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SIMULTANEOUS DETERMINATION OF RESIDUAL NSAIDs AND ANTI-BIOTICS IN RAW MILK BY RP-HPLC

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Keywords:

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ABSTRACT: The presence of Non-steroidal Anti-Inflammatory drugs (NSAIDs) and β -lactam residues in foodstuff constitutes a potential risk to the human health and undesirable effects on consumers. Now-a-days these drug residues are also recognized as an emerging environmental problem. The present work describes the development of a sensitive, reproducible, and accurate method using liquid chromatography with ultraviolet detector for simultaneous determination of the (NSAIDs) and β -lactam antibiotics (paracetamol, nimesulide, amoxicillin, and ampicillin) in buffalo raw milk. The extraction method involves liquid-liquid extraction using diethyl ether and centrifugation followed by evaporation under pressure using Nitrogen. The percentage recovery of the (NSAIDs) and β -lactam antibiotics was 85 and 90 % respectively with relative standard deviations between 0.4 and 0.9%. The limits of quantification (LOQs) for all these compounds were in the range of 1 to 3 ng/ml, which are lower than the Maximum Residue Limits (MRLs) established by the Food and Drug Administration (FDA) for the studied (NSAIDs) and β -lactams in milk, making the method suitable for performing routine analysis. The methods were validated according to Food and Drug Administration guide lines.


INTRODUCTION: Anti-inflammatory drugs and antibiotics are used extensively in animal husbandry to reduce the pain and to prevent infection in animals. Presence of these drug residues in food products can have detrimental effect in humans consuming those products. Hence for consumer safety, withdrawal times should be obeyed, so that these residues are not present above their associated MRL⁴ (Maximum Residue Limit) and prohibited NSAIDs and antibiotics should not be administered to animals producing food for human consumption.

It was observed that LC-MS/MS⁵⁻⁹, HPLC¹¹⁻¹⁶ with fluorescence detection and microbiological¹⁷⁻²⁰ and immunoassay methods have been employed for the determination of NSAIDs and antibiotics in food products. But the present study describes a specific, lucid and sensitive high- performance liquid chromatography method for the determination of residual drugs in buffalo raw milk with UV detection. The methods were validated according to Food and Drug Administration guide lines.

MATERIALS AND METHODS:

INSTRUMENTATION:

The separation was performed on Hyperchrom ODS-BP C₁₈ (250 x 4.6 mm, 5 μ) column. The HPLC system includes HPLC pump (Agilent 1200 Infinity series) and UV detector (Agilent 1200 Infinity series). The software used was EZchrom Elite Compact.

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CHEMICALS:

Paracetamol, Nimesulide, Ampicillin, Amoxicillin were procured from Hetero drugs Private Limited, Hyderabad. The reagents Water, Methanol, Acetonitrile of HPLC grade and Ortho phosphoric acid of Analytical grade were purchased from Merck.

CHROMATOGRAPHIC CONDITIONS:

Isocratic separations were performed using a Hyperchrom ODS-BP C₁₈ column (250 x 4.6 mm, 5 μ). The mobile phase was composed of methanol: acetonitrile: phosphate buffer pH-4.5 [40:40:20% V/V] for NSAIDs and phosphate buffer pH 3.5: acetonitrile (87.5:12.5% V/V) for antibiotics at a flow rate of 1mL/min. Paracetamol, Nimesulide, Amoxicillin, Ampicillin were eluted at 2.9, 4.4, 3.75, and 7.21 minutes respectively with a run time of eight minutes for NSAIDs and nine minutes for antibiotics. The detector was operated at 276 nm for NSAIDs and 215 nm for antibiotics. Analyses were carried out at ambient temperature with an injector volume of 20 μ L and the typical chromatogram presented in Fig 1 – 3.

