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Method Development of Quality Control for Pharmaceutical Products

Rajia, Sultana

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METHOD DEVELOPMENT OF QUALITY CONTROL

FOR PHARMACEUTICAL PRODUCTS

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BY

SULTANA RAJIA

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INSTITUTE OF BIOLOGICAL SCIENCES
UNIVERSITY OF RAJSHAHI
RAJSHAHI-6205
BANGLADESH

Dedicated

To

My Beloved Son

Anirban Hasan

And

Respected Parents

DECLARATION

I do hereby declare that the materials embodied in this thesis entitled "Method Development of Quality Control for Pharmaceutical Products" prepared for submission to the Institute of Biological Sciences (IBSc), University of Rajshahi, Bangladesh for the degree of Doctor of Philosophy, are the original research works of mine and have not been previously submitted for the award of any degree or diploma anywhere.

08.10.13

(**Sultana Rajia**)

Signature of the candidate

CERTIFICATE

This is to certify that the thesis entitled "Method Development of Quality Control for Pharmaceutical Products" has been prepared by Sultana Rajia under my guidance and supervision for submission to the Institute of Biological Sciences (IBSc), University of Rajshahi, Bangladesh for the degree of Doctor of Philosophy. It is also certified that the materials included in this thesis are the original works of the researcher and have not been previously submitted for the award of any degree or diploma anywhere.

I have gone through the draft of the thesis and found it acceptable for submission.

08.10.13

(Dr. Md. Anwar Ul Islam)

Signature of the supervisor

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Sultana Rajia

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ABSTRACT

Chloramphenicol eye drops collected from different manufacturers were exposed to sunlight and induced to heat and their potency values were determined by UV spectrophotometric method that produced incorrect high values because of the presence of degradation products. In separate experiments, the aqueous preparations of chloramphenicol (without any excipient) were subjected to 14 hours of sunlight exposure and 6 hours of heat induced at 100°C turning the sample colour from yellowish to deep yellow. These heat-degraded and light-exposed samples showed false-positive higher potency values as well as decreased efficacy against *Salmonella typhi*, *Shigella dysenteriae*, *Pseudomonas aeruginosa*, *Streptococcus agalactiae* and *Bacillus cereus* by agar disc diffusion method. The λ_{max} values in the UV-spectrum of the standard, sunlight-exposed and heat-induced samples within the wavelength range 200 - 400nm were found at 278nm, 270nm and 274nm respectively indicating that the chloramphenicol aqueous solutions became degraded and converted into other degradation products. Different experiments suggested that direct measurement of chloramphenicol content by UV-spectrophotometer is not a correct approach. So, a combination of measurement methods performed by separation of the active fractions in TLC followed by UV-spectrophotometric method was proposed and also proposed a new methods for potency determination of chloramphenicol using *E.coli DH5α* through agar disc diffusion method.

In another study, a simple, reproducible and economical UV-spectrophotometric method was developed for the determination of ciprofloxacin from its tablet dosages form. The Standard curve of ciprofloxacin in the media of 0.1N HCl and distilled water were obtained by plotting absorbance versus concentration where

calibration curve was found to be linear (R2>0.99) with optimum value of standard error for the entire analytical medium used. The percentage of potency of ciprofloxacin HCl in tablets from different companies were measured by this newly developed UV-spectrophotometric method. Comparing to the standard (99.77%), the values obtained (98.26%, 98.60% and 98.77%) were satisfactory indicating that UV-spectrophotometric method is perfect to determine the potency of ciprofloxacin tablet by taking the absorbance at 277nm for 0.1 N HCl at and 276 nm for distilled water as a diluting solution and these methods were found satisfactory results for the analysis of ciprofloxacin hydrochloride under the stress condition. A relative study for potency determination of ciprofloxacin tablet by high performance liquid chromatographic technique (HPLC) for the same companies showed a comparable results. So this method for the potency determination by UV-spectrophometer could be used when HPLC is not available.

Chapter One

INTRODUCTION

INTRODUCTION

1.1 Development of methods and their validation:

Method validation is the process used to confirm that the analytical procedure employed for a specific test is suitable for its intended use. Results from method validation can be used to judge the quality, reliability and consistency of analytical results; it is an integral part of any good analytical practice. It is the process of defining an analytical requirement, and confirms that the method under consideration has performance capabilities consistent with what the application requires. Use of equipment that is within specification, working correctly and adequately calibrated is fundamental to the method validation process. Likewise the operator carrying out the studies must be competent in the analysis under study and have sufficient knowledge of the method/analysis to draw conclusions from the observations as the validation work proceeds. Quite often method validation evolves from method development and so the two activities are often closely tied, with the validation study employing the techniques and steps in the analysis as defined by the method development.

1.2 Necessity of method validation:

Analytical methods need to be validated or revalidated when it is necessary to verify that its performance parameters are adequate for use for a particular analytical problem. For example:

a) For a Method just developed
b) Revised method or established method adapted to a new problem;
c) When a review of quality control indicates an established method is changing with time;

d) When an established method is used in a different laboratory, with different analysts or with different equipment

e) Demonstration of the equivalence between two methods, e.g. a new method and a standard. Certain areas of analytical practices, such as in clinical chemistry will specify validation requirements relevant to the method. This ensures that particular validation terminology together with the statistics used is interpreted in a manner consistent within the relevant sector. Official recognition of a method may require characterization using a collaborative study.

1.3 Strategy for the validation of methods:

The validity of a specific method should be demonstrated in laboratory experiments using samples or standards that are similar to unknown samples analyzed routinely. The preparation and execution should follow a validation protocol, preferably written in a step by step instruction format. This proposed procedure assumes that the instrument has been selected and the method has been developed. It meets criteria such as ease of use; ability to be automated and to be controlled by computer systems; costs per analysis; sample through put; turn around time; and environmental, health and safety requirements.

(a) Development of a validation protocol, an operating procedure or a validation master plan for the validation

(b) Development of a specific validation project that defines owners and responsibilities

(c) Development of a validation project plan

(d) Adjustment of the application, purpose and scope of the method

(e) Description of the performance parameters and acceptance criteria

(f) Characterization of the validation experiments

(g) Verification of relevant performance characteristics of equipment

(h) Qualify materials, e.g. standards and reagents for purity, accurate amounts and sufficient stability

(i) Performing pre-validation experiments

(j) Adjustment of method parameters or/and acceptance criteria if necessary

(k) Performing full internal (and external) validation experiments

(l) Development of SOPs for executing the method in the routine

(m) Description of criteria for revalidation

(n) Characterization of types and frequency of system suitability tests and/or analytical quality control (AQC) checks for the routine

(o) Documentation of validation experiments and results in the validation report

In many cases, methods are developed and validated in service laboratories that are specialized in this task. When the method is transferred to the routine analytical laboratory, care should be taken that the method and its critical parameters are well understood by the workers in the departments who apply the method. A detailed validation protocol, a documented procedure for method implementation and good communication between the development and operation departments are equally important. If the method is used by a number of departments, it is recommended to verify method validation parameters and to test the applicability and usability of the method in a couple of these departments before it is distributed to other departments. In this way, problems can be identified and corrected before the method is distributed to a larger audience. If the method is intended to be used by just one or two departments, an analyst from the development department should assist the users of the method during initial operation. Users of the method should be encouraged to give constant feedback on the applicability and usability of the method to the development department. The latter should correct problems if any arise. A method should be developed with the

goal to rapidly test preclinical samples, formulation prototypes, and commercial samples. As the methods development and validation processes advance, the information gathered is captured in the design and subsequent improvement of the method. Ideally, the validation protocol should be written only following a thorough understanding of the method's capabilities and intended use. The validation protocol will list the acceptance criteria that the method can meet. Any failure to meet the criteria will require that a formal investigation be conducted.

1.4 Dependence of analytical performance characteristics on the methods of analysis:

The required validation parameters, also termed *analytical performance characteristics*, depend upon the type of analytical method. Pharmaceutical analytical methods are categorized into five general types:

[a] Identification tests

[b] Potency assays

[c] Impurity tests: quantitative

[d] Impurity tests: limit

[e] Specific tests.

The efficient development and validation of analytical methods are a critical elements in the development of pharmaceuticals. Success in these areas can be attributed to several important factors, which in turn will contribute to regulatory compliance. Experience is one of these factors - both the experience level of the individual scientists and the collective experience level of the development and validation department. A strong mentoring and training program is another important factor for ensuring successful methods development and validation. Companies must maintain an appropriate level of expertise in this important dimension of developing safe and effective drugs through an efficient quality control division.

1.5 Role of quality control division in the development of pharmaceutical analysis methods:

Quality control division is the heart of any pharmaceutical industry as it is an essential operation of the drug manufacturing companies. It is a system of routine technical activities, to measure and control the quality of the produced drugs. These drugs may be either new entities or partial structural modification of the existing one. It is expected that a quality control division will conduct the efficient and sophisticated pharmaceutical analysis methods regardless of the analyte class or composition of the product formulation. Sometimes small pharmaceutical companies cannot follow the existing analytical procedures for a number of particular drugs due to the expensive reagents and solvents. Under this condition, standards and analytical procedures for those drugs may not be available in the pharmacopoeias and there are scopes to develop the procedures.

1.6 Degradation of drugs and formation of degradation products:

Drug degradation occurs by a large number of possible reactions that includes hydrolysis, oxidation or degradation by light. This is because of the chemistry of many of the functional groups in drug molecules and the ubiquitous presence of water and oxygen [23]. Even when factors such as water, oxygen and light have been controlled, degradation will still occur, but at a reduced rate. The environmental factors that are known to influence the extent and rate of degradation are heat, moisture, oxygen, light and pH changes. Oxygen in the presence of light causes the oxidation of many pharmaceutical compounds, especially light-sensitive formulation [62]. To predict the shelve-lives of the final products, information about the stability of drug components and drug formulations is essential. Chemical stability studies are conducted under normal storage or experimental conditions or under exaggerated conditions (accelerated

stability testing) and the results extrapolated to normal condition [18]. The procedure usually involves the monitoring of the rate of decomposition of the parent compound or the rate of formation of the degradation product [47].

1.7 Degradation of chloramphenicol encourages to develop a new assay method:

The degradation of drugs like chloramphenicol takes place through mixed chemical pathways and mechanism, but initiated by light (Fig. 1). The rate of decomposition of chloramphenicol may therefore be dependent on the source of light and the light intensity [60].

Chloramphenicol is a broad spectrum antibiotic with wide range of clinical applications for systemic and topical uses [49]. Commercial chloramphenicol preparations are packed in different type of containers, ranging from plain glass, transparent plastics, colored plastic to amber colored bottles. The chemistry of the photo-degradation products of chloramphenicol aqueous solution at room temperature (21-30°C) and in the presence of sunlight, suggest that under the influence of light and water the drug undergoes oxidation, reduction and condensation reactions. The photolysis degradation products were isolated and identified [44, 71]. The photochemical degradation of chloramphenicol in various solvents was investigated and the following photolysis products were isolated: hydrochloric acid, 4-nitrobenzaldehyde, 4-nitrobenzoic acid, 4,4-azoxybenzoic acid and 2-amino-1-(4-nitrophenyl) propane-1,3-diol, as indicated by thin layer chromatography. From a later work concerning the quality control of a pharmaceutical preparation (0.24% eye drops), it has been found that under the influence of sunlight nitroso compounds are formed; p-nitrobenzene, p-nitrobenzaldehyde and p-nitrosobenzoic acid. After one year storage in refrigeration (0-4°C), it has been demonstrated that there is nearly 20% less breakdown of chloramphenicol content to the hydrolysis product 2-amino-1-(4-nitrophenyl) propane-1,3-diol in topical eye drops, when compared

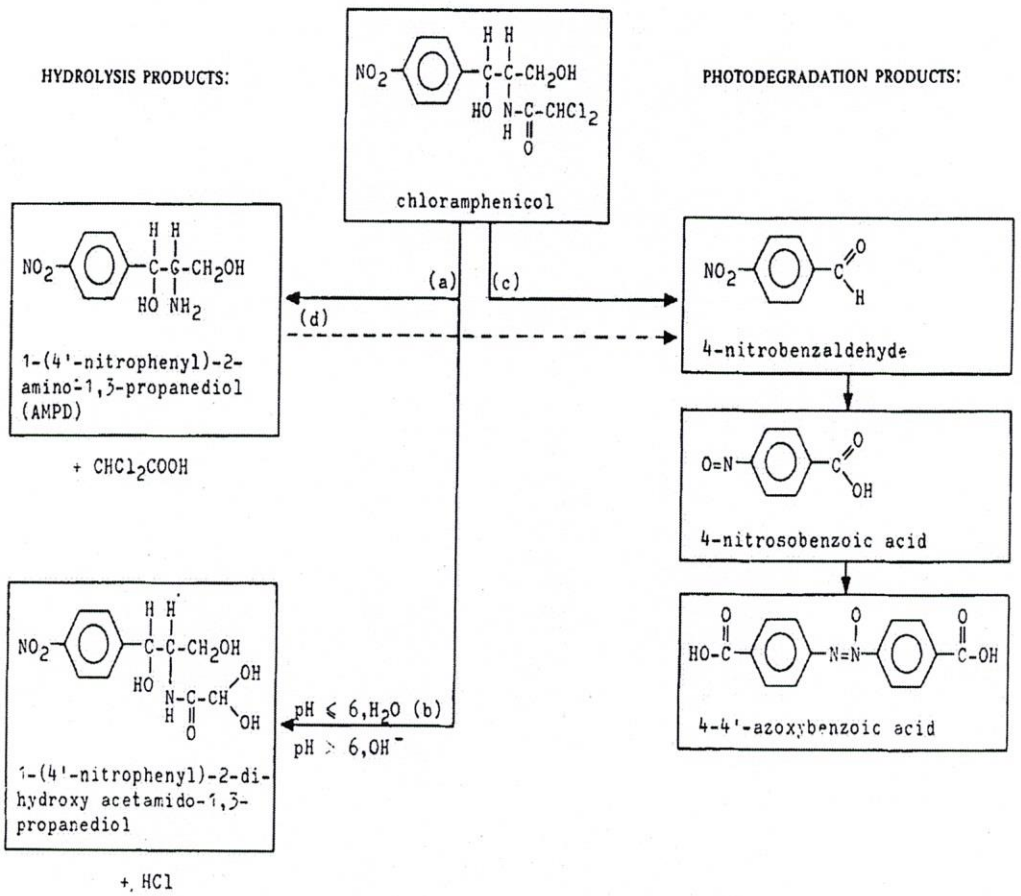


Fig. 1: Degradation pathways of chloramphenicol in aqueous solutions.

with drops stored for the same length of time at room temperature (20-25°C) [44]. The degree of thermal degradation would presumably be even greater at higher room temperature, as found in tropical countries. Knowledge of decomposition of chloramphenicol is essential in order to access the storage conditions of chloramphenicol preparations. There is also no gain in saying the fact that instability posses serious for drug products distributed and used in tropical climates because of extremes of temperature and humidity conditions [61]. Such instability may decrease the therapeutic activity of the preparation or cause the appearance of a toxic substance formed as a degradation product. If the antimicrobial activity of these drugs cannot be assured, then problems are created for patients, doctors and the world at large, due to inadequate

therapeutic responses and the theoretical development of antimicrobial resistance. In Bangladesh, the average temperature during the summer is around 40°C. The light and heat sensitive drug products can be subjected to the exposure of prolonged heat and sunlight. Thus the light sensitive products like Chloramphenicol become exposed to the degradation factors causing decreased potency.

Most of the pharmaceutical companies in Bangladesh follow the UV-spectrophotometric method for the chemical assay of chloramphenicol 0.5% eye drops according to British Pharmacopoeia. It is necessary to investigate whether the UV-spectroscopic method is valid for the determination of potency of chloramphenicol 0.5% eye drops or not. If a validated and cost-effective colorimetric method can be developed then many small pharmaceutical companies will be benefited.

1.8 Development of analytical methods for ciprofloxacin:

The number of drugs introduced into the market is increasing every year. Very often there is a time lag from the date of introduction of a drug into the market to the date of its inclusion in pharmacopoeias. This happens because of the possible uncertainties in the continuous and wider usage of these drugs, reports of new toxicities (resulting in their withdrawal from the market), development of patient resistance and introduction of better drugs by competitors. Resistance to antibiotics is mainly driven by the selective pressure imposed by their inappropriate use. Especially in developing countries like Bangladesh, people do not have the minimal awareness of resistance, antibiotics and infections. They want symptomatic relief to which the health professionals respond by prescribing antibiotics for quick recovery. Cross-infection of hospitalized patients with such organisms is also problematic and relationships between patterns of antibiotic use and resistance trends have been repeatedly demonstrated [10, 57, 59, 80]

Infections with drug-resistant microorganisms causes extra cost of health care, extended stay in the hospital, sudden or prolonged health complications and increased rate of mortality [29, 42].

So another objective of this study is to develop a simple, shorter and more cost-effective analytical method for simultaneous determination of such antibiotics like ciprofloxacin. The standard curve of Ciprofloxacin in the media of 0.1N HCl and distilled water are obtained by plotting absorbance versus concentration where calibration curve may be found to be linear with optimum value of standard error for the entire analytical medium used. The maximum absorbance in the media of distilled water for ciprofloxacin will be checked at wavelength 190 - 400nm. These findings can become useful to determine the content of ciprofloxacin by UV spectrophotometric method.

1.9 Aims of the present study:

(a) Development of new method for determining the potency of chloramphenicol eye drops.

(b) Development of a simpler UV-spectrophotometric method for the simultaneous determination of ciprofloxacin tablet.

1.10 Research plan for determining the potency of chloramphenicol eye drops:

(a) Analysis of the potency of the marketed eye drops after controlled sunlight-induced degradation

(b) Study of changes in efficacy of the products before and after the treatment

(c) Determination of the antibacterial activity of sunlight-induced and heat-degraded eye drops by agar disc diffusion method

(d) Observation of any potency change in sunlight and heat-induced degraded aqueous solutions of Chloramphenicol at 200 - 400nm wavelength by observing their UV-spectra

(e) Detection of degradation of chloramphenicol aqueous solution by thin layer chromatography (TLC)

(f) Development of a new moderated method for the potency determination of chloramphenicol by combining TLC with UV spectrophotometric method

(g) Investigation of a new method for potency determination of chloramphenicol using agar disc diffusion method

1.11 Research plan for development of a simpler UV-spectrophotometric method for the simultaneous determination of ciprofloxacin tablet.

(a) Study of standard curves for ciprofloxacin using different media

(b) Comparative analysis of standard ciprofloxacin with light-exposed and heat-degraded ciprofloxacin

(c) Potency determination of ciprofloxacin samples (in different solvents) from a number of companies by UV-spectrophotometer

(d) Comparing the potency of Ciprofloxacin using H_2O and 0.1N HCl solvents by UV-spectrophotometric method and HPLC method

1.12 General discussion on chloramphenicol:

1.12.1. Discovery:

Chloramphenicol was first described as a new antibiotic produced by cultures of actinomycetes isolated from soil [27]. The soil samples from which the strains were isolated were collected from a mulched field near Caracas, Venezuela (strain ATCC 10712) and from a compost soil on the horticultural farm of the Illinois Agricultural Experiment Station at Urbana (strain ATCC 10595), respectively. It was demonstrate that this actinomycete was a new species [28]. Chloramphenicol was also isolated from the soil actinomycete *Streptosporangium viridogriseum* var. kofuense by Tamura *et al* [77] and from the marine snail *Lunatia heros* (moon

snail) by Price *et al* [67]. The dynamics of the synthesis of chloramphenicol were studied under laboratory conditions by Legator & Gottlieb [50], who showed that the peak concentration of chloramphenicol in the culture medium was reached hours after the growth peak of the microorganisms. Accumulation of the antibiotic was not intracellular. Addition of chloramphenicol to the culture medium inhibited the synthesis of the antibiotic.

Chloramphenicol is a prototypical broad-spectrum antibiotic effective against a wide variety of Gram-positive and Gram-negative bacteria, including most anaerobic organisms, rickettsiae, chlamydia and anaplasmae. Chloramphenicol was originally isolated from the soil organism Streptomyces venezuelae in 1947, but is now produced synthetically. Three common forms are used for systemic therapy, depending on the route of administration; a free base form of chloramphenicol, chloramphenicol palmitate and chloramphenicol succinate. Other formulations are also available for topical use. Chloramphenicol usually acts as a bacteriostatic, but at higher concentrations or against some very susceptible organisms it can be bactericidal. The antibiotic activity of chloramphenicol results from its interference with protein synthesis in invading microbes. It is used for the treatment of serious infections when the micro-organism is resistant to less toxic antibiotics and also when its ability to penetrate to the site of the infection is superior to less toxic alternative antibiotics. It is used in the treatment of human infection with *Salmonella typhi* (typhoid) and other forms of salmonellosis, and other life-threatening infections of the central nervous system and respiratory tract [64]. In veterinary medicine, chloramphenicol is used for the treatment of a variety of infections in animals, particularly those caused by anaerobic bacteria or those that are resistant to other antimicrobial agents. Chloramphenicol in animals is well absorbed by both oral and parenteral routes [66].

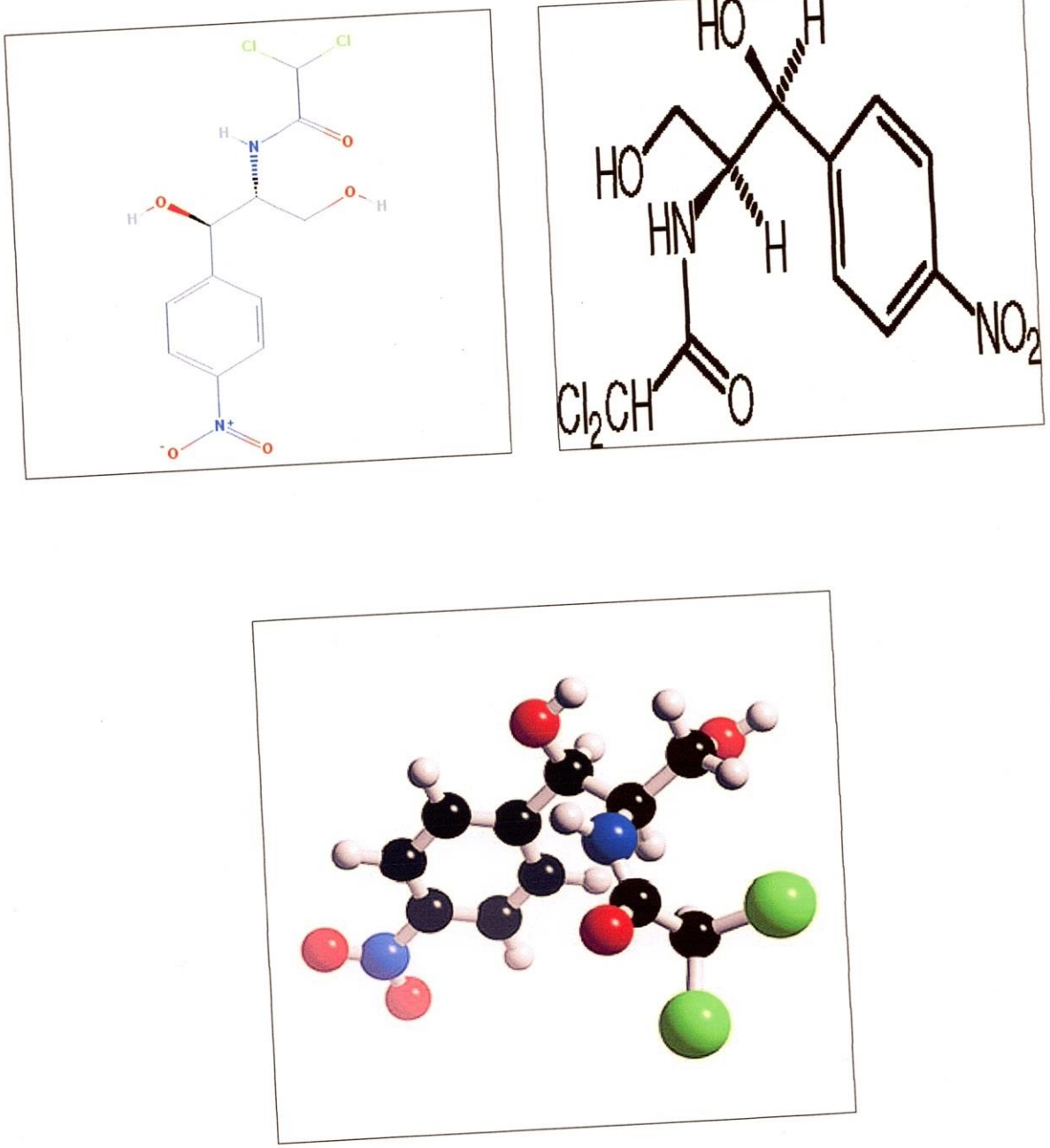


Fig. 2: General and three dimensional structure of chloramphenicol ($C_{11}H_{12}C_{l2}N_2O_5$)

The systematic (IUPAC) name of chloramphenicol is 2,2-dichloro-N-[1,3-dihydroxy-1-(4-nitrophenyl)propane-2-yl] acetamide. As shown in Fig. 2, it contains a nitrobenzene ring, an amide bond, and an alcohol function. The presence of chlorides in biologically produced organic molecules is unusual. The nitrobenzene is relevant because it leads to the formation of aromatic amines which may be carcinogenic. The amide is hydrolyzed by some resistant bacteria leading to inactivation. The alcohol serves as a functional group facilitating the formation of esters that improve chloramphenicol's water solubility.

1.12.2 Synonyms:

The IUPAC name of chloramphenicol is: 2,2-dichloro-N-[(1R,2R)-1,3-dihydroxy-1-(4-nitrophenyl)propan-2-yl]acetamide.Chloramphenicol ($C_{11}H_{12}C_{l2}N_2O_5$) also known as by the following names:

(1) D-(-)-threo-1-(p-nitrophenyl)-2-dichloroacetamido-1,3-propanediol

(2) 2,2-dichloro-N-[2-hydroxy-1-(hydroxymethyl)-2-(4-nitrophenyl) ethyl]acetamide

(3) D-threo-N-dichloroacetyl-1-p-nitrophenyl-2-amino-1,3-propanediol

(4) D(-)-threo-2-dichloroacetamido-1-p-nitrophenyl-propanediol

(5) D-threo-N-(1,1'-dihydroxy-1-p-nitrophenylisopropyl) dichloroacetamide

(6) D-(-)-threo-2,2-dichloro-N-[2-hydroxy-alpha-(hydroxyl-methyl)-p-nitrophenyl] acetmide

(7) D-(-)-threo-paranitrophenyl-1-dichloroacetamido-2-propanediol-(1,3)

1.12.3 Chemical synthesis:

Chloramphenicol can be synthesized by condensation of para-nitrobenzoyl chloride with ethyl malonate to give para-nitroacetophenone, followed by

bromination in acetic acid to form para-nitro bromoacetophenone, and reaction this with hexamethylene tetramine, followed by hydrolysis to give para-nitro aminoacetophenone; subsequent acetylation of the amine group. Treatment with aluminium isopropylate reduces the keto group to a secondary alcohol, and, after deacetylation, condensation of the amine group with methyl dichloroacetate gives chloramphenicol [4]. Chemical synthesis of chloramphenicol usually includes a resolution step to separate stereo isomers. In Japan, production by a fermentation process has also been described. The process resulted from the discovery and isolation of a new strain of microbe and does not require separation of stereoisomers [5].

A fermentation process has been described that does not require separation of stereoisomers [41]. The first commercial production of chloramphenicol in the United States was reported in 1948. U.S. production of chloramphenicol was estimated to be greater than 908 kg (2,002 lb) in 1977 and 1979. U.S. imports for these years were estimated at 8,150 kg (17,970lb) and 8,200 kg (18,080 lb), respectively (HSDB 1995).

Chloramphenicol is produced in Brazil, China, Chechoslovakia, the Federal Republic of Germany, Hungary, Italy, India, Israel, Japan, Mexico, Romania, South Africa, Spain and the USSR and has also been produced in France, Switzerland, the United Kingdom and the USA.

Table 1: Physical and chemical properties of chloramphenicol.

Properties	Information	Reference
Chemical name	Acetamide, 2,2-dichloro-N-[2-hydroxy-1-(hydroxymethyl)-2-(4-nitrophenyl)ethyl]	BP
Color	white to grayish white or yellowish white	[16]
Odor	Having a faint odor	BP
Taste	Bitter burning	[40]
Solubility	Slightly soluble in water(0.25%), freely soluble in alcohol, in propylene glycol, in acetone, and in ethyl acetate	USP
Optical Rotation	Is reasonably stable in neutral or moderately acid solutions. Its alcohol solution is dextrorotatory and its ethyl acetate solution is levorotatory.	USP
Physical state	Fine, crystals, needle-like crystals or elongated plates.	USP
Melting point	148 to 150□ c	http: //chemicalland21.com/lifescience/phar/CHLORAMPHENICOL.htm
pH	Neutral to litmus	[40]
Vapor pressure (mm Hg)	1.73	[40]
Half-life in human	1.6 to 4.6 hours	[40]
Spectroscopy data	UV,IR, NMR and Mass spectra have been reported	
Reactivity	The nitro group is readily readily reduced to the amine	

1.12.4 Types of chloramphenicol:

1. Chloramphenicol (C11H12Cl2N2O5.{R-9}) - Acetamide, 2,2-dichloro-N-[2-hydroxy-1-(hydroxymethyl)-2-(4-nitrophenyl)ethyl]-, [R-(R*},R*)]-{R-9}

2. Chloramphenicol palmitate(C27H42Cl2N2O6.{R-9}) - Hexadecanoic acid, 2-[(2,2-dichloroacetyl)amino]-3-hydroxy-3-(4-nitrophenyl) propyl ester, [R-(R*,R*)]-.{R-9}

3. Chloramphenicol sodium succinate (C15H15Cl2N2NaO8.{R-9}) - Butanedioic acid, mono[2-[(2,2-dichloroacetyl)amino]-3-hydroxy-3-(4-nitrophenyl)propyl]ester, monosodium salt, [R-(R*,R*)]-.{R-9}

1.12.5 Solubility of chloramphenicol:

In purified water, the solubility of chloramphenicol is only 0.25%. Several approaches such as pH adjustment, complexation, micellar solubilization, cosolvancy were successfully employed to enhance the solubility.

Table 2: Solubility of chloramphenicol in different solvent (ChemFinder 2000, HSDB 1995).

Solvent	**Solubility**
Water at 25c	Slightly soluble, 2.5 mg/ml.
Propylene glycol	150.8 mg/ml.
50% Acetamide	5%
Chloroform	Soluble
Methanol	Very soluble
Ethanol	Very soluble
Butanol	Very soluble
Ethyl acetate	Very soluble
Acetone	Very soluble
Ether	Soluble
Benzene	Insoluble
Petroleum ether	Insoluble
Vegetable oils	Insoluble

1.12.6 Solubility and other information about chloramphenicol palmitate and chloramphenicol sodium succinate:

Chloramphenicol Palmitate USP - Insoluble in water; freely soluble in acetone and in chloroform; soluble in ether; sparingly soluble in alcohol; very slightly soluble in hexane.

Chloramphenicol Sodium Succinate USP—Freely soluble in water and in alcohol.

Molecular weight:

Chloramphenicol palmitate—561.54 {R-9}

Chloramphenicol sodium succinate—445.18 {R-9}

Description:

Chloramphenicol Palmitate USP - Fine, white, unctuous, crystalline powder, having a faint odor.

Chloramphenicol Sodium Succinate USP - Light yellow powder .

1.12.7 Types of chloramphenicol used in dosage forms:

Chloramphenicol (as base): Tablet, Capsule, Ear drops, Eye Drops

Chloramphenicol palmitate: oral suspension

Chloramphenicol sodium succinate: for injection formation.

1.12.8 Biosynthesis of chloramphenicol:

The biosynthetic route of chloramphenicol starts with the general shikimate pathway for assembling aromatic structures. It then branches at chorismic acid to generate p-amino-phenylalanine, which serves as an advanced precursor of the p-nitrophenylserinol moiety of chloramphenicol [35, 51]. 3c-O-Acetyl-chloramphenicol, which is commonly formed from chloramphenicol by many resistant bacteria, has also been isolated from the antibiotic-producing organism. It

has been suggested that it is a protected intermediate in chloramphenicol biosynthesis, implicating acetylation as a self-resistance mechanism in the producing organism [32]. 3c-O-Acetyl-chloramphenicol esterase activity was detected in cell-free extracts of strains of *Streptomyces venezuela*, other *Streptomyces spp.* and *Streptosporangium viridogriseum var. kofuense*, which produced chloramphenicol [58].

