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Development and Validation of a Microbiological Assay for the Quantification of Marketed Chloramphenicol Eye Drops

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Abstract: *A simple, sensitive, accurate and cost-effective agar disc diffusion method was developed to estimate the potency of marketed 0.5% chloramphenicol eye drop samples. The assay was based on the inhibitory effect of chloramphenicol 0.5% eye drops against Staphylococcus aureus (ATCC 29213). Mean potency values for chloramphenicol eye drops determined by this bioassay method and high performance liquid chromatography (HPLC) were compared. Linearity, precision, accuracy, specificity and recovery of the method were calculated whether its efficiency was evaluated by checking sunlight-induced (24 hours) and heat-degraded samples (at 100ºC for 6 hours) and comparing the values obtained by HPLC. Potency values determined by this method and HPLC were estimated to be 98.62±0.12% and 98.95±0.24%, respectively. A linearity value (r) of 0.9882 was found in the selected range of 3-30µg/ml whether the precision was 99.87 with a relative standard deviation (RSD) of 0.13%. Potency values for light-induced and heat-degraded chloramphenicol drop samples calculated by HPLC were 78.41±0.74% and 64.52±0.54% whereas values determined by the bioassay were similar (79.82±1.38% and 65.73±1.30%, respectively). Therefore, results validated the proposed assay as a suitable and low-cost method to quantify the potency and bioactivity of chloramphenicol in pharmaceutical samples.*

Keywords*: Agar diffusion assay, chloramphenicol eye drop, heat-degraded, sunlight-induced, low-cost method*

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I. Introduction

Antibiotics are life-saving drugs that fight infections by killing or slowing down the growth of bacteria. Chloramphenicol (**Figure 1**) is a broad spectrum antibiotic with a wide range of clinical applications [1]. It was the first clinically useful antibiotic and in Bangladesh, it is one of the most popular drugs for the treatment of bacterial eye conjunctivitis. It works by inhibiting the growth of bacteria. For the optimum performance of antibiotics, quantification of the actual concentration of antibiotic preparations is essential as overdose of these drugs can cause death [2]. Even in very low concentrations, these drugs show bactericidal or bacteriostatic activities against a broad range of bacteria [3]. Therefore, the actual concentration of active constituent in antibiotic preparations can affect their efficacy.

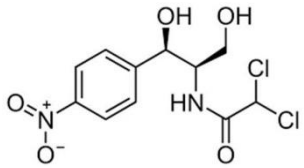

Figure 1: Chemical structure of chloramphenicol ($C_{11}H_{12}Cl_2N_2O_5$ = 323 g mol^{-1}) [Source: Internet]

Efficacy of a number of drugs like chloramphenicol becomes hampered due to drug degradation. It occurs by hydrolysis, oxidation or degradation by light due the chemistry of many of the functional groups in drug molecules and the ubiquitous presence of water and oxygen [4]. Many pharmaceutical compounds, especially light-sensitive formulations become readily oxidized when they get exposed to light and oxygen [5].

Antibiotics are the most misused and overused pharmaceutical product as the frequency of their use is relatively high, especially in developing countries like Bangladesh [6, 7]. Overuse of antibiotics already increased the number of multidrug-resistant bacteria throughout the world. To combat the rise of antibiotic resistant bacteria and practise safe use of antibiotics, correct measurement of potency and bioactivity of antibiotics is mandatory. Chemical and

biological methods can be used for the determination of potency of antibiotics. Chemical methods such as capillary electrophoresis, ultraviolet (UV) spectrophotometers, high performance liquid chromatography (HPLC) have been used conventionally for the quantitative determination of chloramphenicol.

In Bangladesh, drugs are often unavailable in government hospitals and people have no choice but to buy drugs from private retail drug shops. Irrational use of drugs has become a big problem in this country. Majority of the shop owners do not have any formal training in maintenance, supply and storage of drugs [8]. As a result, proper storage conditions like freezing, keeping in a temperature range of 18-29ºC and packaging are not maintained. Chloramphenicol eye drops should be stored in refrigerators (2°C to 8°C) and away from direct heat and sunlight during packaging. But in this country, the climate is generally marked with high temperature (reaching 40°C in summer) and a number of drug retail shops located in remote areas cannot afford to use freezers. So, the light and heat sensitive drug products like chloramphenicol can be subjected to the exposure of prolonged heat and sunlight that are responsible for decreased potency and stability. Beside the allied government organizations, pharmaceutical companies should also be responsible to check the stability of these drugs.

