Interaction of Commercially Available Drugs with Pseudomembrane Model Using Micellar Liquid Chromatography and Electronic Spectroscopy

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SESSION 2014-2017

2014-GCU-PhD-CHEM-3

DEPARTMENT OF CHEMISTRY
GOVERNMENT COLLEGE UNIVERSITY
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Interaction of Commercially Available Drugs with Pseudomembrane Model Using Micellar Liquid Chromatography and Electronic Spectroscopy

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BY

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Dedicated to

My wife Razia

My son Ayan

My daughters Mishal & Hania

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LIST OF ABBREVIATIONS

FDC	*Fixed dose combination*
CTAB	*Cetyltrimethylammonium bromide*
MLC	*Micellar liquid chromatography*
m/p	*mobile phase*
DNA	*Deoxyribonucleic acid*
ATP	*Adenocine triphosphate*
CMC	*Critical micellar concentration*
^{o}C	*degree centigrade*
PEO	*Polyethylene oxide*
SDS	*Sodium dodecyl sulphate*
HPLC	*High performance liquid chromatography*
RP-HPLC	*Reversed phase high performance liquid chromatography*
CO_2	*carbon dioxide*
UV	*Ultra violet*
UV-Vis	*Ultra violet and visible*
mM	*milli moles*
pKa	*dissociation constant*
CTAC	*cetyltrimethylammonium chloride*
ICH	*International conference on harmonization*
ODS	*Octadecyle sulphate*
KH_2PO_4	*Potassium dihydrogen phosphate*
LOD	*Limit of detection*
LOQ	*Limit of quantitation*
NSAIDs	*Non steroid anti inflammatory drugs*
NaCl	*Sodium chloride*
NaOH	*Sodium hydroxide*
K_2HPO_4	*Dipotassium hydrogen phosphate*
BDS	*Base deactivated silica*
NaH_2PO_4	*Sodium dihydrogen phosphate*
Na_2HPO_4	*Disodium hydrogen phosphate*

LIST OF PUBLICATIONS

1. **Waqar Azeem**, Peter John, Muhammad Faizan Nazar, Muhammad Ashfaq, Islam Ullah Khan, Shahzad Sharif, Atif Riaz. Fixed-dose combination antibiotics interacting with quaternary ammonium disinfectant: Insights from spectral and chromatographic measurements. *J. Sol. Chem*. 47 (2018) 1048-1059

2. Muhammad Faizan Nazar, **Waqar Azeem**, Alina Kayani, Muhammad Zubair, Peter John, Asif Mahmood, Muhammad Ashfaq, Muhammad Nadeem Zafar, Sajjad Hussain Sumrra, Muhammad Naveed Zafar. pH-dependent antibiotic gatifloxacin interacting with cationic surfactant: Insights from spectroscopic and chromatographic measurements. *J. Sol. Chem*. (2018) DOI 10.1007/s10953-018-0811-3

3. **Waqar Azeem**, Peter John, Muhammad Faizan Nazar, Islam Ullah Khan, Atif Riaz, Shahzad Sharif. Spectral and chromatographic characterization of fixed dose combination norfloxacin and metronidazole interacting with cetyltrimethylammonium bromide. *J. Mol. Liq*. 244 (2017) 135-140

4. Muhammad Faizan Nazar, **Waqar Azeem**, Usman Ali Rana, Muhammad Ashfaq, Aref Lashin, Nassir Al-Arifi, Hafiz Muhammad Abd Ur Rahman, Azwan Mat Lazim, Asif Mahmood. pH-dependent probing of levofloxacin assimilated in surfactant mediated assemblies: Insights from photoluminescent and chromatographic measurements. *J. Mol. Liq*. 220 (2016) 26-32

Abstract

Micro-heterogeneous surfactant assemblies solubilize and encapsulate the active drug molecules and consequently protect them from the adverse environmental conditions. As pseudomodel of biomembranes, the associative structures of surfactant molecule are very useful for researchers to determine their role in cellular interactions. The present study reveals the molecular interactions of potential antibiotics (fixed dose combinations as well as single drugs) with cetyltrimethylammonium bromide (CTAB, a quaternary ammonium surfactant). Micellar liquid chromatography (MLC), differential absorption and emission spectrometry were performed to probe the drug–CTAB association whereas interaction modes of drug–surfactant were quantified by determining binding capacities and related Gibb's free energies at various pH conditions. The binding values of drug–CTAB obtained from micellar liquid chromatography measurements are found to be in good agreement with as measured by electronic spectroscopy. The fixed dose combination (FDC) drugs, (norfloxacin + metronidazole), (ofloxacin + ornidazole) and single drug sparfloxacin were studied at physiological condition pH 7.4. Gatifloxacin and levofloxacin were also studied at two pH values pH 7.4 and pH 5.5. Most fluoroquinolones solubilize in the aqueous medium. However for better bioavailability, improved efficacy and to overwhelm the lipophilic barrier, a physiological medium (pH 7.4) is useful for diffusion through phospholipid membrane. The spectral-luminescent measurements of these drugs interacting with cationic micelles were investigated as function of CTAB concentration from pre micellar to post micellar region. The results indicated potential solubilization of drugs in the peripheral region of micelles that may facilitate their controlled release. The values of the binding capacities of drug-micelle system have verified these results.

CHAPTER NO. 1

1. INTRODUCTION

Biological membranes are very complex and dynamic structures composed of phospholipid bilayer with inserted protein molecules. Structural integrity of biomembranes ensures their protective role, transportation and various other active life functions. Biological activity of drugs arises as a result of binding to active sites in the membrane bound protein [1-2]. Majority of drugs come across plasma membranes and they undergo various sorts of interactions with the biomembranes while reaching their targets. Mostly drugs interact with the receptor sites in the biological membranes as agonist or antagonist that play regulatory role. Drug substances bring changes in the biological functions by their chemical actions. In order to interact they should have appropriate electrical charges, size, and atomic composition. Beside these, several chemical forces may result in the temporary binding of the drug molecules with the receptor sites. Since drug-receptor association is reversible, covalent bonding is very strong and could not be reversed. Electrostatic interactions are weaker in nature than covalent bonds and are quite common in drugs-receptor bonding. These forces are very strong in case of permanently charged ionic molecules. Hydrophobic interactive forces are very weak and are most important sort of interactive forces among non-polar hydrocarbon groups of the drugs receptor sites [3-5]. The highly complex phospholipid membrane structures and the dynamic nature of lipid-protein and lipid-lipid interactions in the biological membranes, make this biophysical interaction of drugs with membranes very difficult to probe. Therefore an artificial and simple membrane system that mimics natural biomembranes is developed to investigate these drug-membrane interactions [6].

Drugs are classified into several classes that include quinolones, fluoroquinolones, NSAIDs, steroids, anthelmintics, antihistamines, antibacterials (nitroimidazole) etc. The quinolones and fluoroquinolones are synthetic antibiotics having excellent broad spectrum and potent bactericidal action against the lethal pathogens responsible for a number of infections [7-8]. Newly developed fluoroquinolones have broad spectrum of activity. Fluoroquinolones due to their improved pharmacokinetics, excellent

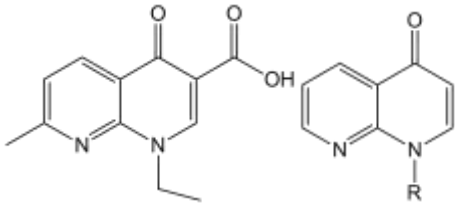
antibacterial activity and lesser side effects have been widely accepted for several clinical applications like:

- Treatment of lower and upper respiratory tract infections [9-11].
- They have been proven very successful in the safe and effective treatment of the sexually transmitted diseases [12].
- They are the drug of choice for many of the uncomplicated and complex skin tissue diseases [13-14].
- Extensively used in the management of the urinary tract infections [15].
- Fluoroquinolones are very effective in controlling number of eye, ear and throat infections and diseases [16].
- Bones and joints infections are very effectively treated by the fluoroquinolones [17].

1.1 Structure of quinolones and fluoroquinolones

The era of fluoroquinolones begun with the discovery of nalidixic acid, which was accidently found during the purification process of an antimalarial drug chloroquine [18-19]. Nalidixic acid is 1, 8 naphthyridone having core nucleus 4-quinolone [20]. It was very useful in many uncomplicated urinary tract infections caused by the gram negative bacteria but was very weak against the gram positive bacteria which urge the need to develop other effective molecules. Later on several structural modifications were carried out with the passage of time to improve the effectiveness of the nalidixic acid. This has given rise to many other first generation quinolones like oxonilic acid, cinoxacin and pipemidic acid. These drugs, although exhibited improvements over the nalidixic acid but they could not prove to be significant until the synthesis of fluimequine, the first mono fluoroquinolone. Fluimequine has a fluorine atom at the C-6 position and showed significant and improved gram positive activity than the previous compounds [21-24]. Later on introduction of a fluorine atom at C-6 and a piprazinyl group at position C-7 developed a new compound Norfloxacin and a new potent class of compounds called fluoroquinolones, with enhanced activity against the gram positive organisms.Efforts were continued to improve the spectrum of the fluoroquinolones when an amino group was introduced at the position C-5 which

enhanced the antibacterial activity as in sparfloxacin having fluorine at position C-6 and an alkylated piprazinyl group at C-7.In addition, acyclopropyl group



(A) (B)

Fig. 1.1 Structures of nalidixic acid (A) and naphthyridone core (B)

was introduced at N-1 position that further improved the activity of the drugs as in ciprofloxacin, moxifloxacin and gatifloxacin etc. In gatifloxacin, a methoxy group was been substituted at N-2 position that boosted up drug efficacy against the anaerobes [25].

1.2 Mechanism of action

Quinolones act by inhibiting replication process of the bacterial DNA. In bacteria, an enzyme topoisomerase replicate the process of unwinding and cleavage of bacterial DNA strands. It permits the single DNA strand to be complimentary with the new one. This process allows the coiling and replication of the bacterial genome. The quinolones thus inhibit this process by restraining the topoisomerase from unwinding and ultimately dislocating the replication process and finally leading to the cell death. [26-30].

1.3 Classification and examples of fluoroquinolones

Fluoroquinolones are grouped in generations according to their activity and pharmacokinetics [31-32].

Table 1.1 Classification of fluoroquinolones

Generation	Examples
First	Oxonilic acid, Nalidixic acid, Fluimequine, Cinoxacin.
Second	Lomefloxacin, Ofloxacin, Norfloxacin, Enoxacin, Pefloxacin.
Third	Sparfloxacin, Temafloxcin, Pazufloxacin, Levofloxacin.
Fourth	Moxifloxacin, Gatifloxacin, Gemifloxacin, Clinafloxacin.

1.3.1 Norfloxacin

Norfloxacin is white to pale yellow in color, photosensitive, crystalline and hygroscopic powder. It is sparingly soluble in water, acetone, alcohol, chloroform and freely soluble in acetic acid. It is insoluble in ether. Chemically norfloxacin is described as 1 – Ethyl – 6 – fluoro – 1,4 – dihydro – 4 – oxo – 7 – [piperazin – 1 – yl] quinoline – 3 – carboxylic acid [33].

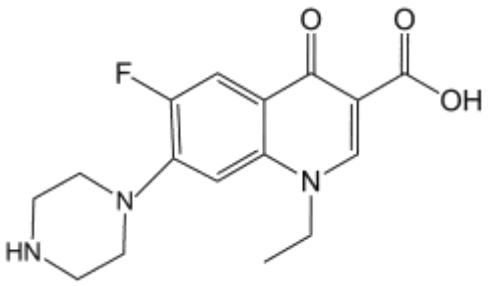


Fig. 1.2 Structure of norfloxacin

1.3.2 Ofloxacin

Ofloxacin is light yellow to pale yellow crystalline powder or crystals. It is very slightly soluble in water, methyl alcohol and alcohol. It is sparingly soluble in chloroform. Chemically ofloxacin is described as (±) – 9 – Fluoro – 2,3 – dihydro – 3 – methyl – 10 – [4-methyl – 1 – piperazinyl] – 7 – oxo – 7H – pyrido [1,2,3 – de] – 1,4 – benzoxazine – 6 – carboxylic acid [33].

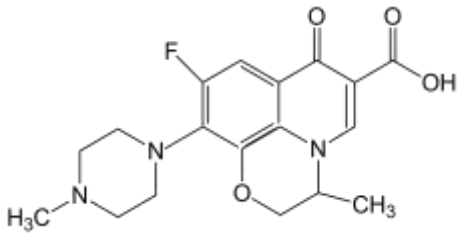


Fig. 1.3 Structure of ofloxacin

1.3.3 Gatifloxacin

Gatifloxacin is white crystalline powder. It is slightly soluble in methanol, sparingly soluble in alcohol and partially soluble in cold water. Chemically gatifloxacin is described as (±) – 1 – Cyclopropyl – 6 – fluoro - 1,4 – dihydro – 8 methoxy – 7 – [3 – methyl – 1 – piperazinyl] – 4 – oxo – 3 – quinoline carboxylic acid sesquihydrate [33].

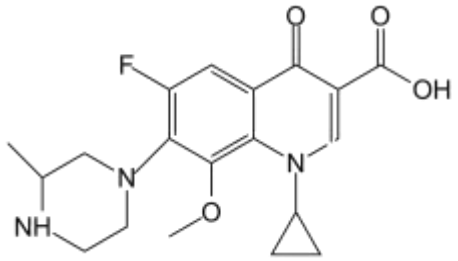


Fig. 1.4 Structure of gatifloxacin

1.3.4 Sparfloxacin

Sparfloxacin is light yellow crystalline powder. It is soluble in methanol, chloroform and ethanol. Solubility of sparfloxacin in water is less than 1mg mL^{-1}. Chemically it is described as 5 – Amino – 1 – cyclopropyl – 7 – [cis- 3,5 – dimethyl piperazin – 1 – yl] - 6,8 – difluoro - 1,4 – dihydro – 4 oxoquinoline – 3 – carboxylic acid [33].

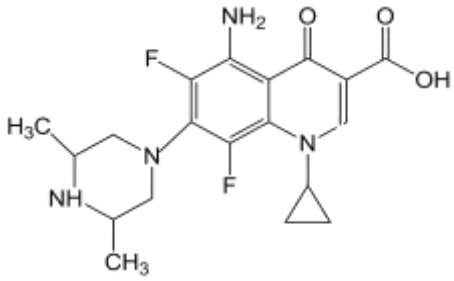


Fig. 1.5 Structure of sparfloxacin

1.3.5 Levofloxacin

Levofloxacin is light yellowish, white to yellow white crystalline powder or crystals. It is soluble in dimethylsulfoxide, acetic acid and sparingly soluble in methanol, water and acetone. Levofloxacin is not soluble in n-octanol and glycerin. Chemically levofloxacin is described as (–) – (S) – 9 – Fluoro – 2,3 – dihydro – 3 – methyl – 10 – [4-methyl – 1 – piperazinyl] – 7 – oxo – 7H – pyrido [1,2,3 – de] – 1,4 – benzoxazine – 6 – carboxylic acid [33].

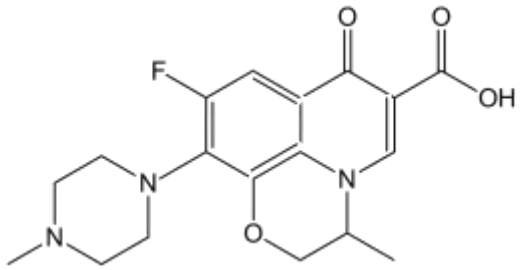


Fig. 1.6 Structure of levofloxacin

1.4 5-Nitroimidazole drugs

Gastrointestinal tract infections are very common worldwide caused by the aerobic bacteria and certain protozoa [34-35]. 5-Nitroimidazole derivatives are group of compounds which have been extensively and widely used to treat these infections [36]. These compounds also play a vital therapeutic function in the treatment of cancer as photo sensitizers [37-38]. They have also been used in the anti-tubercular

therapy [39]. The important widely used 5-nitroimidzoles are metronidazole, ornidazole, secnidazole and tinidazole. The use of 5-nitroimidazole as drugs was started by the discovery of azomycin, a 2-nitroimidazole. Due to its weak activity spectrum it was soon replaced by another most effective molecules metronidazole [40]. Metronidazole is most widely studied. It is most potent for most protozoal infections like giardiasis [41]. Metronidazole has successfully been used as drug of choice for amoebiasis, trichomiasis, balatidiasis and other aerobic bacterial infections like Vincent's disease. In post-operative infections, prophylactically in colonic and gynaecological operations, helicobacter pylori infections, clostridium difficile and after the bowel surgery, metronidazole is mostly prescribed. The activity spectrum of metronidazole give it an edge over other antibacterial drugs due to its potency against gram positive, gram negative bacteria, protozoa and some nematodes. Therefore these drugs have exclusive cytotoxic actions relevant to the species. 5-nitroimidazoles are further classified as antifungal, antimycobacterial, trypanocidal, anti-HIV active and antileishmanial [42-45]. Ornidazole is another widely used 5-nitroimidazole compound effective in antiprotozoal diseases, especially effective against crohn's in bowel resection [46-47]. Tinidazole has actions against parasitic invasion and has been used successfully. Secnidazole is very specific in action against the atopobium vaginae and dientamoebiasis [48-49].

1.5 Mechanism of action of 5-nitroimidazoles

5-Nitroimidazole make their entry inside the aerobic and anaerobics by diffusing through the membrane. An enzyme pyruvate ferredoxin oxidoreductase in the mitochondria reduces the 5-nitoimidazoles, so that their structure is altered. Normally this enzyme provides ATP molecules through oxidation-decarboxylation of pyruvates. Electrons are accepted by hydrogen ions during this cycle but these electrons are now taken up by the 5-nitroimidazoles nitro group. The reduced molecule of 5-nitroimidazole generate a concentration gradient inside the bacterial cell. This causes more drug molecules enter inside the bacterial cell. More and more free radicals and intermediate compounds formation inside bacterial cell create toxicity. The reduced

drug molecule and other free radical react with the bacterial DNA. This causes the disruption of DNA helix and consequently death of the cell [50].

1.5.1 Metronidazole

Metronidazole is white or yellow odorless, crystalline powder. It is slightly soluble in ether and chloroform but sparingly soluble in alcohol and water. Chemically metronidazole is described as 2 – [2 – Methyl – 5 – nitroimidazol – 1 – yl] ethanol [33].

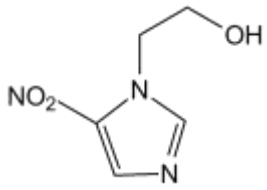


Fig. 1.7 Structure of metronidazole

1.5.2 Ornidazole

Ornidazole is white or yellow, crystalline in nature. It is soluble in chloroform and methanol. Chemically it is 1 – Chloro – 3 – [2 – methyl – 5 – nitroimidazol – 1 – yl] propan – 2 – ol [33].

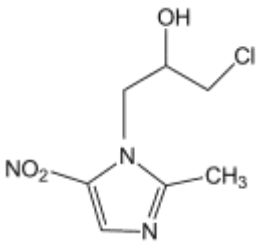


Fig. 1.8 Structure of ornidazole

1.6 Fixed dose combination therapy

When two or more active pharmaceutical ingredients are combined in a single dose with a fixed quantity then this combination is called fixed dose combination (FDC) and therapy is referred as fixed dose combination treatment or therapy. It is

worldwide accepted that for the effective treatment of a disease, drugs should combined in a single dose. FDC is very successful in management of chronic diseases. The risk of noncompliance to the treatment is reduced by 24-26% by combination therapy and it has been proven by the clinical outcomes [51]. Combination therapy has advantages over single therapy such as:

- FDC active drugs have different mechanisms of actions.
- Their pharmacokinetics is not substantially different.
- They have enhanced compliance.
- They have greater efficacy and reduced cost

The spectrum of side effects in combination therapy is decreased as amounts of individual drugs in the combination are reduced with similar therapeutic effects. The combined drugs thus work on the assorted receptors and augment the overall effect of the medication. Some fixed dose combinations of fluoroquinolones and 5-nitoimidazole drugs are ciprofloxacin and tinidazole, ofloxacin and ornidazole and norfloxacin and metronidazole etc. They have been extensively used in the gastrointestinal tract infections and proved very successful. The accumulative anti-infective effect of the quinolones and the 5-nitroimidazole not only enhances the effectiveness but also reduces the span of the treatment. There are also some disadvantages of the FDC therapy that include inflexibility in the ratio of the combination drugs, toxicity and unjustified over the counter FDC formulation that have side effects [52-53].

1.7 Surfactants

The word surfactant has come from surface active agent and belongs to very important class of chemical compounds, amphiphilic. They have undergone very extensive studies over the last few decades. They have been used in almost every field of daily life [54-55]. Of all the principal applications of the surfactants they are used as complex-forming agents. Amphiphilicity, enhanced solubility, lowering of surface tension and foaming ability are some of the remarkable qualities of the surfactants [56-58]. Over the past few decades, numerous surfactants molecules have been

developed. The reason for the wide deployment of surfactants in industry is their exceptional ability to influence the properties of surfaces and interfaces and imposing impact on the products. Surfactants have assorted applications and have great practical importance in various industrial applications in agrochemical, pharmaceutical, laundry, petroleum industry, fuel additives, mineral ores, paint production, adhesives, coatings, photographic films and lubricants etc. They have broad applications in medical field, environment and healthcare applications. The unusual properties of the surfactants in aqueous solutions are due to the presence of bipolar moieties in molecules i-e hydrophilic heads and hydrophobic tails. The polar ionic heads strongly interact with the aqueous phase and get solvated through dipole-dipole interactions and dipole-ion association. They form an organized single layer at the interface and exhibit surface activity in which they decrease the interfacial or surface tension of the medium. In lower concentrations, surfactants behave as normal electrolytes in aqueous solutions but concentrated solutions behave differently which is explained by the formation of surfactants self assembled structures called micelles, the aggregates of the monomers. At lower concentration, monomers adsorb at the surface of water at air/water interface with their heads points into the water and hydrophobic tails points out of water. In micelle, the hydrophobic portion of the monomer associates to the inside of the micelle, whereas hydrophilic part associate with the aqueous medium. At a certain higher concentration called critical micellar concentration (CMC), micelles are formed, which are about 50-100 monomers per micelle [59-60]. This thermodynamic micellar formation process in aqueous phase is considered a compromising ability of the alkyl chain to avoid the energetic unfavorable contact with the aqueous phase and ionic part of the monomer to remain contacted with the water environment. In the presence of the hydrophobic interactions, monomers adsorb onto the interfaces and establish a dynamic equilibrium between adsorption and desorption. The physical and chemical properties of all the surfactants remarkably differ above and below the critical micellar concentration. The CMC usually reveals poor dependence on temperature and pressure. However CMC value of some surfactants is observed to increase linearly with temperature above 100^{o}C. The solubility of most surfactants increases strongly above a specific temperature that is termed as Kraft point. Below that temperature

surfactants are poorly soluble and show no micellization. Above Kraft point, a substantial amount of surfactant is dispersed in the micelles. The non ionic surfactants show no Kraft point as they lessen their solubility as the temperature is increased and also lose surface active properties. CMC of a surfactant can be determined by various physical techniques like measuring conductivity, surface tension, detergency and osmotic pressure etc. When these values are plotted against the concentration of surfactants, a break line in the curve indicates the formation of micelles [61]. The micelle formation phenomenon in the aqueous medium is established by thin balance of weak forces, like electrostatic, steric, hydrophobic, Van der Walls and hydrogen bonding interactions. The major attractive forces that come from the hydrophobic effect are associated with the hydrophobic tails and opposite repulsive forces, which are associated with electrostatic and stearic interaction of the polar head groups. However, the concentration at which micellization starts, primarily depends upon the balance of forces promoting and opposing micellization process [62-63]. The decrease in the CMC of a particular surfactant enhances the stability of the micelles. This is particularly very important in pharmacology, where dilution with large blood volume during intravenous administration, only surfactant micelles with small CMC value will survive.

Micelles that belong to surfactant, which have large CMC values will dissolve to their monomers and their contents may sediment in blood [64]. There are supposed to be different loci of drug solubilization within a micelle. Hydrophilic drugs adsorb at the micellar surface. Drugs that have intermediate solubility locate at intermediate places within a micelle, like, between hydrophilic heads of polyethylene oxide (PEO) micelles and inside the palisade layer, that is, between head group and first few carbons of the hydrophobic tail of the outer core. The hydrophobic drugs may adsorb completely in the inner hydrophobic core of the micelle. The various solubilization positions inside a micelle are due to the fact that some physical characteristics like polarity, micro-viscosity and degree of hydration are not uniform throughout the micelles [65-67]. Surfactants are categorized into three classes. Ionic, non ionic and zwitterionic.