1.12.9 Mechanism of action:

Chloramphenicol is bacteriostatic antibiotic (that is, it stops bacterial growth). It is a protein synthesis inhibitor, inhibiting peptidyl transferase activity of the bacterial ribosome, binding to A2451 and A2452 residues in the 23S rRNA of the 50S ribosomal subunit, preventing peptide bond formation (Fig. 3). While chloramphenicol and the macrolide class of antibiotics both interact with ribosomes, chloramphenicol is not a macrolide. Chloramphenicol directly interferes with substrate binding; macrolides sterically block the progression of the growing peptide.

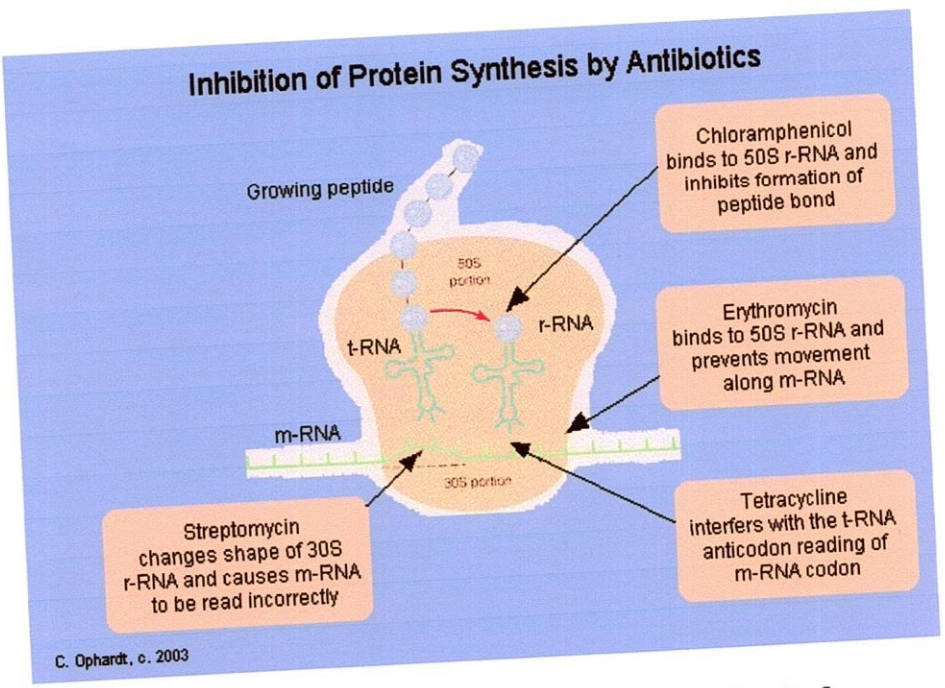


Fig. 3: Mechanism of action of chloramphenicol

1.12.10 Resistance:

Except for rickettsial organisms, resistance to chloramphenocol is increasing. There are three mechanisms of resistance to chloramphenicol: reduced membrane permeability, mutation of the 50S ribosomal subunit and elaboration of chloramphenicol acetyltransferase. It is easy to select for reduced membrane permeability to chloramphenicol *in vitro* by serial passage of bacteria, and this is the most common mechanism of low-level chloramphenicol resistance. High-level resistance is conferred by the *cat*-gene; this gene codes for an enzyme called chloramphenicol acetyltransferase which inactivates chloramphenicol by covalently linking one or two acetyl groups, derived from acetyl-S-coenzyme A, to the hydroxyl groups on the chloramphenicol molecule. Acetylation of the hydroxyl group, hydrolysis of the amide group or reduction of the nitro group prevents chloramphenicol from binding to the ribosome. Resistance-conferring mutations of the 50S ribosomal subunit are rare. Plasmids coding for acetyltransferase may be transferred by transduction resulting multiple drug resistance to chloramphenicol, tetracycline, and streptomycin.

1.12.11 Pharmacokinetics:

(A) Absorption

Because of the poor water solubility of chloramphenicol, dissolution (and hence bioavailability) is not uniform. Free chloramphenicol is rapidly absorbed after IV (Intra venous) and PO (***per os***) administration.

Chloramphenicol palmitate (prepared as a suspension), is intended for oral administration. The ester is hydrolyzed by lipases in the small intestine prior to absorption of the chloramphenicol. The palmitate ester is inactive.

Chloramphenicol succinate (intravenous administration), is a water soluble dose form. This ester must be hydrolyzed to release active chloramphenicol. Hydrolysis occurs in plasma, liver, lungs, and kidneys. IM injection is not recommended because absorption and hydrolysis is inconsistent and incomplete.

1.12.12 Distribution:

Chloramphenicol is ideally distributed throughout the body. Volume of distribution varies with species. For humans and horses it is approximately 1 L/kg. For the cat, values as high as 2.36 L/kg have been reported. CSF levels are 21-50% of those in plasma, but in the presence of inflammation, this may reach 89%. Liver (and bile), kidney (and urine), milk, and placenta all have high concentrations. Therapeutic concentrations are even found in eyes. Protein binding is in the middle range, 25-60% in humans.

1.12.13 Elimination:

Chloramphenicol is eliminated primarily by biotransformation. In humans, as much as 90% is eliminated as the chloramphenicol glucoronide conjugate. The majority of the remaining 10% is eliminated in the kidney by glomerular filtration. This is sufficient to produce therapeutic concentrations in the urine. In other species, e.g., dog and rat, urinary elimination is still dominant, but increased amounts are elimination in bile as aromatic amines. Even in humans, as much as 10% may be eliminated unchanged in the bile, but much of this is reabsorbed and ultimately eliminated via the urine.

1.12.14 Therapeutic uses:

The original indication of chloramphenicol was in the treatment of typhoid, but the now almost universal presence of multi-drug resistant *Salmonella typhi* has meant that it is seldom used for this indication except when the organism is known to be

sensitive. Chloramphenicol may be used as a second-line agent in the treatment of tetracycline-resistant cholera.

Chloramphenicol is active against the three main bacterial causes of meningitis: *Neisseria meningitidis*, *Streptococcus pneumoniae* and *Haemophilus influenzae*. In the West, chloramphenicol remains the drug of choice in the treatment of meningitis in patients with severe penicillin or cephalosporin allergy and GPs are recommended to carry intravenous chloramphenicol in their bag. In low income countries, WHO recommended to use oily chloramphenicol as a first-line drug to treat meningitis.

Because of its excellent BBB penetration (far superior to any of the cephalosporins), chloramphenicol remains the first choice treatment for staphylococcal brain abscesses. It is also useful in the treatment of brain abscesses due to mixed organisms or when the causative organism is not known.

Chloramphenicol has been used in the U.S. in the initial empirical treatment of children with fever and a petechial rash, when the differential diagnosis includes both *Neisseria meningitidis* septicaemia as well as Rocky Mountain spotted fever, pending the results of diagnostic investigations. Chloramphenicol is also effective against *Enterococcus faecium*, which has led to it being considered for treatment of vancomycin-resistant enterococcus.

1.12.15 Interaction with other drugs:

Drug interaction problems involving chloramphenicol stem from two basic properties. First, therapeutic dosages of chloramphenicol can depress hepatic biotransformation of other drugs. Second, problems arise when a bacteriostatic drug is combined with bactericidal drugs. Chloramphenicol is a potent inhibitor of the cytochrome P_{450} isoforms CYP2C19 and CYP3A4 in the liver [65].

Use of the drugs like phenobarbital, amoxicillin, erythromycin, clindamycin, tylosin, chlorpropamide, doxercalciferol, entacapone, paricalcitol, phenobarbital, phenytoin, ramelteon, rifampin, tolbutamide, warfarin etc. may interfere with the activity of chloramphenicol. Besides, vaccines, B- vitamins and iron can also interact with chloramphenicol. Phenytoin, primidone, phenobarbital, cyclophosphamide etc may last longer than expected if used concurrently with chloramphenicol.

1.12.16 Dosing (for the treatment of bacterial infections):

Dose is based on the body weight of the patients.

For oral dosage forms capsules and suspension:

Adults and teenagers - The usual dose is 12.5 milligrams (mg) per kilogram (kg) (5.7 mg per pound) of body weight in every six hours.

Infants (up to 2 weeks of age) - The usual dose is 6.25 mg per kg (2.8 mg per pound) of body weight in every six hours.

Infants 2 weeks of age and older - The usual dose is 12.5 mg per kg (5.7 mg per pound) of body weight in every six hours; or 25 mg per kg (11.4 mg per pound) of body weight in every twelve hours.

For injection dosage form:

Adults and teenagers - usual dose is 12.5 mg per kg (5.7 mg per pound) of body weight in every six hours.

Infants up to 2 weeks of age: usual dose is 6.25 mg per kg (2.8 mg per pound) of body weight in every six hours

Infants 2 weeks of age and older: usual dose is 12.5 mg per kg (5.7 mg per pound) of body weight in every six hours; or 25 mg per kg (11.4 mg per pound) of body weight in every twelve hours.

1.12.17 Concerns and cautions:

Before taking chloramphenicol, the following conditions must be taken under consideration:

- anemia or other blood disorders
- dental problems
- glucose 6-phosphate dehydrogenase (G6PD) deficiency
- liver disease
- kidney disease
- other chronic illness
- porphyria
- an unusual or allergic reaction to chloramphenicol, other antibiotics or preservatives
- pregnant or trying to get pregnant
- breast-feeding

1.12.18 History of antimicrobial susceptibility tests performed against chloramphenicol:

Test of susceptibility for chloramphenicol against *Haemophilus influenzae* by disk diffusion method was described by G Kronvall *et al* and Snell JJ *et al* from different countries [48, 72].

G Kronvall *et al* performed the test at six different regions in Sweden- A total of 601 clinical isolates of *Haemophilus influenzae* isolated in Sweden were tested for chloramphenicol susceptibility by using agar dilution MIC determinations and disk diffusion tests. For seven strains MICs were 4 micrograms/ml or higher, and for one strain the MIC was 2 micrograms/ml. All eight strains produced chloramphenicol acetyltransferase. For the remaining 593 strains, MICs were less than or equal to 1 microgram/ml, and the MICs for 50% and 90% of the strains were both 0.5 microgram/ml. Disk diffusion tests carried out by using revised interpretive criteria introduced in 1984 by the Swedish Reference Group for Antibiotics correctly identified the 593 strains as susceptible and the 8 strains as resistant. Quality assessments were performed in 29 clinical microbiology laboratories. The revised criteria for chloramphenicol disk diffusion testing gave rise to false resistance results in some laboratories. The interpretive accuracy improved when the inter-laboratory variation was compensated for by using adjusted breakpoints. Such revision was possible through peak correction, single-strain regression analysis, and standard curve regression analysis. Peak-corrected breakpoints improved the accuracy from an overall incidence of false-resistant isolates of 4.4% to 2.3%. Single-strain regression analysis and standard curve regression analysis provided laboratory- and species-specific breakpoints which reduced false resistance rates of 0.14% and 0%, respectively.

Six strains of *Haemophilus influenzae* were distributed to 417 United Kingdom laboratories who were asked to test susceptibility of the strains to chloramphenicol. The incidence of reports recording sensitive strains as resistant was 1% chloramphenicol and resistant strains as sensitive was 20% chloramphenicol as described by Snell JJ *et al* [72].

Antimicrobial susceptibility testing of *Streptococcus pneumoneae* was defined by Snell JJ *et al* in 1988 [73]. Six strains of *Streptococcus pneumoniae* were

distributed to 405 United Kingdom laboratories who were asked to test the susceptibility of the strains to penicillin, tetracycline, chloramphenicol and erythromycin and to provide details of methodology to test the standards of susceptibility testing. High error rates were seen only in failure to detect moderate resistance to penicillin (12%) and resistance to chloramphenicol (16%). Increased error rates were associated with several methods or practices. These included the use of certain culture media; failure to standard disc the inoculum; inoculation by loop rather than by swab; failure to use control organisms; failure to measure zone sizes; the use of discs containing a high content of penicillin to test susceptibility to penicillin, and the use of high content discs for testing erythromycin, tetracycline, and chloramphenicol.

1.12.19 Literature review:

Aboshiha J *et al (*1994) showed that chloramphenicol is thermo-labile [1], and it is recommended in the UK that chloramphenicol preparations are stored under refrigeration as per manufacturers' instructions, in order to minimize thermal degradation [52]. After 1 year's storage in refrigeration (0–4^{0}C), it has been demonstrated that there is nearly 20% less breakdown of chloramphenicol content in topical eye-drops to the hydrolysis product 2-amino-1-(4-nitrophenyl) propane-1,3-diol, when compared with drops stored for the same length of time at room temperature (20–25^{0}C) [44]. The degree of thermal degradation would presumably be even greater at higher room temperatures, such as found in tropical countries. Chloramphenicol is therefore best transported and stored in a 'cold chain' (i.e a temperature-controlled supply chain). However, the refrigeration of generic chloramphenicol eye drops as observed anecdotally in several sites throughout India was often not applied. Such breakdowns in the cold chain are not new; indeed the same problem was faced in India during the 1950s and 1960s in the effort to eradicate smallpox by mass immunization with thermo-labile vaccines [17, 69].

Chloramphenicol molecule is quite small (MW = 323.13 Da) and sufficiently stable under ordinary storage conditions, the presence of a nitrobenzene moiety makes this compound scarcely soluble in water. As a result, hydrophilic prodrugs (e.g., chloramphenicol sodium succinate and chloramphenicol palmitate) or oily vehicles are generally used for the preparation of pharmaceutical compositions [31, 74]. But due to possible increase of solubility and antimicrobial activity, Zuorro A *et al* proposed that, the possibility of including Chloramphenicol in water-soluble cyclodextrins (CDs) is explored and using the resulting complex a formulation of aqueous eye drops at high antibiotic concentration is possible [81]. Two chemically modified β-cyclodextrins, 2-hydroxypropyl-β-cyclodextrin (HPβCD) and methyl-β-cyclodextrin (MβCD) were used. It was observed on the UV absorption spectrum of chloramphenicol that the effect of the presence of increasing amounts of HPβCD and MβCD increased the aqueous solubility of chloramphenicol linearly and the antibiotic activity of CHL is not impaired upon CD complexation. Secondly, no significant difference appeared in the behavior of CHL-HPβCD and CHL- MβCD complexes.

Bakare-Odunola *et al* performed experiments by exposing aqueous solutions of chlorampenicol, reference solutions and eye drops to sunlight, ultraviolet (UV) radiation at 365nm wavelength and red light for varying lengths of time [7]. The kinetics of decomposition was studied using thin layer chromatography (TLC) techniques and UV spectrophotometric methods of analysis. The half-lives ($t½$) of decomposition were 20.47h, 22.02h and 1052. 0 h; the rate constant (K) values were 3.39 × 10-2 h^{-1} 3.15 × 10-2 h^{-1} and 0.07× 10-2 h^{-1} in sunlight, UV light and in red light respectively. The average half-lives of decomposition were 21.34 ± 2.70 and 19.66 ± 1.16, 861.36 ± 87.95h; the rate constant (K) values were 3.29 ± 0.42 × $10\text{-}2^{h\text{-}1}$, 3.52 ± 0.21 × $10\text{-}2^{h\text{-}1}$ and 0.08 ± 0.01 × $10\text{-}2^{h\text{-}1}$ for the chloramphenicol preparations in sunlight, UV light and in red light respectively. The time taken for the half reactant to decompose ($t½$) was higher compared with in sunlight or UV

light. The rate constant of decomposition in red light was also lowered in red light than in sunlight or in UV light. The chloramphenicol preparations were more stable in red light than in sunlight or UV light. The study showed the importance of proper storage condition of pharmaceuticals.

The Formulary of the Dutch Pharmacists (FNA) includes formulations for Chloramphenicol eye drops 0.25% and 0.5% [The latter practically corresponded to British Pharmaceutical Codex [13]. The preparations are used for treatment of superficial infections. In the 0.25% eye drop solution, boric acid is used to obtain iso-osmosis. The pH of the solution is 4.7. At that pH, the solubility of chloramphenicol is not high enough to prepare a 0.5% solution. A solubility of 5 gm/l of chloramphenicol is achieved by increasing the pH to 7.2 using borax, which had a solubilizing effect in addition to a pH increasing effect. This solubilizing and even stabilizing effect has been mentioned in the literature [53].

James and Leach showed that the increased solubility of chloramphenicol in the presence of borax was not only due to the influence of borax on the pH of the solution but also to the formation of a (2:1) chloramphenicol-borax complex [44].

In a review of chloramphenicol degradation, it was shown that considering the eye drop medium and the circumstances of preparation and storage [76]. The principal degradation reaction and products might be expected in the preparation under investigation (Figure 1).

1. Amide hydrolysis with the formation of 2-Amino-1-(4-nitrophenyl) propane-1, 3-diol (AMPD) and dichloroacetic acid. In solutions with pH< 7, this reaction majorly describes the degradation of chloramphenicol if there is no light influence. The rate of amide hydrolysis is pH-independent in the region of 2 to 7. The degradation reaction is a general acid-base

catalyzed hydrolysis which is a first order reaction with respect to the drug, and independent of the ionic strength of the medium [39].

2. Hydrolysis of the covalent bonded chlorine of the dichloroacetamide moiety with the formation of hydrochloric acid. The rate of the reaction below pH = 6 is so small that it can be neglected in comparison to the amide hydrolysis. Above pH = 6 the catalytic action of hydroxyl ions becomes increasingly important [38].
3. Photodegradation causes the formation of several products. This kind of degradation was investigated by Shih [71] and recently by Mubarak *et al* [56]. At light exposure during several hours the aqueous solution slowly turns yellow to yellowish brown due to oxidation, reduction and condensation reactions. 4-Nitrobenzaldehyde, 4-nitrobenzoic acid and 4,4-azoxybenzoic acid are formed successively. The formation of 4-nitrobenzaldehyde appeared to be, quantitatively, the most important reaction in the relatively mild circumstances during our investigation.
4. Oxidation of AMPD to 4-nitrobenzaldehyde with the formation of formaldehyde, formic acid and ammonia has been suggested by Saba *et al* [68].

Heward *et al* studied the stability of Chloramphenicol eye drops BPC by TLC [36]. They found a 2% loss after heating at 100°c for 30 minutes, a 10% loss after $4\frac{1}{2}$ months at 20°c and a 5% loss after 10 months at 4°c.

UV-Visible and infrared analysis of chloramphenicol and riboflavin and there mixtures were analyzed [46]. The UV-Vis spectral measurements are carried out on chloramphenicol and riboflavin. The sample is dissolved in methanol and a solution of 2% concentration is prepared and a smooth spectrum is obtained. The spectral recording shows a peak at 278nm for chloramphenicol. In the case of riboflavin, absorbance was measured at 446, 270 and 222nm with maximum absorbance values of 0.16, 0.43 and 0.39.

An HPLC method was described for the simultaneous determination of chloramphenicol and its degradation products, such as AMPD and 4-nitrobenzaldehyde [11]. The amount of degradation of chloramphenicol eye drop solutions containing boric acid and borax, at pH 4.7 and 7.2 respectively, was determined at 4, 21, 100 and 120°C. At pH 7.2, they found a decrease of chloramphenicol as 23%, 5%, 15% and 1% at 120°c for 20min, 100°c for 30 min, 21°c for 53 weeks and 4°c for 53 weeks respectively. At pH 4.7, they found a decrease of chloramphenicol as 9%, 4%, 44% and 11% at 120°c for 20min, 100°c for 30 min, 21°c for 53 weeks and 4°c for 53 weeks respectively. During their investigations, they found that the extent of degradation depends on the applied sterilization method, the storage temperature, the composition of solution and the influence of light during preparation.

Chatzitakis *et al* investigated the photolytic degradation of chloramphenicol in aqueous heterogeneous solutions containing n-type oxide semiconductors as photocatalysts [22]. The disappearance of the organic molecule follows approximately a pseudo-first-order kinetics according to the Langmuir-Hinshelwood model. It was observed that when TiO_2, P-25 and ZnO were present as photocatalysts, quantitative degradation of the chloramphenicol occured after 4 hours of illumination. During this time, the dechlorination of the substrate was complete, while the organic nitrogen was recovered in the form of nitrate and ammonium ions. The effect of temperature on the degradation rate of chloramphenicol showed similar apparent activation energies for all the photocatalysts. The initial apparent photonic efficiency of the photo-oxidation and the mineralization under various experimental conditions have been calculated, while the Kirby-Bauer disc diffusion method showed a 100% reduction of the drug activity after 90 min of photocatalytic treatment.

Assay: A request had been received to replace the current UV assay method in the chlocamphenicol preparation monographs by an HPLC method on the 2nd meeting of the Expert Advisory Group on Antibiotics was held at Market Towers, 1 Nine Elms Lane, London SW8 5NQ on Wednesday 20th February 2008.

The maximum limit of the breakdown product permitted in the BP for 0.5% w/v chloramphenicol eye drops intended for use in the UK is 0.040% w/v of 2-Amino-1-(4-nitrophenyl) propane-1,3-diol. This limit is arrived at by consensus among the British Pharmacopoeia Commissions Expert Advisory Groups, Panels and Working Parties and with the cooperation of manufacturers, and reflects the quality of medicinal products licensed for sale with UK [9]. 2-Amino-1-(4-nitrophenyl) propane-1, 3-diol is very toxic product. It may cause cancer, heritable genetic damage, risk of impaired fertility, possible risk of harm to the unborn child, bone marrow toxicity and disturbance or loss of vision [14].

Al-Rimawi F *et al* proposed the development of a simple and stability-indicating liquid chromatographic method (HPLC methods) is for the analysis of chloramphenicol and its related compounds [3]. They used a reversed-phase C18, 5 μm, 150mm length, and 4.6mm inner diameter column maintained at ambient temperature to separate chloramphenicol and 2-amino-1-(4-nitrophenyl) propane-1,3-diol. Regarding the mobile phase, a mixture of sodium pentanesulfonate solution, acetonitrile, and glacial acetic acid was used. In order to improve the separation and peak symmetry, the mobile phase composition was varied until optimum composition was selected (85: 15: 1, v/v). Isocratic elution at flow rate of 2.0mL/min has been employed in this study. Wavelength of 278 was selected to be used for UV detection. The column was kept at room temperature during this study. After this optimization, this method has been used for the separation of chloramphenicol from 2-amino-1-(4- nitrophenyl) propane-1,3-diol . A good separation with adequate resolution has been obtained.

The activity of chloramphenicol was explained against *Klebsiella pneumonia* C3 (O1: K66, producing porins OmpK35 and OmpK36) and a set of isogenic mutant derived from it lacking the O antigen of lipopolysaccharide (LPS), capsular K antigen, or one or both porins were determined [34]. They said that susceptibility to chloramphenicol did not change after capsule, antigen O or porin mutations. The decreased activity of chloramphenicol observed in strain KT5002 and the mutant derivatives from it was caused by the presence of plasmid pPH1JI.

The effects of chloramphenicol were suggested on *Chlamydia trachomatis* infection in neonatal conjunctivitis [37]. It was found that 26 of 127 infants with chlamydial conjunctivitis had previously received chloramphenicol eye drops. This treatment had delayed the onset and reduced the degree of edema, congestion and discharge compared with infected infants with no 'first-line' chemotherapy, but eye swabs remained positive in 22 (85%) of the chloramphenicol treated infants.

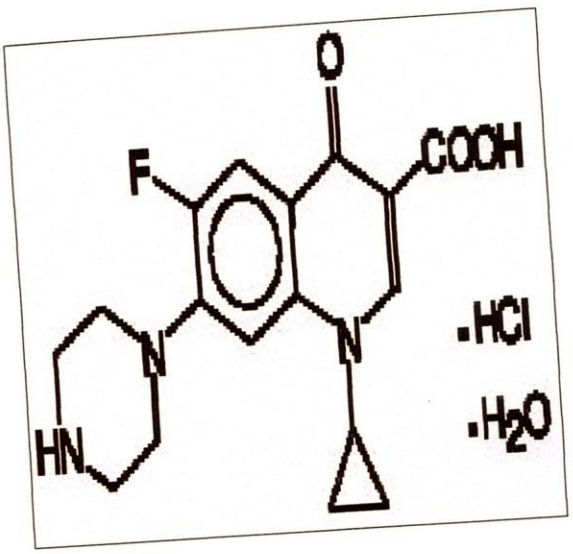
1.13. General discussion on the ciprofloxacin HCl:

1.13.1. Discovery:

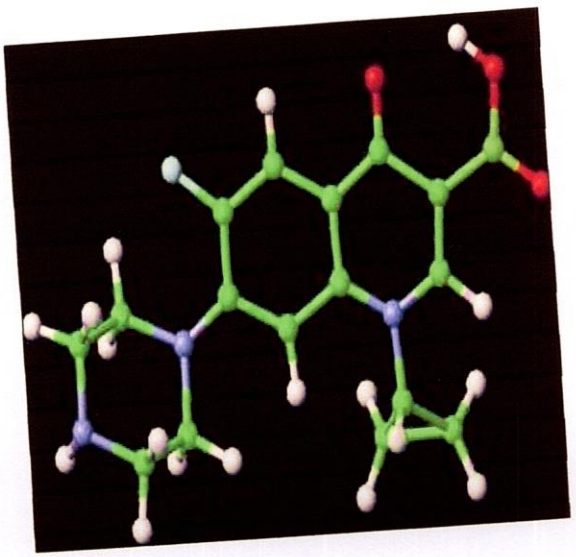
Quinolones: Since their discovery in the early 1960s, the quinolone group of antibacterial drugs has generated considerable clinical and scientific interest. Nalidixic acid, the first quinolone to be developed, was obtained as an impurity during the manufacture of quinine. Since this time, many derivatives have been synthesized and evaluated for their antibacterial potency. Two major groups of compounds have been developed from the basic molecule: quinolones and naphthyridones. Manipulations of the basic molecule, including replacing hydrogen with fluorine at position 6, substituting a diamine residue at position 7 and adding new residues at position 1 of the quinolone ring, have led to enhanced antibacterial efficacy [54]. The patent history for ciprofloxacin makes reference to a 1982 European patent (patent number 0049355), as well a German patent dated

21 January 1986. Bayer introduced ciprofloxacin in 1987 and it was later approved by the US FDA on 22 October 1987 for use in the United States to treat specific bacterial infections. In 1991, the intravenous formulation was introduced. The current US patent appears to be held by Bayer, being the assignee. The United States patent was applied for in January 1987, but was not approved until 1996 according to the patent history.

1.13.2 General discussion:



Ciprofloxacin HCl



Three-dimensional structure of Ciprofloxacin HCl

Fig. 4: Structure of ciprofloxacin HCl.

1.13.3 Synonyms:

(a)1-Cyclopropyl-6-fluoro-1,4-dihydro-4-oxo-(1-piperazinyl)-3-quniolinecarboxylicacid

(b)1-Cyclopropyl-6-fluoro-1,4-dihydro-4-oxo-7-(1-piperazinyl)-3-quinolinecarboxylicacid

(c)1-Cyclopropyl-6-fluoro-4-oxo-7-(1-piperazinyl)-1,4-dihydro-3-

quinolinecarboxylicacid

(d)3-Quinolinecarboxylicacid,1,4-dihydro-1-cyclopropyl-6-fluoro-4-oxo-7-(1-piperazinyl)-hydrochloride

(e) 3-Quinolinecarboxylic acid, 1-cyclopropyl-6-fluoro-1,4-dihydro-4-oxo-7-(1-piperazinyl)- monohydrochloride

1.13.4 Chemical synthesis:

Physical and chemical properties of ciprofloxacin HCl

Appearance: pale yellow, crystalline powder, slightly hygroscopic.

Physical State: Solid

Solubility of ciprofloxacin HCl: soluble in water (3.5 g/dl), slightly soluble in methanol (0.21 g/dl), scarcely soluble in ethanol (0.016g/dL), soluble 0.14g/dL in acetic acid. Practically insoluble in acetone, in ethyl acetate and in methylene chloride.

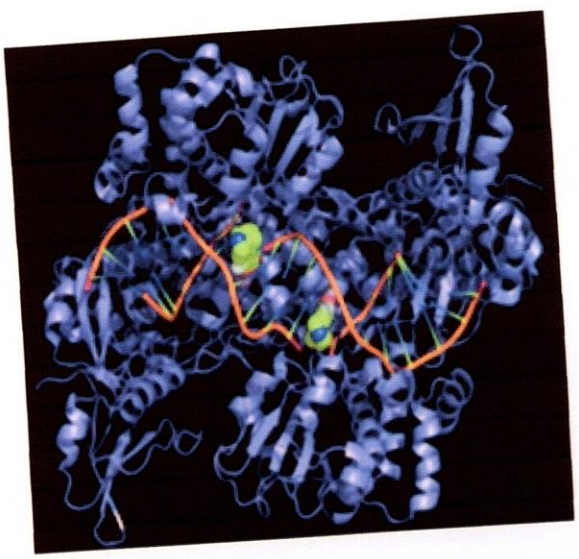
Melting Point	: >300 °C
Boiling Point	: 581.8 °C at 760 mmHg (Predicted)
pH	: between 3.5 and 4.6, except in injection (labeled as concentrated form), its pH is between 3.3 and 3.9 (U.S PHARMACOPEIA)
Storage	: Store at room temperature

Types of chloramphenicol used as dosage form:

Tablet, capsule (as pellet), Ear and Eye drops, Eye ointment, oral suspension and intravenous infusion.

1.13.5 Mechanism of action:

Quinolones can enter cells easily via porin and, therefore, Ciprofloxacin destroys microorganisms by interfering with their DNA gyrase, a type II bacterial topiosomerase necessary for Gram-negative bacterial DNA synthesis. Whereas topoisomerase IV is the target for many Gram-positive bacteria, the antibiotic is unique in that it provides many advantages over other classes of antibiotics of its class, including a low working concentration, a low contamination recurrence rate, and a non-cytotoxic effect on mammalian cells.



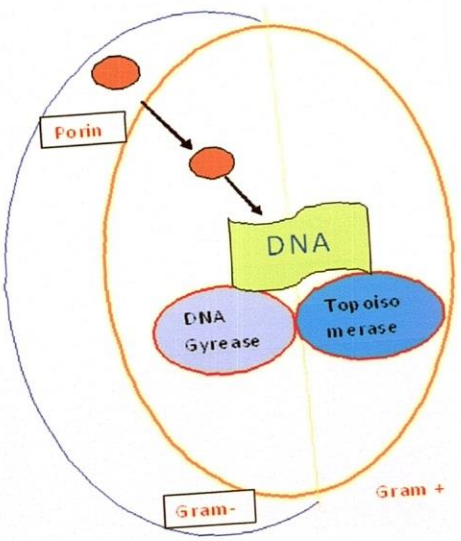


Fig. 5: Mechanism of action of ciprofloxacine.

Ciprofloxacin HCl can also kill bacteria in both the active and inactive growth phases 4. Some compounds in this class have been shown to inhibit the synthesis of mitochondrial DNA. In addition, regular feeding schedules are not interrupted when using the antibiotic are often used to treat intracellular pathogens such as *Legionella pneumophila* and *Mycoplasma pneumoniae*.

The working concentration of ciprofloxacin HCl varies with the organism, cell type, environmental conditions, and growth stage. However, a concentration of 10

µg/ml is active against most strains of bacteria and a number of Mycoplasma strains. Treatment may be continued for 12 to 20 days, after which time the antibiotic is no longer needed in the media.

1.13.6 Mechanism of toxicity:

The mechanisms of the toxicity of fluoroquinolones has been attributed to their interactions with different receptor complexes, such as blockade of the GABA a receptor complex within the central nervous system, leading to excitotoxic type effects and oxidative stress.

1.13.7 Resistance:

Ciprofloxacin is commonly used for urinary tract and intestinal infections (traveler's diarrhea), and was once considered a powerful antibiotic of last resort, used to treat especially tenacious infections. Bacteria become resistance to ciprofloxacin and other fluoroquinolones may evolve rapidly, even during a course of treatment due to by its widespread use to treat minor infections, as well as unapproved uses. Numerous pathogens including *Staphylococcus aureus*, enterococci, *Staphylococcus pyogenes* and *Klebsiella pneumoniae* (quinolone-resistant) now exhibit resistance worldwide. Ciprofloxacin is ineffective against *Treponema pallidum*.