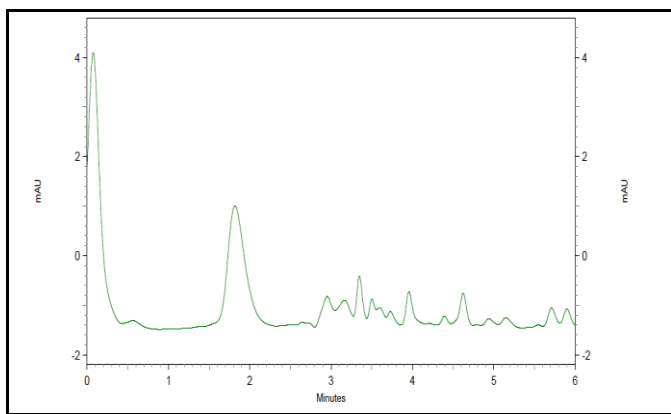


FIG 1: CHROMATOGRAM OF EXTRACTED BLANK

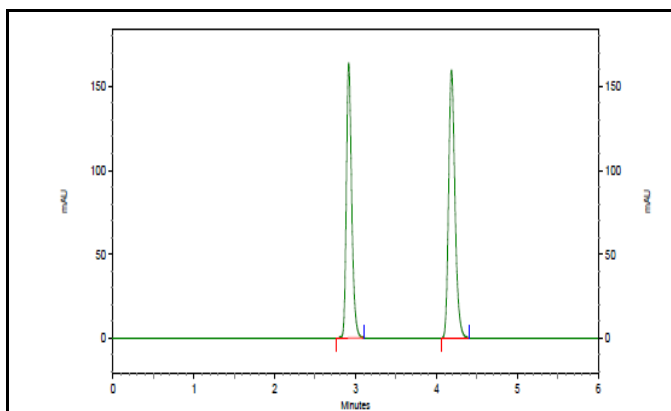


FIG 2: CHROMATOGRAM OF NSAIDs (PARACETAMOL AND NIMESULIDE)

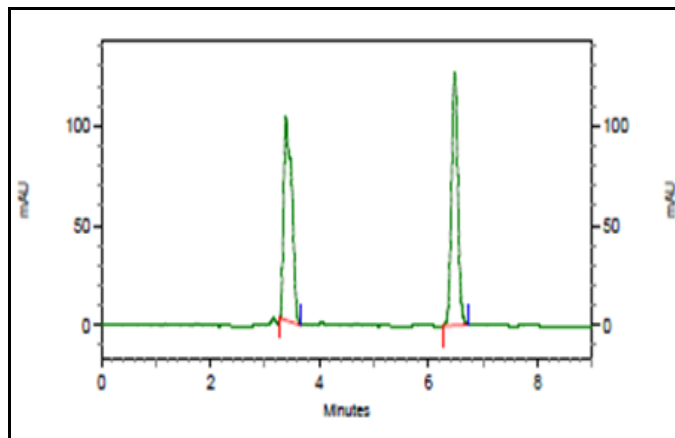


FIG 3: CHROMATOGRAM OF ANTIBIOTICS (AMOXICILLIN AND AMPICILLIN)

SAMPLE COLLECTION:

Milk samples were collected from farm animals (Government veterinary hospital, Guntur, Andhra Pradesh, India) once in a day (early morning) up to ten days (300ml sample volume). Collected samples were refrigerated until analysis.

PREPARATION OF STANDARD SOLUTIONS:

Standard stock solutions of 1mg/mL of NSAIDs and antibiotics were prepared by dissolving 10.0 ± 0.1 mg of each drug in 10mL of methanol and 10mL of water : methanol (90:10 V/V) respectively. NSAIDs (Paracetamol & Nimesulide) working standard mixture concentration was spiked into NSAIDs screened milk to get final concentrations of 10.0-1000.0ng/mL. Antibiotics (amoxicillin and ampicillin) working standard mixture concentration was spiked into antibiotics screened milk to get final concentrations of 10.0-1000.0ng/mL.

SAMPLE EXTRACTION PROCESS:

A volume of 1mL of respective sample was placed in a 5mL polypropylene tube and 3mL of diethyl ether was added to extract the drugs in to organic media. The mixture was vortexed for approximately 15minutes and then left to stand for 10minutes at room temperature.

Then centrifugation was done at 35000 rpm for 20 minutes at 4 °C, the supernatant was collected into a glass tube and the organic mixture was evaporated to dryness under a gentle stream of nitrogen at room temperature.

Finally, the obtained residues were re-dissolved in a volume of 500 μ L of mobile phase and a 100 μ L aliquot was injected into the HPLC system. All the analyses were carried out in triplicate. The same procedure was repeated for 10 days and the results were given in **Fig 4** and **5**. The amount of drug residues found was given in **Table 2** and **3**.