1.7.1 Ionic surfactants

They possess a net charge on the head. Charge could be positive in case of cationic surfactants or negative in case of anionic surfactant. Sulphonates, phosphates and sulphates are commonly used anionic surfactants. These chemical compounds possess permanent negative charge on the head group. Carboxylates are also anionic surfactants, however in this case the net charge on the head totally depends upon the pH of the medium. These surfactants are widely used in cleaning purposes due to their enhanced detergent like properties. Amines are the mostly used as cationic surfactants. Charge on these surfactants could be pH dependent or they may carry a permanent charge. Most extensively used, for example, quaternary ammonium compounds possess permanent charge; however other amine compounds could be neutral or charged based on pH of solution. The cationic surfactants are superb conditioners and are used in number of household utilities. Majority of fabrics and hairs possess surface sites, which are negatively charged, so the positive head of cationic surfactants due to the electrostatic attractions are attached to these negatively charged surfaces, while the hydrophobic tail will tend to align along the fabric or hair surface and hence act like a smooth coating [68].

Table 1.2 Types of surfactant

Class	Examples
Cationic	Trimethyl dodecylammonium chloride Cetyltrimethylammonium bromide (CTAB) Lauryl amine hydrochloride
Anionic	Sodium dodecyl benzene sulphonate Sodium dodecyl sulphate (SDS) Sodium stearate
Non-Ionic	Polysorbate 80 Alkyl phenol ethoxylate Triton x-100 Polyoxyethylene alcohol
Zwitterionic	Dodecyl betaine Lauramidopropyl betaine

1.7.2 Non-ionic surfactants

These are the surfactants which do not possess any type of charge on the head group. The structure of these molecular compounds is fairly simple. Most of these

compounds are only consisted of oxygen, hydrogen and carbon atoms. Alkyl ethoxylates and fatty alcohols are the most commonly used compounds of non ionic surfactants, however in this case the structure of the head and tail can differ significantly. They have been extensively used in detergents, paints, drugs agrochemicals, and cosmetics. They have also been used as dispersing agents.

1.7.3 Zwitterionic surfactants

Zwitterionic surfactants nearly act similar to non ionic surfactants. These compounds are of interest as they possess both charges, negative and positive, in the head moiety. These charges could be due to pH of the solution or may be permanent. Some compounds can be cationic or may be anionic in certain conditions. The commonly used surfactants are betaine and lecithins. They are particularly used as emulsifier and cleansing agents [69-71].

1.8 Surfactant as pseudo-membrane models

Biomembranes constitute the boundary of the cells and are consisted of proteins, lipids and carbohydrates. Lipids are very well studied components of the biomembranes. Lipids are amphiphilic structures with charged heads and hydrophobic tail. They form a fluidic dynamic bilayer structure, on which carbohydrate and protein molecules are embedded and carry out their unique and specific biological functions. Understanding of the biological membrane has always been a challenging assignment due to its complicated structure [72]. Majority of drugs come across plasma membranes and they undergo various sorts of interactions with the biomembranes while reaching their targets. Physiochemically, these interactions are very complex in nature. Pharmacologically active molecules are hydrophobic or hydrophilic in nature. Transport of these molecules through the phospholipids membranes therefore has not been fully understood. These interactions could partially be explored using simplified membrane mimetic such as micelles. These physiochemical interactions of drug molecules with the micelles can be envisioned as an approximation of interaction with plasma membranes. The drug and membranes interactions could either be considered as partitioning or binding phenomenon. These mechanisms are of core importance when the pharmacokinetic and pharmacodynamic

aspects of the drugs are to be evaluated. Micelles solubilize the sparingly soluble drugs, enhance their bioavailability, save the drugs by encapsulation and act as drug carriers to the specific site of action [73-75]. Micelles show thermodynamic stability in their aggregation and dissociation. Biodistribution, transportation and efficacy of drugs are some of the important pharmacokinetic properties of the drugs that can easily be explained by the phenomenon of drug-micelle interaction. Owing to the electrostatic forces of attraction at the molecular level, numerous biological phenomenon take place at the membrane surfaces or within their lipophilic moiety. Lipid head groups are ionizable due to which plasma membranes frequently bear a net charge. This results into a number of diverse binding properties of uncharged and charged molecules bearing ionizable groups. In spite of numerous amounts of research work aimed to comprehend the action mechanism of drug at molecular level, this question remains uncertain. More information is required from membrane model system studies [76-78].

2. INSTRUMENTATION

2.1 High performance liquid chromatography (HPLC)

HPLC is an analytical technique used to analyze and separate single or multiple chemical compounds in a solution mixture. High performance liquid chromatography is an equipment comprise of multiple components i-e detector, pumps, column, column oven, sample injector either manual injector or automatic sample injector, reservoirs to deliver the mobile phase and degasser apparatus. The sample solution containing analytes goes through the column at varying rates owing to their different partitioning behavior between mobile and stationary phase [79].

2.2 Micellar liquid chromatography

Micellar liquid chromatography (MLC) is one of the important alternatives of the conventional high performance liquid chromatography (HPLC) with same primary components of RP-HPLC system that is non polar stationary phase and a polar mobile phase. Mobile phase in conventional RP-HPLC is homogeneous. Mobile phase in MLC is heterogeneous in nature having two characteristic phases, micelles and water-organic solvents that also contains monomers of the surfactant. Monomer

concentration is usually equal to or greater than the CMC of the surfactant. Monomers thus adsorb onto the stationary phase and reduce the silanophilic interactions. Composition of the stationary phase therefore does not change with deliberate differences of micelle concentration in mobile phase. In contrast, in RP-HPLC changing hydro-organic ratios, the conformation and composition of alkyl-bonded stationary phase is altered. There are three fundamental types of partitioning mechanisms of solute in MLC by which retention behavior is controlled i.e. partitioning from bulk into the micelles, and/or partitioning directly from bulk into the stationary phase. They may also directly transfer from micelles present in the mobile phase into the stationary phase. Retention of highly polar molecules is described by the partitioning from aqueous phase into the micelles and alkyl stationary phase. Hydrophobic molecules are supposed to move from micelles in the mobile phase into the stationary phase[80-83]. Efficiency of columns in MLC decreases with time due to very slow mass transfer from stationary phase. Monomer adsorption on the stationary phase and poor wetting alters the characteristics of alkyl stationary phases. Column efficiency in MLC can be improved by three possible approaches.

- Adding small amount of organic modifiers in the micellar mobile phase.
- Rising column temperature.
- Flow rate can also be reduced to increase column performance.

Mostly short-chain organic alcohols like methanol, propanol, and/or acetonitrile are used as modifiers. Modifiers essentially enhance the elution strength and also improve the peak symmetry. Furthermore, addition of triethylamine along with organic modifier further enhances the column efficiency. Similarly, increased temperature increases the kinetics of mass transfer. So working under these conditions possibly improves the column performance in such a way that MLC becomes comparable to conventional RP-HPLC [84-85]. On account of the fundamental analytical parameters that include accuracy, precision, sensitivity, traceability and selectivity, MLC is a technique that fulfills the main analytical objects, in addition to greener aspects. Considering environmental aspects, conventional RP-HPLC consumes excess of organic solvents. MLC is a good substitute of RP-HPLC that not only works well

from economic point of view but also improves the ecological aspects. From sample collection, preparation, separation to the final determination, MLC is supposed to be greener in all steps than conventional RP-HPLC. Mostly mobile phases in MLC are made of aqueous surfactant solutions above their CMC value and a little proportion of the organic modifier (usually 3-15% by volume). CTAB, SDS and Brij-35 are the surfactants which are commonly used in the MLC. SDS is safe for health and is not carcinogenic in case of direct contact with the human skin or either consumed [86]. CTAB is believed to be safe as it is used as disinfectant. It is absorbed through the intestine. The undigested and unabsorbed amount comes out with feces. The absorption through the skin is very slow [87]. Similarly fatty alcohol ethoxylates (Brij-35) surfactant have no effect on eyes and skin and nor on reproduction and genetics. Mobile phases in MLC are therefore considered safer for environment and for the operator. Moreover, propanol, butanol etc. used as modifiers, get retained in the micellar solution and cause less evaporation of these solvents inside the laboratory and environment, making micellar mobile phases more stable. Also they have less toxicity than acetonitrile and methanol. The little content of organic solvents also provides benefit of non-flammability and safety for laboratory work.

Biodegrability is another useful character of surfactants used in micellar mobile phases. CTAB is a quaternary ammonium compound, which is biodegradable through various pathways. In a pathway, N-dealkylation, CTAB is degraded to alkyl residue and triethylamine by monooxygenase activity [88-89]. SDS is aerobically degraded. It has been reported that 70 percent of SDS (C^{14}) was degraded to CO_2 and rest of 30 percent was incorporated into the microbial biomass i-e all of the SDS was utilized for either biomass production or energy. Likewise Brij-35 is an eco-friendly substitute to alkyl-phenol ethoxylates. They are considered readily biodegradable and 80 percent is biodegraded primarily in 28 days. Recycling of hybrid micellar eluents could also be possible in MLC because of the reduced evaporation risk of organic modifiers. Water-acetonitrile effluents can be decomposed to ammonia and acetic acid by treating with excess sodium hydroxide. Thus, waste generated from MLC could be considered a clean waste [90-93]. Regarding sample treatment there are certain advantages of MLC compared to conventional RP-HPLC. For example, a medicine solution can directly

be injected into the system without any special treatment except filtration. Micelles easily extract the drugs, as excipients are normally not solubilized in the micelles. Small quantity of organic solvents can further improve the drug solubility.

In conventional RP-HPLC, repeated sample preparation is needed for the protein based biological samples analysis. This is necessary to remove proteinaceous mass prior to injection. This is required to avoid irreversible adsorption and plugging of packings by the proteins. Precipitation of proteins is a time consuming and tedious exercise and can cause loss of materials. Physiological proteins can preferably be analyzed with MLC by directly injecting them into the system owing to ability of micelles to solubilize protein, serum plasma and urine sample. They otherwise involve hectic sample preparation steps in conventional HPLC and other extraction procedures [94]. MLC mobile phases are well matched with RP-HPLC stationary phases like C_8, C_{18}, phenyl, cyanopropyl etc. MLC is also compatible with various RP-HPLC detection methods such as UV, fluorescence, diode-array detection, chemi-luminescence, electro-chemical detection, phosphorescence, and inductively-coupled-plasma mass spectrometry. Fascinatingly, sometimes micellar mobile phases could improve the detection ability [95-96].

2.3 Binding constant determination by micellar liquid chromatography

The binding constant (K_2) values of drugs were estimated by micellar liquid chromatography using Arunyanart and Cline Love equation 1 [97-98].

$$\frac{1}{k'} = \frac{K_2.[M_m]}{K_1.\varphi.[L_s]} + \frac{1}{K_1.\varphi.[L_s]} \qquad (1)$$

Where k' is capacity factor of solute, M_m is micellar concentration, φ represents phase ratio, k_2 stands for solute-micelle binding constant, K_1 symbolizes solute-stationary phase binding constant and L_s is the concentration of stationary phase sites. Binding constant value (K_2) for the drug is calculated from slope/intercept ratio of the plot $1/k'$ against $[M_m]$.

2.4 UV-Visible spectrophotometry

UV-visible spectroscopy is an important and useful technique in the analytical chemistry. It obeys the Beer-Lambert law according to which when a monochromatic beam of light passes through a solution having absorbing analyte molecules, the rate of decrease of intensity of radiations is proportional to the solution concentration i-e more the number of absorbing analyte molecules absorbing specific wavelength, more is the absorption. UV-visible spectrophotometer composed of various components like light source, monochromator, sample and reference quartz cells, detector and amplifier. The test solution is kept in the sample cell and reference medium is placed in the reference cell. In differential spectral absorbance measurements, drug solution is kept in reference cuvette whereas drug and micellar solution is taken in sample cuvette [99-100].

2.5 Benesi-Hildebrand equation for binding parameter

The binding phenomenon of drug with surfactant is elaborated using differential UV spectroscopic practices. A more specific quantitative and analytical approach to find such binding parameters between surfactant and drug was taken into account by using customized Benesi-Hildebrand equation 2 [101-104] as under.

$$\frac{dC_a}{DA} = \frac{1}{De K_b C_s^{mo}} + \frac{1}{De} \qquad (2)$$

In above equation, $C_a$corresponds to concentration of drug substance, optical path length of the solution (1.0 cm) is represented by d, difference of absorbance between the drug and complex is symbolized by ΔAand attained from differential UV absorbance spectrum. Absorption coefficient difference between free drug and micelle encapsulated drug in aqueous solution is symbolized by $\Delta\varepsilon$. $1/(C_s^{mo})$ represents difference between CMC of the surfactant solution containing drug and total concentration of surfactant. The reliability of above equation (2) was established when $dC_a/\Delta A$ was plotted versus $1/(C_s^{mo})$. The analytical values of the binding constant (K_b, determined from UV spectroscopic measurements) and binding energies (ΔG^o_b) were estimated from the subsequent slope/intercept ratio.

2.6 Standard Gibb's' free energy change

The Gibb's free energy (ΔG^{o}_{b}) for the transportation of drugs from aqueous bulk phase to the micellar interior is calculated by the given equation 3.

$$\Delta G^{\circ}_{b} = -RT\ln K_{b} \qquad (3)$$

T corresponds to the absolute temperature and R is the gas constant. A high negative value of ΔG^{o}_{b} indicates that drug-micelle partitioning process is spontaneous [105].

2.7 Fluorescence spectrophotometry

Fluorescence spectrophotometry is an analytical technique based on the measurement of photo-emission of the radiation from an excited molecule that has been to the higher energy levels by the absorption of the radiations. Fluorescence measurements have advantage over the absorption spectrometry such that fluorescence signals, in-principal, have zero background. Fluorescence detector is also used in liquid chromatography. A fluorescence spectrophotometer has multiple components including excitation source that could be deuterium or xenon lamps, monochromator, sample cell, detector, amplifier and recording devices [99-100].

2.8 Stern -Volmer equation

By employing the fluorescence quenching data, the Stern-Volmer quenching plot of drug-CTAB with varying concentration of CTAB is estimated using the Stern-Volmer relation 4.

$$\frac{I_{\circ}}{I} = 1 + K_{sv}[C_{s}] \qquad (4)$$

I_o and I represent the steady state fluorescence intensity of drug molecules in the absence and presence of surfactant respectively. $K_{sv}$and $[C_s]$ represent Stern Volmer quenching constant and concentration of surfactant respectively. The data obtained by this equation shows that the quenching mechanism that may be caused by the formation of ground state complexes (static quenching) rather than dynamic collisions, supported by the literature [106-108].

2.9 Lineweaver–Burk equation

The binding affinity (K_b) for static quenching is calculated using modified Lineweaver–Burk equation 5.

$$\log[\frac{I_\circ - I}{I}] = \log K_b + n_b \log[C_s] \qquad (5)$$

In equation 5,I_o and I represent the steady state fluorescence intensity of drug molecules in the absence and presence of surfactant respectively [108-109].

2.10 Degree of counter-ion binding

$\beta = S_2 / S_1$, the micellar ionization degree (β) is determined from the ratio of the slope of the micellar posterior zone (S_2) to the slope of the pre micellar zone (S_1). When $\alpha = 1-\beta$, micellar ionization (β) is related to the degree of counter-ion (α).

Aims and objectives of the research work

A brief literature survey has revealed that no significant work has been done in the past to find out interaction of combination drugs with the membrane model system by the micellar liquid chromatography and spectroscopy. Previously research work has been carried out to find out this interaction study only for some single drug molecules involving either micellar liquid chromatography or electronic spectroscopy. In the present study, both micellar liquid chromatography and electronic spectroscopy were used to study the interaction behavior of combination drugs as well as single drugs with membrane model. The emphasis of the present study was to gain in depth and better understanding of the potential assimilation of pharmacologically active drugs into the cationic surfactant cetyltrimethylammonium bromide at various pH conditions. The main objectives of the present work include:

a. In-vitro interaction studies of single and fixed dose combination drugs with the simple membrane model system.

b. Binding and allocation of antibiotic drugs in surfactant micelles.

c. Quantitative determination of drug-micelles binding capacities and associated binding energies.

d. Qualitative analysis of CMC values of cetyltrimethylammonium bromide during drug-surfactant interaction.

e. Quantitative interaction effect of one drug in the presence of other drug in fixed dose combinations.

f. Developing simple micellar chromatographic methods for the combinational and single drugs

CHAPTER NO. 2

LITERATURE SURVEY

Enache *et al.* described a UV-visible spectroscopic procedure for the quantitative determination of water-micelles partitioning coefficient and drug-micelles binding constant for the anti-cancer drug mitoxantrone [74]. Three non-ionic surfactants i-e tween 20, triton x-100 and tween 80 were used. They suggested that at basic pH 10, the interaction between mitoxantrone-micelles was found higher than at pH 7.4. At pH 10, large values of partitioning coefficient corresponds to incorporation of deprotonated drug effectively inside the hydrophobic core of the micelles, rather than at pH 7.4, where mitoxantrone was more predominantly protonated. The authors supposed that drug interaction with non ionic micelles minimized the dimerization of the drug, as only the monomer drug was encapsulated by the micelles.

Caetano *et al.* utilized absorption electronic spectrometry to determine binding of two psychiatric drugs trifluoperazine and chlorpromazine with surfactants of different classes including cetyltrimethylammonium chloride (CTAC, cationic), N-hexadecyle-N,N-dimethyl-3-ammonio-1-propanesulphonate (zwitterionic), triton x-100 and Brij-35 (neutral) [78]. The binding constant and the acid dissociation constant values were calculated using red shift of absorption maxima in alkali or detergent medium. They revealed that dissociation constant value of both drugs changed by interacting with different surfactants. These changes in pKa values, due to the electrostatic attractions, were all positive and direction of shift depends mainly upon the charges on the polar head moiety. The values for the binding capacities obtained for both drugs in different protonation conditions, revealed that electrostatic affinity was necessary for the binding of drugs with the micelles having charges on the head.

Arunyanart *et al.* proposed three phase equilibrium modules, linking capacity factor to micellar concentration in micellar liquid chromatography [98]. The module was helpful in estimation of equilibrium constant of the analyte between bulk aqueous phases. The electrostatic interactions were supposed to be minimized in this module.

They proposed that if equilibrium constant was determined by another method, then this module could be used to find chromatographic capacity factors.

Cudina *et al.* applied UV spectroscopic and micellar liquid chromatographic methods for the determination of valsartan interaction with cetyltrimethylammonium bromide surfactant at physiological pH 7.4 [110]. The cationic micelles effect on acid, base and spectroscopic properties of valsartan was investigated. Valsartan dianions had greater affinity with the cationic CTAB micellar surfaces that were proved by the shift in the acidity constant values of the drug ($pK_a^{water} - pK_a^{micelle} = 1.69$). CTAB-valsartan bindings were quantified by using two mathematical models, water-micelle partitioning constant and valsartan-micelle binding constant. In pseudo-phase model system, the decline in water-micelle constant value with drug concentration found consistent with adsorption phenomenon. Binding constants were estimated from the differential absorbance.

Vicente *et al.* quantified three antiretroviral drugs in plasma by using simple liquid micellar chromatographic technique [111]. They directly dissolved the tablets in micellar phase and filtered. Mobile phase was simple SDS (0.05M) at pH 7 and separation was carried out using C_{18} column at UV wavelength 260nm. Standard ICH guidelines were used to validate the method with precision (less than 6.8 %), accuracy (92.3 to 104.2 %), linearity and linear range (0.25 to 2.5 grams per mL).

Jose *et al.* studied seven antidepressant drugs by HPLC using micellar mobile phase [112]. They used Brij-35 surfactant and compared these results with those obtained using simple mixture of acetonitrile and water as mobile phase. They suggested that these drugs were slightly basic in nature and made stronger attachment with silanoles of alkyl stationary phases. This resulted in more consumption of organic solvents to get suitable retention time. They proposed that use of nonionic surfactant altered the stationary phase to somewhat neutral by covering the surface. This led to decrease the retention time. The authors used Brij-35 (0.02M) at pH 3.

Nazar *et al.* used absorption and emission spectroscopic procedures to determine quantitatively the binding capacities of moxifloxacin with cationic surfactantscetrimonium bromide and mecetronium bromide, at different pH values 7.4 and 6.5 [113]. The authors supposed that solubilization of the drug in the micellar corona was supported by the larger head groups of the surfactant by electrostatic forces of attraction. This was verified experimentally by binding and Stern Volmer quenching constant values. The binding between drug and cationic surfactants was spontaneous in nature. Quenching existed in static mode which was initiated by ground state complex formation. The host-guest mechanism of interaction between moxifloxacin and membrane models (micelles) helped to understand molecular behavior in membrane model system.

Kulikov *et al.* analyzed the opium contents codeine, noscapine, papaverine and morphine with micellar chromatography [114]. Method was validated and was applied effectively on the pharmaceutical injection formulation. They optimized various chromatographic parameters like retention time, resolution, peak asymmetry and efficiency. The authors used mixture of 1-butanol (5%) and SDS (0.1%) as mobile phase at pH 2.5.

Alam *et al.* reported thermodynamic properties of four different amphiphilic drugs imipramine HCl, amitryptiline HCl, chlorpromazine HCl and promethazine HCl with various additives like cetyltrimethylammonium bromide, sodium chloride and triton x-100 [115]. They determined various physical properties like Gibb's free energy change for micelle formation, Gibb's free energy at the water-air interface, excess free energy changes for micellization and standard Gibb's free energy of adsorption.

Vicente *et al.* utilized micellar mobile phase in HPLC to find tamoxifen in tablet formulations at 40^{o}C using C_{18} column [116]. They directly solved samples in mobile phase, filtered and finally injected to avoid the extraction steps. Mobile phase was comprised of a mixture of SDS (0.15M) and pentanol (7%). Fluorescence detection with excitation at 260nm and emission at 380nm was used. Method was validated

using standard ICH guidelines of linearity, precision, accuracy, robustness, specificity and sensitivity.

Ibrahim *et al.* prescribed a validated micellar chromatography procedure for simultaneous quantitative finding of clindamycin phosphate and nicotinamide in a dosage formulation [117]. Micellar mobile phase was a mixture of 0.1M SDS, 0.3 percent triethylamine and 10 percent isopropanol in 0.02M ortho-phosphoric acid (pH 3.0). The experiment was performed on a C_8 Eclips XDB column (150 × 4.6mm) with pore size 5µm. A diode array detector was fixed at 210nm wavelength. Well resolved peaks of nicotinamide and clindamycin with retention time 3.8 and 5.6 minutes were obtained. This method had improved accuracy, low cost and high percentage recovery.

Rizk *et al.* proposed a MLC procedure for finding itraconazole in bulk, human plasma and dosage forms [118]. Mobile phase consisted of SDS (0.1molar), 1-propanol (20 percent), triethylamine (0.3 percent) dissolved in 0.02 molar ortho-phosphoric acid (pH 3.5). Column was cyano µBondapak. UV detector was fixed at 258nm. Different chromatographic parameters including different columns, pH of mobile phase, triethylamine, 1-propanol and SDS concentrations were optimized. The method was found linear over the concentration gradient 16.4 – 320 microgram per mL. The accuracy and validation of the method was comparable with the conventional HPLC method. This method was also found suitable to be applied under different stress conditions.

Fatteh *et al.* investigated interaction behavior of losartam potassium with the non-ionic surfactant triton x-100 [119]. They used buffer 7.4 as physiological medium to investigate their findings. They found that by increasing surfactant concentration, there was a linear and systematic progression in the hypochromic and bathochromic shifts. They used differential absorbance to calculate the binding between drug and micelles and found to be $4.13\pm0.35\times10^5$ M^{-1}. Partition coefficients between micelle and water was calculated using pseudo-phase model and found to be

$2.26\pm0.12\times10^{5}M^{-1}$. The losartam potassium acidity constant also showed a shift during this binding phenomenon.