Widespread veterinary usage of the fluoroquinolones, particularly in Europe, has been implicated. Meanwhile, some *Burkholderia cepacia*, *Clostridium innocuum* and *Enterococcus faecium* strains have developed resistance to ciprofloxacin to varying degrees.

The prevalence of resistance may vary geographically and with time for selected species and local information on resistance is desirable, particularly when treating

severe infections. This information gives only an approximate guidance whether microorganisms will be susceptible for ciprofloxacin or not.

1.13.8 Pharmacokinetics properties:

(A) Absorption: The absorption of ciprofloxacin from different regions of the human gastrointestinal tract was investigated in four healthy males using a remote-controlled drug delivery device (hf-capsule). Significant differences in AUC were observed in the control study (oral administration of ciprofloxacin solution without the hf-capsule = 100%) and after release of ciprofloxacin in the jejunum (geometric mean: 37%), the ileum (mean: 23%), the ascending colon (mean: 7%) and the descending colon (mean: 5%), whereas t_{max} showed no difference for any of the absorption sites. Ciprofloxacin release in the stomach resulted in the greatest AUC (mean: 140%). Thus, it is concluded that the main absorption site of ciprofloxacin is the upper gastrointestinal tract, up to the jejunum. Differences in presystemic metabolism of known drug metabolites along the gut could be excluded, as the pattern of urinary recovery of desethylene-, sulpho-, and oxo-ciprofloxacin and the parent compound was similar for all drug release sites.

Ciprofloxacin given as an oral tablet is rapidly and well absorbed from the gastrointestinal tract after oral administration. The absolute bioavailability is approximately 70% with no substantial loss by first pass metabolism.

(B) Distribution: Protein binding of ciprofloxacin is low (20-30%). Ciprofloxacin is present in plasma largely in a non-ionized form and has a large steady state distribution volume of 2-3 l/kg body weight. Tissue concentrations often exceed serum concentrations in both men and women, particularly in genital tissue including the prostate.

Interstitial ciprofloxacin concentrations were significantly lower than corresponding total serum drug concentrations; the interstitial-to-serum concentration ratios ranged from 0.55 to 0.73 [15].

Ciprofloxacin is present in active form in the saliva, nasal and bronchial secretions, mucosa of the sinuses, sputum, skin blister fluid, lymph, peritoneal fluid, bile, and prostatic secretions. Ciprofloxacin has also been detected in lung, skin, fat, muscle, cartilage, and bone. The drug diffuses into the cerebrospinal fluid (CSF); however, CSF concentrations are generally less than 10% of peak serum concentrations. Low levels of the drug have been detected in the aqueous and vitreous humors of the eye.

1.13.9 Elimination:

Ciprofloxacin is largely excreted unchanged both renally, and to a smaller extent, faecally. The serum elimination half-life in subjects with normal renal function is approximately 4-7 hours.

Excretion of ciprofloxacin (% of dose)	**Oral administration**	
	Urine	**Faeces**
Ciprofloxacin	44.7	25.0
Metabolites (M1- M4)	11.3	7.5

Approximately 40 to 50% of an orally administered dose is excreted in the urine as unchanged drug. After a 250 mg oral dose, urine concentrations of ciprofloxacin usually exceed 200 µg/ml during the first two hours and are approximately 30 µg/ml at 8 to 12 hours after dosing. The urinary excretion of ciprofloxacin is virtually complete within 24 hours after dosing. The renal clearance of ciprofloxacin, which is approximately 300 ml/minute, exceeds the normal glomerular filtration rate of 120 ml/minute. Thus, active tubular secretion would

seem to play a significant role in its elimination. Non-renal clearance of ciprofloxacin is mainly due to active trans-intestinal secretion and metabolism. 1% of the dose is excreted via the biliary route.

Co-administration of probenecid with ciprofloxacin results in about a 50% reduction in the ciprofloxacin renal clearance and a 50% increase in its concentration in the systemic circulation [43].

Although bile concentrations of ciprofloxacin are several fold higher than serum concentrations after oral dosing, only a small amount of the dose administered is recovered from the bile as unchanged drug. An additional 1 to 2% of the dose is recovered from the bile in the form of metabolites. Approximately 20 to 35% of an oral dose is recovered from the feces within 5 days after dosing. This may arise from either biliary clearance or trans-intestinal elimination.

With oral administration, a 500 mg dose, given as 10 ml of the 5% cipro Suspension (containing 250 mg ciprofloxacin/5ml) is bioequivalent to the 500 mg tablet. A 10 ml volume of the 5% cipro Suspension (containing 250 mg ciprofloxacin/5ml) is bioequivalent to a 5 ml volume of the 10% cipro Suspension (containing 500 mg ciprofloxacin/5ml).

1.13.10 Metabolism:

Low concentrations of four metabolites have been reported, which were identified as: desethyleneciprofloxacin (M 1), sulphociprofloxacin (M 2), oxociprofloxacin (M 3) and formylciprofloxacin (M 4) from human urine by Craig counter current distribution and semipreparative high-performance liquid chromatography. Their molecular structures were elucidated by nuclear magnetic resonance and mass spectrometry and confirmed by comparing their spectra with those of authentic synthetic reference compounds [30]. The metabolites display in-vitro

antimicrobial activity but to a lower degree than the parent compound. Additionally, ciprofloxacin is known to be a moderate inhibitor of the CYP 450 1A2 iso-enzymes.

1.13.11 Therapeutic uses:

Ciprofloxacin is a broad spectrum fluoroquinolone antibacterial agent. Since its introduction in the 1980s, most Gram-negative bacteria have remained highly susceptible to this agent in vitro; Gram-positive bacteria are generally susceptible or moderately susceptible.

Ciprofloxacin attains therapeutic concentrations in most tissues and body fluids. The results of clinical trials with ciprofloxacin have confirmed its clinical efficacy and low potential for adverse effects. Ciprofloxacin is effective in the treatment of a wide variety of infections, particularly those caused by gram-negative pathogens. These include complicated urinary tract infections, sexually transmitted diseases (gonorrhoea and chancroid), skin and bone infections, gastrointestinal infections caused by multiresistant organisms, lower respiratory tract infections (including those in patients with cystic fibrosis), febrile neutropenia (combined with an agent which possesses good activity against Gram-positive bacteria), intra-abdominal infections (combined with an antianaerobic agent) and malignant external otitis [25]. It has also been found to show anti-tumour activity against P388 leukemia [79].

It is also used in combination with other specific drugs:

- Complicated intra-abdominal infections (in combination with metronidazole);
- Empirical therapy for febrile neutropenic patients (in combination with piperacillin)

Oral and intravenous fluoroquinolones are approved by the FDA for use in children for only two indications due to the risk of permanent injury to the musculoskeletal system.

Indications include:

- Complicated urinary tract infections and pyelonephritis due to *Escherichia coli*
- Inhalational anthrax (postexposure)

Ciprofloxacin is not recommended for the treatment of *tuberculosis* and to treat community-acquired pneumonia (CAP) as a stand-alone first-line agent due to its modest activity against *Streptococcus pneumoniae*. Other studies have shown that repeated use of fluoroquinolones predicts an increased risk of infection with fluoroquinolone-resistant pneumococci.

Ciprofloxacin may be approved for other uses, or restricted, by the various regulatory agencies worldwide.

1.13.12 Interaction with other drugs:

Davis R *et al* proposed that clinically important drug interactions involving ciprofloxacin are well documented and avoidable with conscientious prescribing. Recommended dosage adjustments in patients with impaired renal function vary between countries; major adjustments are not required until the estimated creatinine clearance is < 30 ml/min/1.73m2 (or when the serum creatinine level is > or = 2 mg/dl) [25].

Ciprofloxacin interacts with drugs and herbal and natural supplements.

- aminophylline
- antacids that contain aluminum, calcium, or magnesium (take ciprofloxacin at least 2 hours before or 6 hours after these medications)
- anti-inflammatories (NSAIDs; e.g., naproxen), not including aspirin
- buffered antiretroviral medications (e.g., didanosine)
- caffeine
- calcium supplements and multivitamins that contain calcium (take ciprofloxacin at least 2 hours before or 6 hours after these medications)
- cyclosporine
- glyburide
- iron supplements and multivitamins that contain iron (take ciprofloxacin at least 2 hours before or 6 hours after these medications)
- methotrexate
- metoclopramide
- oxtriphylline
- phenytoin
- probenecid
- sucralfate (take ciprofloxacin at least 2 hours before or 6 hours after these medications)
- theophylline
- tizanidine
- warfarin

Serum levels of certain drugs metabolized by the cytochrome P450 system is enhanced by concomitant use of some quinolones. Levels of tizanidine and methylxanthines (for example, theophylline and caffeine) may be increased due to ciprofloxacin's interaction with the cytochrome P-450 enzyme CYP1A2.

The Committee on the Safety of Medicines and the FDA warn that central nervous system (CNS) adverse effects, including seizure risk, may be increased when

NSAIDs are combined with quinolones. The interaction between quinolones and NSAIDs is important, because it has the potential for considerable CNS toxicity. The mechanism for this interaction is believed to be due to a synergistic increased antagonism of GABA neurotransmission.

Ciprofloxacin's renal clearance may affect other drugs subject to renal clearance or otherwise affecting the kidney. The use of ciprofloxacin concomitantly with cyclosporine has also been associated with transient elevations in serum creatinine. Renal tubular transport of methotrexate may be inhibited by concomitant administration of ciprofloxacin, potentially leading to increased plasma levels of methotrexate and risk of methotrexate toxicity. Probenecid interferes with renal tubular secretion of ciprofloxacin and produces an increase in the level of ciprofloxacin in serum.

Altered serum levels of the anti-epileptic drugs phenytoin and carbamazepine (increased and decreased) have been reported in patients receiving concomitant ciprofloxacin.

Current or past treatment with oral corticosteroids is associated with an increased risk of Achilles tendon rupture, especially in elderly patients who are also taking the fluoroquinolones. This is the subject of Black box warnings in FDA and BNF labeling for quinolones.

1.13.13 Dosing (infections caused by bacteria):

The dose of this medicine will be different for different patients. Follow your doctor's orders or the directions on the label. The following information includes only the average dosages of this medicine. If your dose is different, do not change it unless your doctor tells you to do so.

The amount of medicine that you take depends on the strength of the medicine. Also, the number of dosages you take each day, the time allowed between dosages, and the length of time you take the medicine depend on the medical problem for which you are using the medicine.

- For oral dosage form (extended-release tablets):
    - For uncomplicated urinary tract infections:
        - Adults—500 milligrams (mg) once a day for 3 days.
        - Children—Use and dose must be determined by your doctor.
    - For urinary tract infections:
        - Adults—500 to 1000 milligrams (mg) once a day.
        - Children—Use and dose must be determined by your doctor.
- For oral dosage forms (suspension or tablets):
    - For infections:
        - Adults—250 to 750 milligrams (mg) two times a day, taken every 12 hours.
        - Children—Use and dose must be determined by your doctor.
    - For urinary tract or serious kidney infections:
        - Adults—250 to 500 milligrams (mg) two times a day, taken every 12 hours.
        - Children—Dose is based on body weight and must be determined by your doctor. The dose is usually 10 to 20 milligrams (mg) per kilogram (kg) of body weight every 12 hours.

- For treatment of anthrax infection (post-exposure):
  - Adults—500 milligrams (mg) two times a day, taken every 12 hours.
  - Children—Dose is based on body weight and must be determined by your doctor. The dose is usually 15 milligram (mg) per kilogram (kg) of body weight every 12 hours.

1.13.14 Concerns and cautions:

Before taking ciprofloxacin make sure your doctor or pharmacist knows:

- pregnant, trying for a baby or breast-feeding.
- tendon problems after taking any other quinolone antibiotic such as ofloxacin, levofloxacin, moxifloxacin, nalidixic acid or norfloxacin.
- kidney problems.
- epilepsy or any other condition that causes fitting.
- *myasthenia gravis* (a muscle weakening disease).
- glucose 6-phosphate dehydrogenase (G6PD) deficiency.
- allergic reaction to this or any other medicine..
- If you are taking other medicines, including those available to buy without a prescription, herbal and complementary medicines

1.13.15 Quality control for susceptibility testing of ciprofloxacin:

Standardized susceptibility test procedures require the use of laboratory control microorganisms to control the technical aspects of the laboratory procedures. For dilution technique, standard ciprofloxacin powder should provide the MIC values according to criteria outlined in the table. For diffusion technique, the 5-µg ciprofloxacin disk should provide the zone diameters outlined in Table 4.

Table 3: Susceptibility test by different antibacterial strains for Quality control.

Strains	MIC range (µg/ml)	Zone Diameter (mm)
Enterococcus faecalis ATCC 29212	0.25–2	-
Escherichia coli ATCC 25922	0.004–0.015	30–40
Haemophilus influenzae ATCC 49247	0.004–0.03[a]	34–42[d]
Pseudomonas aeruginosa ATCC 27853	0.25–1	25–33
Staphylococcus aureus ATCC29213	0.12–0.5	-
Staphylococcus aureus ATCC25923	-	22–30
Neisseria gonorrhoeae ATCC 49226	0.001–0.008[b]	48–58[e]
C. jejuni ATCC 33560	0.06–0.25 and 0.03–0.12[c]	-

(a) This quality control range is applicable to only *H. influenzae* ATCC 49247 tested by a broth microdilution procedure using Haemophilus Test Medium (HTM)1.

(b) *N. gonorrhoeae* ATCC 49226 tested by agar dilution procedure using GC agar and 1% defined growth supplement in a 5% CO2 environment at 35-37°C for 20-24 hours3.

(c) *C. jejuni* ATCC 33560 tested by broth microdilution procedure using cation adjusted Mueller Hinton broth with 2.5-5% lysed horse blood in a microaerophilic environment at 36-37°C for 48 hours and for 42°C at 24 hours, respectively.

(d) These quality control limits are applicable to only *H. influenzae* ATCC 49247 testing using Haemophilus Test Medium (HTM)3.

(e) These quality control limits are applicable only to tests conducted with *N. gonorrhoeae* ATCC 49226 performed by disk diffusion using GC agar base and 1% defined growth supplement.

1.13.16 Literature review:

A kinetic spectrophotometric method was established for the determination of ciprofloxacin in its pharmaceutical dosage forms [42]. It is a less complex and sensitive kinetic spectrophotometric method that was developed and validated for the determination of ciprofloxacin. The method was based on the oxidation of ciprofloxacin with alkaline potassium permanganate to give a green coloured reaction product. The reaction was monitored spectrophotometrically by measuring the absorbance of the reaction product at 610 nm.

A simple and sensitive spectrophotometric method for the determination of ciprofloxacin hydrochloride in tablets was proposed [45]. In this method the solution of drug has been found to give a light reddish orange chromogen with Î²-naphthol in acidic medium, which absorbs at λ_{max} 365 nm. This light reddish color was sufficiently stable to be used for quantitative purposes.

A spectrophotometric method was described for the determination of the antibacterial quinolone derivatives, ciprofloxacin, enrofloxacin and pefloxacin through charge transfer complex formation with three different acceptors [55]. Chloranilic acid (CL) was utilized for their determination, forming charge transfer complex with λ_{max} 520 nm.

Four simple, rapid and reliable methods were evolved by using visual titrimetric, pH metric, conductometric and spectrophotometric techniques that were described

for the determination of ciprofloxacin as bulk drug and in commercial formulations [8]. The methods are based on the neutralization reaction involving the carboxyl group of ciprofloxacin and sodium hydroxide in aqueous medium. Visual titrimetry involves the addition of a measured excess of sodium hydroxide to ciprofloxacin followed by back titration of residual base with hydrochloric acid using bromothymol blue-phenol red mixed indicator. In pH-metry and conductometry, ciprofloxacin solution is titrated directly with sodium hydroxide, the end-point being determined by pH and conductance measurements in spectrophotometry, ciprofloxacin is treated with phenol red - sodium hydroxide mixture and change in absorbance is measured at 560 nm and related to ciprofloxacin concentration.

Rajesh Sharma *et al* proposed three accurate, precise, sensitive and economical procedures for simultaneous determination of ciprofloxacin hydrochloride in tablet dosage form [70]. In the present investigation, 1.0 M urea solution (hydrotropic solubilizing agent) was employed to solubilize, ciprofloxacin is a drug that is poorly water soluble. Fine powder from its tablet is used to carryout spectrometric analysis. The methods employed were derivative spectrophotometry method, area under curve method and multi- component method. The result showed that Beer-lambart's law was obeyed in concentration range of 5-50 µg/ml with good linearity, with a R2 value >.99, for both the drug in all the methods. The recoveries were within 99.42-101.27% for ciprofloxacin hydrochloride.

Colorimetric determination of the fluoroquinolones was briefly described previously [26]. In this study ciprofloxacin, ofloxacin and norfloxacin formed an amber coloured complex with iron (III) nitrate non anhydrate. The complex, which formed instantaneously at room temperature, was stable. The solutions of the complex obeyed Beer's law at 370 nm, the wavelength of maximum absorption of radiation (max).The $A1\%$ for norfloxacin, ofloxacin and ciprofloxacin was 202,

207 and 235 respectively. The formation of the complex was the basis for the quantitative and qualitative determination of the drugs.

Two simple, accurate, precise, reproducible and economical UV spectroscopic methods (A & B) for simultaneous estimation of ciprofloxacin and tinidazole in tablet dosage form have been developed [33]. Method A employs solving of simultaneous equations based on the measurement of absorbance at two wavelengths, 271nm and 318nm which are the λ_{max} values of ciprofloxacin and tinidazole respectively in phosphate buffer (pH 6.8). Method B is based on the principle of Q-Analysis where in the absorbance was measured at 292nm (iso-absorptive point) and 271nm (λ_{max} of ciprofloxacin) in phosphate buffer (pH 6.8). Ciprofloxacin and tinidazole showed linearity at all the selected wave-lengths and obeyed Beer's law in the concentration range of 10-35µg/ml and 10-80µg/ml respectively. Recovery studies for ciprofloxacin and tinidazole were performed and the percentage recovery for both the drugs was obtained in the range of 98.1-99.7% (Method A) and 98.0-100.4% (Method B) confirming the accuracy of the proposed method.

A simple, accurate and sensitive spectroscopic method has been proposed for the assay of ciprofloxacin in tablet by least square treatment of fourier transform infrared spectrometric data obtained at the wave number corresponding to the carbonyl group centered at 1707 cm^{-1} [63]. The method involves the extraction of the active ingredient with methanol followed by phosphate buffer pH 6.0. The excipients in the commercial tablet did not interfere with the assay. The specificity, linearity, detection limits, precision and accuracy of the calibration curve, drug extraction, infrared analysis and data manipulation were determined in order to validate the method. Moreover, the statistical results were compared with other methods for quantification of ciprofloxacin.

Synthesis and spectral studies of novel benzyl derivative of ciprofloxacin was done by using the reaction of 1-cyclopropyl-6-flouro-1, 4-dihydro-4oxo-7-(1-piperazinyl)-3quinolone carboxylic acid (ciprofloxacin) with benzyl chloride in presence of base triethylamine [78]. An introduction of amide group in ciprofloxacin took place to form new benzyl derivative of 1-cyclopropyl-6-fluoro-4-oxo-7-[4-(phenyl carbonyl) piperazin-1-yl]-1,4-dihydroquinoline-3-carboxylic acid (Cip-d) and their Cu(II) and Co(II) complexes were established on the basis of spectral studies 1H NMR, IR, Mass spectroscopy.

Spectrophotometric determination of ciprofloxacin hydrochloride in ophthalmic solution was measured directly at 275 nm by UV-spectrophotometric methods [19]. The calibration graph was linear over the range 2.0 - 7.0 μg/ml with relative standard deviations (RSD) values ranging from 1.55 to 2.47% (n=6). The method was validated through the parameters of linearity, accuracy, precision, limit of detection, limit of quantitation, specificity and robustness according to ICH. The proposed method enabled a quantitative determination of ciprofloxacin hydrochloride in ophthalmic solution.

Adam EHK *et al* described the stability study of ciprofloxacin hydrochloride under stress conditions using reverse phase-high performance liquid chromatography method [2]. This method investigated under different stress conditions of sun light, UV light at 254 nm and some pharmaceutical excipients using HPLC. RPHPLC method were modified that could separate the drug from its degradation products formed under these stress conditions. Degradation was found to occur under sun light and thermal changes that were successfully resolved on a C18column (5S, ODS 25 cm × 4.6 mm, 5μm), utilizing a mobile phase of 0.025 M orthophospharic acid (adjusted to pH 3.0 with triethylamine) and acetonitrile in a ratio of 87:13 respectively. Mobile phase was delivered at a flow rate of 1.5 ml/min. Ultra violet detection was carried out at 278 nm.

Analytical laboratory methods

- In vitro release of ciprofloxacin hydrochloride in presence of various antacids like sodium bicarbonate, calcium hydroxide, calcium carbonate, aluminum hydroxide, magnesium hydroxide, magnesium carbonate, magnesium trisilicate and magaldrate has been studied on BP 2002 dissolution test apparatus. Drug in each case was analyzed either by measuring the absorbance of aliquots at 278 and 316 nm on a UV/VIS spectrophotometer, or by reversed-phase high-performance liquid chromatographic (RP-HPLC) method. These studies were carried out in simulated gastric and intestinal juices for three hours at 37°C. The availability of ciprofloxacin was found to be markedly retarded in presence of all the antacids studied [75].

- Two kinetic spectrophotometric methods were developed for determination of ciprofloxacin (CIP) in a pharmaceutical preparation. The methods are based on oxidation of CIP with potassium permanganate in alkaline media and measurement of the enhancement in the absorbance of manganate ion at 603 nm by spectrophotometry. The calibration graphs were constructed using the initial rate and fixed time methods. The linearity range for concentrations of CIP was found to be 4.0-20.0 μg/ml. The RSD /relative standard deviation/ values for intraday and interday precision were 0.05-0.50 and 0.07-0.63%, respectively. The procedures were applied successfully for determination of CIP in commercial tablets. The results compared well with those from a reference HPLC method. The proposed methods can be recommended for routine analysis of CIP in QC laboratories [6].

- A simple and rapid HPLC method with UV detection was developed for the separation of ciprofloxacin, levofloxacin and moxifloxacin. Chromatography was carried out using a BDS Hypersil C18 (100 x 4.6 mm, 2.4 microm) HPLC column and an isocratic mobile phase consisting of MeOH/25 mM phosphate buffer 28/72 (v/v) at pH 3 and flow rate 1 ml/min^{-1}. The effect of mobile phase variables such as methanol content, pH and buffer concentration on the chromatographic behavior of the three fluoroquinolones was investigated. The retention behavior on a sub 3 microm C18 column was also compared with that on three different calixarene-bonded and on monolithic stationary phases. The results indicate that some differences exist between these three types of stationary phases, particularly in the effect of buffer concentration on the retention mechanism of the three used FQs on calixarene-bonded stationary phases [21].

Chapter Two

MATERIALS AND METHODS

MATERIALS AND METHODS

2.1 Evaluation of efficacy and potency of marketed chloramphenicol 0.5% eye drops after controlled exposure to sunlight:

2.1.1 Collection of chloramphenicol 0.5% eye drops from local market of Bangladesh:

Chloramphenicol 0.5% eye drops were collected from different drug stores of Rajshahi Town. The following parameters for those eye drops of different companies were observed visually: appearance of the preparations, types of containers used, shelf-lives indicated and the storage conditions. The manufacturing and expiry dates, batch numbers, date of collection of the products were recorded (Table 4).

To avoid discrepancy in analysis, code numbers were used for company names. The samples were collected randomly from ten different companies and different drug stores.

Table 4: The company name code of marketed chloramphenicol 0.5% eye drops with other checking parameters.

Code (Company)	**Mfg.Date/ Exp. Date**	**Batch No.**	**Storage condition(specified by company)**	**Collection time**	**Storage condition(During collection)**
ACPC	OCT-10/ OCT- 11	EA0062	Room Temperature	03/03/2011	Normal
NIPC	OCT-10/ OCT-12	101310	Room Temperature	03/03/2011	Normal
IBPC	JAN-10/ JAN-12	767	Room Temperature	03/03/2011	Normal
OPPC	OCT-10/ OCT-13	EYJ035	Room Temperature	03/03/2011	Normal
POPC	SEP-10/ SEP-12	EIF08	Room Temperature	03/03/2011	Normal
REPC	OCT-10/ OCT-13	3610	Below 30°c	03/03/2011	Normal
ASPC	SEP-10/ SEP-12	1010	Below 25°c	03/03/2011	Normal
JAPC	OCT-10/ OCT-12	581	Below 25°c	03/03/2011	Normal
BEPC	OCT-10/ APR-12	OT0023	Below 25°c	03/03/2011	Normal
SQPC	OCT-10/ SEP-12	0100018	2-8°c	03/03/2011	Normal

2.1.2 Appearance of different Sets of chloramphenicol eye drops samples before and after the exposure of sunlight:

For sunlight exposure, the collected chloramphenicol 0.5% eye drops were divided into four different Sets. These were-

Set-1 (Control): One sample from each company was kept in refrigerator with their primary (Eye drops in Low density polyethylene (LDPE) container) and secondary packaging (Eye drops in carton).

Set-2 (Primary and Secondary Packaging): One sample from each company with their primary (Eye drops in LDPE container) and secondary packaging (Eye drops container in carton).

Set-3 (Primary Packaging): One sample from each company only with their primary packaging (Eye drops in LDPE container).

Set-4 (Test Tube): One sample from each company was kept into 10 different clear transparent test tubes.

The samples of Set-2, Set-3 and Set-4 were exposed to direct sunlight for 14 hours. The appearances of samples from different sets were checked visually after controlled exposure to sunlight. The change in color before and after exposure to sunlight was recorded and analyzed.

2.1.3 Potency of the Sunlight-induced degraded marketed chloramphenicol eye drop preparations by UV- spectrophotometric method:

2 ml solution (10 mg equivalent of chloramphenicol) from each preparation were taken in 100ml volumetric flasks. Then 50 ml of distilled water was added to the

volumetric flasks and was shaking for 5 minutes. The total volume was made up to 100 ml with the same solvent and mixed properly. Then 10ml solution was transferred to the 100 ml volumetric flasks and the volume was adjusted. The absorbance of the assay preparation was taken at 278 nm wave length whereas the specific absorbance was at 297 nm. Then the potency of chloramphenicol 0.5% eye drops was calculated using the following calculation.

$$X\ (gm) = Au \times 100 \times 100 / V \times 10 \times 297$$

Here,

Au = Absorbance of the chloramphenicol solution

V = Volume of the sample taken

The potency = 100 x X/ 0.5

= (Y%)

2.1.4 Efficacy of Heat and Sunlight induced degraded chloramphenicol eye drops preparations by disc diffusion assay method:

Principle of disc diffusion assay method (Bauer et al., 1951): One of the most widely used methods to determine the susceptibility of microorganisms to antimicrobial agents is the disc agar-diffusion method. The principle of this method is based on the ability of the compound to diffuse from a confined source through the nutrient agar gel and create a concentration gradient. If the agar is seeded or streaked with a sensitive organism, a zone of inhibition will result where the concentration exceeds the minimum inhibitory concentration (MIC) for the particular organism.

In this method, measured amount of the test samples is dissolved in definite volumes of solvent to give solutions of known concentrations (µg/ml). Then sterile filter paper (BBL, Cocksville, U.S.A) discs having the diameter of 5 mm are

impregnated with known amounts of test substances and are dried. These test material discs as well as standard antibiotic discs are placed on plates containing a suitable medium (nutrient agar) seeded with the test organisms. These plates are kept at low temperature (4°C) for 24 hours to allow maximum diffusion.

The plates are then kept in an incubator (37°C) for 12-18 hours to allow the growth of the organisms. If the test material has antibacterial activity, it will inhibit the growth of microorganisms, giving a clear, distinct zone called as the 'zone of Inhibition'. The antibacterial activity of the test agent is determined by measuring the diameter of the zone of inhibition in terms of mm.

2.1.4.1 Materials:

1. Blank filter paper discs (5 mm in diameter)
2. 12-14 hours old culture of the test bacteria
3. Test tubes
4. Petri dishes (120 mm in diameter)
5. Sterile forceps and cotton
6. Ethyl acetate
7. Inoculating loop
8. Bunsen burner
9. Micropipette (10 and 100 µL)
10. Laminar air flow unit (BIOCRAFT and SCIENTIFIC INDUSTRIES, INDIA)
11. Autoclave (ALP Co. Ltd. KT-30L, Tokyo, Japan)
12. Incubator (OSK 9639 A, Japan)
13. Nutrient agar media (DIFCO)
14. Nutrient Agar Plates
15. Alcohol (95%)
16. Methanol

2.1.4.2 Preparation of discs:

Sterilized filter paper discs having 5 mm in diameter (BBL, Cocksville U.S.A.) were prepared with the help of a punch machine and were taken in a blank Petri dish. Blank discs were first kept in a covered Petri dish and then subjected to dry heat sterilization at 180°C for 1 hour. Later they were kept in a laminar hood under the UV light for 30 minutes. For preparation of 30µg/discs of chloramphenicol preparations, 6µl of each solution were taken by a 10µl micropipette in an aseptic condition. These discs were left in the oven for a few minutes for the complete removal of solvent.

2.1.4.3 Preparation of culture media:

Commercially available nutrient agar medium (DIFCO) were used to demonstrate the antimicrobial activity of test preparations. 2.8gm of the nutrient agar was used to dissolve in 100 ml distilled water for the preparation of culture media. The culture media was sterilized by autoclaving at a temperature of 121°c and a pressure of 15-lbs /sq. inch for 20 minutes.

2.1.4.4 Test organisms:

Two gram-negative bacterial strains *Salmonella typhi* and *Shigella dysenteriae* were taken for the test. These bacterial strains are available in the microbiological research laboratory of the department of Pharmacy, University of Rajshahi. The test organisms were maintained in the nutrient agar medium at 4°C.

2.1.4.5 Preparation of test plates:

Petri dishes and other glass wares were sterilized by autoclaving at a temperature of 121°C and a pressure of 15-lbs /sq inch for 20 minutes. The test organism was transferred from the subculture to the test tube containing 20 ml autoclaved

medium with the help of an inoculating loop in an aseptic area. The test tube was shaken by rotation to get a uniform suspension of the organism. The bacterial suspension was immediately transferred to the sterile Petri dishes in an aseptic area and was rotated several times, at first clockwise and then anti-clockwise to assure homogeneous distribution of the test organisms. The depth of media into each Petri dish (120 mm diameter) was approximately 4 mm. After the medium was cooled to the room temperature, it was stored in a refrigerator at 4°C.

2.1.4.6 Placement of the discs, diffusion and incubation:

Antibacterial investigation was done in a laminar hood and all types of precautions were highly taken to avoid any contamination of the organism under test. UV light was switched on before one hour of working in the laminar hood to avoid any accidental contamination. The sample discs impregnated with the test material were placed gently on the solidified agar plates, freshly seeded with the test organisms with the help of a sterile forceps to assure complete contact with the surface of the medium. The discs were not closer than 30 mm to the edge of the plate and were far enough apart to prevent overlapping of the zones of inhibition. The plates were then inverted and kept in a refrigerator for about 24 hours at 4°C to obtain the maximum diffusion of the test material. Finally, the plates were incubated at 37°C for 12-18 hours. After 12 hours of incubation, the antibacterial activity of the test agents was determined by measuring the diameter of the zones of inhibition in mm with a transparent scale and the results were recorded.

2.1.5 Relationship between the efficacy of different Sets of samples by disc diffusion methods and their estimated potency by UV-spectrophotometric method:

The relationship between the efficacy of different sets of samples by disc diffusion method and their estimated potency by UV-spectrophotometric method for different companies can be determined by combining the data for

the efficacy i.e. the zone of inhibitions of sunlight-induced degraded chloramphenicol 0.5% eye drops against two bacteria with the data for the estimated potency of those samples.

2.2 Investigation the cause of in reverse correlation between efficacy by ager disc diffusion method and potency by UV-spectroscopic method after Heat and Sunlight induced degradation:

2.2.1 Appearance of different Sets of chloramphenicol aqueous solution (without any excipient) before and after the exposure of Sunlight and Heat:

The physical changes of aqueous solutions of chloramphenicol were observed visually after induction of heat and sunlight to check the compatibility of the phenomenon happening with the pharmaceutical preparations of the drug.

