A Validated RP-HPLC Method for Analysis of Chloramphenicol Residue in Pasteurized Milk Sample in Bangladesh

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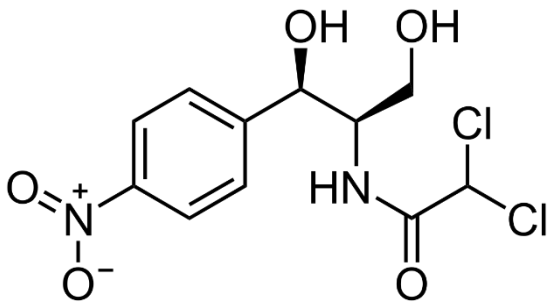
Abstract: The present study of this research was to develop a useful HPLC sensitive method to validate and determine the presence of a trace amount of chloramphenicol in pasteurized milk. A method was developed by using Waters C_{18} (150×4.6 mm, 5µm). The mobile phase was isocratic, composed of phosphate buffer pH 6.8 and acetonitrile (20:80), pumped at a flow rate of 1 mL/minute, and the wavelength was 270 nm. Using deproteinized milk samples and Trichloroacetic acid and acetonitrile, the method was tested for system suitability, accuracy, linearity, precision study, robustness, detection limit, and quantification limit. Finally, determination of chloramphenicol in tested samples was observed. Trace amount of chloramphenicol in Bangladesh is rare. The findings of this research may aid in the investigation of the existence of trace amounts of chloramphenicol in pasteurized milk samples, as well as the fact that the presence of antibiotics in foods poses a serious hazard to human health. From this study, we can conclude that chloramphenicol presence may not be found, but if it was present in a minimal amount, that may have an alarming issue, but the range was very minimal.

Key Words: Chloramphenicol, Pasteurized milk, RP-HPLC, Robustness, Mobile phase, Quantification.

1. INTRODUCTION

Chloramphenicol (CAP), also known as 2,2-dichloro-N-[1,3-dihydroxy-1-(4-nitrophenyl) propan-2-yl], is a type of antibiotic. Acetamide, isolated from *Streptomyces venezuelae*, is a broad-spectrum antibiotic that works against both gram-positive and gram-negative bacteria, as well as other bacteria [1][2]. It is used to treat bacterial meningitis, typhoid fever, rickettsial diseases,[3][4] mastitis in cattle, bacterial conjunctivitis[5]. Due to the presence of both polar and nonpolar groups, it is soluble in organic solvents



(such as acetone, ethanol, ethyl acetate, and methanol), although its solubility in water is only marginally increased [2].

Fig 1: Structure of Chloramphenicol.

The simple CAP moiety is thermostable, resistant to acid or alkaline degradation, and stable to light or oxygen. CAP is the only broad-spectrum antibiotic to be prepared by non-fermentation techniques. CAP is distinctive among natural substances since it has a nitrobenzene group and is a dichloroacetic acid derivative [3]. It comes in a parenteral dose form that is water soluble and contains the inactive prodrug sodium succinate.

Veterinary practitioners use CAP to treat bacterial infections or illnesses and to enhance the diets of animals. A danger to human health is posed by drug residues in milk as a consequence of careless or excessive use of antibiotics, which may induce allergic responses in those who are sensitive, the gray baby syndrome, deadly aplastic anemia, or drug-resistant bacteria. To ensure food safety, the analysis of antibiotic residues is critical. [6] [7] [8]. CAP use in animals that produce food has been banned for the purpose of protecting consumers from the drug's inherent toxicity as well as the potential presence of residues in foods derived from animals [9]. After 72 to 96 hours, 100mg per pound of body weight may induce hazardous effects in newborns [10]. CAP is rapidly absorbed from the GI tract and, within 2-3 hours, peak concentrations of 10–13 µg/mL occur for a 1 g dose. The drug's bioavailability ranges between 75 and 90% [6]. As shown by recent research using CAP [7], antibiotics from milk's complex matrix may be identified using a number of different methods. For foods derived from animals, the European Commission has set a minimum performance level of 0.3 µg kg-1 for CAP [11]. Due to the antibiotic's accessibility and cheap cost in certain nations, however, there is still the possibility of its illegal usage [12]. Unfortunately, it is readily accessible in Asia and widely utilized in