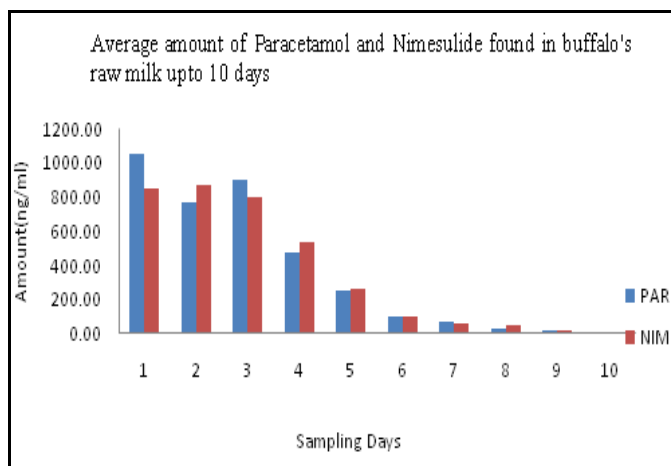


FIG 4: CONCENTRATION (ng/ml)-TIME (days) PROFILE OF PARACETAMOL AND NIMESULIDE ADMINSTRATED BUFFALOS

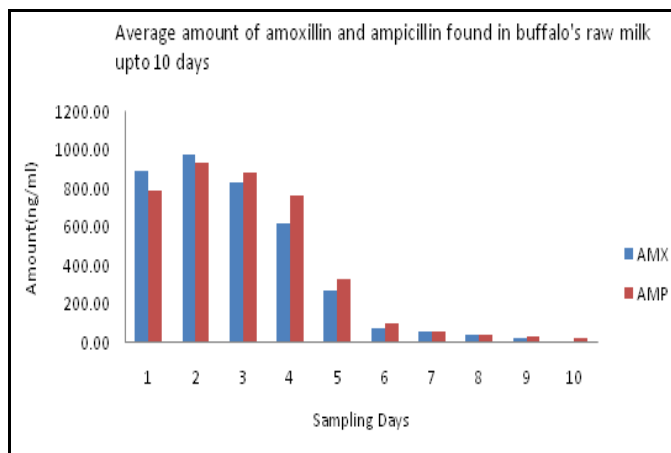


FIG 5: CONCENTRATION (ng/ml)-TIME (days) PROFILE OF AMOXICILLIN AND AMPICILLIN ADMINSTRATED BUFFALOS

RESULTS AND DISCUSSIONS:

The developed analytical method was validated as per US-FDA guidelines.

LINEARITY:

A set of eleven extracted standards ranging between 10.0-1000.0 ng/ml was prepared in screened milk and injected in to HPLC. A very good linearity was generated with excellent regression (> 0.995) and the results are tabulated in **Table 1**.

TABLE 1: CALIBRATION DATA FOR NSAIDS AND ANTIBIOTICS

Name of the Drug	Linearity range (ng/ml)	Regression equation (y = mx)	Correlation coefficient (r ²)
PARACETAMOL	10-1000	y = 12630x	0.9992
NIMESULIDE	10-1000	y = 11791x	0.9994
AMOXICILLIN	10-1000	y = 17772x	0.9961
AMPICILLIN	10-1000	y = 18551x	0.9987

TABLE 2: AMOUNT FOUND (ng/mL) AFTER (10THDAY)

Buffalo Id	Paracetamol (ng/ml)	MRL (ng/ml)	Nimesulide (ng/ml)	MRL (ng/ml)
BUF001	12	30	9	20
BUF002	9	30	6	20
BUF003	14	30	14	20

TABLE 3: AMOUNT FOUND (ng/ml) AFTER (10THDAY)

Buffalo Id	Amoxicillin (ng/ml)	MRL (ng/ml)	Ampicillin (ng/ml)	MRL (ng/ml)
BUF004	6	40	18	40
BUF005	15	40	25	40
BUF006	13	40	32	40

PRECISION:

The precision of the method was determined by measuring the repeatability (intraday precision) and intermediate precision (inter day precision) both expressed as Relative Standard Deviation (% RSD). The precision values are within 2 % RSD and the results were tabulated in **Table 4 – 7**.