Aqueous solution of chloramphenicol (potency 98.6%) was prepared in a concentration of 0.15 mg/ml. Then the prepared solutions were transferred to 9 different test tubes containing 10 ml each. The test tube were divided into three different Sets (n=3) and labeled as follows-

Set-1 (Control): The test tubes were stored in a refrigerator.
Set-2 (Heat): The test tubes were heated in a water bath at 100°c for 6 hours.
Set-3 (Sunlight): The test tubes were exposed to direct sunlight for 14 hours.

2.2.2 UV-spectra of the absorbance values for Standard, Sunlight & Heat-induced degraded aqueous solution of chloramphenicol:

The UV-spectra of control (Set-1), sunlight (Set-3) & heat (Set-2) induced degraded aqueous solution of chloramphenicol were taken at 200-400 nm by a UV-spectrophotometer and the result was recorded.

2.2.3 Potency of Heat (Set-2) and Sunlight (Set-3) induced degraded chloramphenicol aqueous solutions by UV-spectrophotometric method:

The potency of heat and sunlight-induced degraded chloramphenicol aqueous solution was determined by UV-spectrophotometric method and the result of aqueous chloramphenicol solution were recorded.

2.2.3.1 Materials:

1. Measuring cylinder
2. Pipette
3. UV-spectrophotometer
4. Distilled water
5. Volumetric flask
6. Beaker

2.2.3.2 Method:

3 ml solution was taken from Set-1, Set-2 and Set-3 into a beaker and then made 45 ml with the distilled water. The final concentration was 0.01 mg/ml or 10µg/ml. The absorbance of the assay preparations were taken at 278nm wave length recorded. Then the potency of chloramphenicol was calculated using the following calculation.

Calculation:

$$\text{Potency} = \frac{\text{Ab(TS)} \times 15 \times 3 \times 100 \times 45 \times 98.6 \times 100}{\text{Ab(STD)} \times 100 \times 45 \times 15 \times 3 \times 10}$$

Here, Ab (TS) = Absorbance of test sample

Ab (STD) = Absorbance of standard sample

98.6 = Potency of standard solution

2.2.4 The efficacy of Sunlight and Heat-induced degraded pure solution of chloramphenicol:

Efficacies of the aqueous solution of pure chloramphenicol after heat and sunlight exposure were determined by agar disc diffusion method. In this case one gram - positive bacteria *Bacillus cereus* and one gram- negative bacteria *Pseudomonas aeruginosa* was used to check the activity against chloramphenicol aqueous solution (15 µg/ disc) and two gram negative bacteria *Shigella dysenteriae,* & *Salmonella typhi* and one gram positive bacteria *Streptococcus agalactiae* were used to check the activity against chloramphenicol aqueous solution (30 µg/ disc).

After 12 hours of incubation, the antibacterial activity of the test solution was determined by measuring the diameter of the zones of inhibition in mm with a transparent scale and the results were recorded.

2.2.5 Change in absorbance between freshly prepared and 4-days old chloramphenicol solution:

Two chloramphenicol samples were prepared, one was freshly prepared and the other was prepared 4 days before taking the reading.

40 mg chloramphenicol in 100 ml was denoted as 100% (400µg/ml). It was then serially diluted to 50% (200µg/ml), 25% (100 µg/ml), 12.5% (50µg/ml), 6.25% (25µg/ml) and 3.13% (12.5µg/ml). The 4-days old solution was kept in room temperature to find out the continuous degradation in the presence or absence of degradable environment and absorbance values were measured by UV-spectroscopic methods.

2.2.6 Effect of the presence and absence of pure 4-nitrobenzaldehyde in chloramphenicol solution on UV absorbance:

If chloramphenicol becomes degraded, the major photo degraded product is 4-nitrobenzaldehyde that gives false absorbance values whereas the potency of the drug becomes decreased. To test this phenomenon, this experiment on the effects of presence and absence of 4-nitrobenzaldehyde on UV- absorbance values was attempted.

The molecular weight of chloramphenicol is 323.129 and the molecular weight of 4- nitrobenzaldehyde is 151.12. After the photo-degradation of chloramphenicol, 1 mole of chloramphenicol produces 1 mole of 4-nitrobenzaldehyde [43,71] making 323.129 gm of chloramphenicol to release 151.12 gm of 4- nitrobenzaldehyde.

2.2.6.1 Preparation of stock solution for chloramphenicol:

3 mg chloramphenicol was dissolved in 100 ml water (denoted as 100% chloramphenicol solution). So, the concentration has become as 30µg/ml. From this stock solution, 1% , 2%,3% , 5% ,10%, 20%, 30% and 40% chloramphenicol replaced by 4-nitrobengaldehyde from the stock solution of 4-nitrobenzaldehyde

2.2.6.2 Preparation of stock solution for 4-nitrobengaldehyde:

6.42 mg 4-nitrobenzaldehyde was diluted in 100 ml of water (0.0642 µg/ml) to use as stock solution.

2.3 Investigation of a new method for potency determination of chloramphenicol:

2.3.1 Standard curve of pure chloramphenicol:

Different concentrations of chloramphenicol samples were prepared using a stock solution of 150 mg/100 ml denoted as 100% solution. From this solution 90%,

80%,70%, 60%, 50%, 40% ,30% ,20% 10% solutions were prepared by adding distilled water. Then take 1 ml solution from those gradually diluted solution in 30 ml distilled water so the final concentration of 100% solution was 50µg/ml, 90% solution was 45µg/ml, for 80% was 40 µg/ml,70% was 35 µg/ml, 60% was 30 µg/ml, 50% was 25 µg/ml, 40 % was 20 µg/ml, 30% was 15 µg/ml, 20% was 10 µg/ml and 10% contained 5 µg/ml. A concentration-dependent curve of standard chloramphenicol sample was drawn according to the absorbance values measured by the UV-spectrophotometer at 278 nm.

2.3.1.1 Relation in absorbance values of Light-degraded and Heat-induced solution of pure chloramphenicol with Standard chloramphenicol measured by UV-spectroscopic methods:

The absorbance values of the light-induced and heat-degraded solutions having concentrations as 90% (45µg/ml) was measured by UV-spectrophotometer at 278 nm.

2.3.2 Estimation of residual chloramphenicol after Heat and Sunlight-induced degradation by Thin-layer chromatography:

The TLC technique consists of placing a spot of a drug sample on a thin layer of silica attached to a glass plate or aluminum foils. The separation depends on the relative affinity of compounds towards stationary and mobile phase. The compounds under the influence of mobile phase (driven by capillary action) travel over the surface of stationary phase. During this movement the compound with higher affinity to stationary phase travel slowly while the others travel faster. Thus separation of components in the mixture is achieved.

2. 3.2.1 Apparatus:

TLC plate of size 20cm × 5cm, TLC chamber, Micropipette. UV lamp
Chemicals: *Chloroform, Methanol*

2. 3.2.2 Chromatographic condition:

Test method: Thin Layer Chromatography (TLC)

Test plate: TLC plate of size 20cm × 5cm

Coating material: 0.25 mm layer of silica gel mixture

Mobile Phase: A mixture of Chloroform, methanol and Water (90:10:1).

Separation technique: Ascending

2.3.2.3 Procedure:

150 mg chloramphenicol was dissolved with distilled water in a 100 ml volumetric flask and divided into three sample groups. One of those kept in refrigerator as a control, Other exposed in sunlight for 14 hours and the third one were kept in 100°C in a water bath for 6 hours.

TLC plates were activated at 120^0C for 30 minutes. 3 sample spots, each containing 90 µg in 60 µl from the test tubes were applied on one TLC plate. Then the plate was placed into a suitable chromatographic chamber and were developed the chromatogram in a solvent system consisting of a mixture of chloroform, methanol and water (90:10:1) until the solvent front moved about three-fourths of the length of the plate. After removal of the plate, it was subjected to dry in air and was examined under the ultraviolet light at 254 nm. The spots appeared in the middle of the plates were identified and marked. All the spots were eluted in test tubes separately with 6 ml of distilled water. The UV-spectrophotometric readings of all the test tubes were taken at 278 nm.

Calculation:

$$\frac{\text{Absorbance of the deg raded sample (required sample)} \times \text{potency of the s tan dard}}{\text{Absorbance of the s tan dard sample}}$$

2. 3.3 Microbiological assay of chloramphenicol by agar disc diffusion method using *E. coli DH5α*:

2. 3.3.1 Standard sample preparation:

150 mg of chloramphenicol was dissolved in a 100 ml volumetric flask with distilled water to use as the stock solution. From this stock solution 90%, 80%, 70%, 60%, 50%, 40%, 30%, 30% and 10% solutions were prepared by adding distilled water.

2. 3.3.2 Preparation of degraded sample:

The stock solution (100%) and 90% chloramphenicol solution were degraded by sunlight exposure for 14 hours and heated at 100°C for 6 hours in an incubator.

2. 3.3.3 Preparation of discs:

For preparation of chloramphenicol containing discs 20µl of each solution were taken by a 20 µl micropipette from the serially diluted standard sample solution **(2.3.3.1)** in amounts like 30µg/disc(indicated in plate as 100%), 27 µg/disc(90%), 24 µg/disc(80%), 21 µg/disc(70%), 18 µg/disc(60%), 15 µg/disc(50%), 12 µg/disc(40%), 9 µg/disc(30%), 6 µg/disc(20%) and 3 µg/disc(10%), in aseptic condition. These discs were dried in the oven for a few minutes to remove the solvent completely.

2. 3.3.4 Test Organisms: *E.coli DH5α*

2.3.3.5 Preparation of media:

The culture media and all the glassware including petri dishes were sterilized by autoclaving at a temperature of 121°C and a pressure of 15-lbs/sq. inch for 20 minutes. The test organism was transferred from the subculture to LB (Luria-Bertani broth) media. 10 gm of trypton, 5 gm of yeast extract and 10 gm of NaCl

was dissolved in 950 ml of deionized water. The pH was adjusted to 7.0 by using 1N NaCl with a final volume of 1 liter. The solution was allowed to cool down to 55°C and stored at room temperature or at 4°C.

2.3.3.6 Procedure:

The sample discs impregnated with the standard and degraded chloramphenicol were placed gently on the solidified agar plates, freshly seeded with the test organisms with the help of a sterile forceps to assure complete contact with the surface of the medium. The discs were not closer than 30 mm to the edge of the plate to prevent overlapping of the zones of inhibition. The plates were then inverted and kept in a refrigerator for about 24 hours at 4°C to obtain the maximum diffusion of the test material. Finally, the plates were incubated at 37°C for 12-18 hours. After 12 hours of incubation, the antibacterial activity of the test agents was determined by measuring the diameter of the zones of inhibition in mm with a transparent scale and the results were plotted into concentration VS zones of inhibition.

2.3.4 High Performance Liquid Chromatography (HPLC) is used for crosschecking of agar disc diffusion methods:

2.3.4.1 Principle:

A prepared standard solution & sample solution are injected consequently into a suitable chromatographic column, together with the solvent system, to separate chloramphenicol from formulation products. The content of chloramphenicol present in sample is calculated by comparing both the peak area of active chloramphenicol present in standard preparation and sample preparation.

2. 3.4.2 Reagent required:

1. Glacial acetic acid
2. Methanol

Preparation of buffer:

Take 2 ml Glacial acetic acid in 1000 ml purified water. Filter with 0.2µ membrane filter and sonicate for 10 minutes.

Organic Phase	:	Methanol filter with 0.2µ membranes filter and sonicate for 10 minutes.
Mobile Phase	:	Buffer: Organic Phase = 55:45
Diluting solution	:	50% Methanol

2. 3.4.3 Standard preparation:

Accurately weigh about 150 mg of chloramphenicol WS into a 100 ml volumetric flask. Then solution were sonicate for 10 minutes. Dilute to volume with the same. Then solution was serially diluted into 10 %, 30%, 60%, and 90 % by adding distilled water and divided into three sample set. One kept in refrigerator as control, other exposed direct sunlight for 14 hours and the third one had heated at 100^0C for 6 hours. Then each solution was diluted 200 µl in 1800 µl with diluting solution. Filter through 0.2µ-disc filter. Call this solution A.

2. 3.4.4 Sample preparation:

The light and heat degraded solution was then prepared 90% with distilled water and then diluted 200 µl in 1800 µl with diluting solution. Filter through 0.2µ-disc filter. Call this solution B.

2.3.4.5 Chromatographic System:

Apparatus	:	Shimadzu LC-MS solution integrated with SPD10A VP spectrophotometic UV Detector.
Column	:	Nucleosil 100-5 $C_{18,}$ 250 X 4.6 mm, 5µ or equivalent
Temperature	:	35^0 C
Flow rate	:	1.0 ml /minute
UV detection wavelength	:	278 nm
Retention Time	:	15.0 minutes (Approx.)
Load / Inject Volume	:	10 µl by valve injection.

2. 3.4.6 Procedure:

Place vials containing standard preparation A and sample preparation B into the tray of the auto sampler of Shimadzu HPLC. Run the instrument and record the chromatogram. Calculate the quantity of chloramphenicol in the sample using the following equation:

Calculation:

Chloramphenicol content, mg /ml

$$= \frac{A}{B} \times P$$

Where:

A = Peak area of chloramphenicol Hydrochloride in sample (Solution A)

B = Peak area of chloramphenicol Hydrochloride in Standard (Solution B)

P = Potency of standard.

2.4. Developing a new methods for potency determination of ciprofloxacin from ciprofloxacin tablet by UV- spectrophotometric method:

2.4.1 Study of standard curves for ciprofloxacin using different media:

2.4.1.1 Chemicals and Reagents:

Raw material of ciprofloxacin was collected from Pharmadesh Laboratories Ltd, Dhaka, Bangladesh. The potency of ciprofloxacin was 99.77%. All other ingredients used were of analytical grade.

2.4.1.2 Instruments:

HPLC (LC-MS 2020 Shimadzu), UV-Visible spectrophotometer-1650, Electric balance, Digital pH meter

2.4.1.3 Preparation of 0.1N HCl:

0.83 ml of 37% pure concentrated HCl was taken in a 100 ml volumetric flask. The volume was 86 made up with distilled water and the content is thoroughly mixed it by shaking and approximately 0.1N HCl solution was prepared.

2.4.1.4 Sample preparation of standard curve from standard ciprofloxacin HCl in the media of 0.1N HCl acid and distilled water:

2mg/ml of stock solution was prepared by dissolving 100 mg of ciprofloxacin HCl in 50ml of water having pH of 1.0 to 1.2 and pH 4.5 to 6.0 respectively in 0.1N HCl and in distilled water. Standard solution of various concentrations (5, 10, 15, 20, 25 µg/ml) were prepared by dilution from each stock solution. The wavelength of maximum emission (λ_{max}) of ciprofloxacin in each media was found by scanning them over the UV range of 190nm to 400nm. Concentration vs. absorbance was plotted and standard curve was prepared by scanning the standard solutions of ciprofloxacin at 277nm in the media of 0.1N HCl and at 276 in the media of distilled water.

2.5 The comparison of potency of ciprofloxacin of three different companies using 0.1 N hydrochloric acid, distilled water as solvent and HPLC methods:

2.5.1 The potency of ciprofloxacin from tablet of three companies determined by UV-spectrophotometric method using 0.1N HCl as solvent:

Ciprofloxacin tablets were collected from three different companies and the potency was determined by UV-spectrophotometric method using 0.1 N HCl as solvent and follow the following procedure.

2.5.1.1 Standard preparation:

100 mg ciprofloxacin hydrochloride was dissolve accurately weigh 100 mg of ciprofloxacin hydrochloride WS into a 100 ml volumetric flask. Add about 60 ml of 0.1 N HCl solution & sonicate for 5 minutes. Dilute to volume with the same. Then 5 ml was taken in another volumetric flask

2.5.1.2 Sample preparation:

Twenty tablets were grinded in mortar by using pestle. An equivalent weight of the standard ciprofloxacin was taken in100 ml volumetric flask, sonicated for 20 minutes. Then 5 ml was taken in another 100 ml volumetric flask. The potency was determined by an UV-spectrophotometer at 277 nm for 0.1N HCl acid.

Calculation:

$$\text{Potency} = \frac{\text{Ab(TS)}}{\text{Ab(STD)}} \times \text{Potency of s tan dard solution}$$

Here, Ab (TS) = Absorbance of sample

Ab (STD) = Absorbance of standard sample.

99.77 = Potency of standard solution

2.5.2 The potency of ciprofloxacin from ciprofloxacin tablet of three companies determined by UV-spectrophotometric method using distilled water as solvent:

Standard and sample preparation follow the previous procedure **(2.5.1.1and 2.5.1.2)**, only use distilled water instead of 0.1 N HCl as a diluting solvent and the λ_{max} of this preparation is at 276 nm.

2.5.3 The potency of ciprofloxacin tablet of three companies (previously measured by UV-spectrophotometer) determined by HPLC method:

2.5.3.1 Reagents:

1. Phosphoric Acid
2. Acetonitrile
3. Triethylamine

Preparation of Buffer:

Take 1.68 ml Phosphoric acid in 1000 ml purified water, mix well. Adjust pH to 3.0±0.1 with Triethylamine. Filter with 0.2µ membrane filter and sonicate for 5 minutes.

Organic Phase:

Acetonitrile, Filter with 0.2µ membrane filter and sonicate for 5 minutes.

Mobile Phase:

Buffer : Organic Phase = 87:13

Diluting Solution:

Purified Water

2.5.3.2 Chromatographic System:

Apparatus: Shimadzu HPLC-LC solution integrated with SPD10A VP spectrophotometic UV Detector.

Column:	Nucleosil C18, 250 X4.6 mm, 5µ or equivalent.
Flow Rate:	1.5 ml/minute
Temperature:	30^0 C
Wavelength:	278 nm
Inject Volume:	10 µl
Retention Time:	20 minutes (Approximately)

2.5.3.3 Standard Preparation:

Accurately weigh about 27.5 mg of ciprofloxacin Hydrochloride WS into a 100 ml volumetric flask. Add about 60 ml of diluting solution & sonicate for 5 minutes. Dilute to volume with the same. Filter the resultant solution through 0.22µ-disk filter. Call this solution B.

2.5.3.4 Sample Preparation:

Twenty tablets were grinded in mortar by using pestle. An equivalent weight of the standard ciprofloxacin 27.5 mg was measured in 100 ml volumetric flask. Add 60 ml-purified water. Shake 250 rpm for 10 minutes by shaker. Sonicate for 15 minutes. Make volume with the same. Filter the resultant solution through 0.22µ-disk filter. Call this solution A.

2.5.3.5 Procedure:

Place vials containing standard preparation B and sample preparation A into the tray of the auto sampler of Shimadzu HPLC. Run the instrument and record the

chromatogram. Calculate the quantity of ciprofloxacin in the sample using the following equation.

Calculassions:

Ciprofloxacin content (mg/tab).

$$\frac{A \times C \times 100 \times P \times Avg.wt \times 100}{B \times 100 \times Sample\,wt.(mg) \times 500}$$

Where:

A = Peak area of ciprofloxacin HCl in sample (Solution A)

B = Peak area of ciprofloxacin HCl in Standard (Solution B)

C = Weight of standard taken in mg

P = Potency of standard as ciprofloxacin. (As such)

2.5.4 Relative study of UV-spectrophotometric method and HPLC method for the potency determination of ciprofloxacin tablets of three companies:

The relationship between the estimated potency from the UV-spectroscopic method and the estimated potency by HPLC method for three different companies were determined.

2.6 To evaluate the UV-spectroscopic methods for potency determination of ciprofloxacin tablet use another five different companies:

2.6.1 The potency of ciprofloxacin from its tablet of another five different companies determined by UV-spectrophotometric method using 0.1N HCl as solvent:

Sample preparation same as 2.5.1(included 2.5.1.1 and 2.5.1.2)

2.6.2 The potency of ciprofloxacin tablet of another five different companies determined by UV-spectrophotometric method using distilled water as solvent:

Same as upper procedure (2.6.1) use only distilled water as solvent replaced by 0.1 N HCl

2.7 To investigate the interference of degraded product on UV-spectra and absorbance of ciprofloxacin hydrochloride after Heat and Sunlight exposure:

2.7.1 Comparative analysis of Standard ciprofloxacin with Light-exposed and Heat-degraded ciprofloxacin when diluting solvent uses 0.1 N HCl and distilled water:

100 mg of standard ciprofloxacin HCl as active material was taken in 100 ml volumetric flask. 0.1N HCl and distilled water were used as solvent to complete the experiment separately. It was shake and made up to the mark. 9 transparent test tubes were taken each containing 10 ml of the solution. 3 of those tubes were kept in refrigerator as a control, 3 were exposed in sunlight for 14 hours and the other 3 test tubes were kept in 100°C in a water bath for 6 hours. The potency was observed by UV-spectrophotometer.

Calculation:

$$\frac{\text{Absorbance values of the degraded sample}}{\text{Absorbance values of the standard sample}} \times \text{potency of the standard}$$

Chapter Three

RESULTS AND DISCUSSION

RESULTS AND DISCUSSION

3.1 Evaluation of efficacy and potency of marketed chloramphenicol 0.5% eye drops after controlled exposure to sunlight:

3.1.1 Appearance of different Sets of chloramphenicol eye drops samples before and after the exposure of sunlight:

Before the exposure, all the chloramphenicol eye drop samples from ten different companies were clear colorless solutions that turned into opaque, slightly yellow or deep yellow color after the exposure to direct sunlight for 14 hours with their primary, secondary packaging and without packaging in the clear test tubes. Appearances of different sets of samples before and after exposure of sunlight are given in Table 5 and Figure 6.

(a) **(b)** **(C)**

Figure 6: Appearances of different sets of chloramphenicol eye drop samples of different companies before and after the exposure to sunlight for 14 hrs, where a) sample in trasparent test tubes (Set-4), b) samples in containers with its primary and secondary packaging (Set-2), c) samples in only primary packaging (set-3).

Table 5: Appearance of different Sets of chloramphenicol eye drops samples before and after the exposure of sunlight. Set-2, samples in containers with its primary and secondary packaging, Set-3, samples in containers(only with primary packaging), Set-4, sample in trasparent test tubes.

Code	Set-2		Set-3		Set-4	
	Before exposure	After exposure	Before exposure	After exposure	Before exposure	After exposure
ACPC	Clear colorless solution	Opaque	Clear colorless solution	Slightly yellow	Clear colorless solution	Deep yellow color
NIPC	Clear colorless solution	Opaque	Clear colorless solution	Slightly yellow	Clear colorless solution	Deep yellow color
IBPC	Clear colorless solution	Opaque	Clear colorless solution	yellow	Clear colorless solution	Deep yellow color
OPPC	Clear colorless solution	Opaque	Clear colorless solution	yellow	Clear colorless solution	Deep yellow color
POPC	Clear colorless solution	Opaque	Clear colorless solution	Slightly yellow	Clear colorless solution	Deep yellow color
REPC	Clear colorless solution	Opaque	Clear colorless solution	yellow	Clear colorless solution	Deep yellow color

Code	Set-2		Set-3		Set-4	
ASPC	Clear colorless solution	Opaque	Clear colorless solution	Slightly yellow	Clear colorless solution	Deep yellow color
JAPC	Clear colorless solution	Opaque	Clear colorless solution	Slightly yellow	Clear colorless solution	Deep yellow color
BEPC	Clear colorless solution	Clear colorless solution	Clear colorless solution	Opaque	Clear colorless solution	Deep yellow color
SQPC	Clear colorless solution	Clear colorless solution	Clear colorless solution	Opaque	Clear colorless solution	Deep yellow color

From Table 5 and Figure 6, it is evident that before sunlight exposure, appearances of all the sample solution from different sets were clear and colorless. But after 14 hours of sunlight exposure, for set-2, most of the sample solutions turned into opaque while BEPC and SQPC samples retained their clarity. The samples for Set-3 turned to slightly yellow but BEPC and SQPC samples became opaque as the primary packaging could not protect the solution from sunlight in spite of opaque LDPE bottles. In case of Set-4, all samples became deep yellow due to the sensitivity of chloramphenicol to light. It was found that packaging is very important factor for their stability to maintain the shelf life. So, chloramphenicol eye drops must be packaged in opaque or amber colored light protected containers.

3.1.2 Potency of the Sunlight-induced degraded marketed chloramphenicol eye drop preparations by UV- spectrophotometric method

The potency of sunlight-induced degraded chloramphenicol 0.5% eye drop preparations by UV- spectrophotometric method are given in the Table 6.

Table 6: Potency of sunlight-induced degraded marketed chloramphenicol eye drop preparations. Set-1 stored in refrigerator with primary and secondary packaging (Control).Set-2 contain LDPE containers and secondary packaging, Set-3 in only primary packaging (LDPE containers) and Set-4 kept in transparent test tubes.

Code	Sets	Absorbance	Potency (%)
ACPC	Set-1	0.335	112.79
	Set-2	0.352	118.52
	Set-3	0.363	122.22
	Set-4	0.413	139.05
NIPC	Set-1	0.298	100.34
	Set-2	0.308	103.70
	Set-3	0.318	107.07
	Set-4	0.328	110.44
IBPC	Set-1	0.320	107.74
	Set-2	0.338	113.80
	Set-3	0.347	116.83
	Set-4	0.404	136.03

Code	Sets	Absorbance	Potency (%)
OPPC	Set-1	0.298	100.34
	Set-2	0.320	107.74
	Set-3	0.351	118.18
	Set-4	0.363	122.22
POPC	Set-1	0.322	108.42
	Set-2	0.329	110.77
	Set-3	0.334	112.50
	Set-4	0.417	140.40
REPC	Set-1	0.305	102.69
	Set-2	0.322	108.42
	Set-3	0.342	115.15
	Set-4	0.385	129.63
ASPC	Set-1	0.287	96.63
	Set-2	0.295	99.32
	Set-3	0.319	107.41
	Set-4	0.370	124.58

Code	Sets	Absorbance	Potency (%)
JAPC	Set-1	0.292	98.32
	Set-2	0.305	102.69
	Set-3	0.322	108.42
	Set-4	0.365	122.89
BEPC	Set-1	0.316	106.39
	Set-2	0.325	109.43
	Set-3	0.338	113.80
	Set-4	0.385	129.63
SQPC	Set-1	0.302	101.68
	Set-2	0.307	103.37
	Set-3	0.314	105.60
	Set-4	0.370	124.58

From Table6, it was observed that all the samples stored in the refrigerator (Set-1) have the potency near 100% except JAPC where the potency of Set-1 is only 75.75%. When the samples were exposed to the sunlight with their primary and secondary packaging (Set-2), the potency increased in comparison to Set-1. The potency of SQPC, POPC and BEPC increased a little bit comparing other companies as their primary and secondary packaging are strong enough to protect

the samples from sunlight. When the samples were exposed to the sunlight with only their primary packaging (Set-3), the potency increased sharply. Potency values of samples that were kept in transparent LDPE container (IBPC, OPPC and REPC) were found to be abruptly increased. Samples with a minimum potency increase were from SQPC, BEPC and POPC. When the samples were exposed to the sunlight in clear transparent test tubes (Set-4), increase of potency reached to the maximum.

As a result of photo-degradation of chloramphenicol eye drops, the color of chloramphenicol solution turns into yellow indicating its change into another color producing product. Photo degradation reaction leads to the formation of several products. Similar incidents of degradation was investigated by Shih and Mubarak *et al* [71,55]. The potency of chloramphenicol determined at 278nm for Set-1 solution did not degrade. For Set-2, there is a minor change of potency as the solution became yellowish. For Set-3, the color of solution changed to yellow due to the photo degradation of chloramphenicol solution. So when the potency was determined at 278 nm, the remaining chloramphenicol and degradation product of chloramphenicol jointly gave the absorbance. As a result, the potency level increased promptly. Furthermore, when the samples were exposed to direct sunlight in transparent test tubes, the color of the solution turns into deep yellow. So there was a sharp rise in the absorbance values whereas the potency also became increased.

Table 7: Percentages of increase of the estimated potency for different marketed chloramphenicol eye drops determined by UV-spectrophotometric method.

Code	Sets	% of estimated potency increase	Remarks
ACPC	Set-1 to Set-2	5.08	Clear solution, opaque LDPE bottle.
	Set-1 to Set-3	8.36	Slightly yellow color, opaque LDPE bottle.
	Set-1 to Set-4	23.28	Deep yellow color
NIPC	Set-1 to Set-2	3.34	Clear solution, opaque LDPE bottle.
	Set-1 to Set-3	6.70	Slightly yellow color, opaque LDPE bottle.
	Set-1 to Set-4	10.06	Deep yellow color
IBPC	Set-1 to Set-2	5.62	Clear solution, Transparent LDPE bottle.
	Set-1 to Set-3	8.43	Yellow color, transparent LDPE bottle.
	Set-1 to Set-4	26.25	Deep yellow color
OPPC	Set-1 to Set-2	7.37	Clear solution, Transparent LDPE bottle.
	Set-1 to Set-3	17.78	Yellow color, transparent LDPE bottle
	Set-1 to Set-4	21.80	Deep yellow color

Code	Sets	% of estimated potency increase	Remarks
POPC	Set-1 to Set-2	2.17	Clear solution, opaque LDPE bottle.
	Set-1 to Set-3	3.76	Slightly yellow color, opaque LDPE bottle.
	Set-1 to Set-4	29.43	Deep yellow color
REPC	Set-1 to Set-2	5.58	Clear solution, transparent LDPE bottle.
	Set-1 to Set-3	12.13	Yellow color, transparent LDPE bottle
	Set-1 to Set-4	26.23	Deep yellow color
ASPC	Set-1 to Set-2	2.78	Clear solution, opaque LDPE bottle.
	Set-1 to Set-3	11.16	Slightly yellow color, opaque LDPE bottle
	Set-1 to Set-4	28.92	Deep yellow color
JAPC	Set-1 to Set-2	4.44	Clear solution, opaque LDPE bottle
	Set-1 to Set-3	10.27	Slightly yellow color, opaque LDPE bottle.
	Set-1 to Set-4	24.98	Deep yellow color

Code	Sets	% of estimated potency increase	Remarks
BEPC	Set-1 to Set-2	2.85	Clear solution, opaque LDPE bottle
	Set-1 to Set-3	6.96	Slightly yellow color, opaque LDPE bottle.
	Set-1 to Set-4	21.84	Deep yellow color
SQPC	Set-1 to Set-2	1.65	Clear solution, opaque LDPE bottle
	Set-1 to Set-3	3.85	Slightly yellow color, opaque LDPE bottle.
	Set-1 to Set-4	22.52	Deep yellow color

In some cases, there was a presence of slightly high potency values of Set-1 or control sample solutions. Probably this happened due to the addition of overage by the manufacturers during formulation of chloramphenicol eye drops.

Different percentages of estimated increase of potency for different Sets determined by the UV-spectrophotometric method as compared to Set-1 (control) from different companies are shown in table 7:

For Set-2, the potency increased from 1 to 3% for SQPC, POPC, ASPC and BEPC. For ACPC, NIPC, JAPC, IBPC and REPC the values were between 3 to 6% but for OPPC, it was more than 7%.

For Set-3, the potency increased 3 to 7% for SQPC, POPC, NIPC and BEPC. For ACPC, ASPC, JAPC, IBPC and REPC it was 8 to 13% but for OPPC it was more than 17%.

In case of Set-4, the percentage of estimated potency increased more than 21 % for all companies. From the above result it became clear that this increase in potency values depends on photo-degradation reactions. When the samples (Set-4) were exposed to the direct sunlight, much amount of color-forming photodegradation products was produced. As the color of pure chloramphenicol and photodegradation products was the same, the chromophores gave absorbance values at the same wavelength, resulting potency values more than the actual values.

3.1.3 Efficacy of Heat and Sunlight induced degraded chloramphenicol eye drops preparations by disc diffusion assay method:

To observe the change in efficacy after sunlight exposure of marketed chloramphenicol eye drop preparations, the agar disc diffusion test was performed. After 12 hours of incubation, the antibacterial activities of sunlight-induced degraded chloramphenicol eye drops were determined by measuring the diameter of the zones of inhibition in mm with a transparent scale. The zones of inhibition and percentage of decrease in efficacy compared to the controlled preparations (Set-1) against *Salmonella typhi* and *Shigella dysenteriae* are given in Table 8.

Table 8: Zones of inhibition for sunlight-induced and heat-degraded chloramphenicol eye drops formed against *Salmonella typhi* and *Shigella dysenteriae*.