Kord *et al.* proposed a micellar electrokinetic chromatographic and micellar liquid chromatographic procedure to find out micellar-solute binding constant of various chemical compounds [120]. The data showed that micellar electrokinetic chromatography could be a substitute for the MLC for finding binding constant. Both techniques were corresponding regarding practical range of pH.

Zaghbani *et al.* studied the interactive behavior of different cationic surfactants with eriochrome blue black R spectrophotometrically [121]. Experiment was conducted with n-alkyltrimethylammonium C_nTAB in premicellar to postmicellar concentration, where n =12, 14, 16, 18. They reported that dye absorbance shifted towards higher wavelength as alkyl chain length of surfactant increased and similarly dye-micelles binding constant also increased. This was confirmed by the fact that surfactant with longer alkyl chain length solubilized more dye.

Jaipang *et al.* determined sildenafil citrate with simple micellar HPLC method in pharmaceutical preparation [122]. Isocratic separation was carried out using C_{18} column with SDS (8.2mM) solution in acetate buffer having pH 4. UV detector was adjusted at 298 nm. Linearity range was 125-500 microgram per mL. LOD and LOQ were 35 and 106 microgram per mL respectively.

Nazar *et al.* published the interaction mechanism of N-m-tolylbenzamide with cetyltrimethylammonium bromide and sodium dodecyl sulphate [123]. They utilized fluorescence and UV spectroscopy to quantify the thermodynamic properties from pre micellar to post micellar surfactant concentration. Different thermodynamic parameters like micelles-water partitioning coefficient, micelle-N-m-tolylbenzamide binding constant, counter ion binding, binding capacities were determined using the different mathematical models. They hypothesized on the basis of data that there were more interactions of drug with cetyltrimethylammonium bromide surfactant than SDS. Based on the values obtained for binding capacities of drug-micelle combination

and Stern Volmer quenching constant, drug molecules were supposed to be at single binding site with each surfactant micelle and expressed static quenching mechanism instead of collision quenching.

Walash *et al.* prescribed a novel MLC procedure for the estimation of binary combination of drugs norfloxacin and tinidazole in plasma and dosage form [124]. For plasma analysis the proposed method did not require any protein precipitation before injection. Separation was done on a ODS Waters symmetry column at UV 275 nm wavelength and 1mL per minute flow rate. Mobile phase was sodium dodecyl sulphate (0.15 molar), triethylamine (0.3 percent) and n-propanol (5 percent). pH was adjusted to 4.0 with orthophosphoric acid. Linear calibration curves were obtained for norfloxacin and tinidazole over concentration ranges 1-28 and 1.5-42 microgram per milliliter respectively. Quantification limits were also calculated for norfloxacin and tinidazole 0.7 and 1.0 microgram per milliliter respectively.

Milonowska *et al.* carried out physiochemical properties of some amines i.e histamine, tyramine, dopamine, 2-phenylethylamine, octopamine and adrenaline by micellar liquid chromatography [125]. The main focus of the research was to see effect of pH of mobile phase and concentration of the surfactant on the retention of analyte molecules. The authors investigated the effect of surfactant concentrations using different mobile phases having various concentrations of sodium dodecyl sulphate in buffer pH 7.4. Organic modifier used was acetonitrile (20% v/v).

Nisar *et al.* described a UV-visible spectroscopic technique to study partitioning and solubilization of some organic cyclohexenone carboxylate compounds with ionic surfactants, sodium dodecyl sulphate and cetyltrimethylammonium bromide [126]. They determined partitioning coefficient (29.714×10^3 to 5.46×10^6) between micelles and aqueous phase by differential spectroscopy. Partitioning standard free energy was also estimated and found -25 to -38 kJ mol^{-1} which indicated the system stability. The experimental data revealed that the organic cyclohexenone carboxylate compounds had more interaction with the cetyltrimethylammonium bromide than sodium dodecyl sulphate.

Dong *et al.* applied micellar mobile phase on a cold cough preparation to separate chlorpheniramine, pseudoephedrine and paracetamol with C_{18} column using programmed UV detection at 210 nm for first two molecules and 300nm for Paracetamol [127]. Mobile phase was Brij-35, KH_2PO_4 and methanol mixture at pH 3. Organic proportion was only 4%. The method was validated under standard parameters of precision, recovery, stability, linearity, LOD and LOQ. UV absorption by Brij-35 was compared with that of CTAB and SDS.

Nazar *et al.* accomplished gemifloxacin interaction with cetrimonium bromide and mecetronium bromide as mimetic membranes at molecular level. UV absorption and fluorescence measurements were carried out to study biophysical research [128]. Encapsulation of gemifloxacin molecules inside the micellar cages were quantified using mathematical model. Fluorescence quenching and differential spectroscopic techniques were made into use to calculate related free energies and binding parameters. The authors hypothesized interaction of the drug in the peripheral region of the micelles with electrostatic interactions.

Mondal *et al.* reported interaction of anionic surfactant with the cationic dye safranine T [129]. At low concentration, anionic surfactant made complexes with the dye. The authors reported that anionic surfactants formed charge transfer complex with dye beyond the CMC concentration. They observed maximum decline of absorbance of dye due to SDS and minimum decline in the intensity of fluorescence due to sodium N-dodecanoyl-N-methyl alaninate. Very small interaction was shown by cationic surfactants and no interaction by CTAB.

Hirek *et al.* presented a transmission electron and optical spectroscopic method to characterize two types of cat-anionic micelles for sodium dodecyl sulphate and cetyltrimethylammonium bromide with different surface charge ratios [130]. These cat-anionic micelles were used as membrane models to study the interaction of NSAID drug, piroxicam, with the bio-membranes. They reported that mean sized mixed micelles were quite bigger than those micelles that have uniform charged head

groups. The mixed micelles that have more concentration of CTAB (positive heads) were bigger in size and polar in nature than those having higher ratios of the negative head group (SDS). They further hypothesized that diverse and dynamic biomembrane surface charges could play a significant role to select a specific drug to its target.

Vicente *et al.* proposed a micellar method for separation of tamoxifen and its four derivatives i-e N–desmethyl-tamoxifen, 4-hydroxytamoxifen, tamoxifen-N-oxide and endoxifen in plasma taken from the patients of breast cancer [131]. Mobile phase was consisted of SDS (0.08M) and butanol (4.5%) at pH 3 with column temperature 40^{o}C. The authors employed fluorescence detection with emission and excitation wavelengths 380 and 260nm respectively. Initially sample was irradiated for 20 minutes with UV radiations to get photo-cycled fluorescent-derivatives. Samples were simply diluted and directly injected after filtration to avoid mess up steps. Procedure was validated using ICH guidelines.

Belal *et al.* prescribed a micellar liquid chromatographic method to find out levofloxacin hemihydrates and ambroxol hydrochloride, simultaneously, in plasma and tablet dosage form [132]. The average recoveries in dosage form were found to be 100.20±1.64% and 100.72±1.11% for levofloxacin and ambroxol respectively. Plasma samples did not require any prior preparation before injection. The method was also applicable in levofloxacin spiked plasma analysis with average recovery 100.10±1.14%. ICH guidelines were followed for statistical analysis of the experimental data. The method presented good linearity ranges 1-44 and 1-20µg/mL, LOD was 0.26 µg/mL and 0.07 µg/mL and LOQ was 0.80 µg/mL and 0.20µg/mL for levofloxacin and ambroxol respectively.

Martinez *et al.* accomplished analysis of different beta-blockers celiprolol, atenolol, labetalol, nadolol, acebutolol in urine by micellar chromatography using fluorometric detection [133]. Micellar mobile phase was a mixture of triethylamine (1%), propanol (15%) and 0.1 molar sodium dodecyl sulphate (pH=3). Triethylamine, propanol and low pH improved the peak symmetry. Peaks were resolved within 15 minutes. The

method also separated the metabolite peaks along with principal peak of metoprolol and propanolol in the urine sample of a healthy volunteer. The metabolite peaks weredegradation peaks of metoprolol and propanolol. Limit of detection was found to be between 3-30 ng/mL.

Wang *et al.* used various surfactants (cetyltrimethylammonium bromide, Brij-35 and sodium dodecyl sulphate), their concentrations and different pH of the micellar mobile phases to explain their effects on retention of various types of antihypertensive medicine on ODS column [134]. The stationary phases in conventional reversed phase HPLC became modified with micellar mobile phases. These modified stationary phases in MLC supposed to be analogous to biomembranes and presented electrostatic and hydrophobic sites for interactions. The authors proposed activity-retention association of various surfactants and drugs quantitatively and also explained comparison with organic-aqueous mobile phases. Correlation between toxicity of drug and log of retention factor was determined.

Algaba *et al.* proposed a quick MLC procedure for the estimation of antihistamines alone and also in combination with other drugs dextromethorphane, caffeine, acetaminophen, pyridoxine and guaifenasin as well [135]. Separation was done on ODS column with CTAB surfactant using organic modifier as n-butanol/n-propanol. The method was successfully applied on ointments, suppositories, syrups and tablets with recovery of 100±10%. Relative standard deviation was less than 5 percent with LOD less than 1µg/mL.

Banito *et al.* studied the effect of concentration, nature of different surfactants (CTAB, SDS and Brij-35) and pH of mobile phases on the retention behavior of barbiturates on modified stationary phase [136]. They proposed quantitative relationship for structure-activity and structure-retention for barbiturates with various surfactants. The data was also matched with that obtained with aqua-organic mobile phases. The authors suggested that monomers of the surfactants get adsorbed on the stationary phase and altered their hydrophobic characteristics, as a consequence generated new hydrophobic and electrostatic interacting sites. These modified

chromatographic surfaces structurally resembled with phospholipid biomembranes to study partitioning processes.

Cline Love *et al.* illustrated the effect of ionic and non ionic micelles on retention order of different analytes in serum plasma [137]. The electrostatic interactions in non-ionic micellar mobile phases with the analyte molecules were very simple. Therefore their retentions follow the mathematical models. Quantitative determination of the drugs in the serum plasma was carried out by direct injecting the plasma into the chromatograph, without any pressure build up or clogging. The authors suggested that anionic micelles behaved similarly but in cationic micelles precipitation of proteins was reported. Drug-micelles binding constant and CMC value of Brij-35 was chromatographically determined and reported. The authors demonstrated the effectiveness of the non-ionic micellar mobile phases in the estimation of acetaminophen, theophylline, quinidine, quinine, barbiturate, morphine, cocaine carbamazepine and codeine.

Hosseinzadeh *et al.* studied an absorption spectroscopic method to demonstrate the behavior of procaine hydrochloride with various surfactants, CTAB, SDS and triton x-100 [138]. The interaction behavior of non ionic and ionic surfactant with the drug was carried out at pH=6.8 at 29 Celsius temperature. Water-micelles partition coefficients were determined with pseudo phase model. They reported that CTAB micelles dissolved more drug than SDS (partition coefficient=166 and 96 for CTAB and SDS respectively). No significant solubility in triton x-100 was observed due to poor interactions with the drug molecules. They investigated electrostatic and hydrophobic interactions to estimate binding constants. It was found that the positively charged drug showed more interaction with anionic surfactant (SDS) than with CTAB (K_b= 128 for CTAB and 175 for SDS respectively).

Rakesh *et al.* contributed a UV absorption spectrophotometric procedure to look into the interaction of quaternary ammonium surfactant (CTAB) with curcumin (an active ingredient of curcuma *longa*) in pre-micellar to post micellar concentration range at pH=6.4 [139]. They studied premicellar and micellar surfactant range with

conductivity and tensiometery techniques. In lower surfactant concentrations, there occurred a change of absorbance due to attraction between drug and micelles shown by the spectroscopic data. At micellar concentration, the hydrophobic tail of the CTAB got attached to the hydrophobic aryl part of the drug molecule and caused displacement of CTAB head from the β-diketone of curcumin. In post micellar concentration, the curcumin encapsulated with formation of sharp peak at 423 nm.

Gereia *et al.* proposed the use of non ionic surfactant Brij-35 in the micellar liquid chromatographic analysis of flavonoids and sulfonamides, instead of SDS that had been widely used in this technique [140]. Brij-35 was useful over SDS as it was cheap, low toxic, has low background absorbance and high cloud point. They worked with two mobile phases, pure surfactant eluents and other was composed of surfactants and acetonitrile. Both were compared. The polarity of alkyl stationary phase enhanced with Brij-35. The polyoxyethylene length of the Brij-35 having –OH groups interact with phenolic molecules and form H-bonding. Due to this reason aqueous Brij-35 mobile phases were suggested for significant short retention time.

Angel *et al.* described the chromatographic performance of some beta blocker drugs interacting with sodium dodecyl sulphate [141]. They used micellar liquid chromatographic technique as function of concentration of both surfactant and organic solvent. They used sub-micellar water-organic mode with high organic concentration and reduced surfactant concentration. Secondly they used micellar and sub-micellar mode having high ratios of both the organic phase and surfactant. When surfactant is in high ratio, the monomers adsorbed on the stationary phase and interact strongly with the cationic basic drug molecules. It increased retention time of the analytes. This strong interaction improved the solubility by which the analyte molecules directly transfer from micellar assemblies to alter stationary phase.

Cudina *et al.* applied UV spectroscopic and micellar liquid chromatographic methods for the determination of quinapril interaction with cetyltrimethylammonium bromide surfactant at pH 8 [142]. The cationic micellar effect on acidic, basic and spectroscopic properties was investigated. They concluded that anionic quinapril

have greater affinity with the cationic CTAB micellar surfaces. It was proved by the shifts in the acidity constant values ($pK_a^{water} - pK_a^{micelle} = 1.39$) of the drug. CTAB-quinapril bindings were quantified by using two mathematical models, water-micelle partitioning constant and quinapril-drug binding constant. Binding constant was found to be ($2.3\pm0.4\times10^3$ mol^{-1} dm^3). In pseudo-phase model system, the decline in water-micelle constant value with drug concentration found consistent with adsorption phenomenon. Binding constants were estimated from the differential absorbance.

Tiwary *et al.* accomplished the thermodynamic behavior of CTAB with or without the drug tyrosine hydantoin using fluorescence, UV visible, surface tension and conductometric techniques at different temperature i-e 294, 313, 318K [143]. The results showed that there were strong interactions. CMC of CTAB decreased by increasing drug concentration in solution. Fluorescence spectroscopy was used to evaluate the aggregation number. Other thermodynamic properties of micellization including excess free energy change, standard Gibb's free energy, enthalpy change, entropy change and activity coefficients (interaction parameter) were also estimated.

Gilabert *et al.* used high performance liquid chromatography to describe some biological properties of various non-steroidal anti-inflammatory drugs using Brij-35 as surfactant in mobile phase [144]. They used quantitative-retention-activity module to explain the pharmacokinetic characteristics of these drugs.

Erdinc *et al.* accomplished the behavior of epirubicin with various surfactants CTAB, triton x-100, SDS and tween 20 from premicellar to postmicellar concentration using absorption spectroscopy [145]. Below the CMC, Jobs' method was used to determine the complex formation constant between drug and SDS. Above the CMC, the binding constants with micelles were determined with Benesi Hildebrand equation. The drug-nonionic micelle interaction was proved to be stronger than the ionic surfactants in the order tween-20 > triton x-100 > SDS. The effect of glucose and sodium chloride on the binding was also investigated. The presence of both additives decreased the CMC of all surfactants. However the binding of drug-micelles was lowered in sodium chloride and increased in the presence of glucose. They reported decrease in complex

formation equilibrium constant value in the presence of NaCl than in pure hydro-environment.

DeLuccia *et al.* used direct plasma injection mode in liquid chromatographic technique to conclude therapeutic properties of some drugs (carbamazepine, acetaminophen, phenobarbital, chloramphenicol, acetaminophen, theophylline and procainamide) interacting with SDS [146]. They did not use any precipitation or drug extraction step before injection. Drugs were also analyzed by enzyme multiplied immunoassay technique and were well in agreement with MLC. Retention and chromatographic behavior of drugs changed as the pH of mobile phase and SDS concentration varied.

Gilabert *et al.* reported a novel model for retention of local anesthetic compounds [147]. They used micellar liquid chromatography as function of physio-chemical and experimental variables. Non ionic surfactant was used. They described the hydrophobicity and their mole fraction in charged form and presented a comparison with those reported earlier. Retention of molecules in MLC was controlled by the electrostatic and hydrophobic forces and ionic compounds experience these forces. Correlations between capacity factor to anesthetic potency along with log of capacity factor and structural parameters were measured. They also determined qualitative and quantitative structure-activity-relationship for these compounds.

Cudina *et al.* applied UV spectroscopic and micellar liquid chromatographic methods for the determination of hydrochlorothiazide interaction with cetyltrimethylammonium bromide surfactant at pH 10.5 [148]. The cationic micellar effect on acidic, basic and spectroscopic properties of hydrochlorothiazide was investigated. They reported that the dianionic hydrochlorothiazide have greater affinity with the cationic CTAB micellar surfaces, that was proved by the shifts in the acidity constant values ($pK_a^{water} - pK_a^{micelle} = 0.46$) of the drug. CTAB-hydrochlorothiazide bindings were quantified by using two mathematical models, water-micelle partitioning constant and hydrochlorothiazide-micelle binding constant. Binding constant was found to be ($1.17 \pm 0.16 \times 10^3 M^{-1}$). Using pseudo phase

model system, the partition coefficient (6.18±0.64×104 M^{-1}) between bulk water-micelle was estimated from the differential absorbance (355nm) dependence on the surfactant concentrations.

Ahsan *et al.* presented a conductometric measurement method. They estimated interaction of different fluoroquinolone drugs with cationic surfactant cetyldimethylethylammonium bromide in water medium. Drugs used were ciprofloxacin HCl, levofloxacin hemihydrate and lomefloxacin HCl [149]. Temperature was fixed between 298.15 to 318.15 K. In all cases they always got two CMC values as a result of surfactant-drug interactions. In all cases Gibb's free energy values were negative. They presented that the nature of interactions between drugs and surfactants in water medium were of hydrophobic and electrostatic in nature which was indicated by the values of ΔS^0_m and ΔH^0_m.

Choudhary *et al.* reported an isothermal calorimetric procedure for the quantitative measurements of various thermodynamic aspects of interaction between quaternary ammonium surfactant (CTAB) and drugs (neomycin, lincomycin, diclofenac sodium and naproxen sodium) [150]. They investigated the influence of drugs on the micellar formation properties of the surfactant. The partitioning mechanism addressed in this research was based on the kind of interaction of the drug molecules with the monomers and micelles of the surfactant. Various parameters that were estimated were standard enthalpy changes, partitioning constants, stoichiometry, and molar entropy changes.

Waseef *et al.* proposed a validated micellar HPLC method for simultaneous estimation of three antidiebetes drugs gliclazide, metformin and nateglinide [151]. Mobile phase was SDS (0.12M), n-propanol (10%) triethylamine (0.3%) with pH adjusted to 5.6. Column was C_{18} and UV detector was selected at wavelength 254nm. The developed method was very effectively applied on the pharmaceutical formulations and percent reveries were 100.31, 99.66 and 100.08 respectively.

CHAPTER NO. 3

3.1 Reagents, chemicals and solvents

The distilled water used during the analysis and experiments. Various chemicals, solvents and reagents were used throughout the work. These includes Cetyltrimethylammonium bromide (Merck), Sodium hydroxide (Merck), KH_2PO_4 (AppliChem), K_2HPO_4(Merck), NaH_2PO_4 (Merck), Na_2HPO_4(Merck), Ortho phosphoric acid (Merck), Ofloxacin, Ornidazole, Sparfloxacin, Metronidazole, Norfloxacin, Gatifloxacin, Levofloxacin, (Lahore Chemicals & Pharmaceutical Works) distilled water (Lahore Chemicals & Pharmaceutical Works), Acetonitrile (HPLC) (Merck), Methanol (Merck). All reagents chemicals and solvents were of HPLC and analytical grades.

3.2 Analytical instruments

pH meter	WTW, Germany
Sonicator	Ulmasonic, Germany
Balance (0.1mg – 220g)	Sartorius, Germany
HPLC	Hitachi, Japan
UV-Visible spectrophotometer	Shimadzu, Japan
Fluorescence spectrophotometer	Shimadzu, Japan

3.3 Metronidazole and norfloxacin combination

3.3.1Micellar liquid chromatographic (MLC) measurements

Phosphate buffer solution (0.05M) was prepared by dissolving 6.80g KH_2PO_4 per liter distilled water in volumetric flask. Dilute NaOH solution was prepared by dissolving approximately 8g of NaOH per 100mL of water. Dilute orthophosphoric acid solution was prepared by dissolving 10mL of phosphoric acid per 100mL of distilled water. Dilute NaOH and dilute orthophosphoric acid solutions were used to adjust the pH of the buffer solutions.

Norfloxacin and metronidazole stock solution (2mg mL^{-1}) was prepared by accurately weighing 500mg of each drug in same 250mL volumetric flask and volume was made upto mark with phosphate buffer pH 7.4. To prepare working sample solution (0.4mg mL^{-1}), 10mL stock solution was transferred to another 50mL volumetric flask and volume was made upto mark with phosphate buffer pH 7.4. The nylon filter papers 0.45µm (Millipore) were used for the filtration of final solutions.

Surfactant stock solution (100mM) was prepared by dissolving 3.65g of cetyltrimethylammonium bromide (CTAB) per 100mL of phosphate buffer pH 7.4. Varying molar concentrations of surfactant (3, 5, 7, 9, and 11mM) were made from stock solution before the measurement with phosphate buffer pH 7.4. For mobile phase, each CTAB concentration and acetonitrile was mixed in a proportion (100:15 v/v) and filtered through 0.45µm membrane filter (Millipore).Development of MLC method development studies were carried out at HPLC system of following set up. The experimental conditions for HPLC system are summarized below in Table 3.1

Table 3.1 HPLC conditions for metronidazole and norfloxacin combination

HPLC Unit	Hitachi Japan
Pump system	Hitachi L-7100
Detector Unit	Hitachi UV-Visible L-7400
Injection Unit	Hitachi Auto-Sampler L-7200
Mobile Phase	CTAB and Acetonitrile (100:15 v/v)
m/p flow	1.0 mL min^{-1}
Column	Purospher STAR RP-18 column (250 × 4.6mm,5µm)
Wavelength	290nm

3.3.2 UV-Visible spectrophotometric measurements

Drug-surfactant interactions were studied using UV-Visible spectral measurements. A Shimadzu UV-1800 double beam UV-Visible spectrophotometer was utilized for these measurements. Differential UV measurements were performed by placing drug-CTAB solution in sample cuvette whereas drug solution was kept in the reference cuvette (1.0 cm quartz cell). Surfactant stock solution (100mM) was prepared by dissolving 3.65g of cetyltrimethylammonium bromide (CTAB) per 100mL of phosphate buffer pH 7.4. Varying molar concentrations of surfactant (0.1, 0.3, 0.5, 0.7, 0.9, 1, 3, 5, 7, 9, and 11mM) were made from stock solution with phosphate buffer pH 7.4 before the measurement.

For the spectral measurements, separate stock solution was prepared for norfloxacin and metronidazole. For norfloxacin stock solution (0.16mg mL^{-1}), 160 mg drug was accurately weighed in 1000mL volumetric flask and volume was made upto mark with phosphate buffer pH 7.4. Working sample solutions of norfloxacin having concentration 2.0×10^{-5} M were prepared by diluting 20mL of stock solution to 500mL in volumetric flasks with each surfactant concentration. For metronidazole stock solution (0.0856mg mL^{-1}), 85.6mg drug was accurately weighed in 1000mL

volumetric flask and volume was made upto mark with phosphate buffer pH 7.4. Working sample solutions of metronidazole having concentration 2.0×10^{-5} Mwere prepared by diluting 20mL of stock solution to 500mL in volumetric flasks with each surfactant concentration. Both the drugs follow Beer's law to all concentrations applied in the present work. All solutions were stored at room temperature and all measurements recorded at temperature 25 ± 0.1^{o}C.The stability of drug solutions was checked periodically for one week at room temperature and was found stable. However, fresh solutions were prepared before experiments.

3.4Ofloxacin and ornidazole combination

3.4.1Micellar liquid chromatographic (MLC) measurements

Ofloxacin and ornidazole stock solution (2mg mL^{-1}) was prepared by accurately weighing 500mg of each drug in same 250mL volumetric flask and volume made upto mark with phosphate buffer pH 7.4. To prepare working sample solution (0.4mg mL^{-1}), 10mL stock solution was transferred to another 50mL volumetric flask and volume was made upto mark with phosphate buffer pH 7.4. The nylon filter papers 0.45µm (Millipore) were used for the filtration of final solutions. Mobile phase and HPLC conditions were used as described in section 3.3.1 and Table 3.1 however detection was made at 292 nm.