dairy and aquaculture. Accurate and sensitive testing procedures are required to monitor and manage the residual level of CAP [13]. For the purpose of determining the concentration of CAP in various samples, a number of analytical techniques, including liquid chromatography, liquid chromatography–mass spectrometry, gas chromatography (GC), gas chromatography–mass spectrometry (GC–MS), capillary zone electrophoresis, spectrophotometry, and chemiluminescence, have been utilized [14]. There have been a number of published reports on the topic of milk safety and purity using screening techniques for the identification of antibiotic residues [15]. The scope of the present research was to identify and compare the results of raw milk analysis for the detection of CAP residues by HPLC analysis available in the Bangladeshi market. CAP was selected because of the serious effects it has on public health when exposed to it, and because of how often it is used in veterinary medicine in Dhaka, particularly for intramammary administration.

2. MATERIALS AND METHODS

2.1 Materials and Reagents.

A pure drug sample of chloramphenicol was collected from Incepta Pharmaceuticals Ltd. Here, HPLC (Shimadzu SPD-20A Prominence UV/VIS Detector). Di potassium hydrogen phosphate, potassium di hydrogen phosphate, sodium hydroxide, Ortho phosphoric acid and trifluoracetic acid were purchased from Merck, Germany. HPLC grade Acetonitrile, methanol and water were supplied by ACI Labscan, Korea.

2.2 Sample.

Pasteurized milk was collected from the local market in Dhaka, Bangladesh.

2.3 HPLC Instrumentation and Conditions.

The HPLC system consists of a pump (LC-5A, Shimadzu Corporation, Tokyo, Japan) with a spectrophotometric detector (SPD-20A, Shimadzu Corporation, at a working wavelength of 270 nm, column waters C18, 150X4.6 mm, 5-micron particle size), a manual injector fitted with a 10 microliter. Data collection was done with software for data handling. Validation procedures were achieved on the Lithosphere 10m (C_{18}) column. The mobile phase consisted of phosphate buffer (pH 6.8) and acetonitrile (20:80), pumped at a flow rate of 1 ml/min.

2.4. Preparation of Stock Solution:

10 mg of CAP was accurately weighed and taken into a 100 mL dried and cleaned volumetric flask. Then, with acetonitrile, volume up to 100 mL and sonicate for 2 minutes. The volumetric flask was labeled as a stock solution. The concentration of the solution is 100 µg/mL.

2.5 Preparation of Mobile Phase.

Although many mobile phases were attempted, the following mobile phase produced an acceptable outcome. The mobile phase was made of phosphate buffer (pH 6.8) and acetonitrile (20:80), and 0.45 membrane filters were used to filter it.

2.6 Preparation of Standard Solutions and Calibration Curve.

A precise weight of 10 mg of CAP was transferred to a 100 mL dry and cleaned volumetric flask. Then add acetonitrile to a volume of 100 mL and sonicate for 2 minutes. A stock solution was identified in the volumetric flask. The solution has a concentration of 100 µg/mL. Then, 1 mL of the stock solution was added to a 10 mL volumetric flask and the volume was increased to 10 mL using the same solvent. Thus, the solution has a concentration of 10 µg/mL. Similarly, standard solutions containing 10- 90 µg/mL were made. Each volumetric flask was labeled with the appropriate concentration. For future use, the standard solutions were stored at -18°C. The calibration curves were plotted as a relationship between the drug concentration and the peak area response.