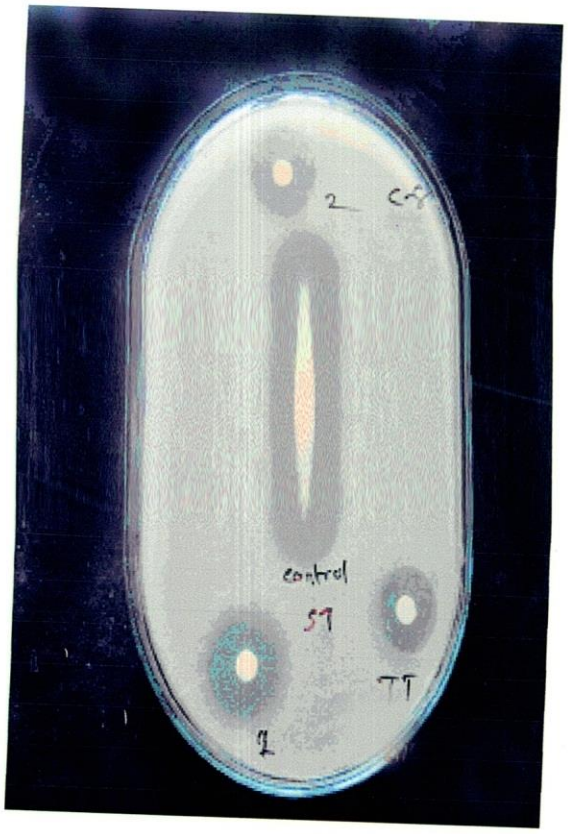
Code	Sets	*Salmonella typhi*		*Shigella dysenteriae*	
		Zone of Inhibition	Decrease of efficacy compared to Set- 1 (%)	Zone of Inhibition	Decrease of efficacy compared to Set- 1 (%)
ACPC	Set-1	20	-	22	-
	Set-2	19	5	20	9.09
	Set-3	19	5	18	18.18
	Set-4	18	10	15	31.81
NIPC	Set-1	21	-	22	-
	Set-2	20	4.76	20	9.09
	Set-3	18	14.28	20	9.09
	Set-4	16	23.80	18	18.18
IBPC	Set-1	22	-	22	-
	Set-2	20	9.09	20	9.09
	Set-3	20	9.09	20	9.09
	Set-4	18	18.18	18	18.18
OPPC	Set-1	21	-	22	-
	Set-2	18	14.28	20	9.09
	Set-3	15	28.57	17	22.72
	Set-4	15	28.57	15	31.81
POPC	Set-1	21	-	22	-
	Set-2	20	4.76	20	9.09
	Set-3	20	4.76	18	18.18
	Set-4	17	19.04	17	22.72

Code	Sets	*Salmonella typhi*		*Shigella dysenteriae*	
		Zone of Inhibition	Decrease of efficacy compared to Set- 1 (%)	Zone of Inhibition	Decrease of efficacy compared to Set- 1 (%)
REPC	Set-1	18	-	20	-
	Set-2	17	5.55	18	10
	Set-3	15	16.67	16	20
	Set-4	13	27.77	14	30
ASPC	Set-1	20	-	21	-
	Set-2	18	10	19	9.52
	Set-3	16	20	18	14.28
	Set-4	15	25	15	28.57
JAPC	Set-1	10	-	10	-
	Set-2	9	10	10	0
	Set-3	7	30	8	20
	Set-4	6	40	6	40
BEPC	Set-1	22	-	24	-
	Set-2	18	18.18	23	4.16
	Set-3	18	18.18	21	12.5
	Set-4	16	27.27	20	16.67
SQPC	Set-1	24	-	24	-
	Set-2	20	16.66	22	8.33
	Set-3	20	16.66	22	8.33
	Set-4	18	25	20	16.67

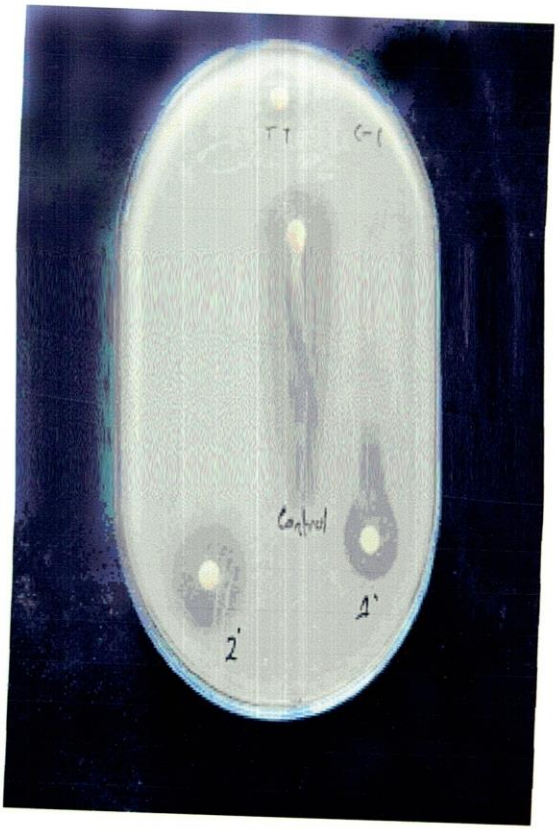
From Table 8, it was observed that all the samples from the Set-1 were highly sensitive to both test organisms. The zones of inhibition of Set-2 sample solution for each company against *Salmonella typhi* and *Shigella dysenteriae* decreased as compared to the Set-1 sample solution for the same company. The zones of inhibition for Set-3 sample solution for each company found decreased comparing to Set-1 and Set-2 sample solution. Minimum antibacterial activity of Set-4 sample solution for each company was found as compared to the all other Sets.

The percentage of decrease in efficacy of Set-2 sample solutions of different companies as compared to Set-1 varied from 5 to 18%. As compared to control, the percentage of decrease in efficacy of Set-3 solutions varied from 5 to 28% whereas for Set-4 solutions, it was 10 to 32%.

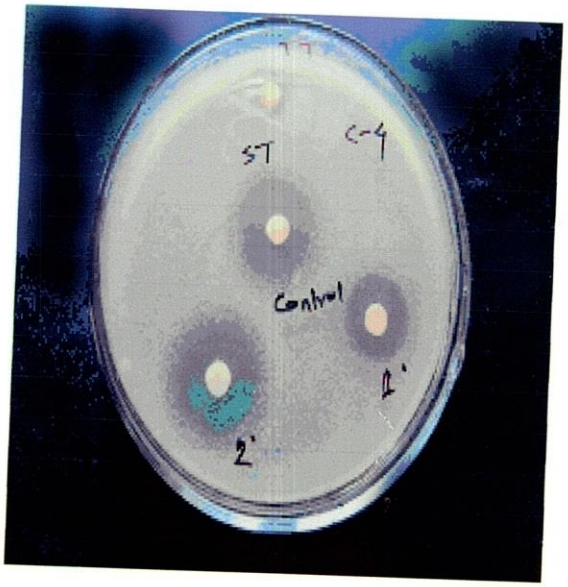
After collection of chloramphenicol eye drops, the Set-1 samples were subjected to store in a refrigerator, so probably no or minimum degradation of chloramphenicol occurred and their zone of inhibition against *Salmonella typhi* and *Shigella dysentereriae* was maximum (Figure 7 and Figure 8).



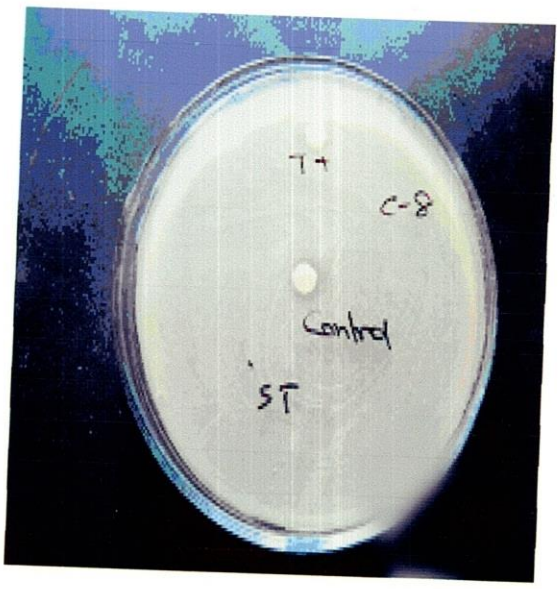
a) C8: SQPC, Control (ST): Set-1, 2: Set-2, 1: Set-3, TT: Set-4



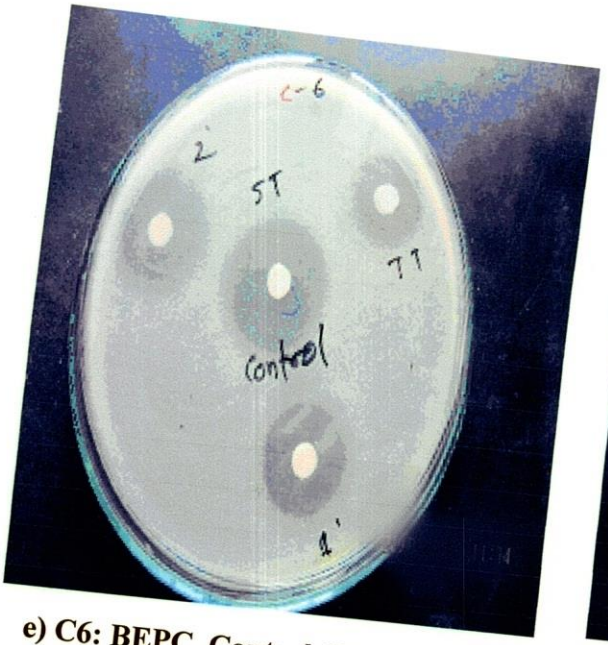
b) C1: POPC, Control: Set-1, 2: Set-2, 1: Set-3, TT: Set-4



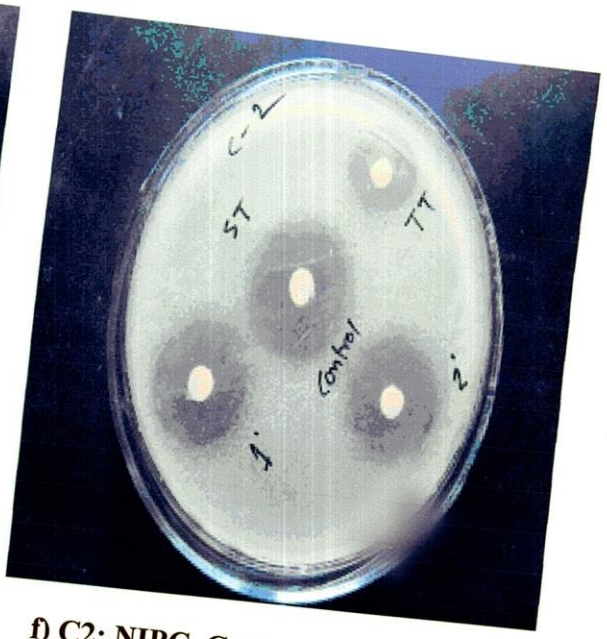
c) C4: IBPC, Control (ST): Set-1, 2: Set-2, 1: Set-3, TT=Set-4



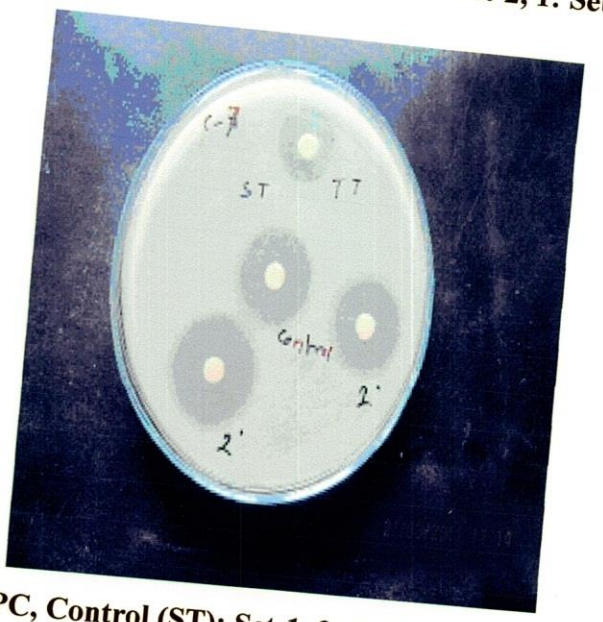
d) C8=JACC, Control: Set-1 TT: Set-4



e) C6: BEPC, Control (ST): Set-1, 2: Set-2, 1: Set-3, TT: Set-4



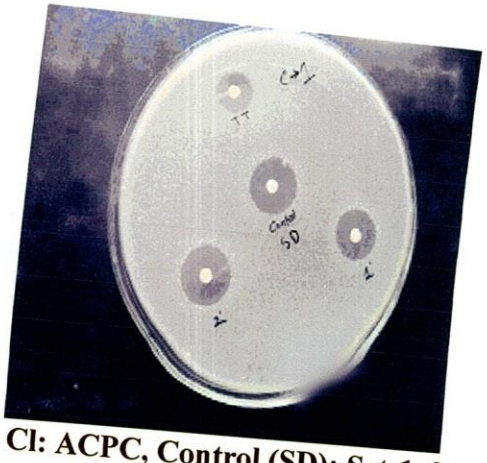
f) C2: NIPC, Control (ST): Set-1, 2: Set-2, 1: Set-3, TT: Set-4



g) C7: REPC, Control (ST): Set-1, 2: Set-2, 1: Set-3, TT: Set-4

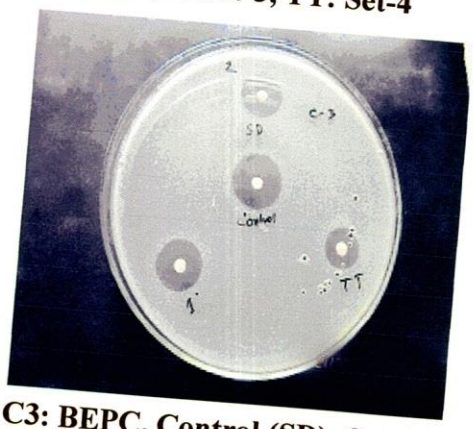
Figure 7: Activity of different Sets of chloramphenicol samples against *Salmonella typhi.* Set-1 was the control samples kept in refrigerator; Set-2 was sunlight-induced samples in LDPE bottle with secondary packaging; Set-3 was sunlight-induced samples in LDPE bottle; Set-4 was sunlight-induced samples in clear transparent test tube.

When the samples were directly exposed to sunlight with their primary packaging, photodegradation reaction occurred and the zone of inhibition also decreased. But as those companies (IBPC, OPPC and REPC) used transparent LDPE bottles, activities were decreased comparing with those using opaque LDPE bottles. When the samples were directly exposed to sunlight within some transparent test tubes, the solutions became deep yellow and most of the chloramphenicol solutions were converted into color producing degradation products. So the zones of inhibition of the Set-4 sample solutions were less than those of the other Sets.

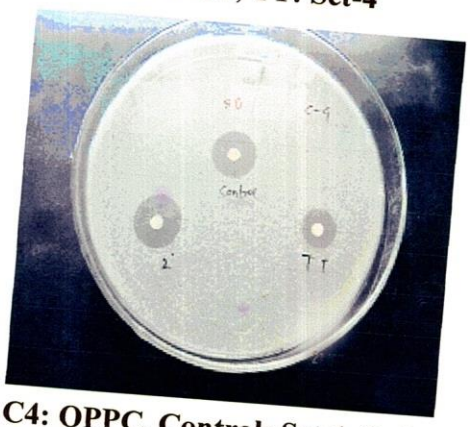


a) Cl: ACPC, Control (SD): Set-1, 2: Set-2, 1: Set-3, TT: Set-4

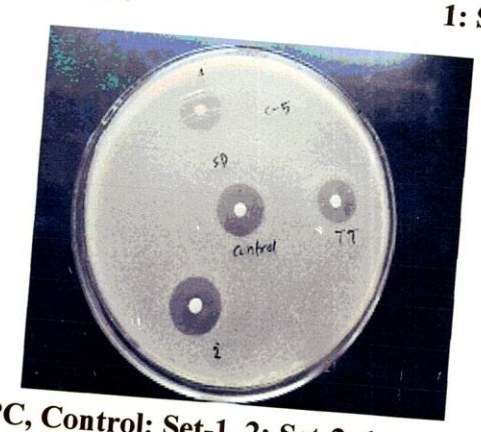
b) C2: SQPC, Control: Set-1, 2: Set-2, 1: Set-3, TT: Set-4



c) C3: BEPC, Control (SD): Set-1, 2: Set-2, 1: Set-3, TT: Set-4



d) C4: OPPC, Control: Set-1, 2: Set-2, 1: Set-3 TT: Set-4



e) C5: POPC, Control: Set-1, 2: Set-2, 1: Set-3, TT: Set-4

Figure 8: Activity of different sets of chloramphenicol samples against *Shigella dysenteriae*. Set-1 were the control samples kept in refrigerator; Set-2 were sunlight-induced samples in LDPE bottle with secondary packaging; Set-3 were sunlight-induced samples in LDPE bottle; Set-4 were sunlight-induced samples in clear transparent test tube.

It was observed that the zones of inhibition for Set-1 were bigger for all marketed chloramphenicol eye drops than other Sets against *salmonella typhi*. Among them, the Set-1 sample for SQPC showed high sensitivity to the bacteria and produced the largest zones of inhibition compared to the products of other companies like BEPC, IBPC and POPC. All other company products were moderately sensitive except JAPC that was not so active against *Salmonella typhi*. This may have happened due to low quality active materials or insufficient amount of the active materials during manufacturing process.

After 14 hours of sunlight exposure, the Set-2 samples (with primary and secondary packaging) showed slightly lower activity as compared to Set-1 samples. The decrease in efficacy for ACPC, NIPC, POPC and REPC were comparatively lower than samples from other companies.

In case of Set-3 samples (only with primary packaging) from ACPC, IBPC, POPC and JAPC the efficacy decreased lesser than ASPC, OPPC and NIPC. It became evident that when the samples were exposed to direct sunlight in clear transparent test tubes, activity of all samples became greatly reduced.

Against *Shigella dysenteriae* it was observed that the zone of inhibition for all the samples for Set-1 was maximum. For Set-2 it was decreased more than 4 to 9% and Set-3 it decreased above 9 to 22% and incases of Set-4 it was greater 31%.

From the above discussion it can be concluded that chloramphenicol is a highly light-sensitive drug and efficacy of the samples decreased with the intensity of

sunlight exposure. As a result, the activity of chloramphenicol was surely reduced when those were not preserved maintaining standard conditions. Due to the formation of inactive products, the chloramphenicol eye drops became ineffective and there is a possibility of developing resistance that will exert serious health hazards to the patients.

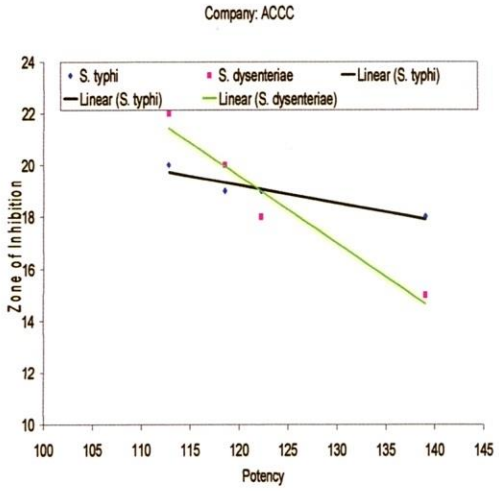
3.1.4 Relationship between the efficacy of different Sets of samples by disc diffusion methods and their estimated potency by UV-spectrophotometric method:

With the increase in percentage of potency, the efficacy against *Salmonella typhi* and *Shigella dysenteriae* became decreased, especially in case of Set – 4 for all the companies (Table 9).

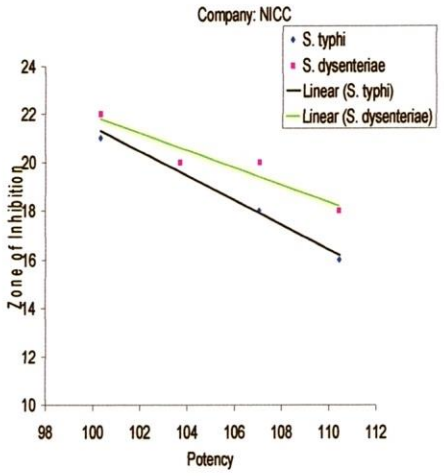
Table 9: Comparative study of percentages of estimated potency increase by UV-spectrophotometer and decrease of efficacy by disc diffusion methods.

Code	Sets (Compared to Set-1)	% of estimated potency increase by UV-spectrophotometer	*S.typhi*	*S.dysenteriae*
			Efficacy decreased (%)	Efficacy decreased (%)
ACPC	Set-2	5.08	5	9.09
	Set-3	8.36	5	18.18
	Set-4	23.28	10	31.81
NIPC	Set-2	3.34	4.76	9.09
	Set-3	6.70	14.28	9.09
	Set-4	10.06	23.80	18.18
IBPC	Set-2	5.62	9.09	9.09
	Set-3	8.43	9.09	9.09
	Set-4	26.25	18.18	18.18
OPPC	Set-2	7.37	14.28	9.09
	Set-3	17.78	28.57	22.72
	Set-4	21.80	28.57	31.81
POPC	Set-2	2.17	4.76	9.09
	Set-3	3.76	4.76	18.18
	Set-4	29.43	19.04	22.72

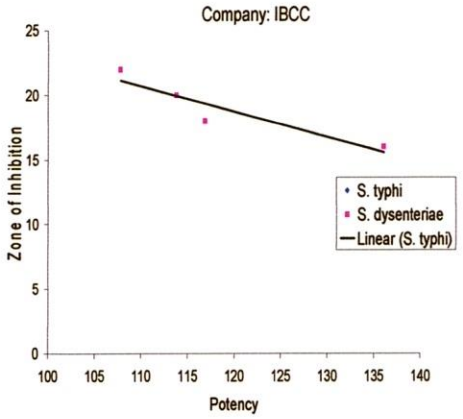
Code	Sets (Compared to Set-1)	% of estimated potency increase by UV-spectrophotometer	*S.typhi*	*S.dysenteriae*
			Efficacy decreased (%)	Efficacy decreased (%)
REPC	Set-2	5.58	5.55	10
	Set-3	12.13	16.67	20
	Set-4	26.23	27.77	30
ASPC	Set-2	2.78	10.00	9.59
	Set-3	11.16	20	14.28
	Set-4	28.92	25	28.57
JAPC	Set-2	4.44	10	0
	Set-3	10.27	30	20
	Set-4	24.98	40	40
BEPC	Set-2	2.85	18.18	4.16
	Set-3	6.96	18.18	12.5
	Set-4	21.84	27.27	16.67
SQPC	Set-2	1.65	16.66	8.33
	Set-3	3.85	16.66	8.33
	Set-4	22.52	25	16.67


Company: ACCC
S. typhi
S. dysenteriae
Linear (S. typhi)
Linear (S. typhi)
Linear (S. dysenteriae)
Zone of Inhibition
Potency

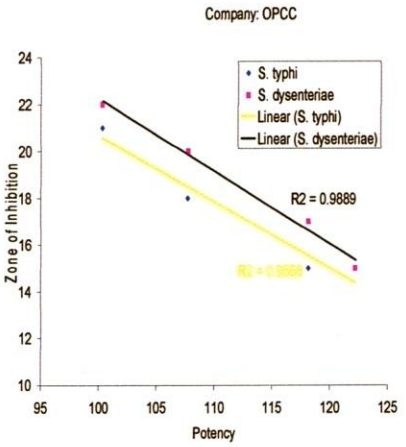
a


Company: NICC
S. typhi
S. dysenteriae
Linear (S. typhi)
Linear (S. dysenteriae)
Zone of Inhibition
Potency

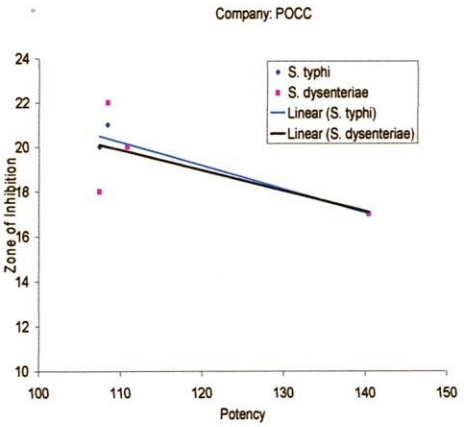
b


Company: IBCC
S. typhi
S. dysenteriae
Linear (S. typhi)
Zone of Inhibition
Potency

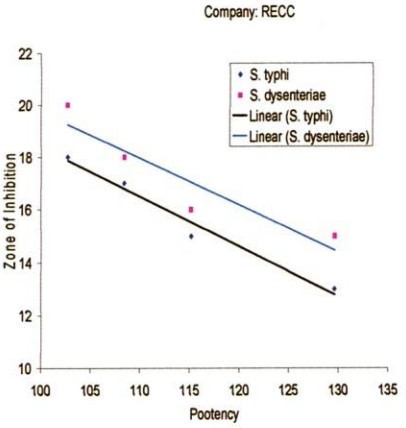
c


Company: OPCC
S. typhi
S. dysenteriae
Linear (S. typhi)
Linear (S. dysenteriae)
R2 = 0.9889
Zone of Inhibition
Potency

d


Company: POCC
S. typhi
S. dysenteriae
Linear (S. typhi)
Linear (S. dysenteriae)
Zone of Inhibition
Potency

e


Company: RECC
S. typhi
S. dysenteriae
Linear (S. typhi)
Linear (S. dysenteriae)
Zone of Inhibition
Pootency

f

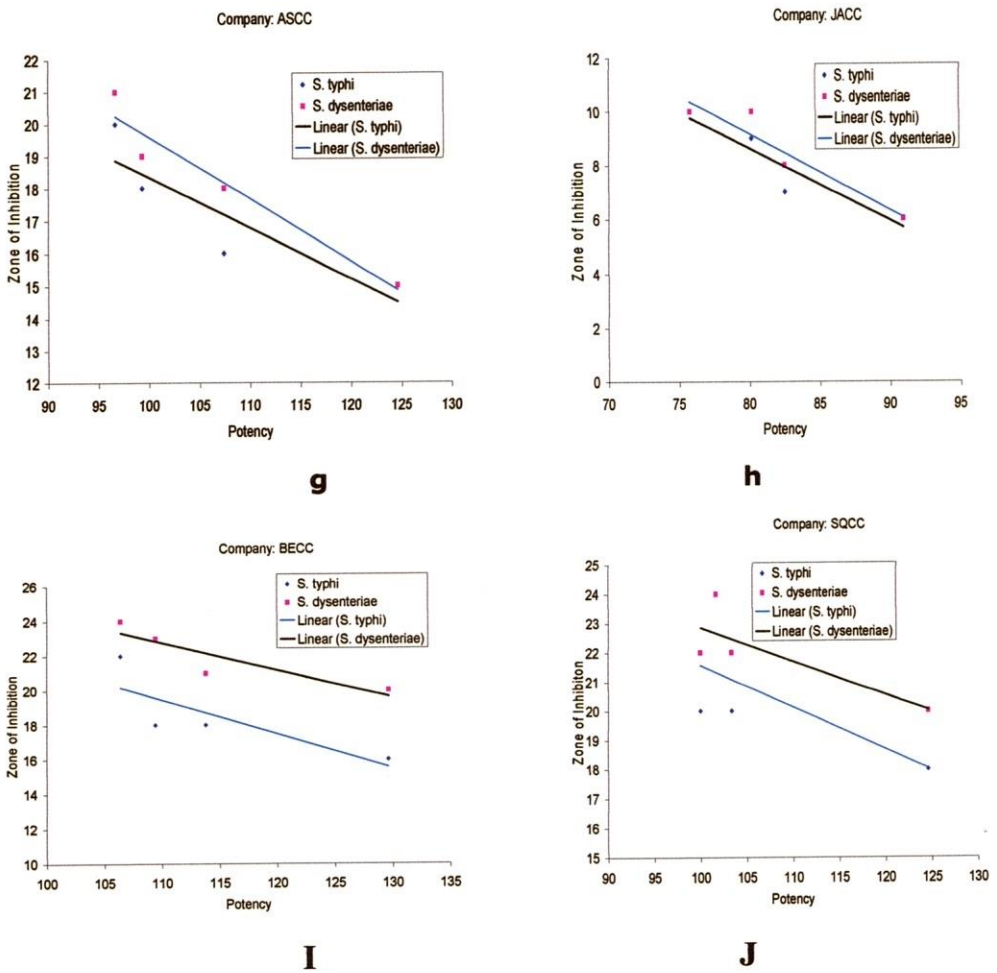


Figure 9: Relationships between the zones of inhibition of different Sets of samples against *Salmonella typhi* and *Shigella dysenteriae* and their estimated potency that was measured by UV-spectrophotometer.

Figure 9 showed that the zones of inhibition of different Sets of samples against *S.typhi* and *S. dysenteriae* were inversely correlated to the estimated potency for different Sets of samples. When the marketed chloramphenicol eye drops from different companies were subjected to sunlight induction, their estimated potency level increased whereas the zones of inhibition became smaller indicating a significant reduction of antibacterial activity.

UV-spectroscopic method for the quantitative analysis of chloramphenicol eye drops is a well known and established method described in British Pharmacopia [Dilute a volume containing 25 mg of chloramphenicol to 250 ml with *water*. Dilute 10 ml to 100 ml with water and measure the absorbance of the resulting solution at the maximum at 278 nm .Calculate the content of $C_{11}H_{12}C_{l2}N_2O_5$ taking 297 as the value of (1%, 1 cm) at the maximum at 278 nm (BP-2010)].

But here we observed a deviation of UV absorbance take place, due to the presence of some excipients other than chloramphenicol itself. For this reason the following experiments were occurred.

3.2 Investigation the cause of in reverse correlation between efficacy by ager disc diffusion method and potency by UV-spectroscopic method after Heat and Sunlight induced degradation:

3.2.1 Appearance of different Sets of chloramphenicol aqueous solution (without any excipient) before and after the induction of Sunlight and Heat:

The appearances of heat and sunlight-induced degraded chloramphenicol aqueous solutions were observed visually after induction of heat and sunlight. We observed that the solution of both Set- 2 and Set- 3 were colorless before the exposure. But after the application of heat for 6 hours in incubator at 100°C, the color of Set-2 changed into yellow when the samples of Set-3 were exposed to sunlight for 14 hrs, the color changed into deep yellow.

Table 10: Appearance of the heat and sunlight-induced degraded chloramphenicol aqueous solution (Set- 1, kept in refrigerator as Standard; Set-2, Heat at 100°C for 6 hrs and Set-3 Sunlight exposure for 14 hrs).

Set	Treatment	Change in appearance	
		Before treatment	After treatment
Set-2	Heat at 100°C for 6 hrs	Clear colorless solution	Yellow color
Set-3	Sunlight exposure for 14 hrs	Clear colorless solution	Deep yellow color

3.2.2 UV-spectra of the absorbance values for Standard, Sunlight & Heat-induced degraded aqueous solution of chloramphenicol:

The UV-spectra of control (Set-1), Sunlight (Set-3) & Heat (Set-2) induced degraded aqueous solution of chloramphenicol were taken within the wavelength 200-400nm by a UV-spectrophotometer. The result is given in the Table 11 and Figure 10.

