers.

Out of the all raw milk samples, vendor milk samples were found highly contaminated with amoxicillin and cloxacillin residues in equal proportion i.e. 10% each followed by mini dairy samples. Five percent of pasteurized milk sample were having amoxicillin residues.

4. Conclusion

The present work was envisaged to standardize and validate the liquid chromatographic methods for detection of penicillins antimicrobials (amoxicillin and cloxacillin) in milk. Total 14% samples were found positive for penicillins residue with high prevalence of residues in raw milk samples.

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