2.7 Preparation of Test Sample Using Pasteurized milk.

Reagents for deproteinization 1) To obtain the clear sample, Trifluroacetic acid (TFA) (0.5 g), Methanol (10 mL), and water (10 mL) were used; and 2) Trifluroacetic acid (TFA) (0.5 g), Acetonitrile (10 mL), and water (10 mL) were used. For two minutes, 50 µL of milk and 100 µL of reagents were mixed in a vortex mixer. After mixing, the solution was centrifuged for 5 minutes to isolate the clear solution.

3. RESULTS.

CAP succinate is rapidly excreted from plasma by the kidneys, which may reduce the bioavailability of the drug because almost 30% of the dose may be excreted before hydrolysis. About 50% of CAP is bound to plasma protein, which is reduced in cirrhotic patients and neonates [3]. CAP resistance is caused primarily by two factors: a) antibiotic inactivation by chloramphenicol acetyltransferase (CAT) enzymes that acetylate the antibiotic, and b) decreased drug absorption in specific gram-negative bacteria [6]. As a result of the recognized danger of aplastic anemia, resistant bacterial strains, hypersensitivity responses, and gastrointestinal issues have been reported. It is not possible to treat humans or animals with CAP because of its high toxicity levels in the body. Lactating cows may be exposed to certain antibiotics due to their usage. In addition to ototoxicity and renal toxicology, there are many more toxicological consequences [16] [17]. The therapeutic range for chloramphenicol in adults is between 5-20 mg/mL, but the therapeutic range for chloramphenicol in newborns is between 3.5-13.9 mg/mL. This difference is due to the lower binding of chloramphenicol in neonates [18].

Recent studies showed that CAP, various techniques should be applied for the identification of antibiotics from complex milk matrix. Extraction of target analytes using a number of organic solvents is followed by SPE (Solid Phase Extraction), solid phase micro extraction, and liquid-liquid extraction, all of which are standard sample preparation procedures. Solid phase extraction using molecularly imprinted polymers as the sorbent material, matrix solid-phase dispersion (MSPD), and dispersive SPE employing the fast, easy, cheap, effective, rugged, and safe (QuEChERS) approach are all examples of current extraction methods employed for the same purpose [7]. In our pasteurized milk sample, CAP was found which might cause antibiotic resistance in the human body after ingesting it orally. The HPLC processes used to determine and confirm the presence of CAP in pasteurized milk were optimized.

3.1 Method Validation.

The HPLC technique was tested for system suitability, accuracy, and linearity in accordance with ICH standards. A system suitability test was carried out by using only diluents (single run) and injecting six consecutive injections of 100 µg/mL of the

standard solution. Separate preparations of 50%, 100%, and 150% concentrations of the analyte were prepared, and chromatograms were obtained of each to determine accuracy. Linearity was evaluated by the analysis of concentrations of 10-90 µg/mL. The developed HPLC technique was used to extract and evaluate the mixtures. By injecting a series of diluted solutions of known concentration, the limits of detection (LOD) and quantitation (LOQ) for analytes might be determined. Values of LOD and LOQ were calculated by using π (standard division) and S (slope of the calibration curve) and by using the equation, LOD = 3.3 π/S and LOQ = 11.1 π/S. The capacity factor (K) is a method for determining an analyte's retention on a chromatographic column. It was calculated using the formulas t_R (retention time in the formula), t_0 (dead time of the column), and $K= (t_R-t_0)/t_0$.

3.2 Linearity

The linearity study was performed for the concentration of 10-90 µg/mL for CAP. Here, the value of CAP curve was measured by using the formula y = 38913x+851371. Here, the minimum area value was found in 10 µg/mL and the maximum value was detected in 90 µg/mL. value of R^2 was found which is R^2= 0.9158. The linearity and range investigations detailed in **Table-1** revealed the anticipated value of assay, height, theoretical plates, USP tailing factor, and capacity factor with a standard variation of 3.4±0.4 minutes for the retention period. Using the same retention time, we determined the LOD and LOQ values µg/mL, with the LOQ value being higher than the LOD value µg/mL.