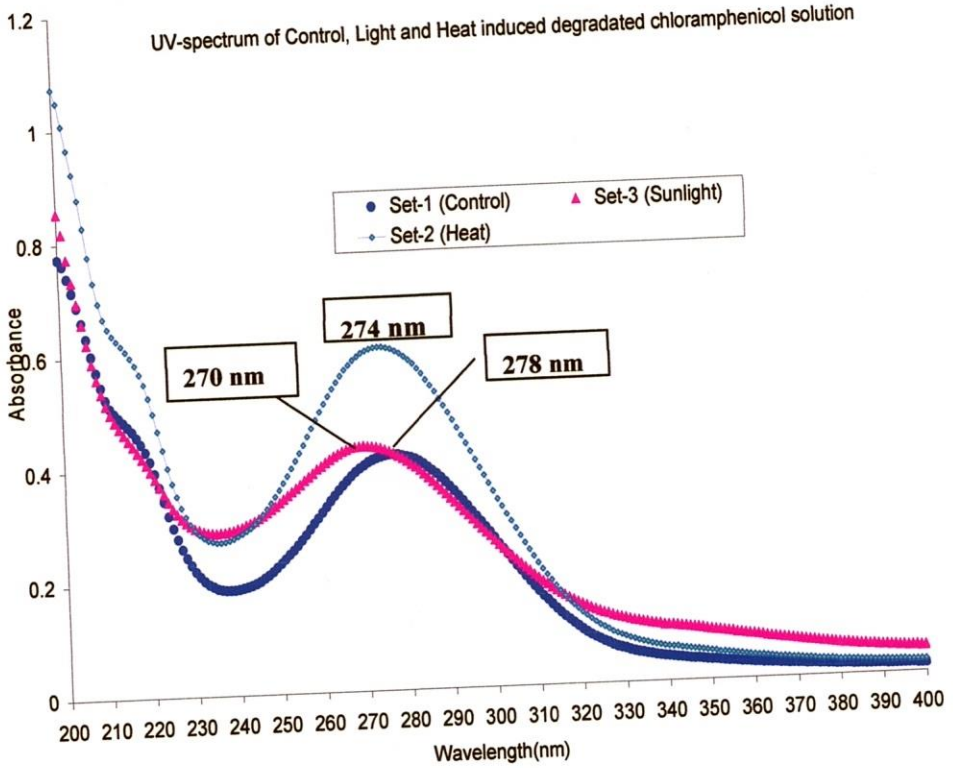


Figure 10: The UV-spectrum of Standard, Heat and Sunlight-induced degraded chloramphenicol aqueous solutions.

From Figure 10, it was evident that λ_{max} for pure chloramphenicol was found at 278 nm but for heat and sunlight-induced degraded chloramphenicol solutions, the λ_{max} became shifted at 274 nm and 270 nm respectively.

The λ_{max} for pure chloramphenicol was 278 nm, so when we took the UV-spectrum of pure chloramphenicol within the range of wavelength 200-400nm, we got the λ_{max} in the accurate position. After heat and sunlight exposure of chloramphenicol solution, hydrolysis and photodegradation reaction took place respectively and chloramphenicol must have converted into one or more degradation products. So

λ_{max} for both heat and sunlight-induced solution became shifted to shorter side from its actual value. (hypsochromic shift or called blue shift).

It was also seen that the peak absorbance of sunlight-induced solution was slightly higher than the peak absorbance of pure chloramphenicol, but it was found to be a lot higher in case of heat-induced solution. At the time of treatment, the concentrations of different Sets were the same, so the expected absorbance of the different Sets after treatment should also remain in the same level. But in practice, the absorbance varied and gave different potency other than the actual value. This phenomenon indicated that chloramphenicol became converted to degradation products. The degradation products of chloramphenicol absorbed UV-light strongly and gave absorbance jointly with chloramphenicol resulting higher absorbance values. The detailed study of absorbance values (within wavelength range 200-400 nm) for standard, heat induced and sunlight-exposed chloramphenicol aqueous solution (Table 11)

Table 11: Absorbance values (within wavelength range 200-400 nm) for standard, heat induced and sunlight-exposed chloramphenicol aqueous solution. The height of UV-spectra of sunlight and heat-induced chloramphenicol solution compared to the UV-spectra of pure chloramphenicol indicates the quantitative change of chloramphenicol.

Wavelength (nm)	Set-1	Set-2	Set-3	Wavelength (nm)	Set-1	Set-2	Set-3
200	0.774	1.074	0.854	249	0.222	0.347	0.33
201	0.763	1.05	0.819	250	0.229	0.358	0.336
202	0.741	1.009	0.774	251	0.236	0.37	0.342
203	0.716	0.966	0.734	252	0.244	0.382	0.348
204	0.69	0.923	0.697	253	0.251	0.395	0.354
205	0.663	0.877	0.66	254	0.259	0.408	0.36
206	0.634	0.828	0.624	255	0.268	0.421	0.366
207	0.603	0.777	0.59	256	0.277	0.435	0.373
208	0.574	0.732	0.562	257	0.287	0.45	0.379
209	0.548	0.696	0.537	258	0.297	0.464	0.385
210	0.526	0.668	0.516	259	0.306	0.478	0.392
211	0.511	0.65	0.5	260	0.316	0.492	0.397
212	0.501	0.638	0.487	261	0.326	0.506	0.403
213	0.493	0.628	0.475	262	0.336	0.519	0.408
214	0.485	0.619	0.464	263	0.345	0.532	0.413
215	0.477	0.609	0.453	264	0.354	0.543	0.417

Wavelength (nm)	Set-1	Set-2	Set-3	Wavelength (nm)	Set-1	Set-2	Set-3
216	0.469	0.598	0.443	265	0.362	0.554	0.421
217	0.458	0.585	0.432	266	0.37	0.564	0.424
218	0.446	0.569	0.421	267	0.378	0.572	0.427
219	0.432	0.551	0.41	268	0.385	0.58	0.428
220	0.415	0.527	0.398	269	0.391	0.586	0.429
221	0.394	0.499	0.385	270	0.396	0.592	0.43
222	0.37	0.468	0.371	271	0.401	0.596	0.429
223	0.345	0.435	0.358	272	0.405	0.599	0.428
224	0.319	0.403	0.344	273	0.408	0.601	0.427
225	0.297	0.376	0.332	274	0.41	0.602	0.425
226	0.277	0.353	0.322	275	0.412	0.602	0.422
227	0.259	0.333	0.313	276	0.413	0.6	0.419
228	0.244	0.317	0.306	277	0.413	0.598	0.416
229	0.231	0.303	0.3	278	0.413	0.594	0.415
230	0.219	0.292	0.295	279	0.411	0.59	0.407
231	0.21	0.284	0.292	280	0.409	0.584	0.402
232	0.202	0.278	0.289	281	0.407	0.578	0.396
233	0.196	0.273	0.287	282	0.404	0.571	0.391
234	0.192	0.271	0.286	283	0.4	0.562	0.385
235	0.188	0.269	0.285	284	0.395	0.553	0.378
236	0.186	0.269	0.286	285	0.389	0.542	0.371

Wavelength (nm)	Set-1	Set-2	Set-3	Wavelength (nm)	Set-1	Set-2	Set-3
237	0.185	0.27	0.286	286	0.383	0.532	0.364
238	0.185	0.272	0.288	287	0.377	0.521	0.357
239	0.185	0.275	0.289	288	0.37	0.508	0.349
240	0.186	0.279	0.292	289	0.362	0.496	0.342
241	0.188	0.283	0.294	290	0.354	0.483	0.334
242	0.19	0.288	0.298	291	0.346	0.47	0.327
243	0.192	0.293	0.301	292	0.337	0.456	0.319
244	0.195	0.3	0.305	293	0.328	0.442	0.311
245	0.199	0.307	0.309	294	0.319	0.428	0.303
246	0.204	0.316	0.314	295	0.309	0.414	0.295
247	0.209	0.326	0.319	296	0.3	0.399	0.287
248	0.215	0.336	0.325	297	0.29	0.385	0.279
298	0.28	0.371	0.272	351	0.034	0.049	0.087
299	0.271	0.357	0.265	352	0.033	0.047	0.086
300	0.261	0.343	0.257	353	0.032	0.047	0.085
301	0.25	0.329	0.249	354	0.031	0.046	0.084
302	0.241	0.316	0.242	355	0.031	0.045	0.083
303	0.231	0.302	0.235	356	0.03	0.044	0.081
304	0.221	0.289	0.228	357	0.029	0.043	0.08
305	0.211	0.276	0.221	358	0.029	0.042	0.079
306	0.202	0.263	0.214	359	0.028	0.041	0.078

Wavelength (nm)	Set-1	Set-2	Set-3	Wavelength (nm)	Set-1	Set-2	Set-3
307	0.192	0.25	0.207	360	0.027	0.04	0.077
308	0.183	0.238	0.201	361	0.027	0.039	0.076
309	0.174	0.226	0.194	362	0.026	0.039	0.075
310	0.165	0.214	0.188	363	0.026	0.038	0.073
311	0.156	0.203	0.182	364	0.025	0.037	0.072
312	0.149	0.193	0.177	365	0.025	0.036	0.071
313	0.141	0.183	0.171	366	0.024	0.035	0.07
314	0.133	0.173	0.166	367	0.024	0.035	0.069
315	0.126	0.164	0.161	368	0.023	0.034	0.068
316	0.119	0.155	0.157	369	0.023	0.033	0.067
317	0.113	0.147	0.152	370	0.022	0.033	0.066
318	0.107	0.139	0.148	371	0.022	0.032	0.065
319	0.101	0.132	0.144	372	0.021	0.031	0.064
320	0.095	0.125	0.14	373	0.021	0.031	0.063
321	0.09	0.119	0.137	374	0.021	0.03	0.062
322	0.085	0.113	0.133	375	0.02	0.029	0.061
323	0.081	0.107	0.13	376	0.02	0.029	0.06
324	0.077	0.102	0.127	377	0.019	0.028	0.059
325	0.073	0.098	0.124	378	0.019	0.028	0.058
326	0.07	0.094	0.122	379	0.018	0.027	0.057
327	0.066	0.089	0.119	380	0.018	0.026	0.056

Wavelength (nm)	Set-1	Set-2	Set-3	Wavelength (nm)	Set-1	Set-2	Set-3
328	0.063	0.086	0.117	381	0.018	0.026	0.055
329	0.061	0.083	0.115	382	0.018	0.026	0.055
330	0.059	0.08	0.113	383	0.017	0.025	0.054
331	0.056	0.077	0.111	385	0.017	0.024	0.052
332	0.054	0.075	0.109	386	0.016	0.024	0.052
333	0.052	0.072	0.108	387	0.016	0.024	0.051
334	0.051	0.07	0.106	388	0.016	0.023	0.051
335	0.049	0.068	0.105	389	0.016	0.023	0.05
336	0.048	0.066	0.103	390	0.016	0.023	0.049
337	0.046	0.064	0.102	391	0.015	0.023	0.049
338	0.045	0.063	0.101	392	0.015	0.022	0.048
339	0.044	0.061	0.099	393	0.015	0.022	0.048
340	0.043	0.06	0.098	394	0.015	0.022	0.047
341	0.042	0.059	0.098	395	0.015	0.022	0.047
342	0.041	0.059	0.097	396	0.015	0.021	0.046
343	0.04	0.057	0.096	397	0.015	0.021	0.046
344	0.039	0.056	0.095	398	0.014	0.021	0.046
345	0.038	0.055	0.094	399	0.014	0.021	0.045
346	0.037	0.054	0.093	400	0.014	0.021	0.045
347	0.037	0.053	0.092				
348	0.036	0.052	0.091				
349	0.035	0.051	0.089				
350	0.034	0.05	0.088				

From the above discussion it might be concluded that the λ_{max} for sunlight and heat induced solution befitted at shorter side means hypsochromic or blue shift and also found hyperchromic shift.

So, it is suggested that UV-spectrophotometric method is inaccurate to determine the potency of chloramphenicol aqueous solution by taking the absorbance at 278 nm.

3.2.3 Potency of Heat (Set-2) and Sunlight (Set-3) induced degraded chloramphenicol in aqueous solutions by UV-spectrophotometric method:

The potency of Heat and Sunlight induced degraded chloramphenicol in aqueous solutions by UV-spectrophotometric method were given in Table 12.

Table 12: Potency of heat and sunlight-induced degraded chloramphenicol aqueous solutions by UV-Spectrophotometric method.

Sets	Absorbance	Potency (%)
Set-1 (Stored in refrigerator)	0.413	98.60
Set -2 (Heated at 100°c for 6 hours)	0.594	141.18
Set -3 (Sunlight exposure for 14 hrs)	0.415	99.07

From this Table 12, it can be observed that the potency of heat-induced solution of chloramphenicol was much higher than the potency of standard chloramphenicol solution. The potency of sunlight-induced chloramphenicol solution was also slightly higher than that of the standard solution.

3.2.4 The efficacy of Sunlight and Heat-induced degraded pure solution of chloramphenicol:

To observe the changes in efficacy after sunlight exposure and heat induction of chloramphenicol preparations, the agar disc diffusion test was performed. After 12 hours of incubation, the antibacterial activity of the test sample (Set-2 and Set-3) was determined by measuring the diameter of the zones of inhibition in mm with a transparent scale. The results of antibacterial activity of the above Sets (15µg/disc) against gram-positive and gram-negative bacteria are given in Table 13.

Table 13: The zones of inhibition (15µg/disc) of heat and sunlight-induced degraded chloramphenicol aqueous solution (15mg/100ml).

Sets	**Zone of Inhibition(mm) (15 µg/disc)**	
	Bacillus cereus	***Pseudomonas aeruginosa***
Set-1 (Test tube in Refrigerator)	14.33 ± 0.57	14.66 ± 0.57
Set-3 (Sunlight for 12 hrs)	11 ± 1	10.66 ± 1.15
Set-2 (Heat at 100°C for 6 hrs)	0 ± 0	0 ± 0

In case of a solution containing 15 mg of chloramphenicol in 100 ml distilled water (15µg/disc), it was observed that, for Set-1, the average zone of inhibition against *Bacillus cereus* was 14.33 and against *Pseudomonas aeruginosa* it was 14.66. In

case of Set-3, the average zone of inhibition against *Bacillus cereus* was 11.00 and against *Pseudomonas aeruginosa,* it was 10.66 which were clearly smaller than Set -1. In case of Set -2, there were no activities against the organisms (Figure 11).

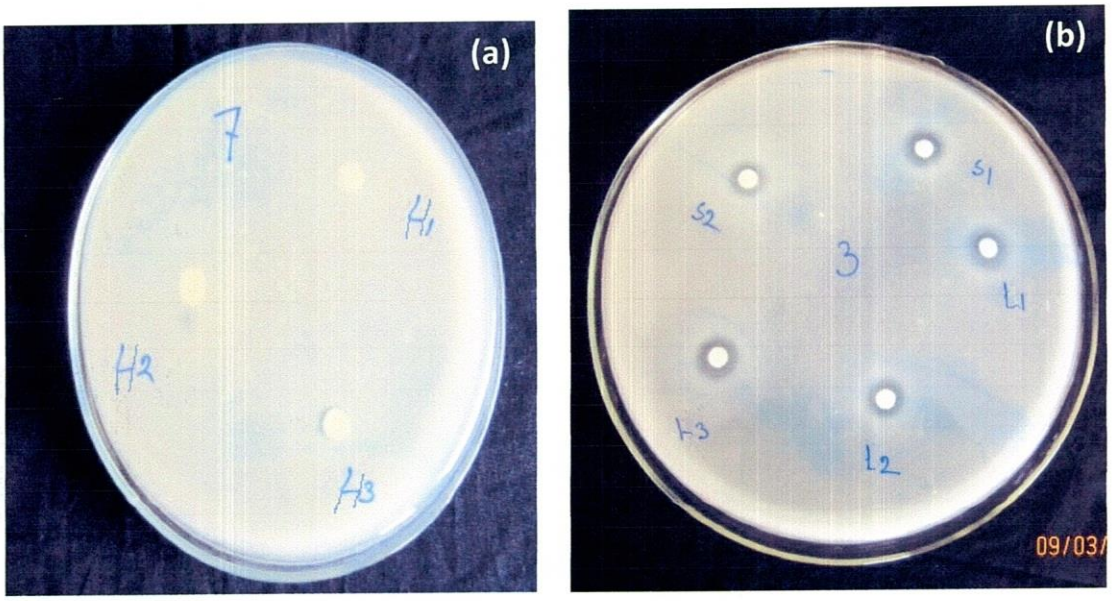
When the concentration of the test disc was increased from 15µg/ disc to 30µg/ disc, the same result was observed (Figure 12). In this case for Set -1, the average zone of inhibition against *Salmonella typhi, Shegella dysenteriae* and *Streptococcus agalactiae* were 22.33, 17.66 and 17.33 respectively. For Set-3, the average zone of inhibition against *Salmonella typhi, Shegella dysenteriae and Streptococcus agalactiae* were 19.00, 14.66 and 14.33 respectively that were slightly smaller than the values obtained for Set -1 (Standard). For Set -2, no activity was found against the organisms observed (Table 14).

This result showed that though the dose was double, the antibacterial effect was not so much affected. When the chloramphenicol solutions were subjected to sunlight exposed, the activity became reduced compared to those solution that were stored in the refrigerator indicating that the heat-degraded products present in chloramphenicol had less or no activity.

Table 14: The zones of inhibition (30µg/disc) of heat and sunlight induced degraded chloramphenicol aqueous solution (15mg/100ml).

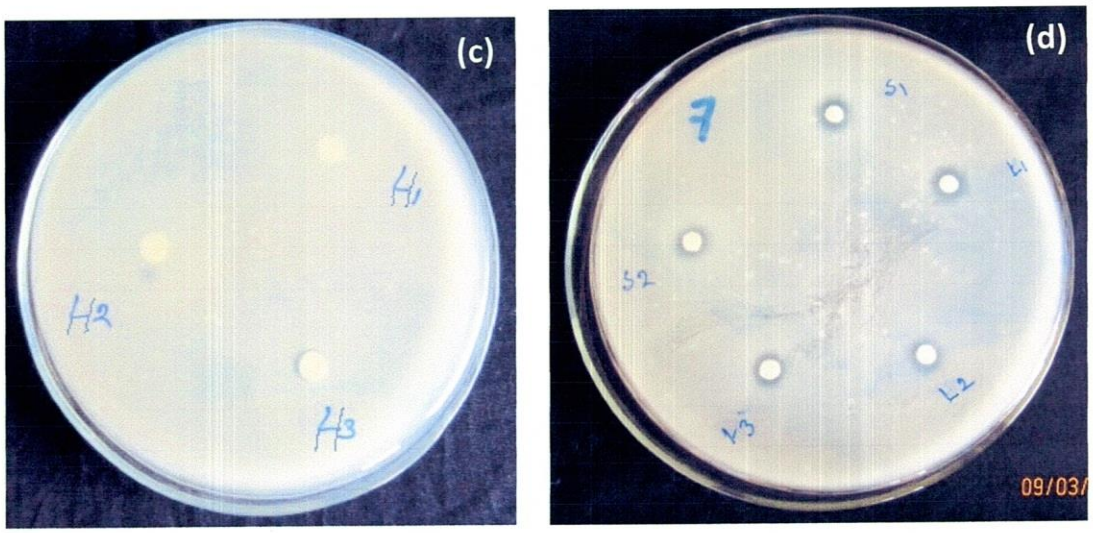
Sets	**Zone of Inhibition(mm) (30 µg/disc)**		
	Salmonella Typhi	***Shigella dysenteriae***	***Streptococcus agalactiae***
Set-1 (Test tube in Refrigerator)	22.33±0.57	17.66±0.57	17.33±0.57
Set-3(Sunlight for 12 hrs)	19± 0	14.66±0.57	14.33±0.57
Set-2 (Heat at 100°C for 6 hrs)	0±0	0±0	0±0

When the solutions were subjected to heat degraded, no activity was observed against any organism although the disc concentration is increased indicating that heat-induced degradation products have no antimicrobial activity.



Antibacterial activity of heat and light induced chloramphenicol aqueous solution against *Bacillus cereus*

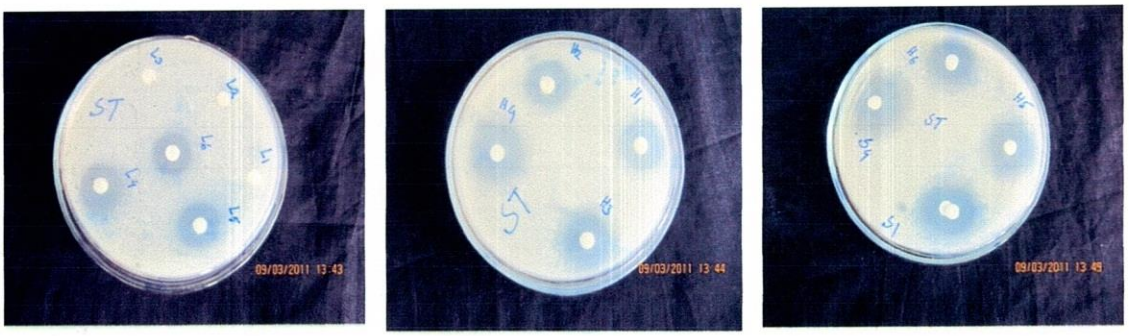
(a)H_1,H_2 & H_3=Set-2 and (b) S_1 & S_2= Set-1, L_1, L_2 & L_3=Set-3



Antibacterial activity of heat and light induced chloramphenicol aqueous solution against *Pseudomonas aeruginosa*

(c)H1,H2 & H3=Set-2 and (d) S1 & S2= Set-1, L1, L2 & L3=Set-3

Figure 11: Antibacterial activity of heat-degraded and light-induced chloramphenicol aqueous solutions (15 µg/ disc) against *Bacillus cereus* and *Pseudomonas aeruginosa.*



Antibacterial activity of heat and light induced chloramphenicol aqueous solution against salmonella typhi.

S1 & S4=Set-1, H1, H2, H3, H4, H5 & H6=Set-2 and L1, L2, L3, L4, L5 & L6=Set-3

S1, H1, H2, H3, L1, L2, L3=15 mg/100 ml of chloramphenicol solution

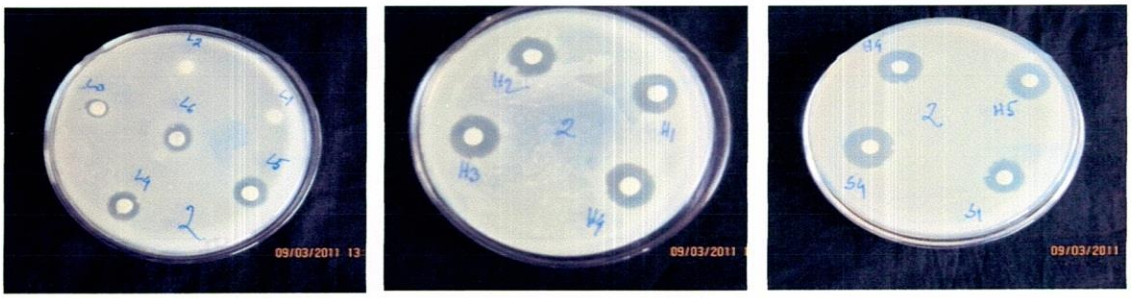
S4, H4, H5, H6, L4, L5, L6= 100 mg/100 ml of chloramphenicol solution

Antibacterial activity of heat and light induced chloramphenicol aqueous solution against *Shigella dysenteriae*

S1 & S4=Set-1, H1, H2, H3, H4, H5 & H6=Set-2 and Li1 L2, L3, L4, L5 & L6=Set-3

S1, H1, H2, H3, L1, L2, L3=15 mg/100 ml of chloramphenicol solution

S4, H4, H5, H6, L4, L5, L6= 100 mg/100 ml of chloramphenicol solution



Antibacterial activity of heat and light induced chloramphenicol aqueous solution against *Streptococcas agalactiae*

S1 & S4=Set-1, H1, H2, H3, H4, H5 & H6=Set-2 and Li1 L2, L3, L4, L5 & L6=Set-3

S1, H1, H2, H3, L1, L2, L3=15 mg/100 ml of chloramphenicol solution

S4, H4, H5, H6, L4, L5, L6= 100 mg/100 ml of chloramphenicol solution

Figure 12: Antibacterial activity of heat-degraded and light-induced chloramphenicol aqueous solutions (30 µg/ disc) against *Salmonella typhi*, *Shigella dysenteriae* and *Streptococcus agalactiae.*

From these results, it can be concluded that the deviation occurred due to the degradation of chloramphenicol, not for any other excipient. So, it raises a question about the validity of the widely established UV-spectroscopic method approved by BP (British Pharmacopeia). So, a newly developed method should be proposed.

3.2.5 Change in absorbance between freshly prepared and 4-days old chloramphenicol solution:

It was observed that the absorbance values steadily increased in stored chloramphenicol solutions in room temperature compared to the freshly prepared solution again indicating its instability when stored and ability to give false impression about its concentrations (Table 15).

Table15: Absorbance of freshly prepared and 4-days old chloramphenicol solution measured by UV-spectrophotometer.

Sample (conc.)	**Freshly prepared (λ_{max}=278 nm)**	**Four days old solution(λ_{max}=278 nm)**
400µg/ml(100%)	2.613	2.635
200µg/ml (50%)	2.613	2.635
100µg/ml(25 %)	2.312	2.408
50µg/ml(12.5%)	1.324	1.324
25µg/ml (6.25%)	0.550	0.659
12.5µg/ml (3.13%)	0.230	0.331

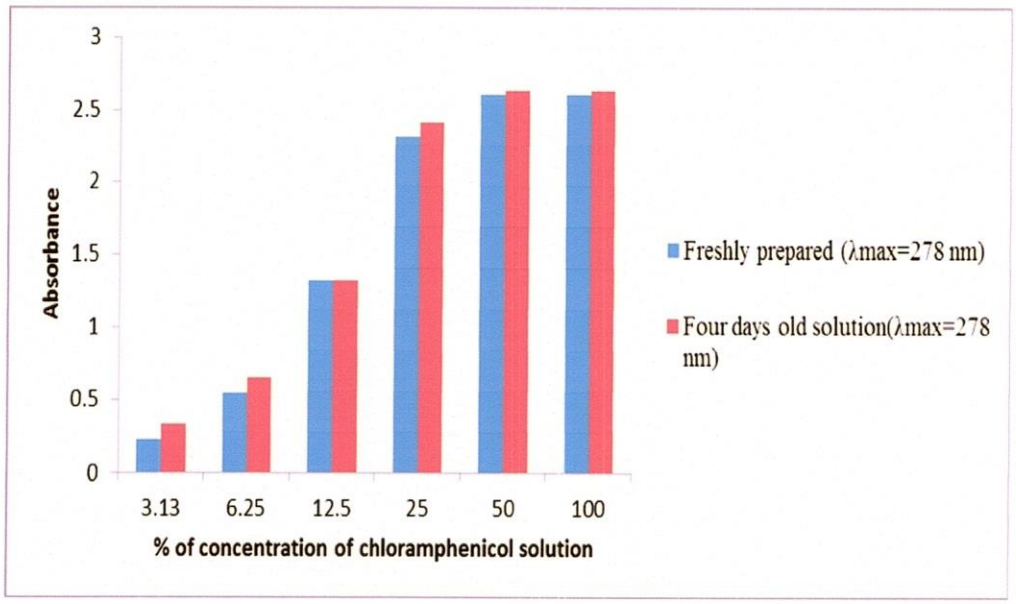


Figure 13: change in absorbance between freshly prepared and 4-days old chloramphenicol solutions.

In higher concentrations 400µg (indicated as100%), 200µg(50%), 100 µg(25%),50 µg (12.5%) in 1 ml of chloramphenicol solution, the absorbance values of 4-days old chloramphenicol solutions were almost same or slightly increased comparing to the values for freshly prepared solutions (Figure 13) . It became much prominent in lower concentrations 12.5 µg (3.13%), 25 µg (6.5%) as the absorbance values for 4 days old solutions became even higher. So, it became evident that the degradation takes place continuously even in room temperature, rather than in hot environment. This Figure also showed that the maximum measureable concentration of chloramphenicol by UV-spectrophotometer is 50 µg/ ml.

3.2.6 Effect of the presence and absence of pure 4-nitrobenzaldehyde in chloramphenicol solution on UV-absorbance:

4-nitrobenzaldehyde is a light degraded product of chloramphenicol. We observed that the potency of chloramphenicol increases when determined by UV-Spectroscopic methods at 278 nm. We assumed that the degradation products of chloramphenicol is responsible for this increased absorbance values. To study the effect of 4-nitrobenzaldehyde on the potency of chloramphenicol, we designed an experiment adding different percentage of 4-nitrobenzaldehyde in replacing same amount of chloramphenicol from the solution. The observed absorbance is shown in the Table 16 and Figure 14

Table 16: Effect of increasing concentration of 4-nitrobenzaldehyde in chloramphenicol aqueous solution.

% added 4-NB in chloramphenicol solution	Absorbance
1	0.609
2	0.618
3	0.625
5	0.65
10	0.693
20	0.787
30	0.886
40	0.992
100%(Pure CPL)	0.595

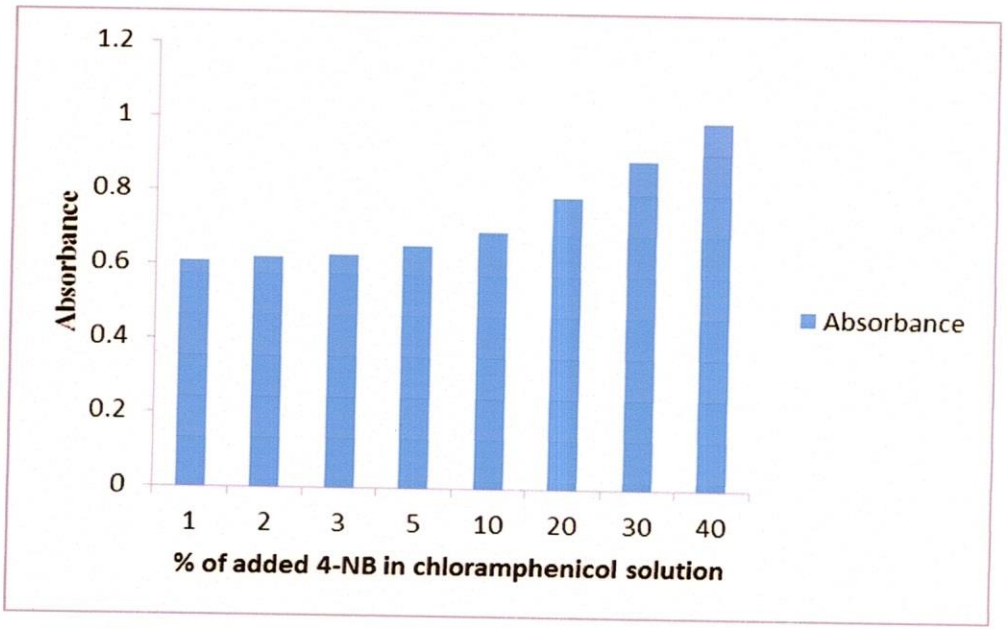


Figure 14: Effect of increasing concentration of 4-nitrobenzaldehyde in chloramphenicol aqueous solution.

So it was clearly seen that, when chloramphenicol was degraded to 4-nitrobenzadehyde it gave higher absorbance values. So if 4-nitrobenzaldehyde is present as a degraded product in any chloramphenicol solution or drug, it gives false-enhancement of potency.

3.3 Investigation of a new method for potency determination of chloramphenicol:

3.3.1 Standard curve of pure chloramphenicol:

The standard curve of chloramphenicol in the media of distilled water were obtained by plotting absorbance versus concentration where the calibration curve was found to be linear.

Table 17: Absorbance of standard chloramphenicol at different concentrations in the media of distilled water at 278 nm respectively.

Concentration (µg/ml)	Absorbance at 278 nm
5	0.145
10	0.291
15	0.408
20	0.531
25	0.663
30	0.792
35	0.928
40	1.041
45	1.191
50	1.266

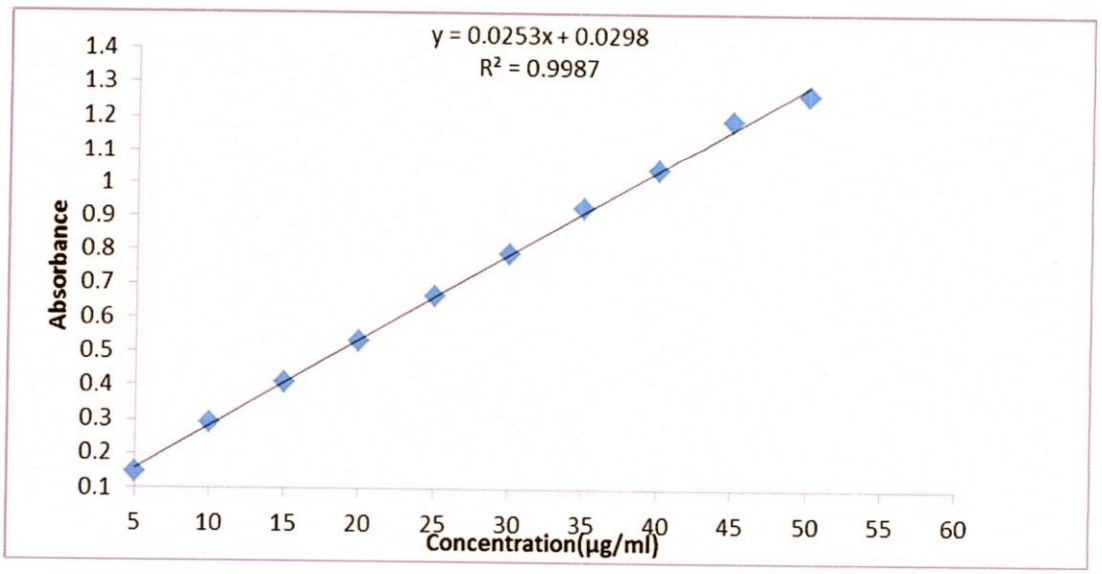


Figure 15: Standard curve of chloramphenicol in the media of distilled water at 278 nm.