TABLE 4: METHOD PRECISION DATA OF PARACETAMOL

S. NO	Conc. (ng/ml)	Peak area			SD	%RSD
		Analyst-1	Analyst-2	Analyst-3		
		1 st day	2 nd day	3 rd day		
1	20	323821	323821	323821	0.001	1.05
		323821	323821	323821	0.002	0.45
		323821	323821	323821	0.005	0.68
2	40	696722	696722	696722	0.002	1.11
		696722	696722	696722	0.002	0.25
		696722	696722	696722	0.005	1.00
3	60	753758	753758	753758	0.004	0.58
		753758	753758	753758	0.001	0.98
		753758	753758	753758	0.005	0.98

TABLE 5: METHOD PRECISION DATA OF NIMESULIDE

S.NO	Conc. (ng/ml)	PEAK AREA			SD	%RSD
		Analyst-1	Analyst-2	Analyst-3		
		1 st day	2 nd day	3 rd day		
1	20	349156	349152	349182	0.004	1.55
		3491541	349150	349162	0.002	0.95
		349152	349152	349154	0.001	1.2
2	40	582060	582070	582040	0.004	1.01
		582060	582030	582560	0.002	0.95
		582060	582060	582030	0.005	1.20
3	60	638814	638817	638816	0.004	1.32
		638813	638811	638814	0.001	1.24
		638817	638817	638816	0.005	0.98

TABLE 6: METHOD PRECISION DATA OF AMOXICILLIN

S.NO	Conc. (ng/ml)	PEAK AREA			SD	%RSD
		Analyst-1	Analyst-2	Analyst-3		
		1 st day	2 nd day	3 rd day		
1	20	473191	473191	473190	0.004	1.45
		473191	473191	473192	0.002	0.75
		473191	473192	473191	0.001	1.2
2	40	1035009	1035009	1035003	0.004	1.06
		1035009	1035008	1035009	0.002	0.95
		1035009	1035009	1035004	0.005	1.01
3	60	1262568	1262566	1262567	0.004	1.32
		1262568	1262568	1262568	0.001	1.14
		1262568	1262569	1262568	0.005	0.78

TABLE 7: METHOD PRECISION DATA OF AMPICILLIN

S.NO	Conc. (ng/ml)	PEAK AREA			SD	%RSD
		Analyst-1	Analyst-2	Analyst-3		
		1 st day	2 nd day	3 rd day		
1	20	1412110	1412110	1412110	0.004	1.55
		1412110	1412110	1412110	0.002	0.95
		1412110	1412110	1412110	0.001	1.2
2	40	1522844	1522844	1522844	0.004	1.01
		1522844	1522844	1522844	0.002	0.95
		1522844	1522844	1522844	0.005	1.20
3	60	1558762	1558762	1558762	0.004	1.32
		1558762	1558762	1558762	0.001	1.24
		1558762	1558762	1558762	0.005	0.98

RECOVERY EFFICIENCY (ACCURACY):

The %recovery efficiency was determined by spiking standard concentration to the previously analysed standards at three different concentration levels. The % recovery was 85% and 90% for NSAIDS and antibiotics respectively and the values are given in **Table 8 – 11**.

TABLE 8: RECOVERY DATA OF PARACETAMOL

S.NO	Un extracted conc.(ng/ml)	Extracted conc. (ng/ml)	% Recovery
1	40	35.23	87.02
2	60	52.25	71.05
3	80	70.28	86.11

TABLE 9: RECOVERY DATA OF NIMESULIDE

S.NO	Un extracted conc. (ng/ml)	Extracted conc. (ng/ml)	% Recovery
1	40	34.23	72.06
2	60	50.25	83.33
3	80	70.28	87.50

TABLE 10: RECOVERY DATA OF AMOXICILLIN

S.NO	Un extracted conc.(ng/ml)	Extracted conc. (ng/ml)	% Recovery
1	40	38.23	95.06
2	60	55.25	91.05
3	80	76.28	95.35

TABLE 11: RECOVERY DATA OF AMPICILLIN

S.NO	Un extracted conc.(ng/ml)	Extracted conc. (ng/ml)	% Recovery
1	40	36.47	90.17
2	60	54.25	90.11
3	80	75.02	93.08

LIMIT OF DETECTION AND QUANTIFICATION:

The LOQ and LOD values were found to be 1 ng/mL and 0.5 ng/mL (for NSAIDs) and 3ng/mL and 0.8ng/mL (for antibiotics) respectively.

CONCLUSIONS: The developed methods are sensitive and reliable using simple liquid chromatography with UV-detection for the simultaneous determination of NSAIDs and β -

lactam antibiotic drug residues in buffalo's raw milk. The sample treatment based on liquid-liquid extraction followed by evaporation allowed purification of the analytes, which yielded extracts ready for fast chromatographic identification and quantification (less than 10 min). The developed methods provided good performance and satisfactory recovery, which suggest that it could be easily applied to the routine quality control analysis of NSAIDs and β -lactams in buffalo raw milk samples at nanogram level concentration approaching the MRLs established under current regulations.

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