3.5 Precision

Table 2 presents the results of an analysis of this method's precision, accuracy, recovery, and absolute matrix effect. The intra and inter assay bias precision and accuracy were expressed as standard deviation (SD) and relative error (RE), respectively, which did not exceed 14.59%. The calculated recoveries and absolute matrix effect values were in the range of 88.87-104.34%. All the results indicated that the assay was reproducible and accurate for the determination of CAP in milk samples. In the precision investigations reported in **Table-2**, six sample injections were utilized to determine the standard deviation and relative standard deviation of CAP. In the **Table-2** accuracy test results, 50% concentration yielded the lowest recovery value, while 150% concentration gave the highest recovery value.

3.7 Robustness Studies

Robustness was tested after intentional alterations of mobile phase composition, i.e., phosphate buffer pH 4.5 and acetonitrile (70:30), phosphate buffer pH 6.8 and acetonitrile (20:80); wavelength was tried at 270 nm, 305 nm and 280 nm; pH 4.5 and 6.8; temperature 30°C and 35°C, in order to examine the necessary changes in the performance of the chromatographic system. The chromatograms of blank with diluents and standard are presented in **Figure 2** and **Figure 3**. **Table-3** shows at first, a 100 µg/mL concentration was tested at the 280 nm wavelength and each sample was given for run 3 times. The retention time was found to be delayed compared to the ICH guideline and also, the area was not satisfactory. Here, methanol as organic mobile phases were tried, but we did not find any satisfactory retention time and area with specific (USP range) theoretical plates. Besides, P^H was also an important factor for a satisfactory area with retention time. The most important thing is that the temperature did not hamper the retention time and area. In **Figure 4**, the HPLC chromatogram of the spiked sample reveals a highest peak value of 3.269, as well as two additional values of 2.202 and 2.399, when the wavelength is 270 nm.

3.6 Deproteinization of milk sample

According to the deproteinization method, peak height was used to find the recovery. The apparent recovery calculation was = Peak height of the drug in deproteinized serum/peak height of the drug (in water) treated with the same deproteinizing solvent used for serumX100 [19]. Here, at first deproteinized milk samples with spiked CAP were measured. Analytic recovery with combination TFA and ACN/MEOH were almost same, that means we can recover the sample by this method. After that, market milk samples were collected and observed under this procedure. Besides, various extraction procedures were carried out using a variety of organic solvents, including ethyl acetate, acetone, chloroform-acetone, and acetonitrile [11] [20] [21] [22] [23] [24] [25] [26] [27].

4. DISCUSSION

Poultry industries and poultry veterinarians use antibiotics to enhance growth and feed efficiency and reduce diseases. The efficient production of poultry has been facilitated by antibiotic use, allowing the consumer to purchase it at a reasonable cost. Usage of antibiotics has also enhanced the well-being of poultry by reducing the incidence of disease. But consumers thought that edible poultry tissues were contaminated with harmful concentrations of drug residues [28]. The People's Republic of China's Ministry of Agriculture requires that CAP be undetectable in all animal products, but particularly in dairy products. This regulation primarily applies to milk products. The majority of food samples contain modest quantities of antibiotic residues and cannot be put directly into the HPLC for analysis. Trace levels of antibiotic residues must be removed and enhanced prior to detection in order to resolve the issues [29]. According to our study, four market samples were observed under the HPLC procedure. **Table 5** provides a discussion of the report that the percent recovery of sample 1 is 0.230±0.0023 with the concentration of 0.24 µg/mL, where the percent recovery of sample 2,3,4 could not be detected. In light of these possible risks, it has been banned in food-producing animals in a number of nations, including the U.S.A, Canada, Australia, the European Union, China and Japan [30]. The use of the prepared stock solution for analytical purposes was validated by using it in the process of determining the concentration of CAP in milk samples. Applying the suggested approach to the samples that had not been spiked and seeing that there was no reaction due to CAP was the first step in establishing that CAP was not present in the samples that had not been tampered with. In order to ensure the health and safety of consumers, it is necessary to develop microanalytical techniques that are sensitive enough to identify antibiotic residues in milk at the ppb level.