Most of the pharmaceutical companies in Bangladesh follow the UV spectrophotometric method provided in the British Pharmacopoeia for the chemical assay of chloramphenicol eye drops. If chloramphenicol becomes degraded, UV spectrophotometric method misinterprets the potency values giving incorrect high values of drug content in the preparations due to the presence of degraded products [7]. As its limitation, this method cannot provide the true indication of biological activity of that drug.

The practice of microbiological bioassay has recently been used for the quantitative determination of antibiotics. Biological method is the most suitable way to calculate the potency of antibiotics as potency and bioactivity of antibiotics can be determined at the same time by the same method [9]. Besides this, microbiological assay does not require specialized equipment or toxic solvents [10-13]. Impurities and related substances do not affect the results of microbiological assay [14, 15].

This study focused on the development and validation of an easy, systematic and cost-effective ager diffusion bioassay for the quantification of potency and bioactivity of marketed chloramphenicol eye drops.

II. Material And Methods

Instrument and reagents

All reagents were at least of analytical reagent grade and purchased from Merck, India. A commercial sample of the chloramphenicol 0.5% ophthalmic solution was purchased from the local market at Rajshahi, Bangladesh. Standard microbiological discs were purchased from BioMaxima SA, Poland. Equipment used for the bioassay study were properly calibrated and validated. The HPLC analysis was performed in a Shimadzu LC-MS integrated with SPD10A VP spectrophotometic UV Detector (Japan). Methanol (Merck, Germany) and milli-Q water (Millipore) of HPLC grade were used in the analysis.

Preparation of various concentrations of sample solutions from chloramphenicol eye drop

30 ml (equivalent to 150 mg) of chloramphenicol 0.5% eye drop was taken in a 100 ml volumetric flask to use it as the stock solution. From this stock solution, various concentrations, 10% (3 µg/ml) to 100% (30 µg/ml) were prepared to apply on standard microbiological disks.

Preparation of light-induced and heat-degraded chloramphenicol eye drop samples

Chloramphenicol eye drop was degraded by sunlight exposure for 24 hours and heated at 100°C for 6 hours in an incubator to prepare sunlight-induced and heat-degraded chloramphenicol eye drop samples, respectively. From those degraded samples, 90% and 100% of chloramphenicol eye drop solution were prepared to apply on disks.

Microbiological assay of chloramphenicol eye drop sample

Staphylococcus aureus (ATCC 29213) was used as the test strain. After subculture, the bacteria were transferred to LB media and agar plates were prepared using the standard protocol [16, 17].The standard microbiological discs soaked with the chloramphenicol eye drop sample solutions and degraded sample solutions were placed on solidified agar plates. The plates were then inverted and incubated at 37ºC for 24 hours. Antibacterial activity of chloramphenicol eye drops was determined by measuring the diameter of the zones of inhibition (in mm) with a transparent scale and the results were plotted as concentration vs zones of inhibition.

Method validation

Potency of chloramphenicol eye drops by microbiological bioassay was validated by determination of the following operational characteristics: linearity, precision and SEM [18-20].

Linearity: Three doses of the reference substance were used to assess the validity of the assay. Linear regression analysis along with least-squares method was performed to evaluate the linearity.

Precision: To determine precision of the method, repeatability and intermediate precision was calculated to express it as relative standard deviation (RSD). The repeatability was checked by assaying three samples of chloramphenicol eye drops on three different days under the same experimental conditions against the chloramphenicol HCl reference standard.

Accuracy: The test was performed over 3 concentration levels, 80, 100 and 120%. Aliquots of 1.2, 1.5 and 1.8 ml of the reference standard solution (100 µg/ml) were transferred into 5 ml volumetric flasks containing aliquots of sample solutions (100 µg/ml). Phosphate buffer solution (pH 6.0) was used to dilute and prepare solutions with final concentrations of 24, 30 and 36 µg/ml, respectively.

Standard Error of Mean (SEM): The standard error of mean was estimated by dividing the relative standard deviation by the standard error.

Comparison of microbiological assay and high performance liquid chromatography (HPLC) method

After developing an agar disc diffusion test method for the potency determination of chloramphenicol, a comparative study was also performed by high performance liquid chromatographic (HPLC) method to highlight the validity and reproducibility of this method.