3.3.1.1 Relation in absorbance values of Light-degraded and Heat-induced solution of pure chloramphenicol with Standard chloramphenicol measured by UV-spectroscopic methods:

The absorbance values of standard chloramphenicol solution increased significantly with concentrations (Figure16). In case of light induced and heat degraded solution for 90% concentration, the potency of it went up to 110.43% and 133.7% respectively. It indicates the increase of potency to 11.83% and 35.1% for light and heat degraded solutions.

Table18: Absorbance of light and heat degraded chloramphenicol solution at 90% (45µg/ml) concentrations in the media of distilled water with standard chloramphenicol at 278 nm respectively.

Concentration (µg/ml)	**Absorbance of standard chloramphenicol at 278 nm**	**Absorbance of light degraded chloramphenicol**	**Absorbance of heat degraded chloramphenicol**
45	1.191	1.351	1.566

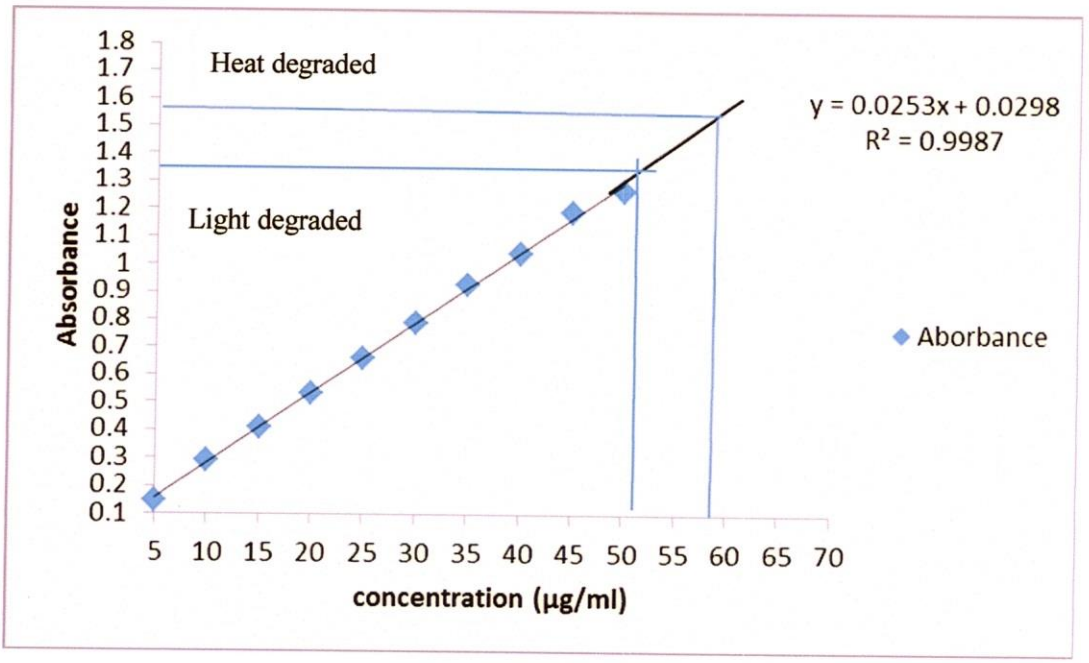


Figure 16: The absorbance values of light and heat degraded solution intercept the standard curve of pure chloramphenicol solution.

3.3.2 Estimation of residual chloramphenicol after Heat and Sunlight-induced degradation by Thin-Layer chromatography:

As shown in the Figure 17 both the sunlight and heat-induced degraded products became separated from their degradation products with a distinguished band on the TLC plate. Elution of the spots of standard, sunlight and heat-induced degraded sample and determination of absorbance results in the amount of residual chloramphenicol after degradation. The values of absorbance of the samples and calculated potencies are shown in the Table19 and Figure19. The Figure19shows scanning spectra of standard chloramphenicol, heat-degraded and light-induced samples. It is observed from these data that the potency of the

standard and degraded samples reduced to a significant level. The measured drug content of standard chloramphenicol after elution from TLC was 91.35% which was reduced to 71.73% when exposed to sunlight (approximately 19.62% less than the standard). In case of heat-degradation, the content was measured as 59.73% which is about 31.62 % less than the standard. It can also be noted that the degradation rate of the sample caused by heat treatment was slightly higher than that exposed to light.

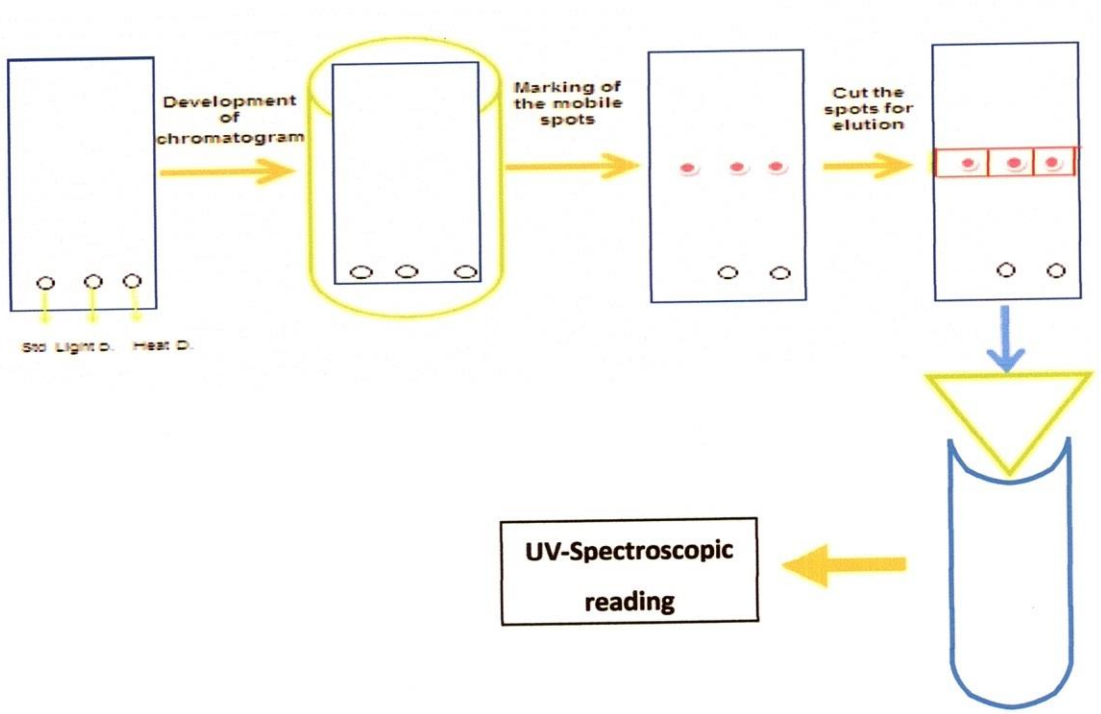


Figure 17: Collection of residual chloramphenicol from the degraded chloramphenicol aqueous solution by Thin Layer Chromatography (TLC).

Table19: UV-absorbance (278 nm) of standard, sunlight and heat-degraded chloramphenicol solutions after separation and elution of the TLC.

Samples	Absorbance	% of content
Standard	0.378	91.35
Light degraded	0.275	71. 73
Heat degraded	0.229	59.73

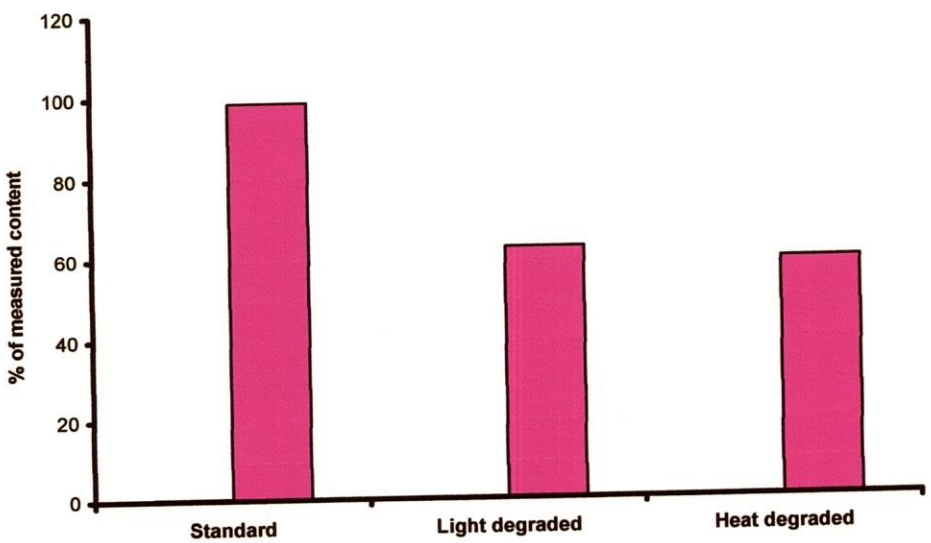


Figure 18: % of measured content of chloramphenicol after sunlight and heat-induced degradation and separation and elution from TLC.

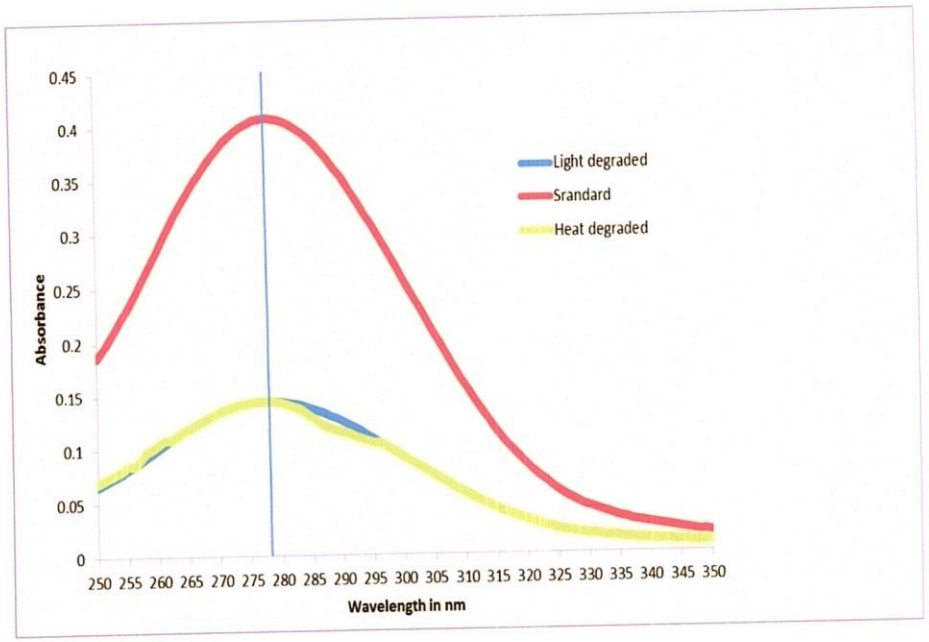


Figure 19: UV-Vis experimental absorption spectrum of eluted fractions of standard, sunlight and heat degraded chloramphenicol solution after thin layer chromatography.

The eluted fractions of the TLC were also analyzed by UV-spectrophotometer over a wide UV range of wavelength (250-350 nm). The resulting UV-absorption spectrum is shown in the Figure19. It is clear from the spectrum that due to sunlight and heat-induced degradation, the content of chloramphenicol was reduced to a significant level when it became separated from the UV-active degradation products by TLC. As observed in the Figure 19 the absorption intensity got decreased after heat and light degradation. So, the measured content of chloramphenicol of the control (standard) was much higher than the degraded sample.

The results of these experiments suggest that direct measurement of chloramphenicol content by UV-spectrophotometer is not a correct approach.

Measurement must be performed by the separation of active fractions in TLC followed by UV-spectrophotometric method.

But, even this newly proposed and valid method for measuring chloramphenicol content in finished products has some limitations, for example, there is a chance of losing a small part of the active drug during the separation and elution process that can lead to an erroneous result.

3.3. 3 Microbiological assay of chloramphenicol by agar disc diffusion method using *E. coli DH5α:*

For the quantitative analysis of chloramphenicol solution, the agar disc diffusion test was performed using *E. coli DH5α.* After 12 hours of incubation, the antibacterial activity of all the samples was measured from the diameter of the zones of inhibition in mm. Figure 20 and Table 20 show the concentration -dependent nature of the zones; that is for chloramphenicol solution containing 3µg per disc, the zone is the smallest and the zones got bigger with the increase of concentrations.

Table 20: Effect of chloramphenicol concentration related the zones of inhibition of standard, heat and sunlight-induced degraded chloramphenicol aqueous solution (150mg/100ml).

Sample	**Zone of inhibition(mm) against E. coli DH5α**	
	30µg per disc (indicated in plate as 100%)	**27µg per disc(indicated in plate as 90%)**
Control	23	21
Light-degraded	19	17
Heat-degraded	16	14

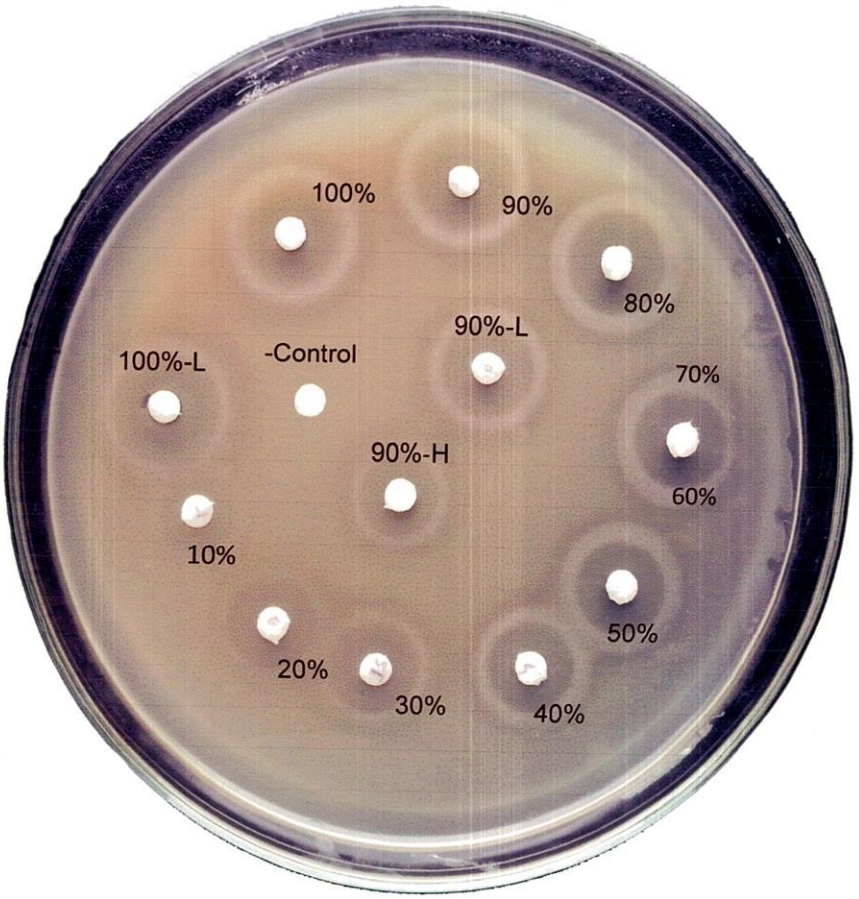


Figure 20: Zones of inhibition of *E. coli DH5α* grown on a Mueller Hinton agar plate. Interpretation of the size of the bacterial "zones of inhibition" is related to the concentration of chloramphenicol applied on the discs. In this plate, the discs contained (clockwise) 3 μg/disc(10%) to 30 μg/disc(100%) of chloramphenicol in addition to light-induced 30 μg/disc [30 μg/disc –L(100%-L)], light-induced 27 μg/disc [27 μg/disc –L(90%-L)] and heat-degraded 27 μg/disc [27 μg/disc-H(90%-H)] samples. A negative control was used (-Control).

The inhibition zones for the discs of 30 μg and 27 μg solutions containing degraded products were also comparatively smaller than the corresponding 30 μg and 27 μg standard chloramphenicol solution containing discs

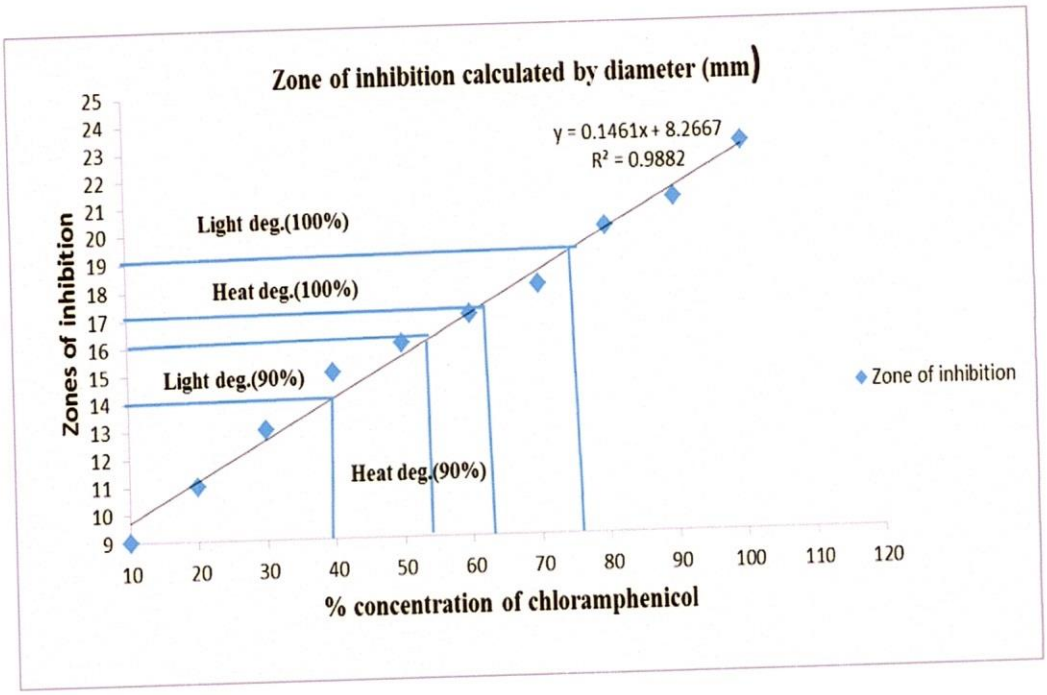


Figure 21(a): Zones of inhibition of light-induced and heat-degraded chloramphenicol samples calculated by diameter.

In this case, the coefficient correlation (R) value was 0.9882. The measurement for zones of inhibition became reduced to 19 mm and 16 mm (100% indicates 30µg per disc) for light induced and heat degraded products respectively whereas for standard, it was 23 mm. It was also observed that for the 90% light induced product, the value came down to 17 mm and for heat degraded sample, it is 14 mm although the standard (90% indicates 27µg/disc) is 21 mm .So the potency for 100% light and heat degraded product let down to 81.48% and 68.59% from 98.6%. Additionally the concentrations of 90% light and heat degraded products significantly reduced to 79.82% and 65.73% respectively.

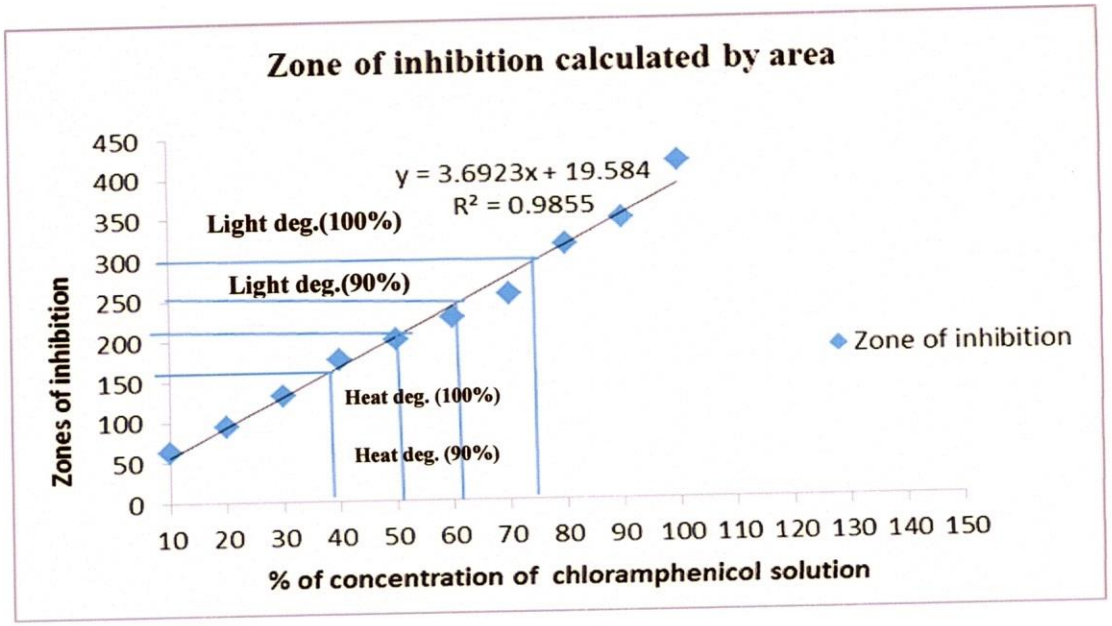


Figure 21(b): Zones of inhibition calculated by area whereas the areas of inhibition were calculated using the diameter values.

In this case, the correlation coefficient (R) value was 0.9855. From Figure 21 (a) a more accurate best fit graph drawn from the data was obtained by plotting the zones of inhibition against diameter values.

3.3.4 High Performance Liquid Chromatography HPLC method is used for crosschecking of agar disc diffusion methods:

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

Standard chloramphenicol sample had a potency of 98.6%. The standard solution and both the 90% light and heat degraded solution of chloramphenicol used for

application on the discs were compared by injecting a 200 µl aliquot into the HPLC column. The chromatographic profile and the profile of disc diffusion methods were found to be nearly similar.

Sample Analysis Report

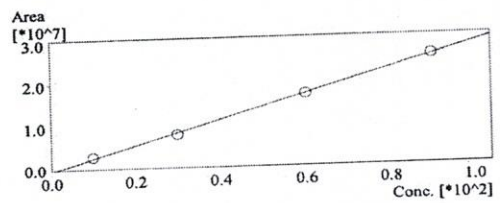
Sample Information

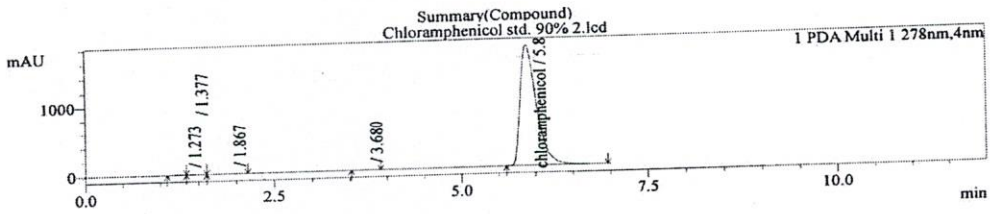
Acquired by	: System Administrator
Date Acquired	: 6/9/2013 12:55:52 PM
Sample Name	: Chloramphenicol
Sample Type	: Standard
Sample ID	: Chloramphenicol std.90%2
Vial#	: 94
Injection Volume	: 20
Data File	: Chloramphenicol std. 90% 2.lcd
Method File	: Chloramphenicol-cal.lcm
Date Processed	: 6/9/2013 1:17:59 PM

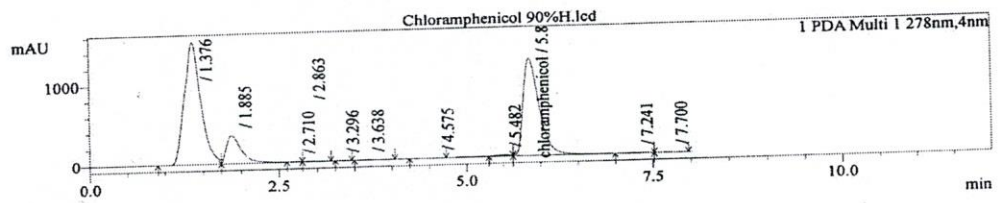
Calibration Curve

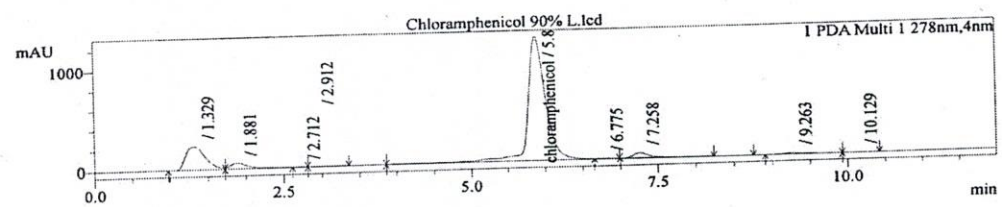
ID#	: 1
Name	: chloramphenicol
Quantitative Method	: External Standard
Function	: f(x)=292529*x-376195
Rr1=0.9994285 Rr2=0.9988573	
FitType	: Linear
ZeroThrough	: Not Through
Detector Name	: PDA

#	Conc.(Ratio)	MeanArea	Area
1	10	2911327	2911327
2	30	7956264	7956264
3	60	17096437	17096437
4	90	26111730	26111730









Title	Ret.Time	Area	Height	Conc.	Unit
Chloramphenicol std.90%	5.890	26111730	1722449	90.548	%
Chloramphenicol heat.90%	5.864	17085420	1217139	59.289	%
Chloramphenicol light.90%	5.883	20764322	1244050	71.011	%

Figure 22: Chromatogram of 90% standard, light and heat degraded product of chloramphenicol at fixed wavelength 278 nm, using 90 µg /ml for each sample. Mobile phase: glacial acetic acid: methanol (55:45, v/v), flow rate1.0 ml/min, injection volume 10 µl. Column: reversed phase C18, 5 µm, 15 cm length, 4.6 mm inner diameter. Peak area for standard, light degraded and heat degraded solution is 26111730, 20694322 and 17085420 respectively.

The important finding of this study is that the calibration curves were linear from 15µg, 45µg ,90 µg, 135 µg of chloramphenicol in 1 ml solution and the correlation coefficients (R) between 0.9994 and 0.9989 (average R = 0.9992). The potency values for 90% light and heat degraded products were calculated by the peak area. It is 78. 41% (in case of agar disc diffusion method, it was 79.82%) for light exposed solution and for heat degraded it is 64.52% (in case of agar disc diffusion method, it was 65.73%).

Table 21: Comparative study of four different methods for quantitative determination of chloramphenicol.

Methods of estimation	**Exposure to degradation factors**	**% of measured chloramphenicol**	**Change in % of content compared to control**
1. UV-Spectrophotometer	Control	98.6	-
	Light	110.43	+11.83
	Heat	133.7	+35.1
2. UV-Spectrophotometer (Separation was performed using TLC method)	Control	91.35	-
	Light	71. 73	-19.62
	Heat	59.73	-31.62
3. HPLC	Control	98.6	-
	Light	78. 41%	-20.19
	Heat	64.52%	-34.08
4. Microbiological assay	Control	98.6	-
	Light	79.82%	-18.78
	Heat	65.73%	-32.87

(+) indicates values increased from the value for control.

(-) indicates values decreased from the value for control.

The percentages of measured chloramphenicol of standard, light induced and heat degraded samples by using four methods are given in Table 21.

From this Table, it is evident that, by using UV- spectrophotometer (method 1) we get increased values for the measurement of degraded chloramphenicol samples whereas other methods (method 2, 3 and 4) provide decreased percentage values comparing the standard sample. So, it can be assumed that the values obtained by method 1 are false-positive that creates more confusion to check the quality of the drug.

In case of method 2, 3 and 4; the values decreased from the standard value reflecting the true situations. In all the cases, application of heat significantly caused more degradation of chloramphenicol in comparison to the application of sunlight.

From the comparison of method 1 and method 2, it comes out that results found using method 2 (combination of TLC method with UV-spectrophotometric method) differs a lot from method 1 (UV-spectrophotometric method). For method 1, the values for light induced and heat degraded solutions went up to 110.43% and 133.7% respectively indicating an increase of potency to 11.83% and 35.1% from that of the control (98.6%). But for method 2, the measured drug content of standard chloramphenicol after elution from TLC was 91.57% that became reduced to 71.87% when exposed to sunlight (approximately 19.7% less than the standard) and 59.73% when degraded by heat (about 31.62% less than the standard).

From the method 3 (HPLC), the potency values for light and heat degraded solutions were calculated from the peak areas. The percentage values decreased to 78.14% and 64.52% respectively whereas for the control it was 98.6%.

In case of method 4 (Microbiological assay), the values for light and heat degraded products significantly reduced to 79.82% and 65.73% respectively. In this case, the value for control was about 98.6%. In this method, the change in percentage values compared to control was the minimum among all the methods discussed here.

As method 3 (HPLC method) is regarded as the most accurate method, the comparison of other methods against this method provide interesting findings. Values obtained in method 1 varied from the HPLC values in case of heat-degraded and light-induced samples. The false-positive results obtained proved it to be considered as an objectionable method.

If the values for all the samples (control, heat-degraded and light-induced) of method 2 are compared with method 3 (HPLC), in each case the values decreased by 7% (98.6 – 91.57 = 7.03, 78.14 – 71.87 = 6.68, 64.52 – 59.73 = 5.76, respectively). Possibly this errors take place because of sample loss or unavoidable errors in handling. It can be assumed that if we add these differences (within a range of 5.76 – 7.03) to the values from method 2, it is possible to get the accurate values same as method 3. Thus method 2 has a potential to be established as a new method that can replace HPLC method after little modification.

On the other hand, method 4 provided almost same values (differed by not more than 1%) obtained from the HPLC method signifying its accuracy. So, this microbiological assay method can replace the more costly HPLC method after a number of trials.

3.4 Development of an assay method for quantitative determination of ciprofloxacin from its tablet dosages form by UV Spectrophotometric method:

3.4.1 Preparation of Standard curve from standard ciprofloxacin HCl in the media of 0.1N HCl acid and distilled water:

The standard curve of ciprofloxacin in the media of 0.1N HCl and distilled water and were obtained by plotting absorbance versus concentration where the calibration curve was found to be linear [67]

Table 22: Absorbance of standard ciprofloxacin HCl at different concentrations in the media of 0.1 N HCl and distilled water at 277 nm and 278 nm respectively.

Concentration (µg/ml)	Absorbance (0.1N HCl at λ_{max}=277nm)	Absorbance (Distilled water at λmax =278nm)
10	0.101	0.080
20	0.213	0.162
30	0.326	0.241
40	0.435	0.326
50	0.535	0.412

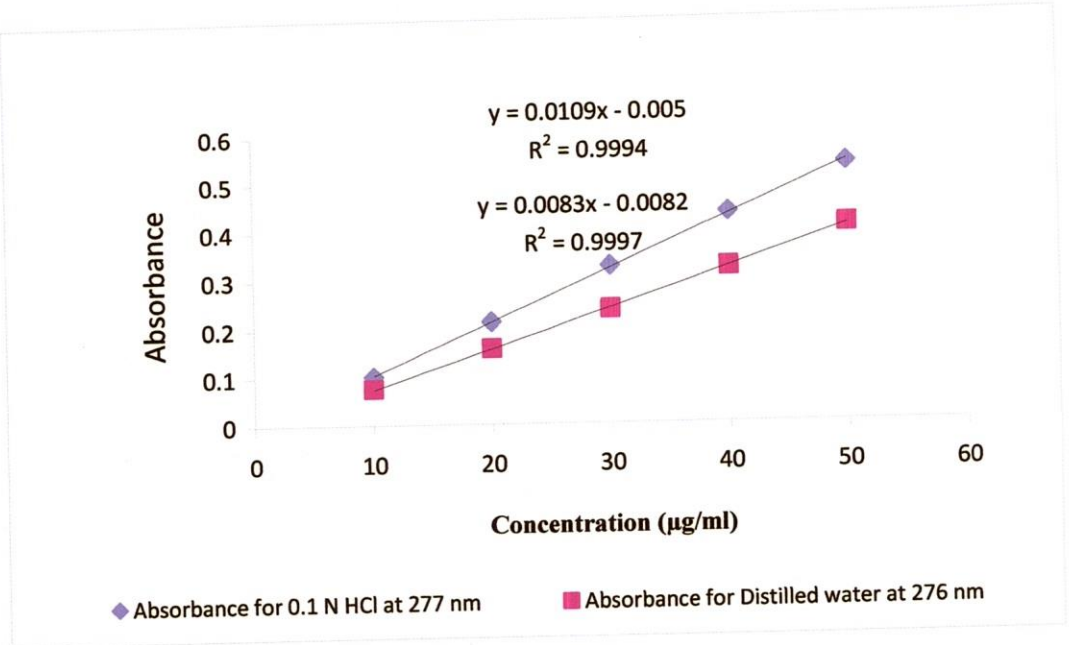


Figure 23: Calibration curve of ciprofloxacin HCl in the media of 0.1N HCl and distilled water.