4. CONCLUSION:

The liquid chromatographic technique is the standard method for drug compound research and chemical detection in body fluids. Validation results showed that the suggested approach for detecting chloramphenicol is straightforward, quick, and robust. Using this validated method, four pasteurized milk samples collected from the local market in Bangladesh were analyzed in search of chloramphenicol and peak responses were found in two samples. Though the percentage of recovery is low, it can be said that a

trace amount of chloramphenicol is present in the samples. Solid phase extraction may improve the percentage of recovery of drugs in samples.

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Tables

Table-1: Results of Linearity and range studies

Parameter	CAP
Retention time (min)	3.4 ± 0.40
Assay (%)	100.13 ± 2.50
Peak height	3539 ± 175.68
No of theoretical plates	4000 ± 40
USP Tailing Factor	1.50 ± 0.10
Capacity factor	2.5 ± 0.30
LOD (ng/mL)	2.89
LOQ (ng/mL)	6.56

Data represent the mean ± SD of 3 experiments.

Table-2: Precision Studies result

Sample	Spiked analyte (µg/mL)	Intra day				Inter day			
		Mean ± S.D	CV%	Accuracy (RE %)	Recovery (%)	Mean ± S.D	CV%	Accuracy (RE %)	Recovery (%)
CAP	50	57.18±7.94	13.45	-14.53	104.34	42.43±6.85	16.22	14.59	88.87
	100	92.50±6.31	6.82	7.67	94.27	97.35±7.51	07.72	10.23	89.65
	150	151.72±18.81	12.39	-1.15	99.20	149.58±10.38	08.12	0.27	98.56

Table-3: Result of Robustness Studies

Parameters	Variables	CAP	
		RT	% Recovery
Flow rate (mL/min)	1	3.2	96.6 ± 4.34
	0.5	6.6	115.70 ± 9.76
Mobile Phase	Acetonitrile	3.1	93.70 ± 7.87
	Methanol	ND	ND
pH	4.4	12.34	108.56 ± 6.07
	6.8	3.4	93.70 ± 7.87
Column (µm)	250X4.6	3.4	102.22 ± 3.43
	150X4.6	3.4	123.70 ± 8.87
Wavelength (nm)	270	3.4	93.70 ± 7.87
	280	7.6	123.70 ± 8.87
	305	ND	ND
Column Tem. (°C)	30	3.4	96.70 ± 4.30
	35	3.4	99.65 ± 1.54

Table-4: Deproteinization of Spike Sample.

Serial	Sample Name	Retention Time	Peak height ratio	% Recovery
Sample-1	Spiked+Trifluroacetic acid+Acetonitrile	2.9	0.88	88
	Spiked+Trifluroacetic acid+Methanol	3.3	0.91	91
Sample-2	Spiked+Trifluroacetic acid+Acetonitrile	3.2	0.95	95
	Spiked+Trifluroacetic acid+Methanol	3.2	0.97	97
Sample-3	Spiked+Trifluroacetic acid+Acetonitrile	3.3	0.75	75
	Spiked+Trifluroacetic acid+Methanol	3.4	0.93	93
Sample-4	Spiked+Trifluroacetic acid+Acetonitrile	3.3	0.84	84
	Spiked+Trifluroacetic acid+Methanol	3.4	0.69	69

N= Run 3 time for each sample.

Table-5: Observation of chloramphenicol in pasteurized milk

Serial No.	Sample name	Retention time	Concentration µg/mL	% Recovery
01.	Sample-1	3.8	0.24±0.024	0.23
02.	Sample-2	ND	ND	0
03.	Sample-3	ND	ND	0
04.	Sample-4	ND	ND	0

ND= Not Detected.