A Nucleosil 100-5 C_{18} (reversed phase C_{18}, 5 µm, 25 cm length, 4.6 mm inner diameter) column was used as the stationary phase. Glacial acetic acid and methanol buffer were mixed in a ratio of 55:45 (v/v) to use as the mobile phase and adjusted with methanol. After filtering it through 0.2µm membrane filter, the mobile phase was degassed and was pumped at a speed of 1 ml/ min. Injection volume for samples and the standard was fixed as 10 µl. The eluents were monitored at 278 nm.

A stock solution containing 300 µg/ml of chloramphenicol HCl was prepared. A working standard solution containing 21 µg/ ml, 24 µg/ ml, 27 µg/ ml and 30 µg/ ml were prepared from the above standard solution. All the stock solutions were covered with aluminum foil to prevent the photolytic degradation until the time of analysis. The light-induced and heat-degraded solutions were then prepared at a concentration of 100% and 90% with distilled water and filtered through 0.2µm disc filters. The amount of standard, sunlight-induced and heat-degraded samples was calculated by comparing their peak areas.

III. Result

Microbiological assay of chloramphenicol eye drop samples

The disc containing 10% of chloramphenicol eye drop solution formed the smallest zone whereas the zones got bigger with increasing concentrations. Comparing to the zone formed by the 90% chloramphenicol eye drop solution, zones formed by the 90% light-induced (90%-L) and heat-degraded (90%-H) chloramphenicol eye drop sample solutions appeared to be smaller. **Figure 2** showed the concentration-dependent nature of those zones. It also showed that the zone for 90%-H was smaller than that of 90%-L, signifying the adverse effect of heat degradation.

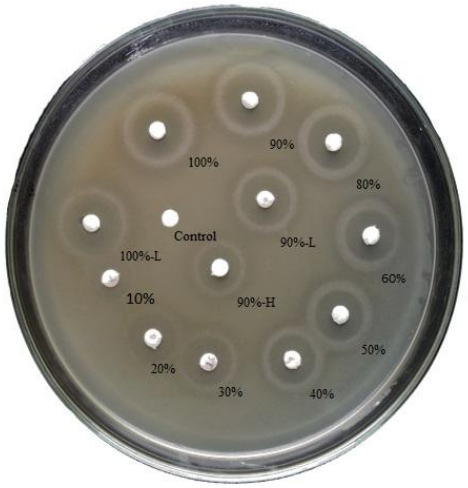

Figure 2: Zones of inhibition of *Staphylococcus aureus* (ATCC 29213) grown on an agar diffusion plate. Discs contain 10% (3 µg/disc) to 100% (30 µg/disc) samples of chloramphenicol eye drop, light-induced 100%-L (30 µg/disc) chloramphenicol eye drop sample, light-induced 90%-L (27 µg/disc) chloramphenicol eye drop sample and heat-degraded 90%-H (27 µg/disc) chloramphenicol eye drop sample. A negative control (with no chloramphenicol) was also used.

Zones of inhibition for light-induced (100%-L) and heat-degraded (100%-H) chloramphenicol eye drop samples became reduced to 19±0.24 mm and 16±0.38 mm, respectively whereas for the control eye drop sample solutions (100%), it was 23±0.29 mm. It was also observed that at the concentration of 90%, values for the light-induced (90%-L) and heat-degraded sample (90%-H) came down to 17±0.80 mm and 14±0.06 mm, respectively.

It was found that potency of the 90% light-induced (90%-L) and 90% heat-degraded (90%-H) chloramphenicol samples let down to 79.82±1.38% and 65.73±1.30%, respectively from the potency value of standard 90% chloramphenicol sample solution (90.18±0.65). Concentration of the standard 100% chloramphenicol sample solution was 98.62±0.12% (**Table 1**).

The test was repeated in four different conditions and concentration levels. The concentration levels were 100% standard chloramphenicol sample, 90% standard chloramphenicol sample, 90% light-induced (90%-L) and 90% heat-degraded (90%-H) sample solutions that were tested on three different days. To calculate the precision, we found RSD values of 0.12%, 0.72%, 1.73% and 1.98%, respectively (**Table 1**).