3.4.2 UV-Visible spectrophotometric measurements

Surfactant stock solution were prepared and diluted to 0.1, 0.3, 0.5, 0.7, 0.9, 1, 3, 5, 7, 9, and 11mM solutionsas described in section 3.3. For the spectral measurements, separate stock solution was prepared for ofloxacin and ornidazole. For ofloxacin stock solution (0.18mg mL^{-1}), 180 mg drug was accurately weighed in 1000mL volumetric flask and volume was made upto mark with phosphate buffer pH 7.4. Working sample solutions of ofloxacin having concentration 2.0×10^{-5} Mwere prepared by diluting 20mL of stock solution to 500mL in volumetric flasks with each surfactant concentration. For ornidazole stock solution (0.11mg mL^{-1}), 110mg drug was accurately weighed in 1000mL volumetric flask and volume was made upto mark with phosphate buffer pH 7.4. Working sample solutions of ornidazole having concentration 2.0×10^{-5} Mwere prepared by diluting 20mL of stock solution to 500mL in volumetric flasks with each surfactant concentration. Both the drugs follow Beer's law to all concentrations applied in the present work. All solutions were stored at room temperature and all measurements recorded at temperature $25\pm0.1^{o}C$.

Drug-surfactant interactions were studied using UV-Visible spectral measurements. A Shimadzu UV-1800 double beam UV-Visible spectrophotometer was utilized for these measurements. Differential UV measurements were performed by placing drug-CTAB solution in sample cuvette whereas drug solution was kept in the reference

cuvette (1.0 cm quartz cell). The stability of drug solutions was checked periodically for one week at room temperature and was found stable. However, fresh solutions were prepared before experiments.

3.5Gatifloxacin

3.5.1Micellar liquid chromatographic (MLC) measurements

Two gatifloxacin stock solutions (2mg mL^{-1}) were prepared separately in phosphate buffers pH 5.5 and pH 7.4. They were prepared by accurately weighing 500mg of drug in separate 250mL volumetric flasks and volume was made upto mark with phosphate buffer pH 5.5 and 7.4 respectively. To prepare working sample solutions (0.4mg mL^{-1}), 10mL of each stock solution was transferred to another separate 50mL volumetric flask and volume was made upto mark with phosphate buffer pH 5.5 and buffer pH 7.4 respectively. The nylon filter papers 0.45µm (Millipore) were used for the filtration of final solutions.

Mobile phase for pH 5.5 and 7.4 were prepared with CTAB solution. CTAB stock solution (100mM) was prepared by dissolving 3.65g CTAB per 100mL of appropriate phosphate buffer solution. Varying molar concentrations of CTAB (3, 5, 7, 9, and 11mM) were prepared from stock solution with phosphate buffer pH 5.5 and pH 7.4 separately before the measurement. Each CTAB concentration and acetonitrile was mixed in a proportion (100:15 v/v) and filtered through 0.45µm membrane filter (Millipore).Development of MLC method was carried out using experimental conditions summarized in Table 3.1. Detection was made at 285nm.

3.5.2 UV-Visible spectrophotometric measurements

Two separate surfactant stock solutions (100mM) were prepared by dissolving 3.65g of cetyltrimethylammonium bromide (CTAB) per 100mL of phosphate buffer pH 5.5 and pH 7.4 separately. Varying molar concentrations of surfactant (0.1, 0.3, 0.5, 0.7, 0.9, 1, 3, 5, 7, 9, and 11mM) were prepared from these stock solutions in both buffers (pH 5.5 and pH 7.4) separately before the measurement.

Stock solutions (0.188 mg mL^{-1}) of gatifloxacin were also prepared separately.

188 mg drug was dissolved in 1000mL volumetric flask and volume was made upto mark with phosphate buffers pH 5.5 and pH 7.4. Working sample solutions of gatifloxacin having concentration 4.0×10^{-5} Mwere prepared by diluting 40mL of

stock solution to 500mL with phosphate buffer pH 5.5 and pH 7.4. The drug follows Beer's law to all concentrations applied in the present work. All solutions were stored at room temperature and all measurements recorded at temperature 25±0.1^{o}C.

Drug-surfactant interactions were studied using UV-Visible spectral measurements. A Shimadzu UV-1800 double beam UV-Visible spectrophotometer was utilized for these measurements. Differential UV measurements were performed by placing drug-CTAB solution in sample cuvette whereas drug solution was kept in the reference cuvette (1.0 cm quartz cell).

3.5.3 Fluorescence spectrophotometric measurements

For the fluorescence measurements, same stock and working sample solutions of gatifloxacin were used that were prepared for UV-Visible spectrophotometric measurements. A Shimadzu fluorescence spectrophotometer RF-6000 was used for the fluorescence measurements. High-resolution electronic bands are obtained at the slit width (ex. 5.0 nm, em. 5.0 nm). The stability of drug solutions was checked periodically for one week at room temperature and was found stable. However, fresh solutions were prepared before experiments.

3.6 Levofloxacin

3.6.1Micellar liquid chromatographic (MLC) measurements

Levofloxacin stock solution (2mg mL^{-1}) was prepared by accurately weighing 500mg of drug in 250mL volumetric flask and volume was made upto mark with phosphate buffer pH7.4. To prepare working sample solution (1mg mL^{-1}), 25mL stock solution was transferred to another 50mL volumetric flask and volume was made upto mark with phosphate buffer pH 7.4. The nylon filter papers 0.45µm (Millipore) were used for the filtration of final solutions.

Surfactant stock solution (100mM) was prepared by dissolving 3.65g CTAB per 100mL of phosphate buffer pH 7.4. Varying molar concentrations of surfactant (1, 3, 5, 7, and 9mM) were manufactured from stock solution with phosphate buffer pH 7.4 before the measurement. Each CTAB concentration and acetonitrile was mixed in a proportion (90:10 v/v) and filtered through 0.45µm membrane filter (Millipore). HPLC conditions are summarized in Table 3.2 below. The chromatographic separation was achieved using Hypersil ODS (250×4.6mm, 5µm) at 35^{o}C.

Table 3.2 HPLC conditions for levofloxacin

HPLC Unit	Hitachi Japan
Pump system	Hitachi L-7100
Detector Unit	Hitachi UV-Visible L-7400
Injection Unit	Hitachi Auto-Sampler L-7200
Mobile Phase	CTAB and Acetonitrile (90:10 v/v)
m/p flow	1.5 mL min^{-1}
Column	Hypersil ODS (250 × 4.6mm, 5µm)
Wavelength	295nm
Column Temperature	35^{o}C

3.6.2 UV-Visible spectrophotometric measurements

Two separate surfactant stock solutions (100mM) were prepared by dissolving 3.65g of cetyltrimethylammonium bromide (CTAB) per 100mL of phosphate buffer pH 5.5 and pH 7.4 separately. Varying molar concentrations of surfactant (0.6, 0.65, 0.69, 0.74, 0.80, 0.87, 0.95, 1.05, 1.18, 1.33, 1.54, 1.82, 2.22, 2.86 and 4.0mM) were made from these stock solutions in both buffers (pH 5.5 and pH 7.4) separately before the measurement.

For the spectral measurements, stock solutions (0.18mg mL^{-1}) of levofloxacin were prepared separately in buffers pH 5.5 and 7.4. A sample of 181 mg drug was dissolved per liter of buffer pH 5.5 and pH 7.4. Working sample solutions of levofloxacin having concentration 4.0×10^{-5} Mwere prepared by diluting 40mL of stock solution to 500mL with phosphate buffer pH 5.5 and pH 7.4 for each surfactant concentration.Drug-surfactant interactions were studied using UV-Visible spectral measurements. A Shimadzu UV-1800 double beam UV-Visible spectrophotometer was utilized for these measurements. Differential UV measurements were performed by placing drug-CTAB solution in sample cuvette whereas drug solution was kept in the reference cuvette (1.0 cm quartz cell).

3.6.3 Fluorescence spectrophotometric measurements

For the fluorescence measurements, same stock and working sample solutions of levofloxacin were used that were prepared for UV-Visible spectrophotometric measurements. A Shimadzu fluorescence spectrophotometer RF-6000 was used for the fluorescence measurements. High-resolution electronic bands are obtained at the slit width (ex. 5.0 nm, em. 5.0 nm).The stability of the drug solutions was checked periodically for one week at room temperature and was found stable. However, fresh solutions were prepared before experiments.

3.7 Sparfloxacin

3.7.1Micellar liquid chromatographic (MLC) measurements

Sparfloxacin stock solution (2mg mL^{-1}) was prepared by accurately weighed 500mg of each drug in 250mL volumetric flask and volume was made upto mark with phosphate buffer pH 7.4. To prepare working sample solution (0.4mg mL^{-1}), 10mL stock solution transferred to another 50mL volumetric flask and volume was made upto mark with phosphate buffer pH 7.4. The nylon filter papers 0.45µm (Millipore) were used for the filtration of final solutions.

For mobile phase, surfactant stock solution (100mM) was prepared by dissolving 3.65g CTAB per 100mL of phosphate buffer pH 7.4. Varying molar concentrations of surfactant (3, 5, 7, 9, and 11mM) were made from stock solution with phosphate buffer pH 7.4 before the measurement. Each CTAB concentration and acetonitrile was mixed in a proportion (100:13 v/v) and filtered through 0.45µm membrane filter (Millipore). The experimental conditions for HPLC system are summarized in Table 3.1. Separations was achieved with Purospher STAR RP-18 column (250 × 4.6mm, 5µm). Mobile phase was (100:13 v/v) CTAB-Acetonitrile. UV-vis detection was made at 290nm.

CHAPTER NO. 4

RESULTS AND DISCUSSION

4.1 Metronidazole and norfloxacin combination

4.1.1 Development and optimization of micellar HPLC procedure

Norfloxacin is an antibacterial drug belonging to fluoroquinolone class. It is potent against gram negative and gram positive bacteria. There are two pKa values of norfloxacin that has been reported in literature, pKa_1 6.30 and pKa_2 8.38. The first pKa value is associated with the carboxylic acid and second pKa value correlates with piperazine ring of norfloxacin [152-156]. Metronidazole has antibacterial action and has been widely used against the anaerobic infections. It acts against numerous protozoa, obligate anaerobic bacteria and facultative anaerobes. Metronidazole has single ionization constant value with pKa 2.49. The ionization constant value (pKa) is an important factor in the optimization of drug studies [157-158]. The norfloxacin and metronidazole combination is commercially available in market in the form of tablets (Norfloxacin 400mg + Metronidazole 500mg) and syrup (Norfloxacin 100mg + Metronidazole 100mg).

The main emphasis of the present studies was to gain in depth a better understanding of the potential assimilation of pharmacologically active drugs norfloxacin and metronidazole into the cationic surfactant cetyltrimethylammonium bromide at physiological pH 7.4. Most fluoroquinolones solubilize in the aqueous medium. However for better bioavailability, improved efficacy and to overwhelm the lipophilic barrier, a physiological medium (pH 7.4) is overall useful for diffusion through phospholipid membranes [159].

To optimize the procedure and to get symmetrically resolved peaks of drug molecules, different organic solvents, and various CTAB concentrations in different molar phosphate buffers and different HPLC columns wereinitially used. Mobile phase was optimized through several experiments. Initially different volume ratios of CTAB solution and methanol (85:15, 95:05, 90:10, 100:10, 100:15) were tested and

various columns Hypersil100 C18 (250 × 4.6mm × 5µm), Hypersil-ODS (250 × 4.6mm × 5µm), Hypersil-BDS C18 (250 × 4.6mm × 5µm), Hypersil-BDS C8 (250 × 4.6mm × 5µm), Purospher STAR RP-18 (250 × 4.6mm × 5µm) were used toobtain best separation, but both analytes showed poor resolution. Purospher STAR RP-18 column provided relatively good peaks but very poor resolution. Using column temperature between 30^{o}C - 35^{o}C did not improve the resolution.

Asymmetry and poor resolution between metronidazole and norfloxacin might be due to the interaction of oxygen atoms of the carboxylic group in norfloxacin and nitro group of metronidazole with the ionic head group of the monomers of CTAB adsorbed on the stationary phase or metal ion impurities present in the column. In order to avoid these problems, methanol was replaced by acetonitrile to increase the polarity of micellar mobile phase.

For optimizing suitable phosphate buffer, a series of trials were carried out with phosphate buffers like KH_2PO_4, K_2HPO_4, NaH_2PO_4, Na_2HPO_4 with various molar concentrations (1M, 0.5M, 0.1M, 0.05M, 0.01M) having pH 7.4. This pH was chosen because it is physiological pH and secondly all RP-columns generally work well between pH ranges 2-8. Symmetrical peaks with appropriate resolution of metronidazole and norfloxacin were obtained with acetonitrile and 0.05 M KH_2PO_4 phosphate buffer (100:15 v/v) on Purospher STAR RP-18 column in contrast to the other stationary phases.

4.1.2 Determination of the binding constant by MLC

In the present study, MLC determination for the fixed dose combination metronidazole and norfloxacin has been investigated. The findings revealed that the surfactant head groups supported the drugs to solubilize in the external part of the micelles, which may probably be more supportive in the drug controlled release. This is very useful to understand physiochemical processes at molecular levels. The chromatographic behaviors of metronidazole and norfloxacin were determined on Purospher STAR RP-18 column (250 × 4.6mm, 5µm) and presented in Fig. 4.1–Fig. 4.3. Estimation of this combination by conventional HPLC procedure in

pharmaceutical formulation is reported in literature [160]. However no MLC method of this combination was reported in literature. The binding constant (K_2) values of the drugs metronidazole and norfloxacin with the CTAB were estimated by Arunyanart and Cline-Love equation 1 given in chapter 1.

Binding constant values (K_2) for the individual drugs norfloxacin-CTAB and metronidazole-CTAB obtained from slope/intercept ratio of the plot (Fig. 4.4) $1/k^{/}$ against [M_m] were 68.36 M^{-1} and 38.75 M^{-1} respectively. The binding constant (K_2) values for the combination norfloxacin-metronidazole-CTAB were K_2=58.13 M^{-1} (norfloxacin) and K_2=34.38 M^{-1} (metronidazole). It revealed that both values correlated with binding constants (K_b) determined from UV spectroscopic measurements given in Table 4.1. Generally, the above data shows the ability of the drug molecules under probe to sense electrostatic environment in phospholipid membranes and related structures. Likewise the Gibb's free energy (ΔG^o_b) values for individual norfloxacin and metronidazole drugs when they move from aqueous phase to the micellar interior region were ΔG^o_b = -10.47 kJ mol^{-1} and ΔG^o_b = -9.06 kJ mol^{-1} respectively, whereas the values of Gibb's free energy for both drugs in combination norfloxacin-metronidazole-CTAB thus obtained were ΔG^o_b= -10.07 kJ mol^{-1} (norfloxacin) and ΔG^o_b = -8.76 kJ mol^{-1} (metronidazole). The nearly equal binding constants (K_2) values and Gibb's free energy (ΔG^o_b) values for free drugs and when the drugs in combination revealed that in combination, both drugs do not interact with each other and show no influential behavior. Drugs independently bind with the surfactant micelles.

4.1.3 Determination of the binding constant by UV spectroscopy

4.1.3.1 Spectroscopic characteristics of norfloxacin and metronidazole

The UV electronic spectra for the drug molecules metronidazole and norfloxacin in aqueous and buffer medium at pH 6.5 and pH 7.4 respectively are presented in Fig. 4.5 and Fig. 4.6. The excitation absorption wavelength of metronidazole was blue-shifted (319.5 to 319 nm) as shown in Fig. 4.5. Norfloxacin exhibited maximum absorption at 272nm in aqueous phase and 271nm in buffer medium. It is evident in

Fig. 4.6 that by changing medium from aqueous to buffer, absorption wavelength was also blue-shifted (272 to 271 nm).

4.1.3.2 Interaction of metronidazole and norfloxacinmolecules with CTAB

Some valuable and useful information about the metronidazole and norfloxacin interacting with the mimic membrane system can be collected from UV studies. However the attributes of interaction were determined from premicellar to postmicellar (0.001Mto 0.011M) concentration of surfactant and successive influence on absorption of metronidazole and norfloxacin molecules. Absorption pattern for metronidazole-CTAB and norfloxacin-CTAB in buffer medium (pH 7.4) with different CTAB concentrations is presented in Fig. 4.7 and 4.8 respectively. These analytical statistics were used to explore the partitioning characteristics of anionic drugs (metronidazole and norfloxacin) within cationic micelles of CTAB. It is evident in the spectra that absorption intensity of the drugs metronidazole and norfloxacin in the premicellar region increased by increasing CTAB concentration. However above the critical micellar concentration (CMC) in the post micellar region, the curves almost levelled off due to maximum inclusion of drug molecules inside the micelle interior (Fig. 4.11 and 4.12).

4.1.3.3 Benesi-Hildebrand equation for binding parameter

To study in-depth the binding phenomenon of drugs with surfactant, differential UV spectroscopic practice was successfully utilized. Using various CTAB concentrations, the differential UV absorption pattern of metronidazole-CTAB and norfloxacin-CTAB in physiological condition (pH 7.4) is presented in Fig. 4.9 and Fig. 4.10 respectively. Another more specific quantitative and analytical approach to find out the binding parameters between surfactant and drug was taken into account by using customized Benesi-Hildebrand equation 2 as given under chapter 1. The reliability of equation 2 was established when $dC_a/\Delta A$ was plotted versus $1/(C_s^{mo})$ and it turned out to be linear for both drugs at pH 7.4 as shown in Fig. 4.13 and Fig. 4.14. The analytical values of the binding constant (K_b, determined from UV spectroscopic measurements) and binding energy (ΔG^o_b determined from the subsequent slope/intercept ratios) are presented in Table 4.1.

4.1.3.4 Gibb's free energy changes

Transportation of metronidazole and norfloxacin drugs from aqueous phase to the micellar interior and their related Gibb's free energy changes (ΔG_b°) were estimated by equation 3. Table 1 showed high negative values of Gibb's energy (ΔG^{o}_{b}), which illustrated that partitioning phenomenon was spontaneous in nature. Literature data available for drug-surfactant combinational structures established that due to electrostatic forces of interactions, drugs associated themselves to cationic surfactant in bulk aqueous solution before they were penetrated inside the micelles [102, 105, 128, 161-164]. In the beginning, it happened by adhering additive-surfactant complex at the micellar surface. Later on drug molecules might re-organize themselves to the interior of themicelles. Because of re-orientation, additives might infiltrate inside the outer region of micellar palisade layer but hydrophobic drugs likely to entrap in peripheral core region. It is however assumed that electrostatic forces of interactions among charges of drug molecules and surfactants generally played a role towards their solubilization. The formation of the ion-pair structure among the anionic drug and cationic micellar head moiety were accomplished from initial spectral changes. The ion-pair complex is widely illustrated in literature. Distinct surfactant amounts may cause variations in spectral characteristics of organic moieties. This will however be associated with equilibria that engaged the surfactant monomers, micelles, and organic moiety, complex between organic moiety and surfactant, and drugs incorporated in micelles [113, 165-170]. Amphiphilic drugs largely reside in the interfacial neighborhood. However when hydrophobic character of drug is increased, it simply pulled deep inside the micellar interior. This in turn reduced their movement on the way to the bulk aqueous region. Therefore, it is imagined that drugs might exist as ion-pair along with monomers of the surfactant and this ion-pair then stick onto the micellar surface, thus assimilating drug molecules into the micellar palisade layer. Sticking of ion-pair on the micellar surface, assimilation and binding of drug inside the palisade layer of the micelles actually is referred as penetration binding.

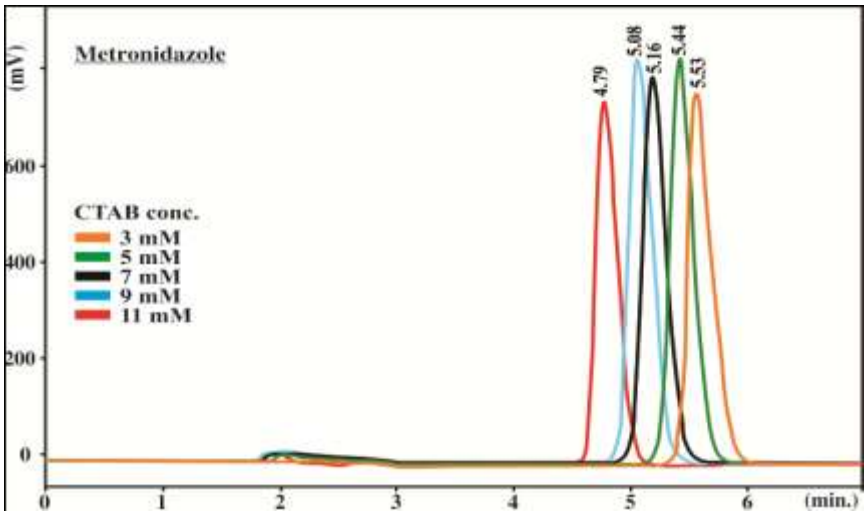


Fig. 4.1 Chromatogram of metronidazole-CTAB with varying concentrations of CTAB at pH 7.4determined on Purospher STAR RP-18 column (250 × 4.6mm, 5µm)

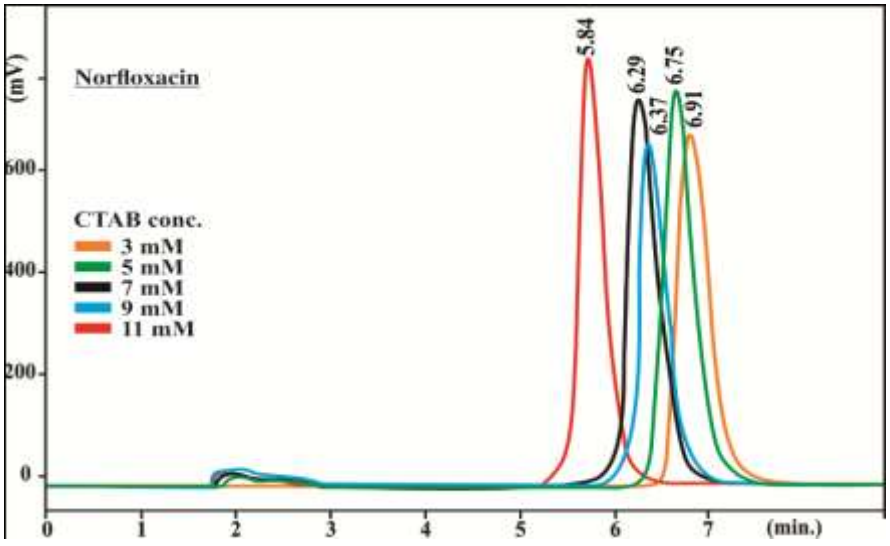


Fig. 4.2 Chromatogram of norfloxacin-CTAB with varying concentrations of CTAB at pH 7.4determined on Purospher STAR RP-18 column (250 × 4.6mm, 5µm)

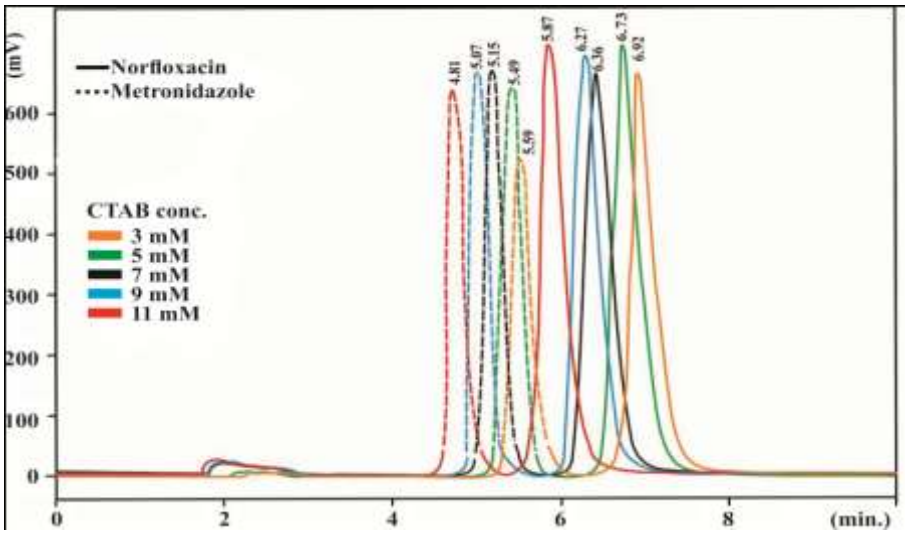


Fig. 4.3 Chromatogram of metronidazole-norfloxacin-CTAB with varying concentrations of CTAB at pH 7.4 determined on Purospher STAR RP-18 column (250 × 4.6mm, 5µm)