Figure captions:

1. Figure 1: Structure of Chloramphenicol.
2. Figure 2: Standard Calibration Curve of Chloramphenicol.
3. Figure 3: HPLC Chromatogram of Blank.
4. Figure 4: HPLC Chromatogram with Standard Chloramphenicol.
5. Figure 5: HPLC Chromatogram of Spike Sample.
6. Figure-5: Chromatogram for the Trace Amount of Chloramphenicol from Sample 1.

1)

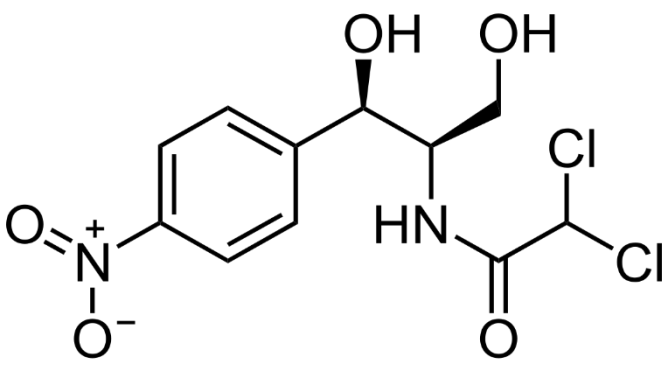

Figure 1: Structure of Chloramphenicol.

2)

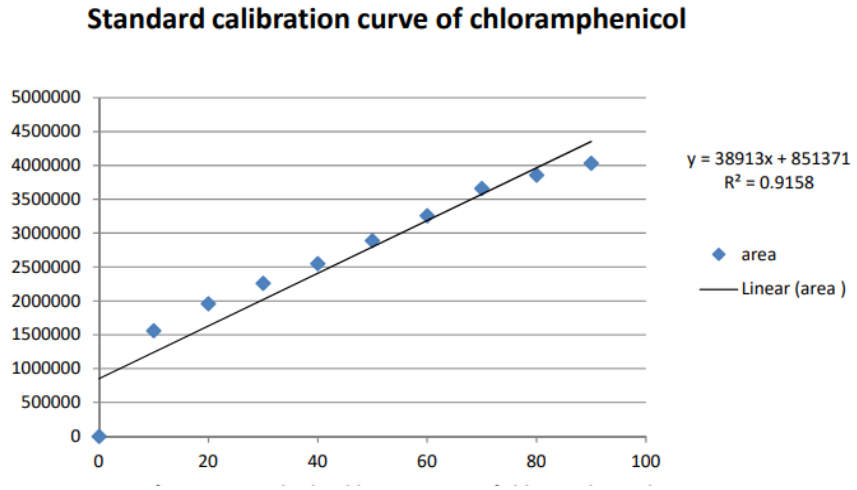

Figure 2: Standard Calibration Curve of Chloramphenicol.

3)

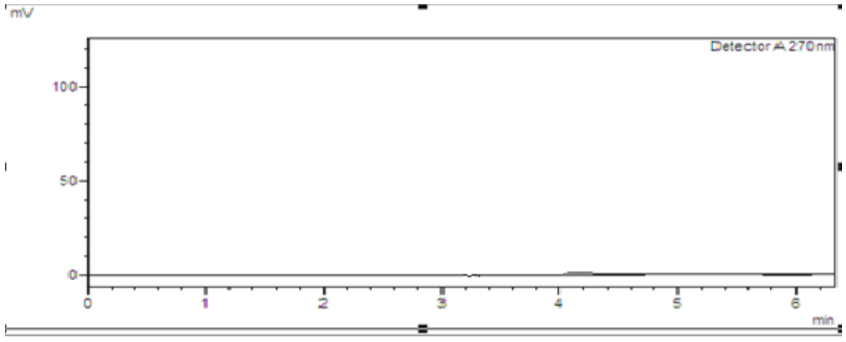

Figure 3: HPLC Chromatogram of Blank.