Table 1: Analysis of potency, precision and variance of determination of chloramphenicol eye drop by the microbiological assay method

Theoretical potency	Days	Potency found (%)	Average potency (%)	SD[a] (%)	RSD[b] (%)	Precision	SEM[c]
100%	1	98.48	98.62	0.1249	0.12664774	99.8733523	0.07
	2	98.72					
	3	98.66					
90%	1	89.55	90.18	0.65	0.72078066	99.2792193	0.38
	2	90.15					
	3	90.85					
90% light-induced (90%-L)	1	78.45	79.82	1.385	1.7351541	98.2648459	0.8
	2	79.83					
	3	81.22					
90% heat-degraded (90%-H)	1	65.81	65.73	1.301	1.9793093	98.0206907	0.75
	2	66.99					
	3	64.39					

[a]SD (Standard Deviation) (n=3), [b]RSD (Relative Standard Deviation) = (SD/ Mean) ×100, [c]SEM (Standard Error of the Mean) = SD/√(n)

The precision of this method was evaluated as 100% and 90% standard, 90% light-induced and heat-degraded solutions. The precision of these samples were 99.87%, 99.28%, 98.26% and 98.02% whereas the coefficient correlation (r) value was found to be 0.9882. The mean accuracy was 98.25 and the RSD (%) value was 0.565 (**Table 2**).

Table 2: Accuracy of the microbiological assay of chloramphenicol eye drops

Run	Amount of Standard (µg/ml)		Recovery (%)[a]	RSD (%)
	Added	Recovered		
R1	1.2	1.185	98.83	0.565
R2	1.5	1.473	98.20	
R3	1.8	1.758	97.72	

[a]Mean of three (03) assays

Comparison of microbiological assay and high performance liquid chromatography (HPLC) method

Standard chloramphenicol solution (100% and 90%) and both the 90% light-induced and heat-degraded solutions of chloramphenicol eye drop were checked using the HPLC. The chromatographic profile and profile of the disc diffusion method were found to be nearly similar.

Figure 3 displays the chromatograms of the chloramphenicol HCl reference standard (100% and 90%), heat-degraded sample (90%-H), and of light-induced sample (90%-L). Peak areas for 100% standard (3A), 90% standard (3B), heat-degraded (3C) and light-induced (3D) solutions were 28631172, 26111730, 17085420 and 20694322, respectively. The main peak at approximately 5.8 min corresponds to chloramphenicol. In case of sunlight-induced and heat-degraded samples, secondary peaks arise, mainly at approximately 7.2 min.

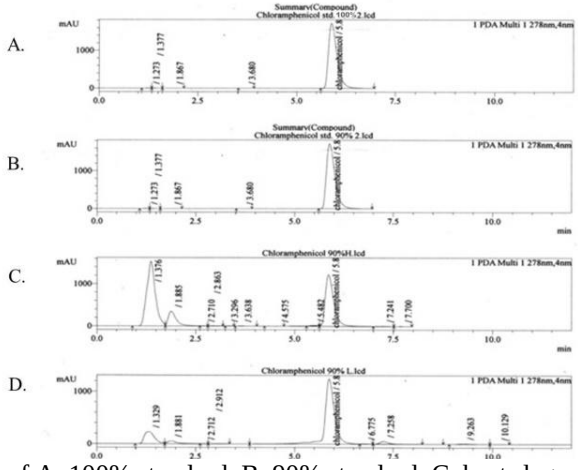

Figure 3: Chromatogram of A. 100% standard, B. 90% standard, C. heat-degraded (90%-H) and D. light-induced (90%-L) products of chloramphenicol at the fixed wavelength of 278 nm

An important finding of this study was that the calibration curves were linear for 21 µg, 24 µg, 27 µg and 30 µg of chloramphenicol in 1 ml solution and the correlation coefficients (r) were between 0.9994 and 0.9989 (average r =0.9992). The potency values for 90% light-induced and 90% heat-degraded chloramphenicol drop samples were calculated by HPLC as 78.41±0.74% and 64.52±0.54% (**Table 3**). In case of the agar disc diffusion method, values for those same light-induced and heat-degraded samples were similar (79.82±1.38% and 65.73±1.30%, respectively).