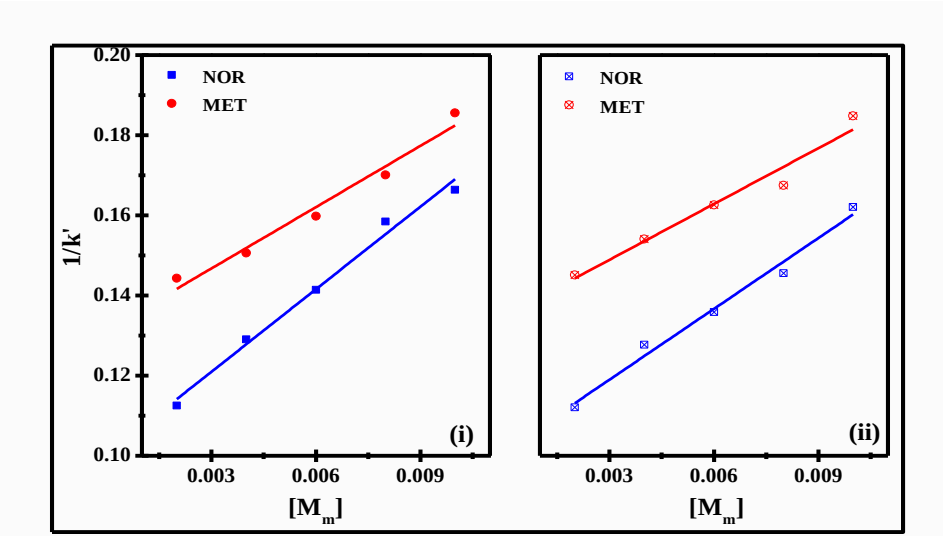


Fig. 4.4 Plot based on Arunyanart and Cline Love relation for binding analysis of drug-CTAB association (i) individual drugs, (ii) combination drugs

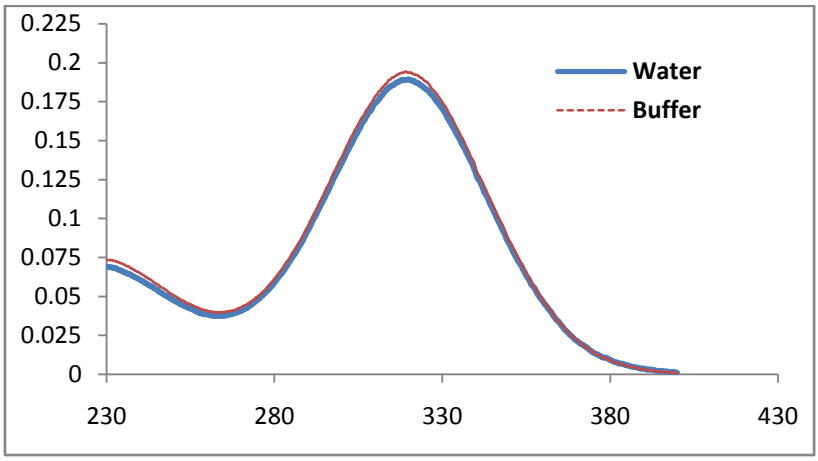


Fig. 4.5 The normalized optical absorption of metronidazole in aqueous (pH 6.5) and buffer (pH 7.4) solution

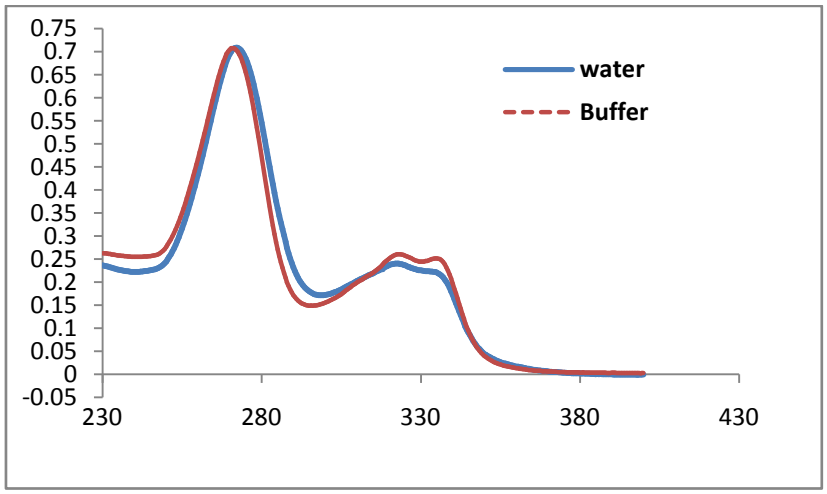


Fig. 4.6 The normalized optical absorption of norfloxacin in aqueous (pH 6.5) and buffer (pH 7.4) solution

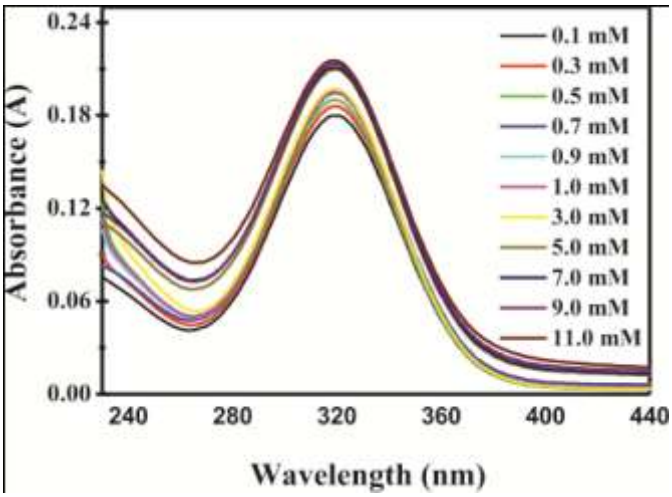


Fig. 4.7 Simple absorption spectra of metronidazole-CTAB with varying concentrations of CTAB at pH 7.4

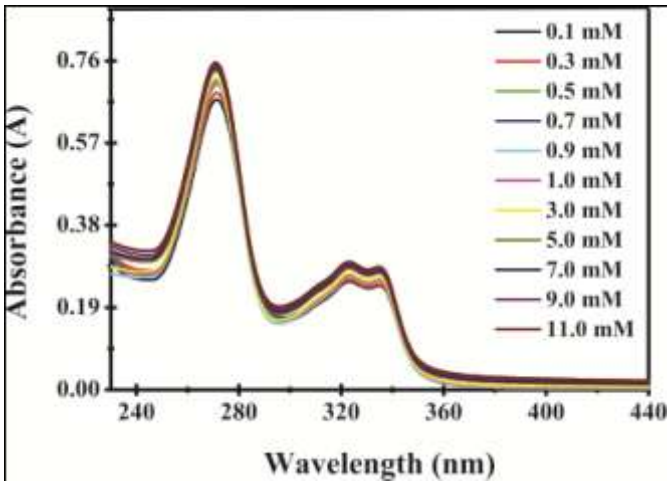


Fig. 4.8 Simple absorption spectra of norfloxacin-CTAB with varying concentrations of CTAB at pH 7.4

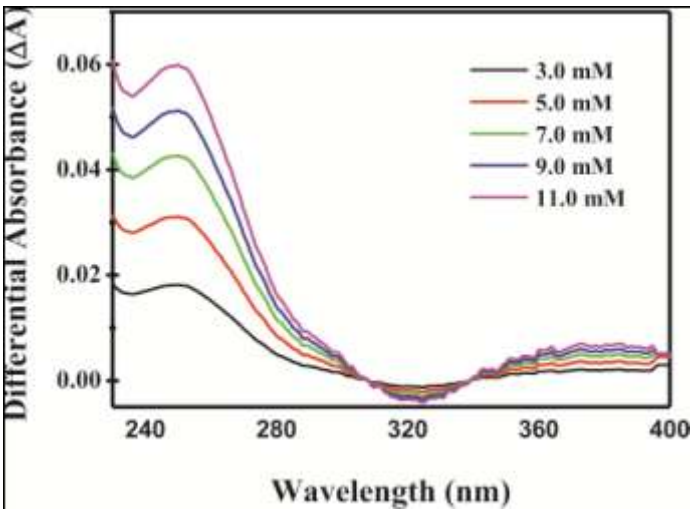


Fig. 4.9 Differential absorption spectra of metronidazole-CTAB with varying concentrations of CTAB at pH 7.4

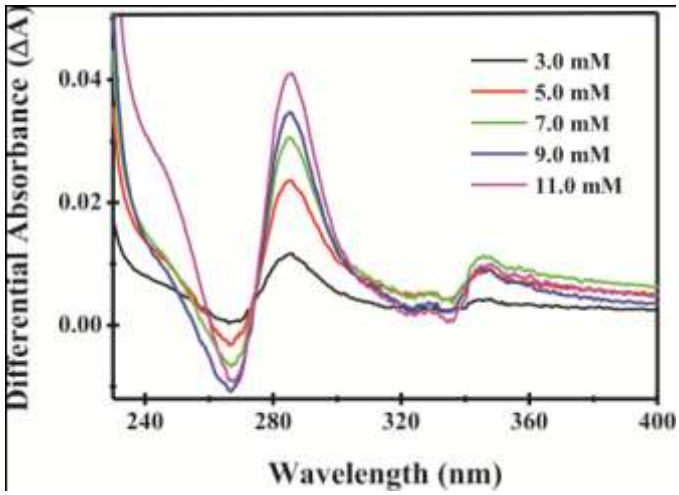


Fig. 4.10 Differential absorption spectra of norfloxacin-CTAB with varying concentrations of CTAB at pH 7.4

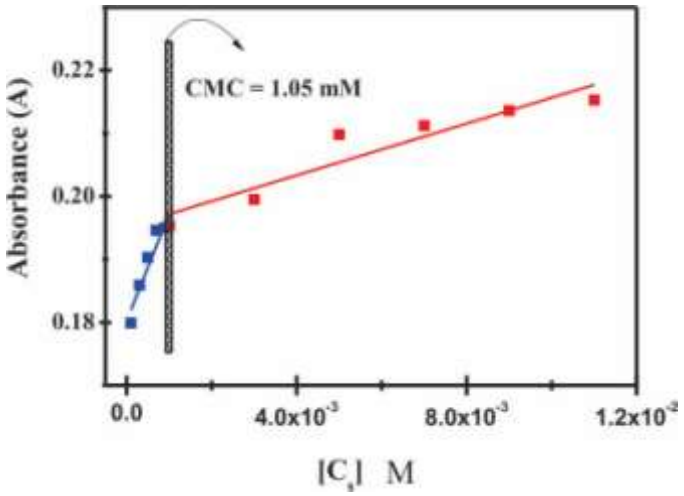


Fig. 4.11 Relation between absorbance of Metronidazole-CTAB with varying concentrations of CTAB

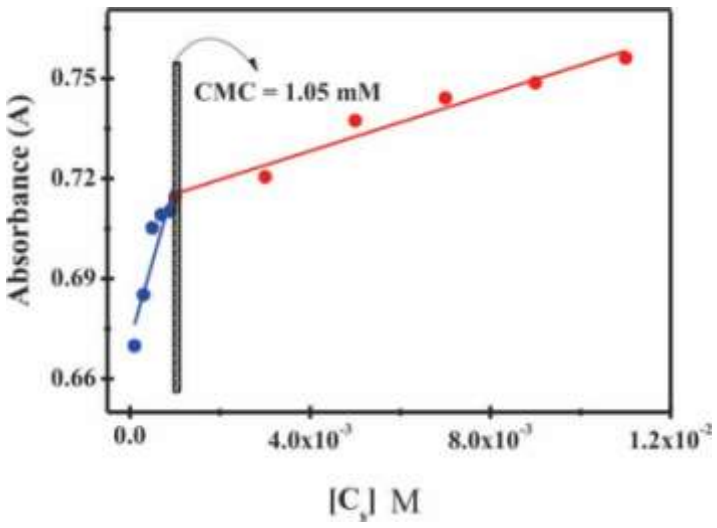


Fig. 4.12 Relation between absorbance of Norfloxacin-CTAB with varying concentrations of CTAB

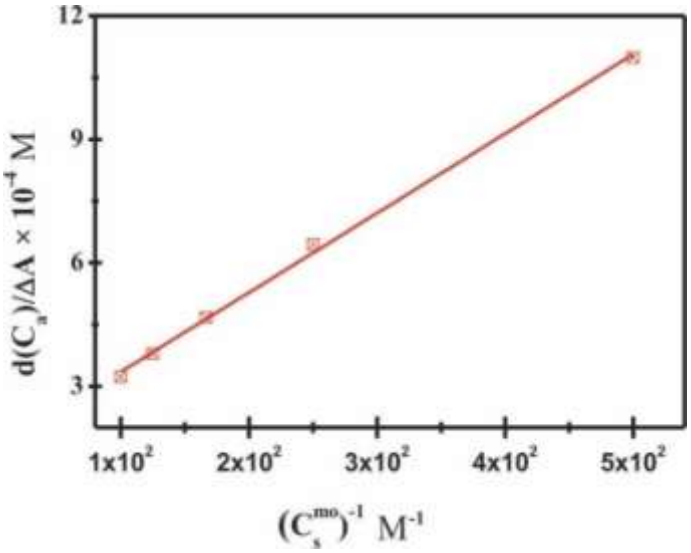


Fig. 4.13 Benesi-Hildebrand binding plot of metronidazole-CTAB

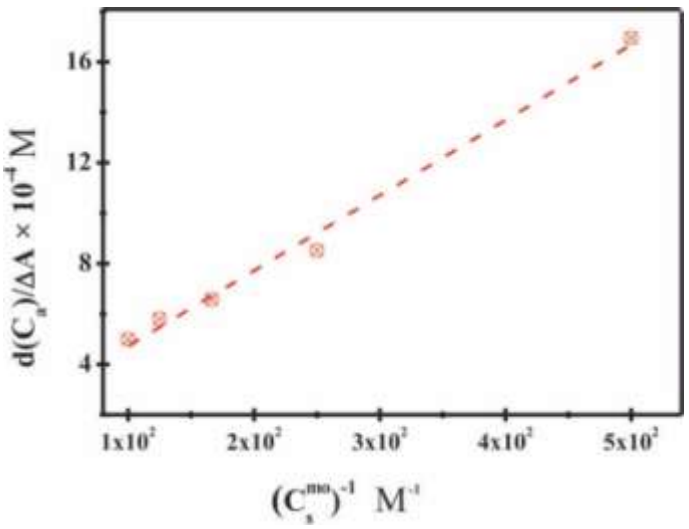


Fig. 4.14 Benesi-Hildebrand binding plot of norfloxacin-CTAB

Table 4.1: Values of K_2, K_b and ΔG^o_b for metronidazole-CTAB and norfloxacin-CTAB at 25±0.1°C

Drug	MLC measurements				UV–Visible measurements	
	$K_{2(M^{-1})}$		$\Delta G^o_{b(kJ\ mol^{-1})}$		$K_{b(M^{-1})}$	$\Delta G^o_{b(kJ\ mol^{-1})}$
	Individual (± RSD %)	In combination (± RSD %)	Individual (± RSD %)	In combination (± RSD %)	Individual (± RSD %)	
Metronidazole	38.75±1.4	34.38±0.9	-9.06±1.7	-8.76±1.8	48.12±0.9	-9.60±1.3
Norfloxacin	68.36±1.3	58.13±1.6	-10.47±1.9	-10.07±1.8	64.57±0.2	-10.33±0.5

Binding constant values (K_2) for the individual drugs metronidazole-CTAB and norfloxacin-CTAB determined by MLC measurements were 38.75 $M^{-1}$and 68.36M^{-1} respectively. The binding constant (K_2) values for the combination metronidazole-norfloxacin-CTAB were 34.38 M^{-1}(metronidazole) and 58.13 M^{-1} (norfloxacin) respectively. Both values correlated with binding constants (K_b) determined from UV spectroscopic measurements

Likewise the Gibb's free energy (ΔG^o_b) values for individual metronidazole and norfloxacin drugs determined by MLC were -9.06 and -10.47 kJ mol^{-1} respectively, whereas the values of Gibb's free energy for both drugs in combination norfloxacin-metronidazole-CTAB thus obtained were -8.76 kJ mol^{-1} (metronidazole) and -10.07 kJ mol^{-1} (norfloxacin) respectively. These value correlated with the Gibb's free energy (ΔG^o_b) valuesobtained from UVmeasurements as given in above Table 4.1.

4.2 Ofloxacin and ornidazole combination

4.2.1 Development and optimization of micellar HPLC procedure

Ofloxacin belongs to fluoroquinolone group of second generation. It is very effective against both gram negative and positive bacteria. It has been reported that ofloxacin has two ionizable functional groups, carboxylic group (pK_{a1}= 6.00) and piperazine ring (pK_{a2}= 8.00). At the physiological pH, both groups dissociate considerably. Ornidazole is a derivative of 5-nitroimadazole. It is used mainly in the anaerobic bacteria and susceptible protozoal infections. Ornidazole has only one ionization constant with pK_{a1}= 2.4 [171-172]. In present study, the potential interaction of the bioactive fixed dose combination (ofloxacin and ornidazole) with micelles formed by the cationic surfactant cetyltrimethylammonium bromide has been illustrated at physiological pH 7.4±0.1.

To develop micellar liquid chromatographic procedure for ofloxacin and ornidazole combination, mobile phase was optimized. Various organic solvents, phosphate buffers and various CTAB concentrations were used. In addition, different stationary phases Hypersil100 C18 (250 × 4.6mm × 5µm), Hypersil-ODS (250 × 4.6mm × 5µm), Hypersil-BDS C18 (250 × 4.6mm × 5µm), Hypersil-BDS C8 (250 × 4.6mm × 5µm), Purospher STAR RP-18 (250 × 4.6mm × 5µm) were chosen for optimization of stationary phase. Initially different ratios of surfactant solution and methanol (85:15, 95:05, 90:10, 100:10, and 100:15) were used on various columns but both peaks got merged. Increasing temperature also did not effect on separation.

Both ofloxacin and ornidazole molecules are anionic in nature. They might have interaction with the cationic monomers of CTAB adsorbed on the stationary phase. There might be present some metal ion impurities in the column and interact with oxygen atoms of the carboxylic group in ofloxacin and nitro group of ornidazole. To avoid these issues, methanol was replaced by acetonitrile to increase the polarity of micellar mobile phase. Suitable buffer concentration was optimized using various phosphate buffers i-e KH_2PO_4, K_2HPO_4, NaH_2PO_4, Na_2HPO_4 with various molar concentrations (1M, 0.5M, 0.1M, 0.05M, 0.01M) having pH 7.4. Both analyte

peaks with appropriate resolution were optimized with acetonitrile and 0.05 M phosphate buffer (KH_2PO_4) with ratio (100:15 v/v) on Purospher STAR RP-18 (250 × 4.6mm 5µm) column.

4.2.2 Determination of the binding constant by MLC

In present study, ofloxacin and ornidazole combination has been studied by micellar liquid chromatography. The results revealed that hydrophobic head supported potential solubilization of drug molecules in peripheral part of the micelles which might prove to be more helpful to their controlled release. These investigations permitted detailed understanding of biophysical process at molecular levels. The retention mechanism of ofloxacin and ornidazole molecules was investigated by MLC on Purospher STAR RP-18 column (250mm × 4.6mm, 5µm) as shown in Fig. 4.15-Fig. 4.17. There was no MLC method for the simultaneous determination of this combination reported before. However simultaneous determination of this combination by conventional RP-HPLC method in bulk and pharmaceutical formulations has been reported [173].

The binding constant values (K_2) for the fixed dose combination ornidazole and ofloxacin have been calculated by Arunyanart-Cline Love equation 1.

The binding constant for ofloxacin-CTAB and ornidazole-CTAB were calculated from the slope/intercept ratio obtained from the linear regression of the $1/k^{/}$ vs $[M_m]$ plot (Fig. 4.18). The values of binding constant for ofloxacin-CTAB and ornidazole-CTAB were K_2=159.32 M^{-1} and K_2=43.94 M^{-1} respectively. The binding constant for both drugs in combination (ofloxacin-ornidazole-CTAB) were K_2=171.41 M^{-1} (ofloxacin) and K_2=45.50 M^{-1} (ornidazole). This analysis revealed that both these values were correlated with binding constant values (K_b) determined from UV-spectroscopic measurements given in Table 4.2.

Generally, the above experimental data showed that the fixed dose combination drugs under study had the ability to sense the electrostatic environment in surfactant micelles as often used mimetic for phospholipid biomembranes and other related

structures. They were capable of firmly binding with electrostatic forces. Electrostatic forces are driving forces in drug-membrane bindings. The charged phospholipid groups of biomembrane have significant contribution in these interactions. The zwitterionic phosphatidylcholine are abundantly common phospholipid head groups in plasma membranes. Similarly there are also sufficient amount of negatively charged phosphatidylglycerol and phosphatidylserine groups. Owing to these charged groups, surface of the biomembranes present a net charge in aqueous medium. It gives rise to various binding properties of charged and uncharged forms of molecules such as drugs which consequently result in the electrostatic interaction between amphiphilic drugs and biomembranes [174-177]. The values of standard Gibb's free energy change (ΔG^{o}_{b}) for the transport of ofloxacin and ornidazole drugs from bulk aqueous phase to micellar region for the individual drugs were ΔG^{o}_{b} = -12.56 kJmol^{-1} and ΔG^{o}_{b} = -9.37 kJmol^{-1} respectively. Whereas the values of Gibb's free energy change for both drugs in combination ofloxacin-ornidazole-CTAB thus obtained were ΔG^{o}_{b}= -12.74 kJmol^{-1} (ofloxacin) and ΔG^{o}_{b} = -9.46 kJmol^{-1} (ornidazole).

4.2.3 Determination of the binding constant by UV spectroscopy

4.2.3.1 Interaction of ofloxacin and ornidazole molecules with CTAB

UV spectroscopic studies of ofloxacin and ornidazole interacting with micelles, in pre micellar to postmicellar surfactant concentrations (0.001Mto 0.011M) have revealed very important and valuable information. Fig. 4.19 and Fig. 4.20 showed the absorption behavior of ofloxacin-CTAB and ornidazole-CTAB respectively in phosphate buffer at pH 7.4±0.1, with increasing concentration of CTAB. This analytical data was utilized for calculating the partitioning properties of anionic ofloxacin and ornidazole within cationic CTAB micelles. In the premicellar concentration, intensity of absorption for ofloxacin and ornidazole increased on increasing the concentration of surfactant. In the postmicellar concentration, above CMC, nearly leveling off the curves revealed the maximum provision of drug molecules inside the micelle interior (Fig. 4.23 and Fig. 4.24).

During the course of integration and encapsulation of fixed dose combination drugs by the micelles, the drug molecules leaned towards the micelle surfaces and

consequently resulted in absorption of light. This phenomenon was attributed to neighborhood surfaces rather than bulk aqueous solution. The micellar interior is a non-polar region in contrast to the polar aqueous bulk solution, therefore amphiphilic ofloxacin and ornidazole drug molecules tend to reside inside the micelles in the vicinity of the polar surface. It was established that the CMC values of the amphiphiles changed in the presence of additives due to the fact that interfacial and micellar characteristics of such compounds in solutions were governed by balance of hydrophobic and hydrophilic interactions [178]. The reported CMC value of CTAB was 0.90 mM [161], however addition of ofloxacin and ornidazole increased the CMC to approximately 1.0 mM and 2.93mM respectively (Fig. 4.23 and Fig. 4.24).The increased CMC can be attributed due to the decline of aqueous phase dielectric constant. This phenomenon leads to the mutual repulsion among the ionic heads within the micelles [179]. As both fixed dose combination drug molecules bear positive charges on nitrogen atom, repulsion was further increased between drug molecules and the monomers of the micelles. Consequently micellization process was opposed and CMC was increased. The drug molecules ofloxacin and ornidazole are therefore capable of intimidating the monomers towards each other, thus forbidding them to combine before CMC. To quantify the interactions among the drugs and surfactant, different binding parameters were suggested.