Table23: Linearity parameter data for 0.1N HCl and distilled water.

Parameter	Ciprofloxacin in the media 0.1 N HCl	Ciprofloxacin in the media distilled water
λ_{max} (nm)	277 (0.1N HCl)	276 nm
Beer's law linearity range(µg/ml)	10-50	10-50
Regression Equation	Y=0.0109x-0.0005	Y=0.0083x-0.0082
Correlation co-efficient (r)	0.999	0.999
Slope	0.0109	0.0083
Intercept	-0.005	-0.0082

The plot of the residuals was normally distributed around the regression line, which reflects the accuracy of the method. For the determination of ciprofloxacin hydrochloride the linear plot was found in the media of distilled water and 0.1N HCl acid. The maximum absorbance was found in the media of distilled water for ciprofloxacin at wavelength 276nm and the calibration curve was to be linear (R2 >0.99) and the maximum absorbance was found in the media of 0.1N HCl for ciprofloxacin hydrochloride at wavelength 277nm. The calibration curve was to be linear (R2 >0.99).

This finding can become useful to determine the content of ciprofloxacin from its tablet dosages form by UV-spectroscopic methods.

3.4.2 The potency of ciprofloxacin in tablet of three companies determined by UV-spectrophotometric using method 0.1N HCl as solvent:

Accurately weighed the 20 tablets for each company and powdered. The powder equivalent to 100mg of ciprofloxacin hydrochloride was transferred to 100 ml volumetric flask and made the volume to mark with 0.1 N HCl. The final concentration of this solution obtain 50µg/ml and determined the respective absorbance at 277nm by UV-spectrophotometric methods. Other dilution obtains for the measurement of % of ciprofloxacin by HPLC methods.

Table 24: The data obtained the absorbance values for determining the potency of ciprofloxacin tablet from three different companies are given below.

Code name	Absorbance values at 277 nm using 0.1 N HCl as a solvent	% of potency measured by UV-spectrophotometer
Standard	0.563	99.77
COM-P	0.553	98.00
COM-B	0.556	98.53
COM-Ph	0.565	100.12

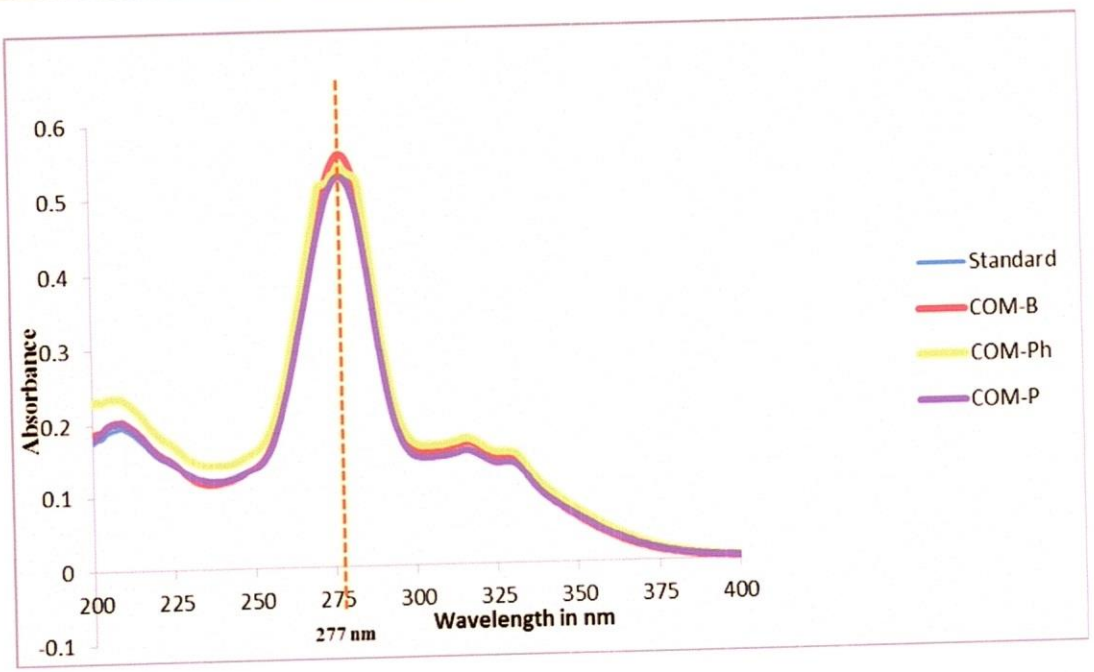


Figure 24: UV-Vis experimental absorption spectrum of ciprofloxacin tablet for three different companies using 0.1N HCl against standard absorption spectra at 277nm.

The values of absorbance and calculated potency of ciprofloxacin HCl in tablet dosages form are displayed in Table 24 Figure24.The absorbance of the samples in a wide range of wavelength under UV-visible range. It is observed from these data that the potency of the ciprofloxacin tablet remain a significant level. The measured % of potency of standard ciprofloxacin HCl was 99.77% and it became 98.00; 98.53, 100.12 % for COM-P, COM-B, COM-Ph, companies respectively

3.4.3 The potency of ciprofloxacin tablet of three companies determined by UV-spectrophotometric method using distilled water as solvent:

The data obtained for determining the potency of ciprofloxacin from three different companies are given below:

Table 25: The data obtained the absorbance values for determining the potency of ciprofloxacin tablet from three different companies are given below.

Code name	Absorbance values at 276 nm using distilled water as solvent	% of Potency measured by UV- spectrophotometer
Standard	0.598	99.77
COM-P	0.577	96.26
COM-B	0.591	98.60
COM-Ph	0.592	98.77

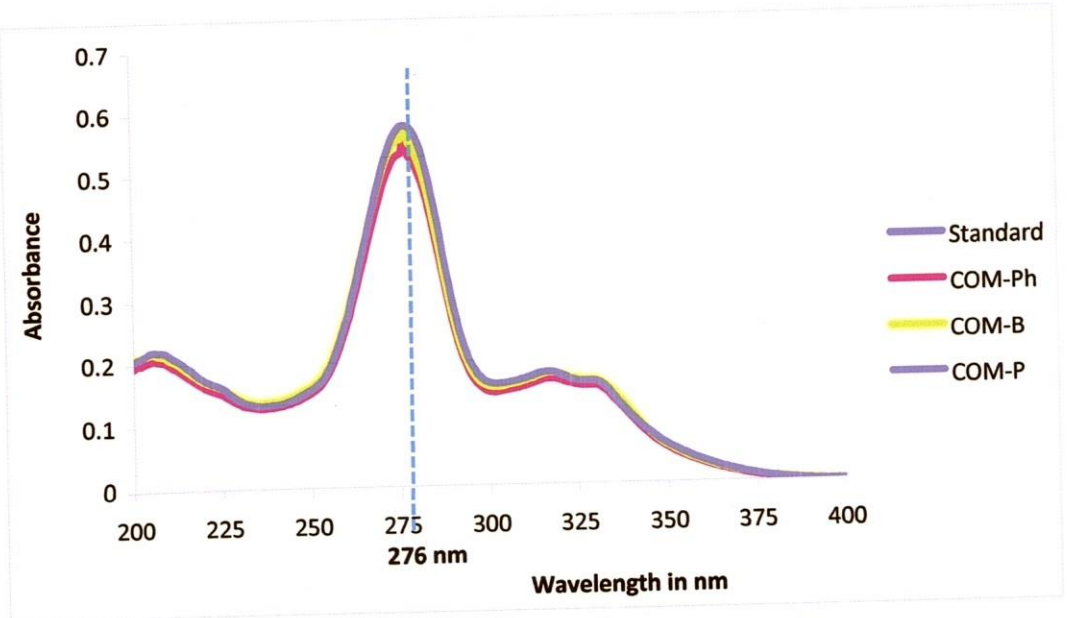


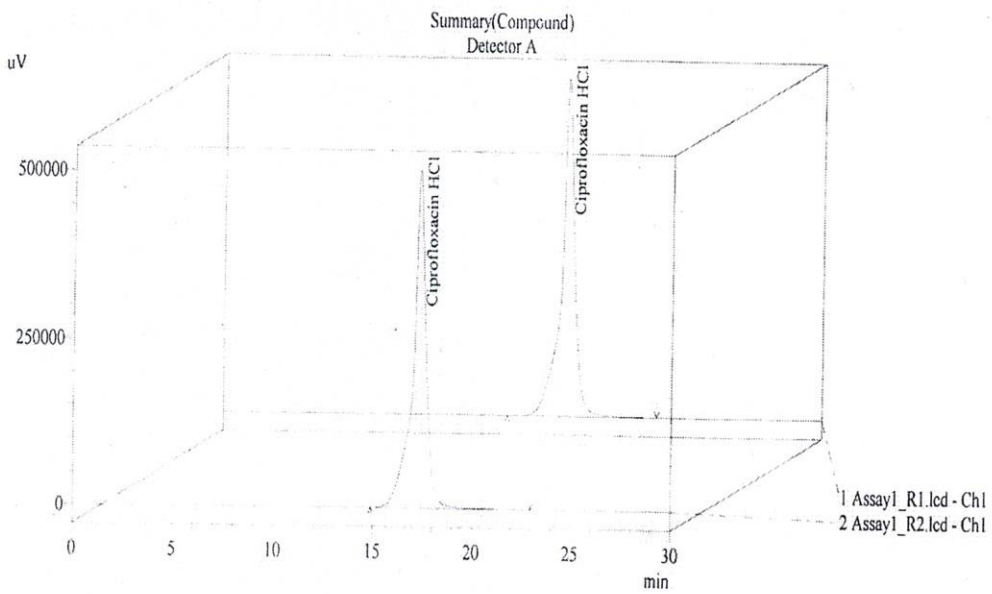
Figure 25: UV-Vis experimental absorption spectrum of ciprofloxacin tablet for three different companies using distilled water against standard absorption spectra at 276nm.

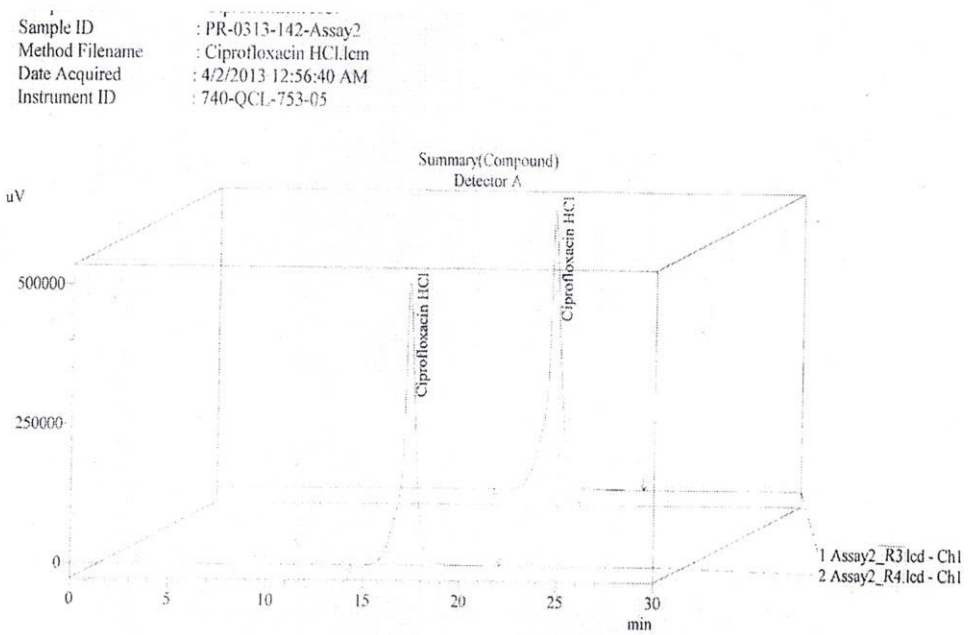
The values of absorbance and calculated potency of ciprofloxacin HCl in tablet dosages form are displayed in Table25 and Figure 25. The measured % of potency of standard ciprofloxacin HCl was 99.77% and it became 98.26, 98.60 and 98.77 for COM-P,COM-B and COM-Ph respectively.

3.4.4 The potency of ciprofloxacin in tablet of three companies (previously measured by UV-spectrophotometer) determined by HPLC method:

After developing an UV- spectrophotometric method for the potency determination of ciprofloxacin, a comparative study was also performed by high performance liquid chromatographic technique (HPLC) to highlight the validity of this method.

Sample Name	: Ciprofloxacin HCl
Sample ID	: PR-0313-142-Assay1
Method Filename	: Ciprofloxacin HCl.lcm
Date Acquired	: 4/2/2013 12:26:13 AM
Instrument ID	: 740-QCL-753-05





Title	Ret.Time	Area	Hight	Tailing Factor
Cipro.HCl Std	17.305	22967935	5313.878	0.854
Assay-Ph	17.294	22999800	5382.792	0.857
Assay-B	17.299	22736367	5287.667	0.854
Assay-P	17.296	22638971	5053.959	0.857

Figure 26: Chromatogram of standard ciprofloxacin HCl with assay-Ph, assay-B assay-P indicated as COM-Ph, COM-B and COM-P of ciprofloxacin at fixed wavelength 278 nm, flow rate 1.5ml/min, injection volume 10µl. Column: Nucleosil C18, 250 X4.6 mm, 5µ or equivalent. Peak area for standard, Assay-Ph, Assay-B and Assay-P are 22967935, 22999800, 22736367 and 22638971 respectively.

So the % of measured potency by this methods were 99.91% for COM-Ph, COM-B and COM-P were 98.76% and 98.57% respectively.

3.4.5 Comparison of potency of ciprofloxacin tablet of three companies determined by UV-spectrophotometric method using 0.1 N HCl and distilled water as solvent beside HPLC methods:

Table 26: Comparison the percentages of estimated potency of ciprofloxacin tablet between UV-spectrophotometer and HPLC method.

Code name	% of Potency measured by UV- spectrophotometer		% of Potency measured by HPLC
	using distilled water	using 0.1 N HCl	
COM-Ph	98.77	100.12	99.91
COM-B	98.60	98.53	98.76
COM-P	96.26	98.00	98.57

The measured potency of ciprofloxacin tablet for COM-Ph; COM-B and COM-P is 98.77; 98.60 and 96.26 % when using distilled water whereas it become 100.12; 98.53 and 98.00 for 0.1 N HCl as a diluting solvent. It is observed from these data that the potency of the ciprofloxacin tablet remain a significant level. We also present a relative study for potency determination of ciprofloxacin tablet by high performance liquid chromatographic technique (HPLC) of the same companies (COM-Ph; COM-B and COM-P) with UV-spectroscopic methods. The % of measured potency by HPLC methods is 99.91;98.76 and 98.34%.The % of potency for COM-Ph is 100.12% and 98.77% for 0.1N HCl and distilled water whereas for HPLC it is 99.91% that is near about at 100.12%. In case of COM-B is 98.53 and 98.60% for 0.1 N HCl and distilled water yet HPLC it is 98.76% and

for COM-P is about 98.00 and 96.26% for 0.1N HCl and distilled water whereas HPLC it is 98.57%.

3.4.6 The potency of ciprofloxacin from ciprofloxacin tablet of another five different companies determined by UV-spectrophotometric method using 0.1N HCl as solvent:

Table 27: The data obtained the absorbance values for determining the potency of ciprofloxacin tablet from five different companies are given below.

Code name	Absorbance values at 277 nm using 0.1 N HCl as solvent	% of Potency measured by UV- spectrphotometer
Standard	0.563	99.77
COM-N	0.559	99.06
COM-S	0.546	96.76
COM-Na	0.144	25.52
COM-I	0.557	98.71
COM-K	0.541	95.87

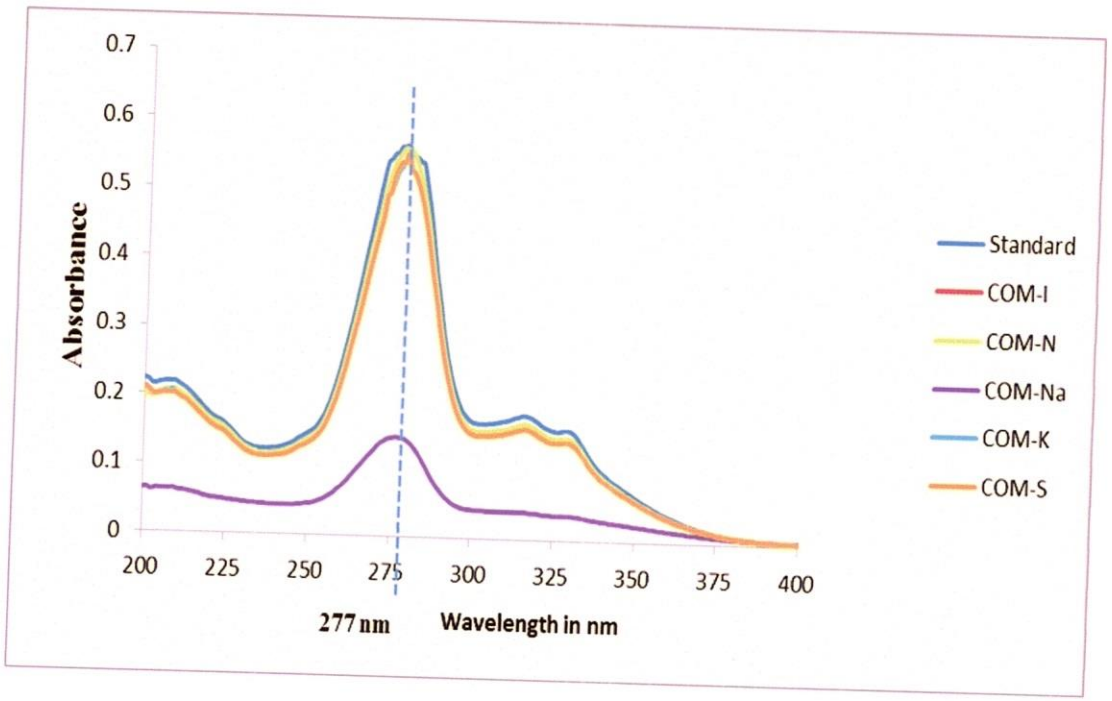


Figure 27: UV-Vis experimental absorption spectrum of ciprofloxacin tablet for five different companies using 0.1N HCl against standard absorption spectra at 277nm.

The values of absorbance and calculated potency of ciprofloxacin HCl in tablet dosages form for another five companies are displayed in Table 27 and Figure 27.The measured % of potency of standard ciprofloxacin HCl was 99.77% and it became 99.06;96.76; 25.52; 98.71; 95.87for COM-N; COM-S; COM-Na; COM-I and COM-K companies respectively. The potency for all the companies (except of COM-Na) had > 95% and the label claim of ciprofloxacin in BP is about (100 ±5) % and USP is(100 ±10)% in tablet dosages form. In case of COM-Na the potency remain at 25.52% this may happened for using low quality active materials or insufficient amount of the active materials during manufacturing process. It remarkable that the optimum absorbance took for all companies was 277 nm.

3.4.7 The potency of ciprofloxacin tablet of five different companies determined by UV-spectrophotometric method using distilled water as solvent:

Table 28: The data obtained the absorbance values for determining the potency of ciprofloxacin tablet from five different companies are given below.

Code name	Absorbance values at 276 nm using distilled water as solvent	% of Potency measured by UV-spectrophotometer
Standard	0.598	99.77
COM-N	0.585	97.60
COM-K	0.575	95.93
COM-Na	0.065	10.84
COM-I	0.588	98.10
COM-S	0.589	98.27

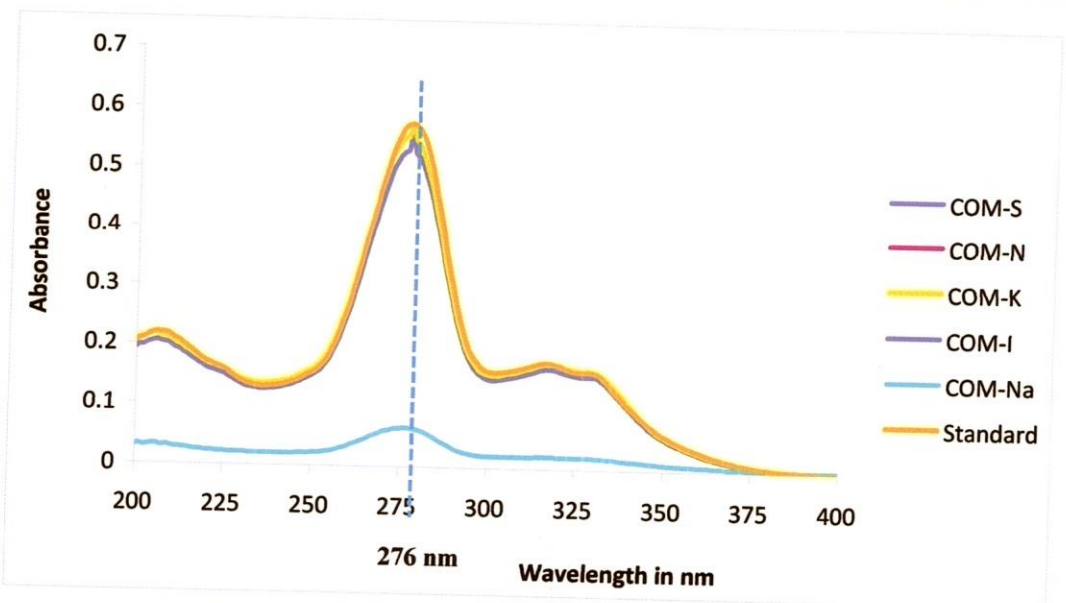


Figure 28: UV-Vis experimental absorption spectrum of ciprofloxacin tablet for five different companies using distilled water against standard absorption spectra at 276nm.

The values of absorbance and calculated potency of ciprofloxacin HCl in tablet dosages form are displayed in Table 28 and Figure 28. The measured % of potency of standard ciprofloxacin HCl was 99.77% and it became 97.60; 95.93; 10.84; 98.10;98.27for COM-N; COM-K; COM-Na; COM-I; COM-S companies respectively. The potency for all the companies (except of COM-Na) had > 95% and the label claim of ciprofloxacin in BP is about (100 ±5) % and USP is(100 ±10)% in tablet dosages form. In case of COM-Na the potency remains at 10.84%. It is remarkable that the optimum absorbance took for all companies were 276 nm.

3.4.8 Comparative analysis of Standard ciprofloxacin with Light-exposed and Heat-degraded ciprofloxacin when diluting solution use 0.1N HCl:

Table 29: Percentages of the estimated potency of standard ciprofloxacin HCl with sunlight and heat degraded ciprofloxacin HCl using 0.1N HCl as solvent.

Samples	**Absorbance values at 277 nm**	**% of Potency measured by UV-spectrophotometer**
standard	0.599	99.77
Sunlight degraded ciprofloxacin HCl	0.53	88.28
Heat degraded Ciprofloxacin	0.231	38.44

For the standard ciprofloxacin HCl solution the absorbance values and potency became 0.599 and 99.77% respectively. In case of sunlight and heat degraded product the potency became reduced to 88.28 and 38.44 respectively (Table 29).

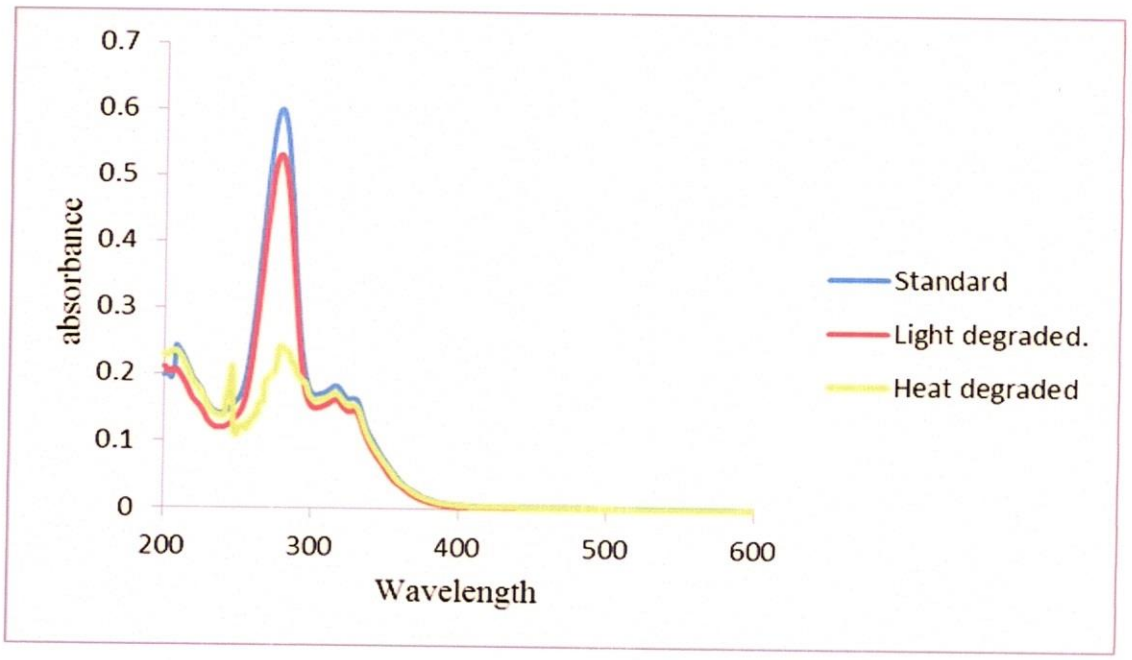


Figure 29: UV-absorbance spectra of standard, sunlight and heat degraded ciprofloxacin HCl using 0.1 N HCl as solvent.

From Figure 29, it was evident that λ_{max} for standard ciprofloxacin HCl was found at 277nm. After sunlight and heat induced solution of ciprofloxacin hydrochloride it was found that the ethylenediamine derivatives as a photo degraded product-(Roy,Jiben *et,al,*)and there was no interference with the peck of the parent compound. So $\lambda_{max}$for sunlight and heat induced solution became reduced from its actual value.

From the above discussion it might be suggested that UV-spectrophotometric method is accurate to determine the potency of ciprofloxacin from its tablet dosages form by taking the absorbance at 277nm for 0.1 N HCl as a diluting solution.

3.4.9 Comparative analysis of standard ciprofloxacin with Light-exposed and Heat-degraded ciprofloxacin when diluting solvent use distilled water:

Table 30: Percentages of the estimated potency of standard ciprofloxacin HCl with sunlight and heat degraded ciprofloxacin HCl using distilled water as solvent

Samples	**Absorbance values at 276 nm**	**% of Potency measured by UV-spectrophotometer**
standard	0.595	99.77
Sunlight degraded ciprofloxacin HCl	0.335	56.17
Heat degraded ciprofloxacin	0.017	2.85

It was found that the standard ciprofloxacin HCl solution the absorbance values and potency became 0.595 and 99.77% respectively. In case of sunlight and heat degraded product the potency became reduced to 56.17and 2.85% respectively in Table 30.

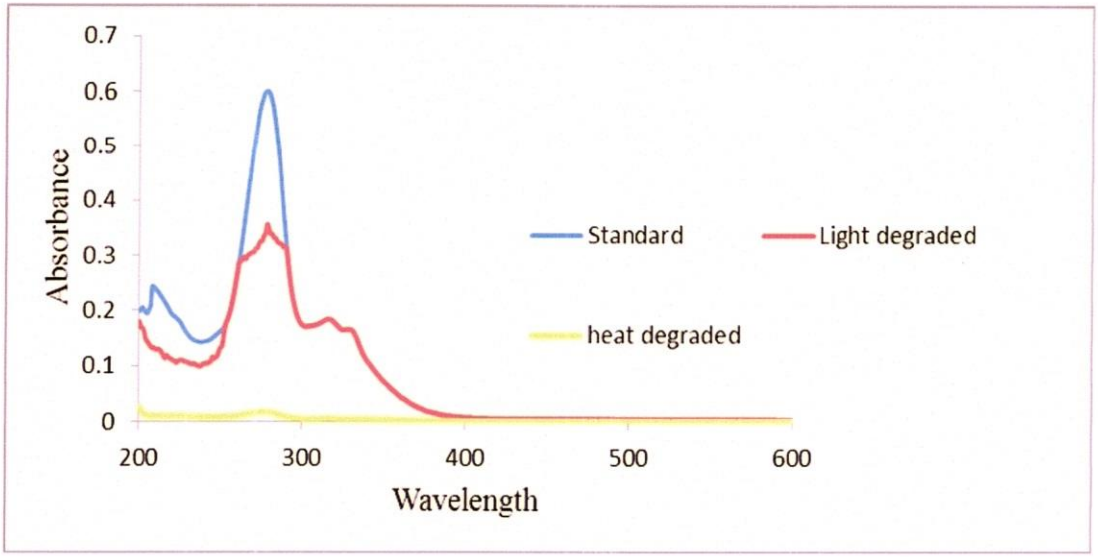


Figure 30: UV-absorbance spectra of standard, sunlight and heat degraded ciprofloxacin HCl using distilled water as solvent.

From Figure 30, it was marked that λ_{max} for standard ciprofloxacin HCl was found at 276nm. After sunlight and heat induced solution of ciprofloxacin hydrochloric acid it was found that the peck of the light degraded solution were shattered whereas the heat degraded solution were smaller than the standard solution and there was no interference with the peck of the parent compound.

The assay showed that the drug content of those product (first three companies and another five companies) to be in agreement with the labeled claim. The proposed method can be successfully applied for assay in tablet dosage forms without any interference by excipient and degraded product.

So from the above discussion it might be suggested that UV-spectrophotometric method is perfect to determine the potency of ciprofloxacin tablet by taking the absorbance at 277nm for 0.1 N HCl at and 276 nm for distilled water as a diluting solution and this methods was found satisfactory results for the analysis of ciprofloxacin hydrochloride under the stress condition.

Conclusion

CONCLUSION

Chloramphenicol drugs should be transported and stored in a 'cold chain' (temperature-controlled supply chain) to maintain their efficacy as it was found to become degraded upon exposure to light and heat. Different experiments suggested that in spite of being less expensive, pharmaceutical companies should not use the UV-spectroscopic method to determine the potency of chloramphenicol according to BP as it seems to be not valid. Until now, HPLC is regarded as the most accurate method. In this study, a combination of TLC method with UV-spectrophotometric method is suggested as a quite prospective method whereas a microbiological assay is also shown to have the potential to replace the more costly HPLC method. So Microbiological assay is proposed a new method for quantitative determination of chloramphenicol.

In another study indicates that, it is possible to determine the content of ciprofloxacin from its tablet dosages form by UV-spectrophotometric method. The functional equation and the extinction coefficient values derived from the calibration curve of reference standard of the pure drugs will enable the analyst to determine the drug content in pharmaceutical dosages forms. In the media of distilled water and 0.1N HCl acid the standard curve of ciprofloxacin showed linearity which concluded that it is possible to determine the content of this drug by UV- spectroscopic method. , it was marked that λ_{max} for standard ciprofloxacin HCl was found at 276nm for distilled water and 277 nm for 0.1 N HCl while using as a diluting solvent .When the values obtained from HPLC method were compared, it was found that this can be used as a reliable method. The UV spectrophotometric method will certainly offer distinct advantage of simplicity, accuracy and sensitivity in analyzing pharmaceutical formulations of ciprofloxacin. So pharmaceutical companies can follow this analytical procedure due to low cost and time-saving formulation.

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