Table 3: Concentration of standard, light-induced and heat-degraded chloramphenicol samples determined by HPLC method

Title	Ret. Time	Area	Height	Conc.
Standard chloramphenicol (100%)	5.830	28631172±1755215	1811332	98.95
Standard chloramphenicol (90%)	5.890	26111730±1697153	1722449	90.55
90% light-induced chloramphenicol (90%-L)	5.883	20764322±608276.8	1244050	71.01
90% heat-degraded chloramphenicol (90%-H)	5.864	17085420±587325.5	1217139	59.29

IV. Discussion

Though chloramphenicol was reported to be a heat-stable antibiotic, this result supported the finding that photolytic degradation eliminates this antibiotic in aqueous media whereas concentration of the heat degraded product got significantly reduced [21, 22]. In similar studies, microbiological assays (using a gram-positive bacteria, *Staphylococcus aureus*) for determining cefuroxime sodium and cefazolin sodium in pharmaceutical formulations were validated with a linearity value of r = 0.9998 and 0.9999, RSD = 1.56% and <2% and accuracy of 101.58% and 99.92%, respectively [23, 24]. So, results of the bioassay method were close to the true concentration value of tested samples. The low RSD value (0.565) confirmed the efficiency of this method whereas reproducible results with a low response variation showed its potential as an independent assay. Such a trend of results can be compared with the results obtained for ceftriaxone sodium determined by another microbiological assay [25]. The standard error mean of those samples (100% and 90% standard, 90% light-induced and heat-degraded solution) were 0.07, 0.38, 0.8, 0.75, respectively (**Table 2**). The lower values of SEM further indicate the accuracy of this method comparable to that of the HPLC method.

It was observed that the decomposition products of chloramphenicol were not microbiologically active. As the impurities and degradation products did not hamper to assess the analyte, this microbiological assay can be recommended to be a precise and handy one.

A previous study on the development and validation of a microbiological assay for orbifloxacin showed linearity (r = 0.9992) and preciseness with a relative standard deviation (RSD) of 2.88% [9]. A novel and rapid microbiological assay reported by the same authors showed good results (linear, r = 0.9994, precise, RSD = 2.06% and accurate, recovery = 99.71%) for ciprofloxacin hydrochloride ophthalmic solution against *staphylococcus epidermidis* [26]. In another agar diffusion bioassay, an ophthalmic solution of azithromycin was checked against *Bacillus subtilis* at a concentration range of 50-200 µg/ml with good linearity value (r = 0.9999) [27]. In this study, r= 0.9882 was found in the selected range of 3-30 µg/ml whether the precision was 99.87 (RSD = 0.13%). The mean accuracy of the microbiological assay for orbifloxacin was 100.31% (RSD = 1.04%) and in case of chloramphenicol eye drop, the mean accuracy was found to be 98.25% with an RSD value of 0.56% (**Table 1**).

In a microbiological assay performed on chlorhexidine digluconate in an aqueous solution, the RSD value for inter-day precision was 2.94% with good linearity (r = 0.9999) whereas the accuracy was 99.03% [28]. Similar to this study, both microbiological method and HPLC method were tried on fluconazole in pharmaceutical injectable preparations [17]. In that case, the mean recovery was found to be 99.25% for HPLC and 98.89% for bioassay. In another microbial bioassay using *Bacillus pumilus* for the quantification of levofloxacin, the method was correlated with HPLC using the same sample and estimated potencies were found to be 100.90% and 99.37%, respectively [29]. In our study on chloramphenicol eye drop, those values were 98.95 (data not shown) and 98.25, respectively. It indicated that these methods have been successfully validated and microbiological method may be considered for the routine analysis of chloramphenicol eye drop.

To maintain the quality of pharmaceutical preparations, a validated analytical method for potency determination is very important. The microbial bioassay method of chloramphenicol 0.5% eye drops for potency determination was found to be a reliable method comparing to the HPLC method. It can offer distinct advantages of simplicity, accuracy and sensitivity in analyzing chloramphenicol eye drop formulation and has not yet been reported for chloramphenicol in any pharmacopeia. Microbiological assay method has the advantage over HPLC method to determine the bioactivity of an antibiotic as well as its potency. It can also be used as a complementary method to HPLC method. Potency of the standard solution of marketed chloramphenicol 0.5% eye drop was found to be 98.62% by this method whereas the HPLC method determined the value to be 98.95%; which are significantly identical.

V. Conclusion

Despite of having some biological errors, microbiological assay has the potential to achieve a precision similar to the HPLC method and results showed that this bioassay method can be reliable for the potency estimation of chloramphenicol eye drops.

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