4.2.3.2Benesi-Hildebrand equation for binding parameter

To further analyze the binding properties of ofloxacin and ornidazole with CTAB, UV differential spectroscopy was successfully used. Fig. 4.21 and Fig. 4.22 showed the differential absorption behavior of ofloxacin-CTAB and ornidazole-CTAB in increasing concentrations of CTAB. To estimate the binding constant between drug and surfactant, modified form of the Benesi-Hildebrand equation 2 was used.The soundness of equation was verified by plotting $dC_a/\Delta A$ versus $1/(C_s^{mo})$ and was proved to be quite linear for each drug at physiological pH 7.4±0.1 (Fig. 4.25 and Fig. 4.26). From the slope/intercept values, binding constant (K_b) and related binding energy (ΔG^o_b) were estimated, as outlined in Table 4.2. The standard Gibb's free energy change (ΔG^o_b) for the transportation of ofloxacin and ornidazole drugs from aqueous bulk phase to the micellar region was determined by the equation 3.

A high negative value of ΔG^{o}_{b} (Table 4.2) indicated the drug-micelle partitioning process was spontaneous.

4.2.3.3 Spectroscopic characteristics of ofloxacin and ornidazole

The UV–Visible spectra of ofloxacin and ornidazole molecules in aqueous medium and buffered medium at pH 7.4±0.1 are shown in the Fig. 4.27 and Fig. 4.28 respectively. The aqueous solution of the ofloxacin molecule showed maximum absorption at 289.5 nm, whereas in buffered medium it showed maximum absorption at 286.5 nm. Fig. 4.27 showed that when medium was changed from aqueous to buffer (pH 7.4), the absorption wavelength of ofloxacin was blue-shifted (289.5 to 286 nm). The free ornidazole molecule showed maximum absorption at 319 nm, which was shifted to 320 nm in buffered medium. Fig. 4.28 showed that when medium was changed from aqueous to buffer (pH 7.4±0.1), the excitation absorption wavelength of ornidazole was red-shifted (319 to 320 nm). Research data for drug-surfactant combinational structures revealed that due to electrostatic interactions, drug molecules combined with the cationic surfactants in bulk solution before penetration inside the micelle [102, 105, 128, 161-164]. Initially this drug-surfactant complex adhered at the surface of micelles and later on these drug molecules might be re-organized themselves to the interior region of the micelles. Consequently during re-arrangement, drug molecules might penetrate inside the outer part of micellar palisade layer. However the hydrophobic drug molecule was still likely to be trapped in external core region. It is hypothesized that the electrostatic interactions initiated from the charges on the amphiphilic drug and the cationic surfactant, both largely participated towards the solubilization. The ion-pair complex formation of anionic drug and head moiety of the cationic micelle were observed from initial spectral changes. Formation of the ion pair complex among anionic organic moieties and cationic surfactants has been extensively reported [113, 165-167]. Different amounts of surfactant may cause variations in spectral characteristics of organic moieties. These variations can be associated with equilibria which ultimately depend upon the surfactant monomers, micelles, organic moiety, organic moiety-surfactant complexes, as well as incorporation of drugs in micelles [168-170]. Amphiphilic drug moieties tend to reside mostly in the interfacial vicinity; however enhancing hydrophobic

character simply pulls the drugs deep inside the micellar interior. This reduces their movement to the bulk aqueous phase. Therefore, it was hypothesized that drugs might exist as an ion-pair with surfactant monomer and sticking onto the micellar surface in the later stages and thus leading to the assimilation of drug into the micellar palisade layer.

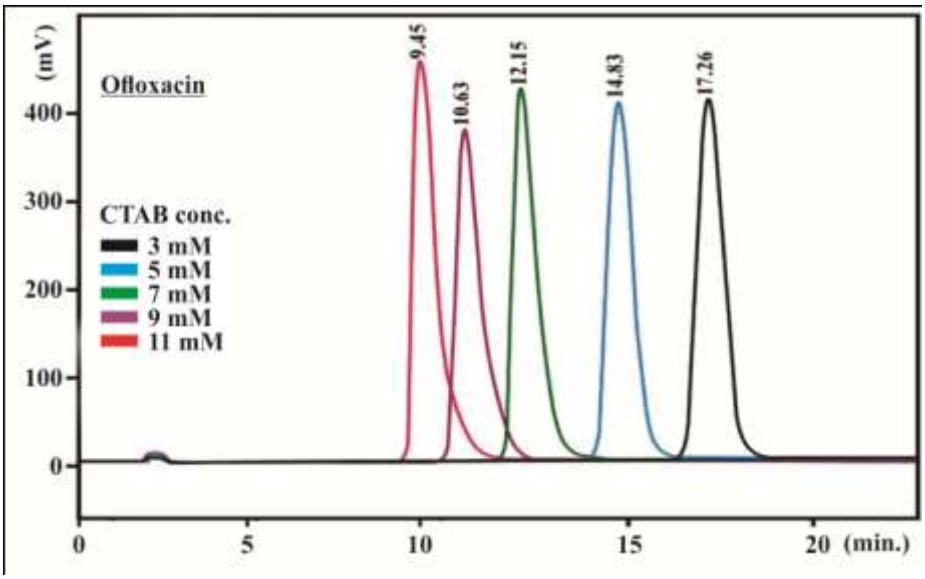


Fig. 4.15 Chromatogram of ofloxacin-CTAB with varying concentrationsof CTAB at physiological pH 7.4 determined on Purospher STAR RP-18 column (250 × 4.6mm, 5µm)

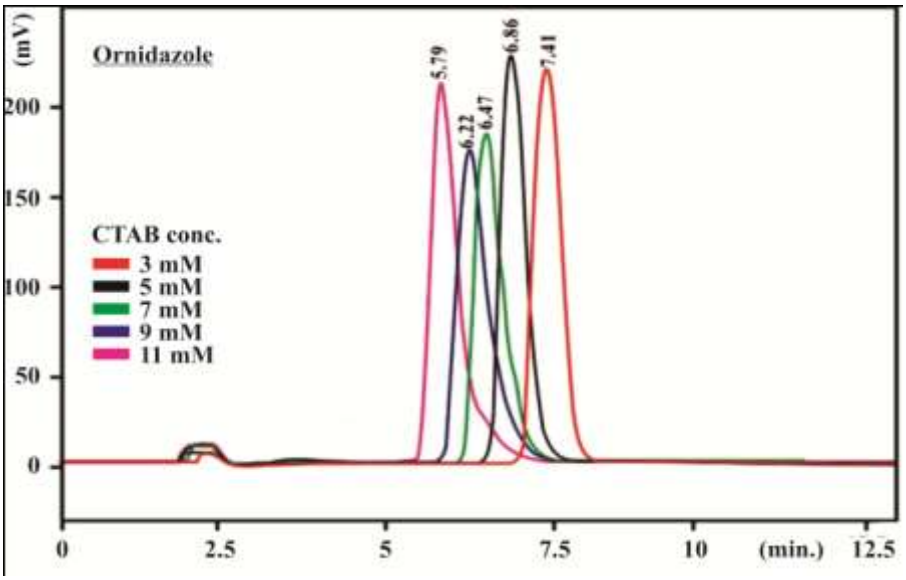


Fig. 4.16 Chromatogram of ornidazole-CTAB with varying concentrationsof CTAB at physiological pH 7.4 determined on Purospher STAR RP-18 column (250 × 4.6mm, 5µm)

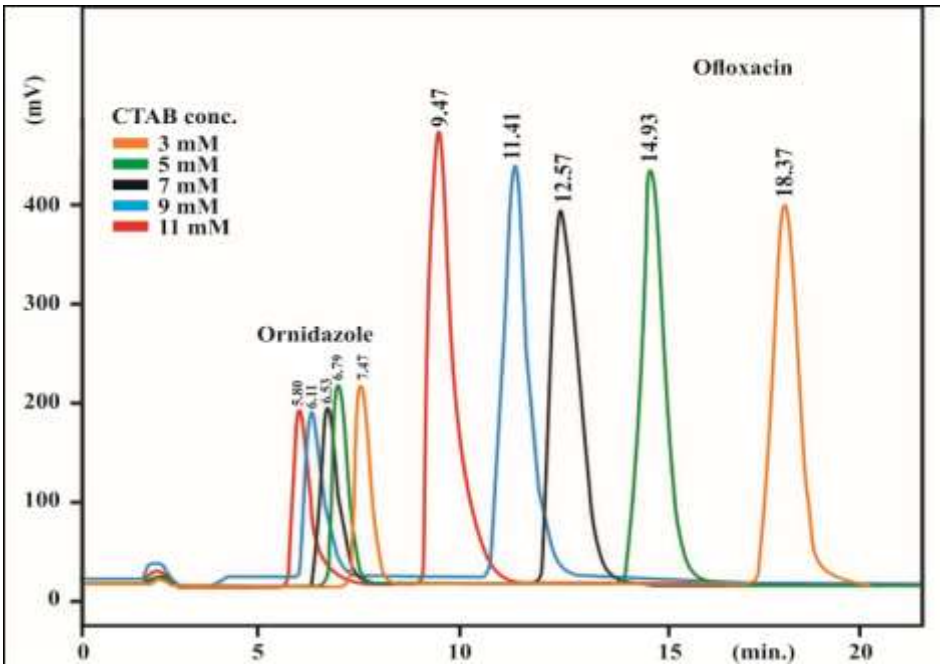


Fig. 4.17 Chromatogram of ofloxacin-ornidazole-CTAB with varying concentrations of CTAB at physiological pH 7.4 determined on Purospher STAR RP-18 column (250 × 4.6mm, 5µm)

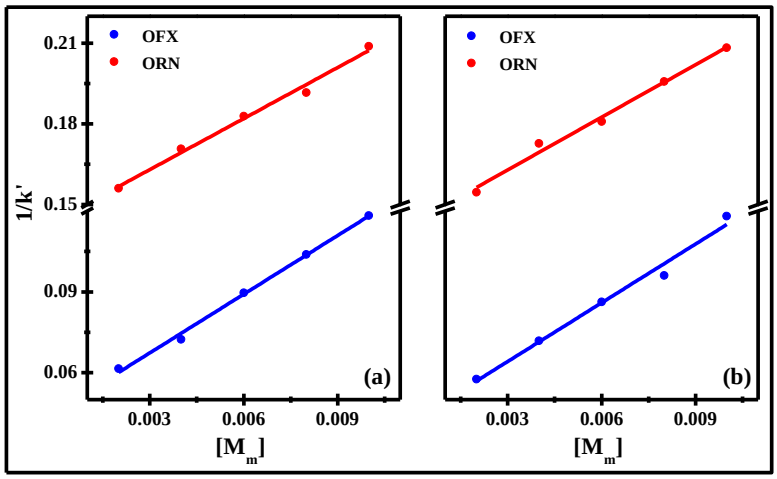


Fig. 4.18 Plot based on Arunyanart and Cline Love equation for binding-analysis of drug-CTAB association (a) individual drugs (b) combination drugs

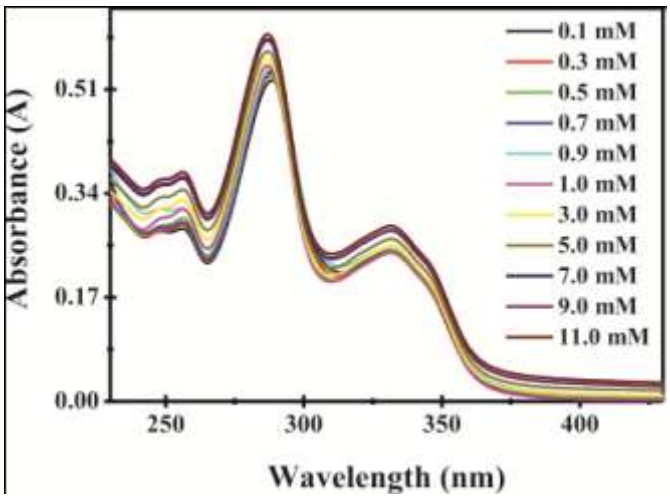


Fig. 4.19 Simple absorption spectra ofloxacin-CTAB with varying concentrationsof CTAB at physiological pH 7.4

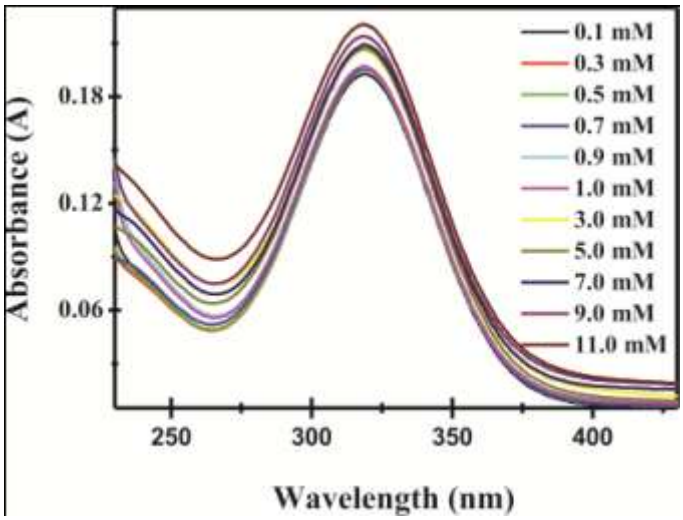


Fig. 4.20 Simple absorption spectra of ornidazole-CTAB with varying concentrations of CTAB at physiological pH 7.4

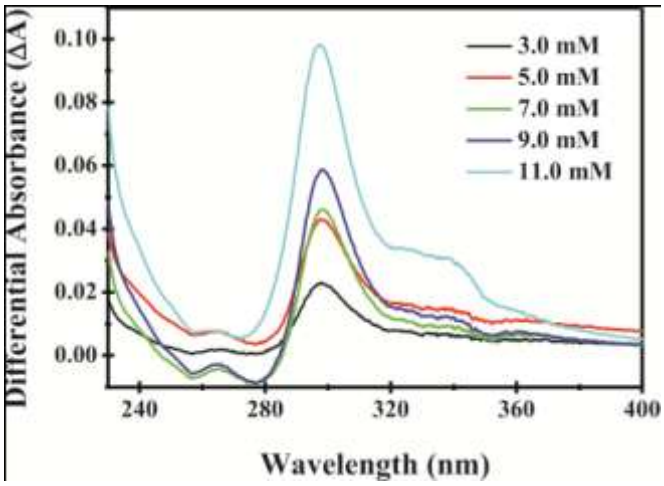


Fig. 4.21 Differential absorption spectra of ofloxacin-CTAB with varying concentrationsof CTAB at physiological pH 7.4

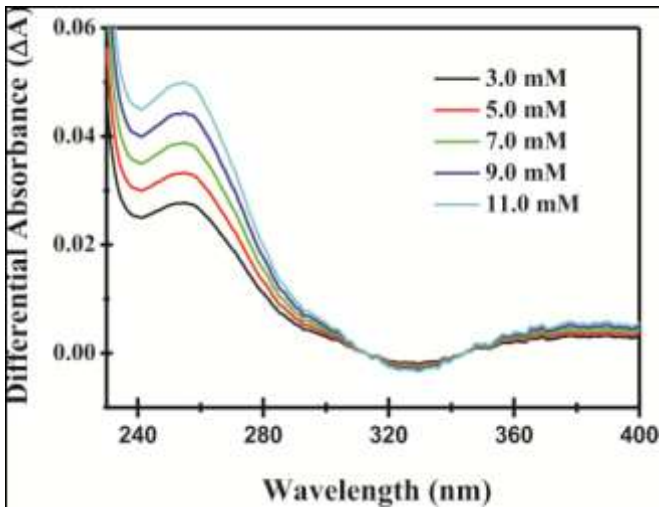


Fig. 4.22 Differential absorption spectra of ornidazole-CTAB with varying concentrations of CTAB at physiological pH 7.4

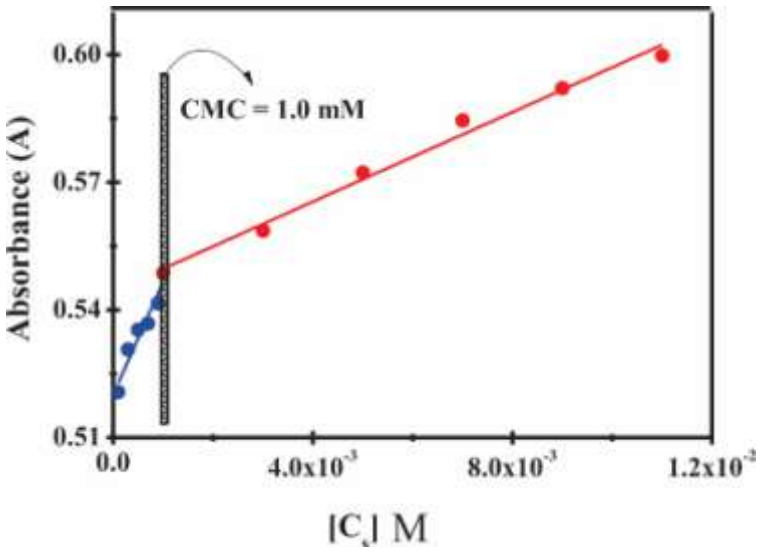


Fig. 4.23 Relation between absorbance of ofloxacin-CTAB with varying concentrations of CTAB

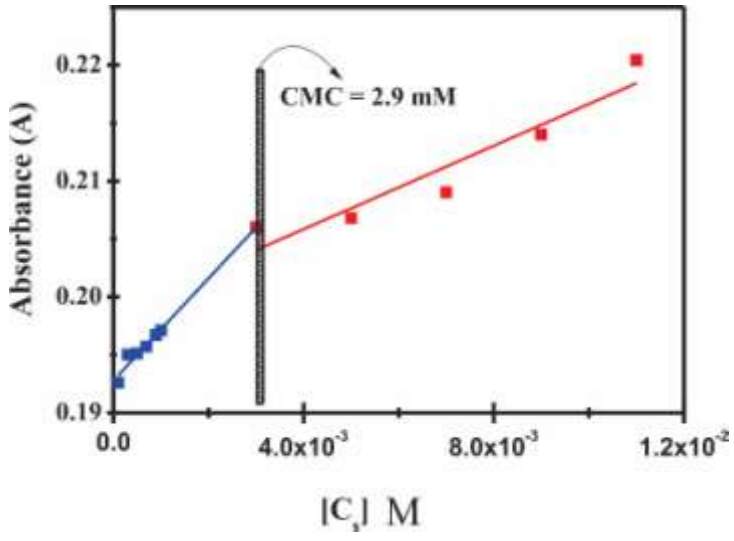


Fig. 4.24 Relation between absorbance of ornidazole-CTAB with varying concentrations of CTAB

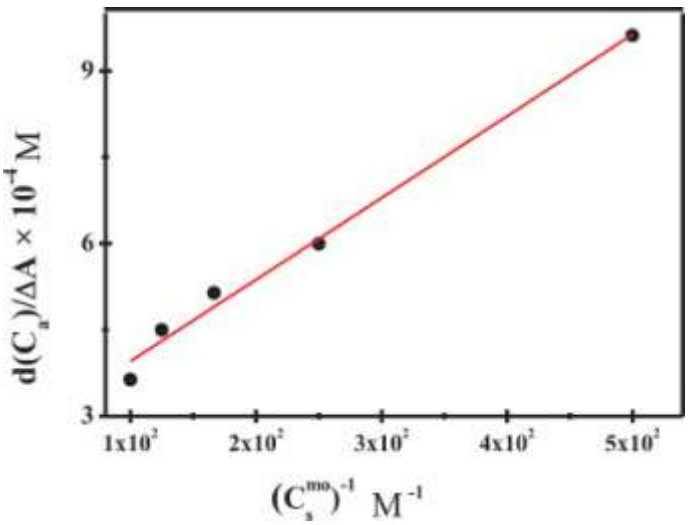


Fig.4.25 Benesi-Hildebrand binding plot of ofloxacin-CTAB

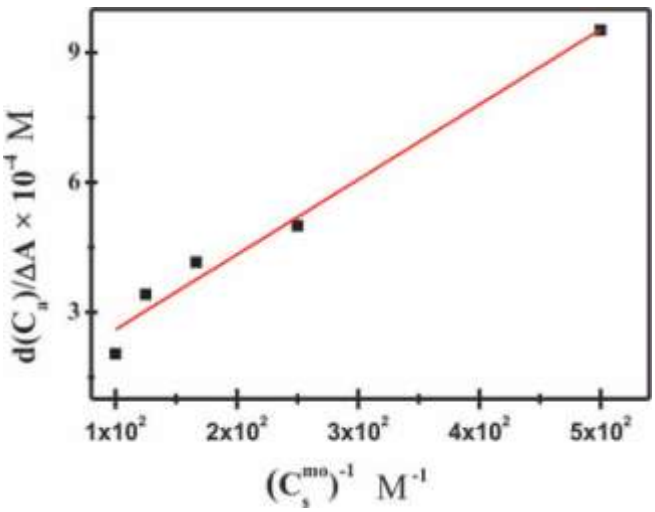


Fig.4.26 Benesi-Hildebrand binding plot of ornidazole-CTAB

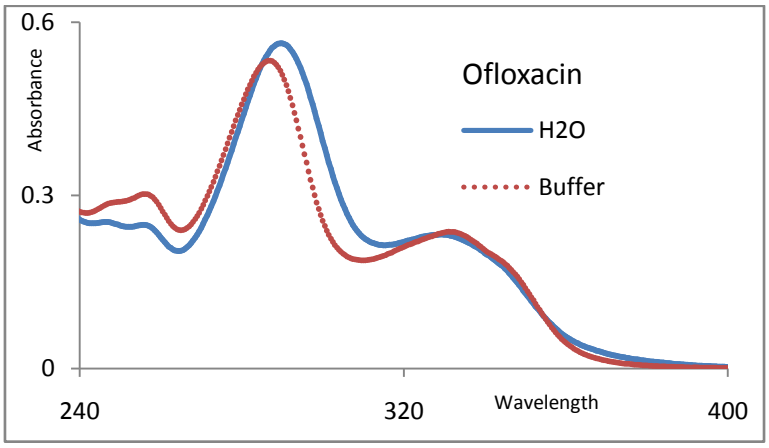


Fig. 4.27 The normalized optical absorption of ofloxacin in aqueous (pH 6.5) and buffer (pH 7.4) solution

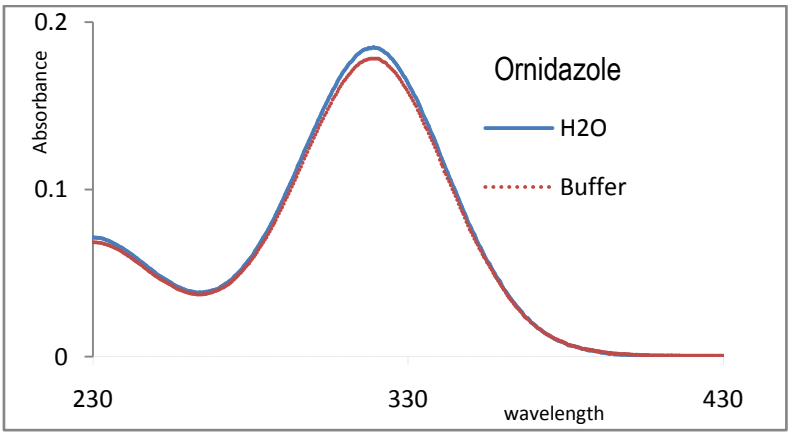


Fig. 4.28 The normalized optical absorption of ornidazole in aqueous (pH 6.5) and buffer (pH 7.4) solution

Table 4.2: Values of K_2, K_b and ΔG^o_b for ofloxacin-CTAB and ornidazole-CTAB at 25±0.1°C

Drug	MLC measurements				UV–Visible measurements	
	$K_{2(M^{-1})}$		$\Delta G^o_{b(kJmol^{-1})}$		$K_{b(M^{-1})}$	$\Delta G^o_{b(kJmol^{-1})}$
	Individual (± RSD %)	In combination (± RSD %)	Individual (± RSD %)	In combination (± RSD %)	Individual (± RSD %)	
Ofloxacin	159.32 ±1.5	171.41±1.8	-12.56±1.1	-12.74±2.1	178.22±2.2	-12.84±1.2
Ornidazole	43.94 ±1.7	45.50±2.3	-9.37±1.3	-9.46±0.6	51.04±1.8	-9.74±1.6

The values of binding constant for ofloxacin-CTAB and ornidazole-CTAB were K_2=159.32 M^{-1} and K_2=43.94 M^{-1} respectively. The binding constant for both drugs in combination (ofloxacin-ornidazole-CTAB) were K_2=171.41 M^{-1} (ofloxacin) and K_2=45.50 M^{-1} (ornidazole). This analysis revealed that both these values were correlated with binding constant values (K_b) determined from UV-spectroscopic measurements given in Table 4.2.