4)

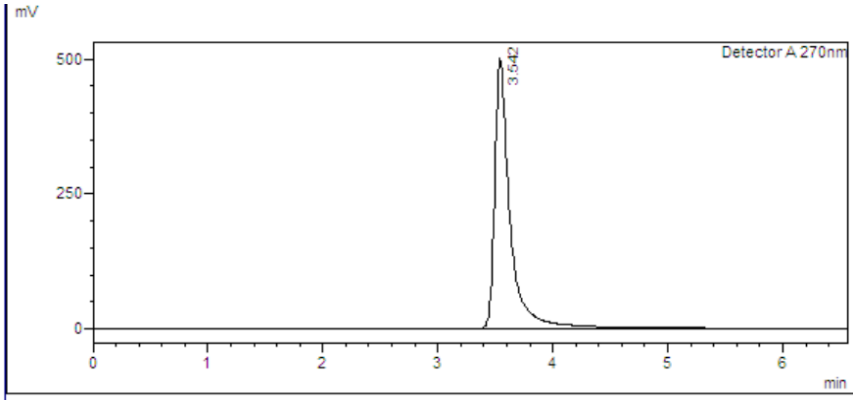

Figure 4: HPLC Chromatogram with Standard Chloramphenicol.

5)

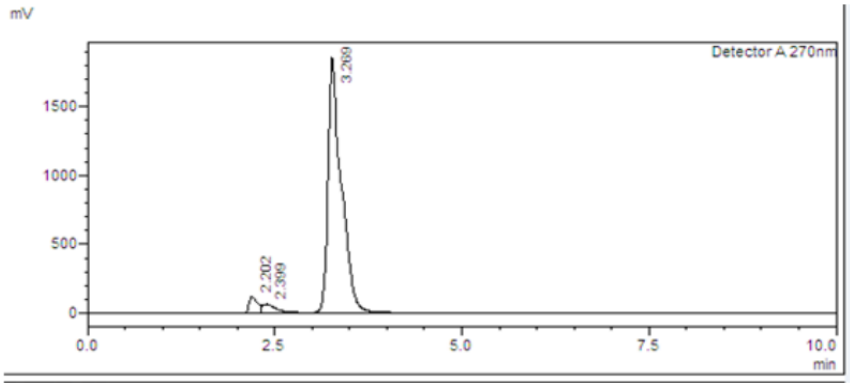

Figure 5: HPLC Chromatogram of Spike Sample.

6)

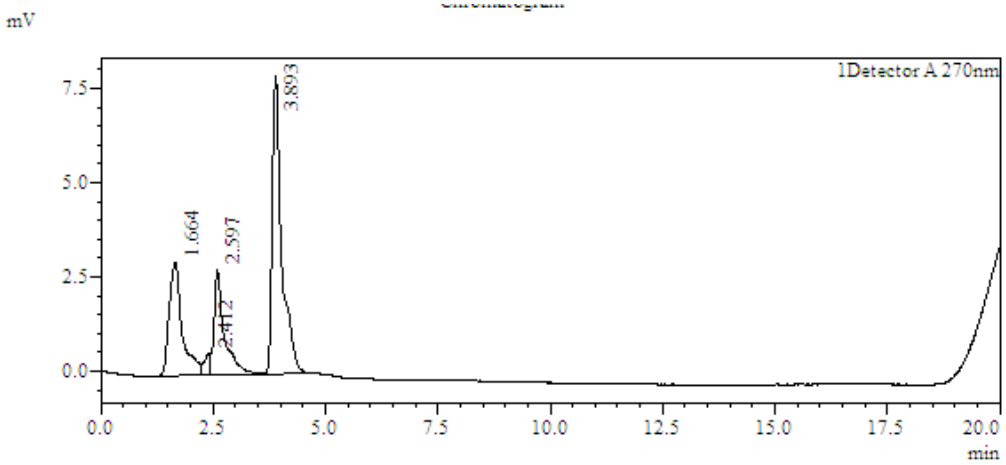

Figure-6: Chromatogram for the Trace Amount of Chloramphenicol from Sample 1.