The values of standard Gibb's free energy change (ΔG^o_b) for the transport of ofloxacin and ornidazole drugs from bulk aqueous phase to micellar region for the individual drugs were ΔG^o_b = -12.56 $kJmol^{-1}$ and ΔG^o_b = -9.37 $kJmol^{-1}$ respectively. Whereas the values of Gibb's free energy change for both drugs in combination ofloxacin-ornidazole-CTAB thus obtained were ΔG^o_b= -12.74 $kJmol^{-1}$ (ofloxacin) and ΔG^o_b = -9.46 $kJmol^{-1}$ (ornidazole) which are in good agreement with the Gibb's free energy valuesobtained from UV measurements.

4.3 Gatifloxacin

4.3.1 Development and optimization of micellar HPLC procedure.

Gatifloxacin is a fourth-generation broad spectrum antibiotic and potent against gram negative and gram positive bacteria. In the present study, solubilization of gatifloxacin in the CTAB micelles at various pH conditions was undertaken. The interaction study between gatifloxacin and CTAB was carried out at two pH values, 5.5 and 7.4. In pH 5.5 solution, the gatifloxacin molecules likely exist in neutral form, while in pH 7.4 solution, the most probable form could be anionic with pK_a=5.6, when most of carboxyl groups of gatifloxacin were deprotonated [113,128]. The significance of this work is the binding and allocation of antibiotic drug in surfactant aggregate. By quantifying the electron absorption, fluorescence emission and micellar liquid chromatography, the molecular interactions between gatifloxacin and CTAB were studied.

Different stationary phases, organic solvents, phosphate buffers and various CTAB concentrations in different phosphate buffers were used to finalize the mobile phase at both pH values 5.5 and 7.4. Surfactant concentrations (in phosphate buffers solution pH 5.5 and 7.4) and methanol were tried in different proportions (85:15, 95:05, 90:10, 100:10, and 100:15). Various columns were used like Hypersil100 C18 (250 × 4.6mm × 5µm), Hypersil-ODS (250 × 4.6mm × 5µm), Hypersil-BDS C18 (250 × 4.6mm × 5µm), Hypersil-BDS C8 (250 × 4.6mm × 5µm), Purospher STAR RP-18 (250 × 4.6mm × 5µm). But peak was very broad and showed severe fronting and tailing. Suitable phosphate buffer and concentration was optimized using various phosphate buffers i-e KH_2PO_4, K_2HPO_4, NaH_2PO_4, Na_2HPO_4 with various molar concentrations (1M, 0.5M, 0.1M, 0.05M, 0.01M) having pH 5.5 and 7.4. The reason for peak broadening and shoulder formation might be due to the interaction of the ionic head group of the monomers of CTAB adsorbed on the stationary phase with the oxygen atoms of the carboxylic group in drug molecules. To increase the polarity of mobile phase methanol was replaced with acetonitrile. Good peak of gatifloxacin was obtained with 0.05M KH_2PO_4 and acetonitrile as organic modifier with ratio (100:15 v/v) on Purospher STAR RP-18 (250 × 4.6mm, 5µm) column at both pH values 5.5 and 7.4.

4.3.2 Determination of the binding constant by MLC

Retention of gatifloxacin by MLC is shown in Fig. 4.29 and Fig. 4.31. Increasing concentration of CTAB was used in mobile phase to determine the retention time of gatifloxacin molecules solubilized in CTAB micelle. With the increasing concentration of CTAB the retention time decreased. According to Jandera and Fischer, gatifloxacin retention followed the behavior of class A compounds [110, 180], i.e. as the concentration of the micelles in the mobile phase increased, retention time was decreased. The value of association or binding constant (K_b) for gatifloxacin molecules with increasing concentration of CTAB was calculated by Arunyanart and Cline Love relation in equation 1.

Binding constant values (K_2) for the gatifloxacin-CTAB at pH 5.5 and pH 7.4 obtained from slope/intercept ratio of the plot (Fig. 4.30 and 4.32) $1/k^{/}$ against $[M_m]$, were found to be 45.54 M^{-1} and 31.68 M^{-1} respectively. It revealed that both values correlated with binding constants (K_b) determined from UV and fluorescence spectroscopic measurements given in (Table 4.3). Likewise the Gibb's free energy change (ΔG^o_b) values for gatifloxacin drug at pH 5.5 and pH 7.4 when it moved from aqueous phase to the micellar interior region were ΔG^o_b = -9.46kJ mol^{-1} and ΔG^o_b = -8.56 kJ mol^{-1} respectively. In general, the above experimental data showed that gatifloxacin molecules had the ability to sense the electrostatic environment in surfactant micelles, such as those commonly used in phospholipid biofilms and other related structures. They were capable of binding strongly with electrostatic forces. Electrostatic force was the driving force for drug-membrane bonding. Overall, the results suggested that gatifloxacin molecules acted as probe for the electrostatic environment in biomembranes and related molecular assemblies.

4.3.3 Spectral characteristics of gatifloxacin molecules interacting with CTAB

To examine the gatifloxacin-CTAB association, the absorption spectra of gatifloxacin with varying premicellar to postmicellar concentration of CTAB were determined at different physiological pH conditions. Fig. 4.33 and Fig. 4.34 displaying the spectral-luminescent behavior of gatifloxacin-CTAB in acidic (pH 5.5) and slight basic medium (pH 7.4) with varying concentration of CTAB respectively.

The results showed that by increasing CTAB concentration, intensity of absorption of gatifloxacin increased in premicellar region whereas leveling-off the curve arises near the postmicellar region after reaching the critical micelle concentration of surfactant. This implied the maximum allocation of gatifloxacin molecules within the micelles interior. The plot of absorbance versus CTAB concentration at various pH conditions are shown in Fig. 4.35. This showed that the CMC of gatifloxacin-CTAB was approximately 1.05 mM at both pH conditions which was slight increased value to the normal CMC of the CTAB i.e. 0.9 mM, as reported in literature [113, 128]. This minimal increase in CMC might be due to decrease in the dielectric constant of the aqueous phase, which might increase the mutual exclusion of ion heads in micelles, thereby opposing the micelle formation and leading to an increase in CMC [179]. The micellar ionization degree (β) was calculated from the ratio of slope of the micellar posterior zone (S_2) to the slope of the premicellar zone (S_1) $\beta = S_2 / S_1$. When $\alpha = 1-\beta$, micellar ionization (β) is related to the degree of counterion (α). The value of counter ion binding was found to be 0.837 and 0.866 at pH 5.5 and pH 7.4 respectively. The observed increase in the degree of counter ion binding to the increased pH could be attributed to an increase in charge density on the surface of surfactant micelle. As the negative charge density increased with increasing pH, the carboxylic acid group in gatifloxacin molecule might be completely ionized [105]. By employing the fluorescence quenching data (Fig. 4.33 and Fig. 4.34) the Stern-Volmer quenching plot of gatifloxacin-CTAB with varying concentrations of CTAB using the Stern-Volmer relation (equation 4) is shown in Fig. 4.36.

The K_{sv} value (in M^{-1}) of gatifloxacin in the Stern-Volmer plot (Fig. 4.36) was 9.96 and 21.61 at pH 5.5 and pH 7.4 respectively. Data showed that the quenching mechanism might be caused by the formation of ground state complexes (static quenching) rather than dynamic collisions, supported by the literature [181-183].

4.3.4 Differential absorbance measurements

The differential UV spectra of gatifloxacin-CTAB at different pH conditions were recorded to quantify the gatifloxacin-CTAB association. Fig. 4.37 and Fig. 4.38 illustrated the differential absorption spectra of gatifloxacin with varying concentration of CTAB in acidic (pH 5.5) and slight basic (pH 7.4) conditions

respectively. Wavelength 297 nm was recorded to be the differential absorption wavelength for both pH conditions. By employing the differential spectroscopic data, Benesi-Hildebrand plot of gatifloxacin-CTAB with varying concentration of CTAB using Benesi-Hildebrand equation 2 is shown in Fig. 4.37 and Fig. 4.38.

Benesi–Hildebrand relation was the characteristic relation which varied linearly with C_s^{mo} under varying physiological pH values (7.4 and 5.5), thereby validating Eq. 2 as a concise measure of binding associations between CTAB and gatifloxacin molecules. The binding affinities (K_b) for static quenching (Figs. 4.33 and 4.34) have been calculated using modified Lineweaver–Burk equation 5.

(Fig. 4.40) shows a plot of log [(I_o-I) / I] versus log [C_s] for CTAB. The binding constant (K_b) obtained from the Benesi-Hildebrand relation and the Lineweaver-Burk relationship is used to determine the free energy change (ΔG^o_b) of gatifloxacin bound to CTAB as,

$$\Delta G_b = -RT\ln K_b$$

The values of the binding constant (K_b) and the associated binding energy (ΔG^o_b) obtained are shown in Table 4.3. The observed K_b value reveals stronger CTAB-gatifloxacin interaction and that the probe is incorporated into the micellar assembly. The mode of K_b andΔG^o_b ΔG_b values for gatifloxacin–CTAB association at various pH conditions were found to be in good agreement with literature values of other fluoroquinolone drugs captured in cationic micelles [105, 113, 123, 128].

The amphiphilic molecules mainly reside in the interfacial neighborhood. As the hydrophobic character of the drug molecules increases they are slightly pulled deeper inside the micelles and this in turn ceases their movement back to the aqueous bulkregion. It is therefore hypothesized that the drug molecule may form an ion-pair with the surfactant monomer. To explain the gatifloxacin–CTAB association, a proposed mechanism is schematically shown in (Fig. 4.41), explaining the ways in which

cationic surfactant monomers may attack the negatively charged gatifloxacin molecule.In the later stage, the adhesion of the ion-pair to the surface of the micelle causes the additive molecules to enter the palisade layer of the micelle. The pH-induced oxidation of gatifloxacin molecules produces a permanent negative charge thus inhibiting self-aggregation of gatifloxacin molecules. This can dynamically assist in the incorporation of gatifloxacin molecules into the outer core in the micelle grid layer, which facilitates the penetration binding.

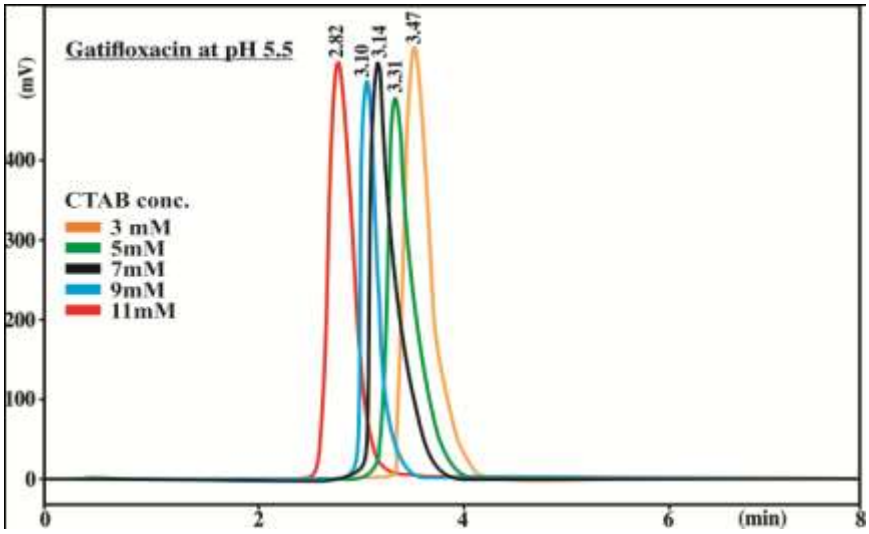


Fig. 4.29 Chromatogram of gatifloxacin-CTAB with varying concentrations of CTAB at pH 5.5 determined on Purospher STAR RP-18 column (250 × 4.6 mm, 5µm)

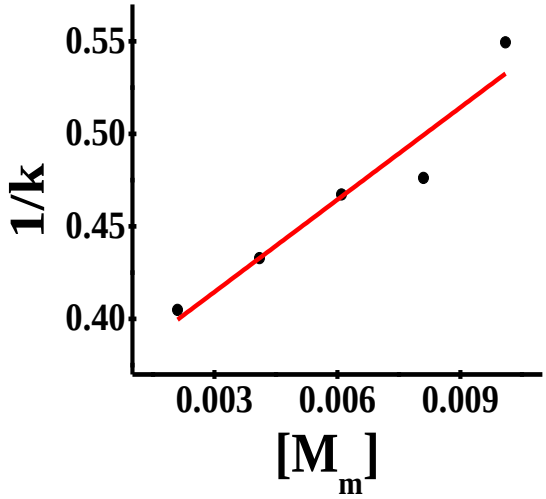


Fig. 4.30 Plot based on Arunyanart and Cline Love relation for binding analysis of gatifloxacin-CTAB association at pH 5.5

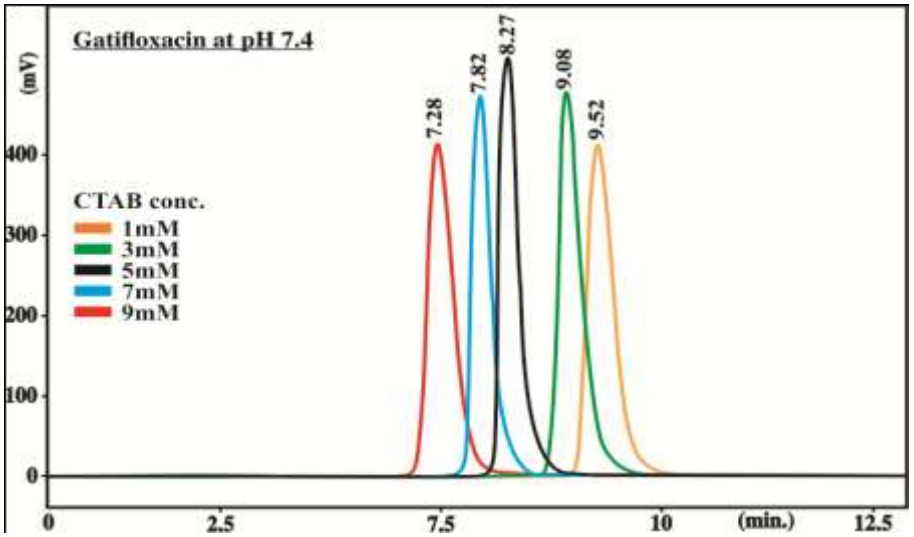


Fig. 4.31 Chromatogram of gatifloxacin-CTAB with varying concentrations of CTAB at pH 7.4 determined on Purospher STAR RP-18 column (250 × 4.6 mm, 5µm)

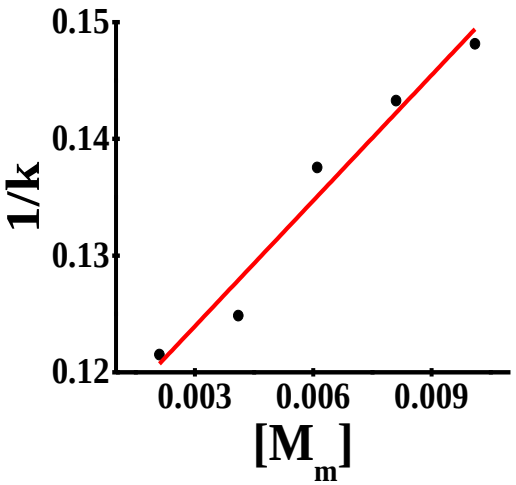


Fig. 4.32 Plot based on Arunyanart and Cline Love relation for binding analysis of

gatifloxacin-CTAB association at physiological pH 7.4

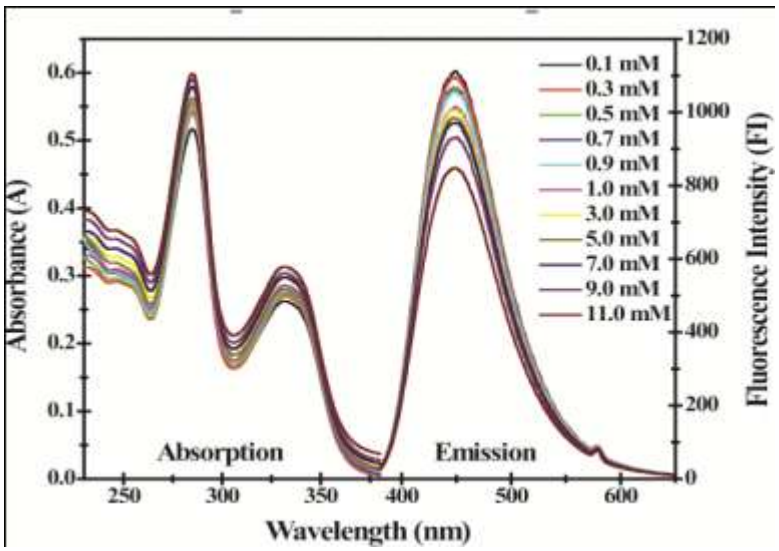


Fig. 4.33 Absorption and fluorescence emission spectra of gatifloxacin-CTAB with varying concentrations of CTAB at pH 5.5

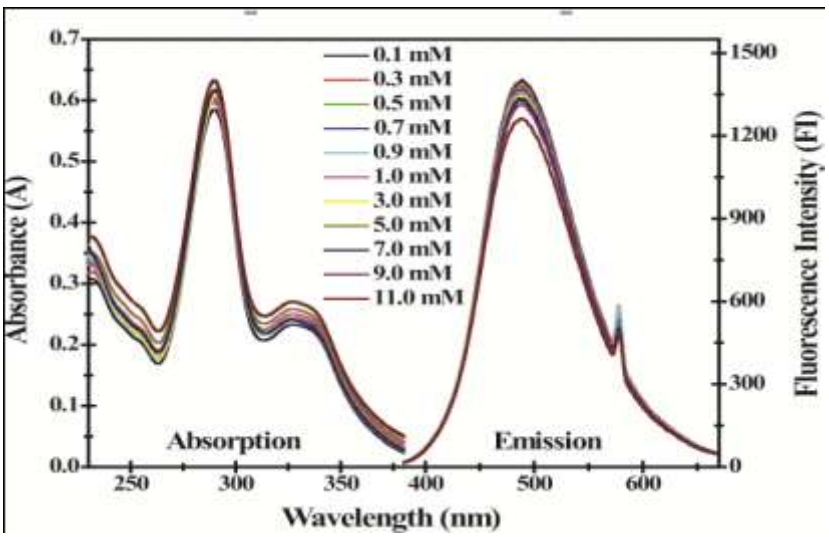


Fig. 4.34 Absorption and fluorescence emission spectra of gatifloxacin-CTAB with
varying concentrations of CTAB at pH 7.4

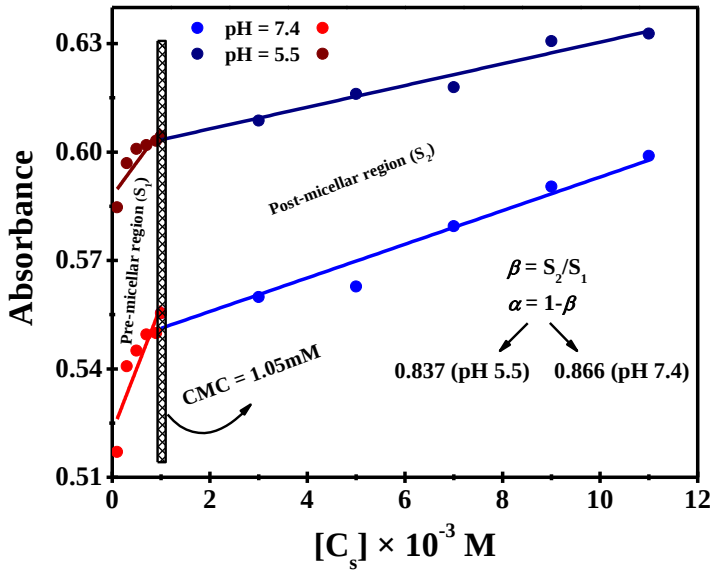


Fig. 4.35 Relation between absorbance of gatifloxacin-CTAB association with varying concentrations of CTAB at different pH condition

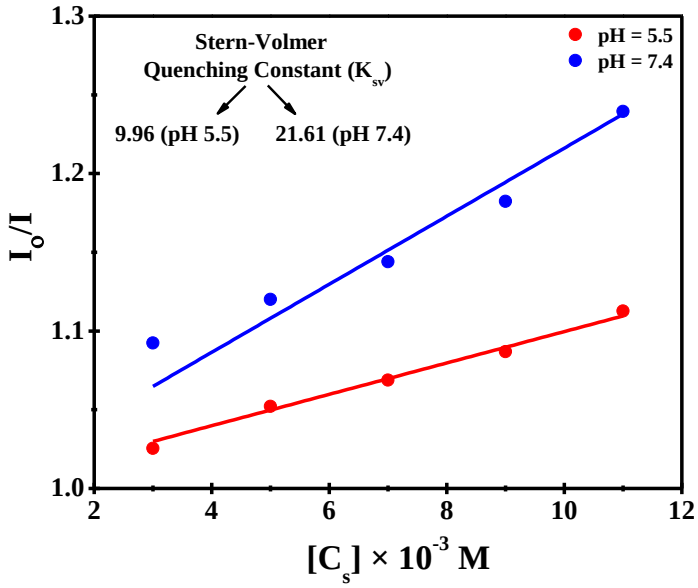


Fig. 4.36 Relation between Stern–Volmer quenching of gatifloxacin-CTAB

association with varying concentrations of CTAB at different pH condition

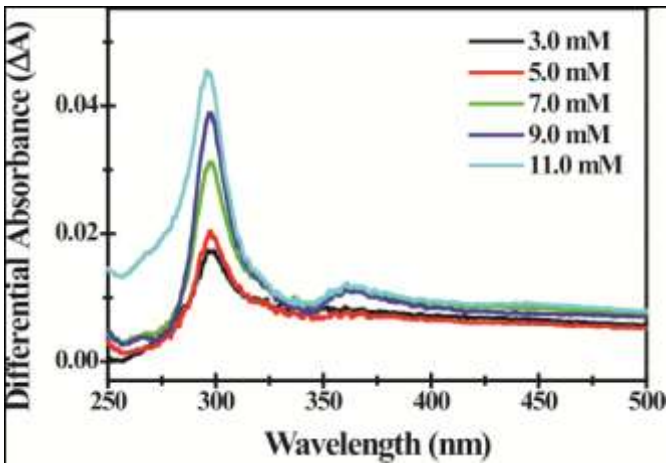


Fig.4.37 Differential absorption spectra of gatifloxacin-CTAB at pH 5.5

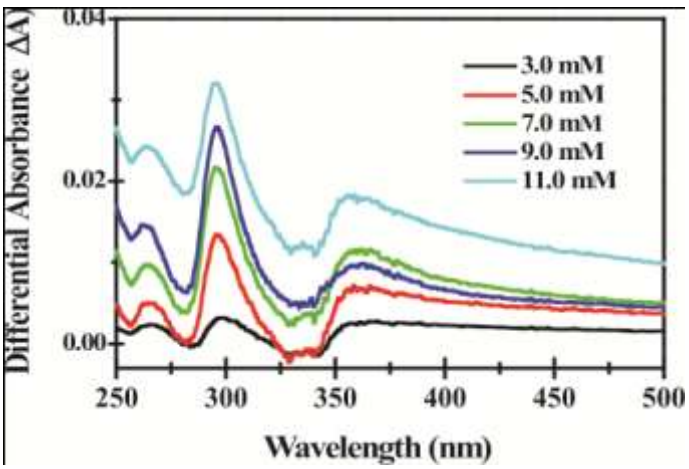


Fig.4.38 Differential absorption spectra of gatifloxacin-CTAB at pH 7.4

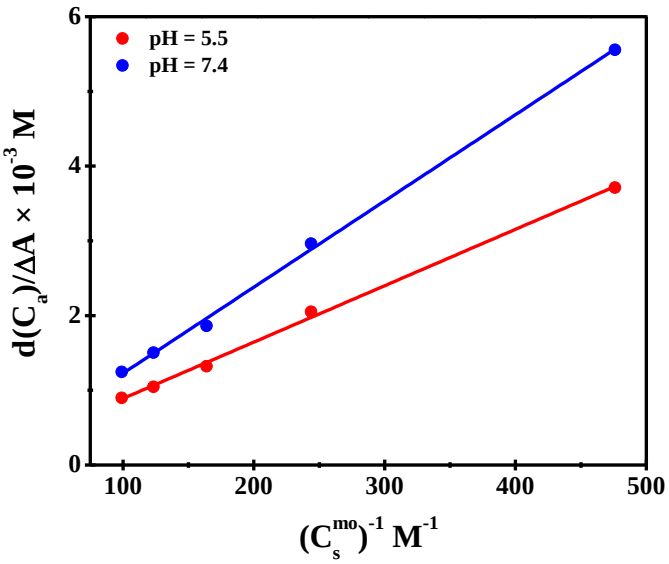


Fig. 4.39 Plot based on Benesi–Hildebrand relation for analysis of gatifloxacin–CTAB associationat different pH condition

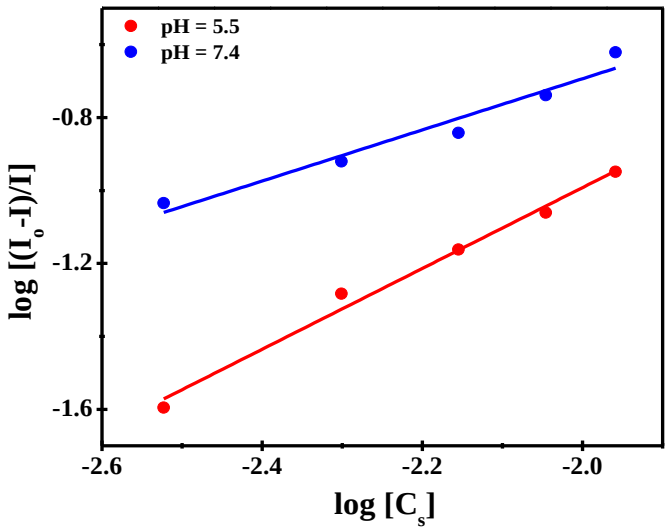


Fig. 4.40 Plot based on Lineweaver–Burk relation for analysis of gatifloxacin–CTAB associationat different pH condition

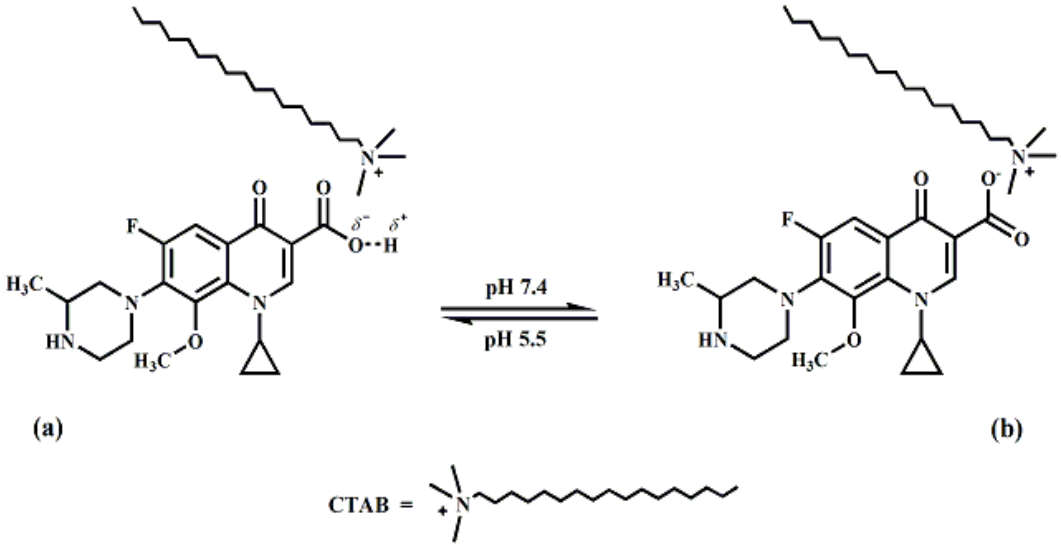


Fig.4.41 Proposed mechanism of gatifloxacin-CTAB association at pH 5.5(a) and pH 7.4(b)

Table 4.3: Values of K_2, K_b and ΔG^o_b of gatifloxacin-CTAB under different pH conditionsat 25±0.1°C

pH	UV-Visible measurements		Fluorescence measurements		HPLC measurements	
	$K_b(M^{-1})$ (± RSD %)	ΔG^o_b (kJ mol^{-1}) (± RSD %)	$K_b(M^{-1})$ (± RSD %)	ΔG^o_b (kJ mol^{-1}) (± RSD %)	$K_2(M^{-1})$ (± RSD %)	ΔG^o_b (kJ mol^{-1}) (± RSD %)
5.5	17.78± 0.08	-7.13± 0.05	16.79 ± 0.09	-6.99 ± 0.05	45.54 ± 0.06	-9.46 ± 0.04
7.4	6.23± 0.11	-4.53 ± 0.06	5.13± 0.11	-4.05 ± 0.08	31.68 ± 0.09	-8.56 ± 0.06

Binding constant values (K_2) for the gatifloxacin-CTAB at pH 5.5 and pH 7.4 obtained from chromatographic measurements were found to be 45.54 M^{-1} and 31.68 M^{-1} respectively. Both values correlated with binding constants (K_b) determined from UV and fluorescence spectroscopic measurements given in Table 4.3. Likewise the Gibb's free energy change (ΔG^o_b) values for gatifloxacin drug at pH 5.5 and pH 7.4 when it moved from aqueous phase to the micellar interior region were ΔG^o_b = -9.46kJ mol^{-1} and ΔG^o_b = -8.56 kJ mol^{-1} respectively. These values are in agreement with binding energy (ΔG^o_b) obtained from UV and fluorescence techniques and are shown in Table 4.3. The observed values revealed stronger CTAB-gatifloxacin interaction and that the probe is incorporated into the micellar assembly.

4.4 Levofloxacin

4.4.1 Development and optimization of micellar HPLC procedure.

Levofloxacin is a potent antibacterial drug. It effectively acts against bacterial infections and bacterial conjunctivitis.In this work the partitioning of levofloxacin molecules in the cationic micelles was investigated at physiological pH 7.4, particularly the allocation of bioactive drug molecules in colloidal fluid was the central point of this work. The ligand-receptor interactionsare often monitored using optical techniques. In this work the extent of molecular interactions of levofloxacin with CTAB was probed by quantifying its chromatographic and spectroscopic data as a function of various concentration of micro-heterogeneous assemblies.

Micellar liquid chromatographic procedure for levofloxacin was developed by using various organic solvents, phosphate buffer, CTAB concentration in different proportions, different HPLC columns Hypersil100 C18 (250 × 4.6mm × 5µm), Hypersil-ODS (250 × 4.6mm × 5µm), Hypersil-BDS C18 (250 × 4.6mm × 5µm), Hypersil-BDS C8 (250 × 4.6mm × 5µm), temperature, flow rate of mobile phase and absorption maximum wavelength. Different ratio of surfactant solution (in various phosphate buffers at pH 7.4) and methanol (85:15, 95:05, 90:10, 100:10, 100:15) were used on various columns but peak was very broad and took long time to elute.This might be due to some electrostatic interaction of oxygen atoms of the carboxylic group in levofloxacin with the cationic monomers of CTAB adsorbed on the stationary phase. There might be metal ion impurities present in the column and interact with oxygen atoms of the carboxylic group in levofloxacin. However to increase the polarity of the mobile phase methanol was replaced by acetonitrile. This change of organic solvent resulted in almost symmetrical levofloxacin peak but still have tailing factor and took longer time to elute. Mobile phase flow rate was also optimized to get peak at shorter time. Experiments were performed with flow rates

from 0.5 to 2.0 mL min^{-1}. The flow rate 1.5 mL min^{-1} was found suitable and produced peaks at shorter retention time. Temperature was also increased slowly from

25^oC - 40^oC to see the elution effect. It was observed that at 35^oC almost symmetrical peak was obtained compared to 25^oC and 30^oC. Absorption maximum was optimized between two wavelength 254 and 295nm. Sharp peaks were detected at 295nm wavelength. Suitable phosphate buffer and concentration were optimized using various phosphate buffers i-e KH_2PO_4, K_2HPO_4, NaH_2PO_4, Na_2HPO_4 with various molar concentration (1M, 0.5M, 0.1M, 0.05M, 0.01M) having pH 7.4. Finally an analytical procedure was optimized with a mobile phase having 0.05M K_2HPO_4 and acetonitrile in a ratio (90:10 v/v), Hypersil ODS (250 × 4.6mm, 5µm) column, flow rate of 1.5 mL min^{-1}, wavelength was 295nm and column oven was thermostated at 35^oC.

4.4.2 Determination of the binding constant by MLC

The retention behavior of the levofloxacin in micellar liquid chromatography was investigated on Hypersil ODS column (250 × 4.6mm, 5µm) and shown in Fig. 4.42. Levofloxacin retention time was determined as per function of lower to higher CTAB concentration, which showed that as the concentration of CTAB increased, retention time was decreased. Arunyanart and Cline Love equation 1 was used to calculate the binding constant (K_2) for levofloxacin interacting with CTAB.

By plotting $1/k^{/}$ versus $[M_m]$ (Fig. 4.43) and from the resultant slope/intercept ratio, the value of levofloxacin-CTAB binding constant $K_2 = 9.72 \times 10^2\ M^{-1}$ was obtained, which was in good agreement with binding constant obtained from photo-luminescent measurement. Likewise the Gibb's free energy change (ΔG^o_b) value for levofloxacin drug at pH 7.4 when it moved from aqueous phase to the micellar interior region was found to be ΔG^o_b = -13.91kJ mol^{-1}.Overall, these results indicated the ability of probe molecules as a reporter for sensing electrostatic environment in biomembranes.

4.4.3 Interaction of levofloxacin molecules with CTAB

The levofloxacin-CTAB association was monitored by estimating the effect of pre to postmicellar concentration of CTAB on the absorption spectra of levofloxacin in physiological pH condition. Fig. 4.44 displayed the spectral behavior of levofloxacin-CTAB in acidic (pH 5.5) and basic (pH 7.4) solution, at different

concentration of CTAB. In premicellar region, the absorption intensity of levofloxacin increased with increasing concentration of CTAB, whereas, in postmicellar region above its critical micelle concentration, the leveling off the curves (Fig. 4.45) indicated the maximum allocation of levofloxacin molecules within the micelle interior. The reported value CMC of the CTAB was 0.90 mM [113, 128]. With the levofloxacin addition, CMC of the CTAB increased to 1.30 mM. This increase was supposed to be due to the decrease of dielectric constant of the aqueous medium. This might increase the mutual repulsion of the ionic heads within the micelles, thus opposed the micellization and resulted in an increased CMC value [179].

The plot of surfactant concentration versus absorbance at varying pH conditions has been shown in Fig. 4.4. The degree of micelle ionization (β) was determined from the ratio of the slope of post-micellar region (S_2) to that of premicellar region (S_1) using relation $\beta = S_2/S_1$. The degree of micelle ionization (β) is related to the degree of counter-ion binding (α) as $\alpha = 1-\beta$. The values of degree of counter-ion binding were found to be 0.789 and 0.934 at pH 5.5 and pH 7.4 respectively. The observed increase in the degree of the counter-ion binding with increasing pH can be ascribed to an increase in the charged density at the surface of each surfactant micelle. Since the negative charge density increased with increasing pH, it might lead to the complete ionization of the carboxylic acid group of the levofloxacin molecules. To quantify the levofloxacin-CTAB interactions, various binding parameters were proficiently predicted. The changes in spectral characteristics of drug molecules with their surrounding environment can be employed to estimate the corresponding partition coefficients and approximate number of drug molecules per micelle. The quantitative results of solubilization ability of the cationic surfactant micelles and micelle/water partitioning coefficient for levofloxacin are summarized in Table 4.4.

4.4.4 Differential absorbance and steady-state fluorescence spectroscopic measurements

To quantify the levofloxacin-CTAB association, the differential absorption and fluorescence emission spectra of levofloxacin in presence of different concentration of CTAB were recorded under different physiological pH

conditions. These measurement provided information related to the binding affinities of drug molecules to the surfactant [180]. Fig. 4.46 and Fig. 4.47 displayed the differential absorption and fluorescence emission spectra of levofloxacin in the presence of various concentration of CTAB in basic (pH 7.4) and acidic (pH 5.5) conditions respectively. The differential absorption peak wavelength of levofloxacin was recorded to be 299 nm at both pH conditions, whereas the fluorescence peak wavelength of levofloxacin was recorded to be 454 nm (at pH 7.4) and 484 nm (at pH 5.5).

4.4.5 Kawamura relation for partitioning of levofloxacin in CTAB microheterogeneous assemblies

To determine the levofloxacin partitioning from aqueous phase to the micellar interior, the differential spectroscopic technique was successfully utilized. The resultant partition coefficient (K_x) values provided the essential information related to the interaction of organic solubilizate with the surfactant micelle and the locus of solubilizate within the micelle. The solubilization of levofloxacin in CTAB micellar media is calculated by using the following Kawamura relation [185] given below.

$$\frac{1}{\Delta A} = \frac{1}{K_c \Delta A_\infty (C_a + C_s^{mo})} + \frac{1}{\Delta A_\infty}$$

By plotting $1/\Delta A$ against $1/ (C_a + C_s^{mo})$ (Fig. 4.48a) and from the resultant slope/intercept ratio, K_c value was determined. The value of partition coefficient $K_x(K_c.n_w)$ and related Gibb's free energy of partitioning (ΔG_p°) are given in Table 4.4. The values of K_x for levofloxacin molecule were found comparable with the reported literature values for some other fluoroquinolone

based molecules [113, 128, 186-187]. The standard free energy change of thetransfer (ΔG^{o}_{b}) of levofloxacin molecules from bulk aqueous phase to micellar region can be calculated using the equation 3.The value of (ΔG^{o}_{b}) for the levofloxacin, using K_x is reported in Table 4.4. The relatively high negative values of (ΔG^{o}_{b}) implied that the process oflevofloxacin partition was

intrinsically spontaneous one. Similar results were reported for the drug-surfactant combinational system earlier [113, 186-190].

4.4.6 Benesi-Hildebrand relation for binding of levofloxacin with CTAB

To examine the binding of levofloxacin molecules with CTAB, the differential spectroscopic technique was successfully utilized. A more qualitative approach, the modified form of Benesi-Hildebrand equation 2, was used to determine the levofloxacin-CTAB binding constant.

The validity of this equation was examined by plotting various property functions (y-axis terms) against C_s^{mo} (Fig. 4.48b). A linear variation for almost every characteristic relation *viz.* (a) Kawamura relation, (b) Benesi–Hildebrand relation, (c) Lineweaver–Burk relation and (d) Stern–Volmer relation under varying pH conditions (pH = 5.5 and pH 7.4) validated respective equation as a true measure of the binding associations between levofloxacin and CTAB molecules. The values of binding constant (K_b) obtained from these plots were used to calculate the respective Gibb's binding energy (ΔG^{o}_{b}) using equation 3 as outlined in Table 4.4.

The values of K_b and hence ΔG^{o}_{b} for levofloxacin molecules were found comparable with the reported literature values for some other fluoroquinolone based molecules connected to cationic micelles [113, 128, 186-187]. Levofloxacin being amphiphile molecule is feasible to reside mainly in the interfacial vicinity. An increased hydrophobicity simply drags the levofloxacin molecules slightly deeper into the micelle interior. This in turn ceases their movement towards the aqueous bulk domain.

It was therefore suggested that the additive molecule may appear as an ion pair with surfactant monomers. At the later stage, the adhesion of this ion-pair to micellar surface leads the incorporation of additive molecule into the palisade layer of micelle.

4.4.7 Stern Volmer relation for quenching of levofloxacin by CTAB

The fluorescence intensity of a probe molecule can be quenched by a number of molecular interactions such as energy transfer, ground state complex formation,

collision quenching, molecular rearrangements and excited state reactions. The inclusion of surfactant, originates the concentration-dependent quenching of the intrinsic fluorescence of levofloxacin. The substantial interactions between levofloxacin and cationic surfactant indicated the existence of a non-fluorescent complex with no obvious shift in the emission maximum of the levofloxacin. Stern Volmer equation 4 was used to analyse the fluorescence quenching data.

The values of K_{sv} of levofloxacin from Stern Volmer plot (I_o/I versus $[C_s]$) (Fig. 4.48d) are shown in Table 4.4. The data revealed that the quenching mechanism is probably initiated by ground-state complex formation (static quenching) in preference to dynamic collision [191]. Since only the excited states of the fluorophores are affected in collisional quenching, no changes in the absorption spectra were observed. However in the ground-state complex formation, the perturbation of the absorption spectrum of the fluorophore has been frequently observed earlier [192].

4.4.8 Lineweaver–Burk relation for binding equilibria between levofloxacin and CTAB

The above results compliment the CTAB as quencher for the intrinsic fluorescence of levofloxacin molecules. The binding affinities (K_b) for static quenching procedure can be calculated using the modified Lineweaver–Burk equation 5 [192-193].Fig. 4.48c displayed the plot of log $[(I_o-I)/I]$ versus log $[C_s]$ for CTAB. The binding constant (K_b) thus obtained was used to calculate the standard free energy change $\Delta G^o_b$of the levofloxacin binding to surfactant. The obtained values of binding constant K_b and the related binding energy $\Delta G^o_b$are listed in Table 4.4. The observed high value of K_b

indicated that the interaction between levofloxacin and CTAB was strong and that the probe molecule was incorporated into surfactant assemblies.

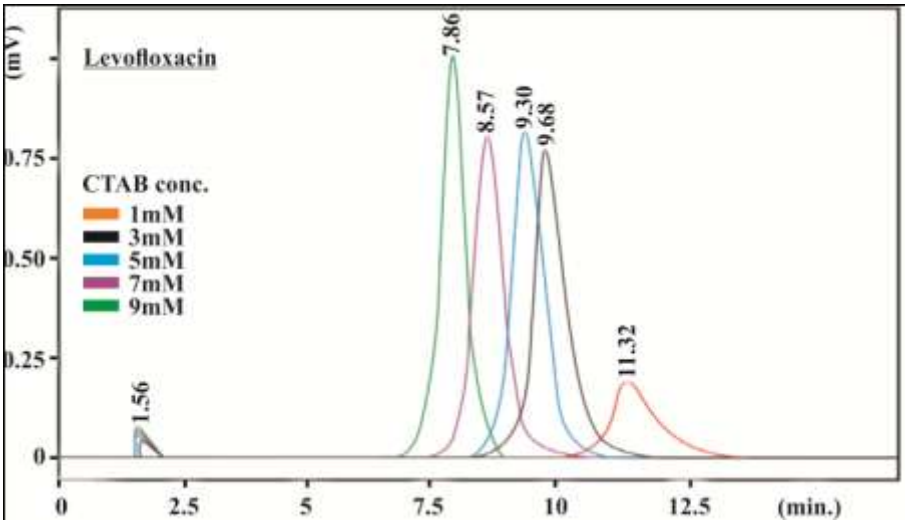


Fig. 4.42 Chromatogramof levofloxacin-CTAB with varying concentrations of CTAB in physiological condition pH 7.4 determined on Hypersil ODS column (250 × 4.6 mm, 5 µm)

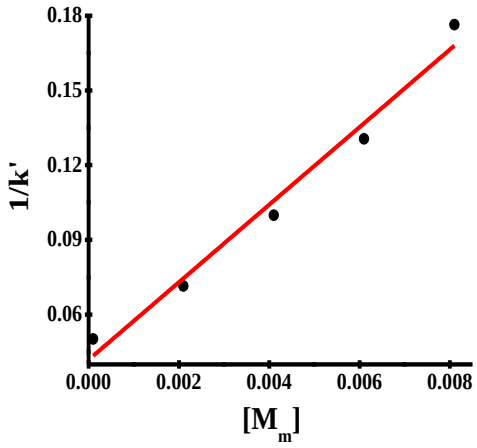


Fig. 4.43 Plot based on Arunyanart and Cline-Love relation for binding analysis of levofloxacin–CTAB association

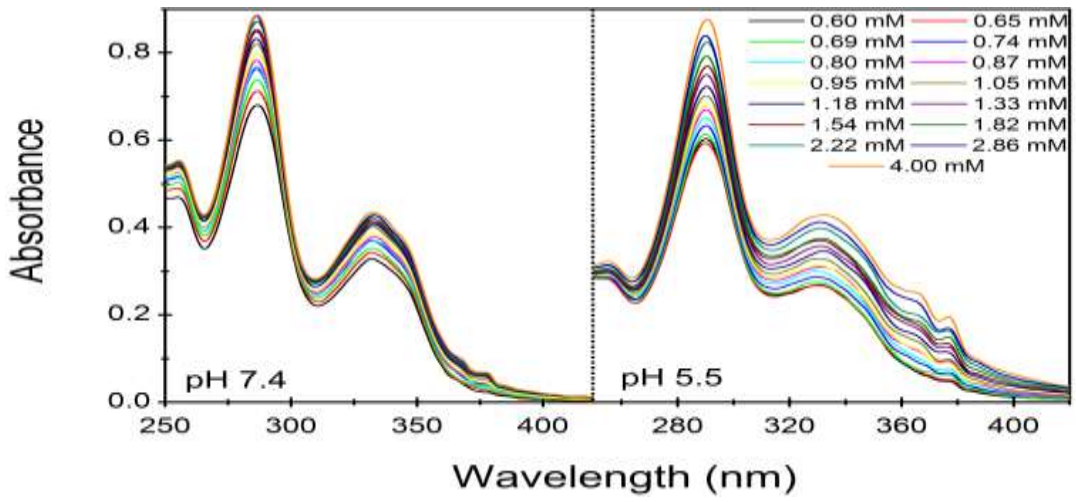


Fig. 4.44 Absorption spectra of levofloxacin-CTAB with various concentrations of CTAB in acidic (pH 5.5) and basic (pH 7.4) condition

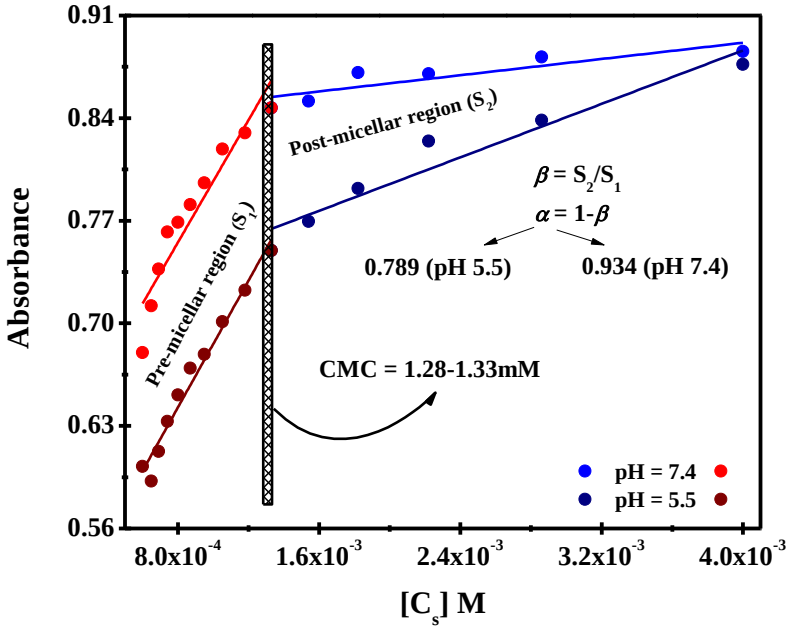


Fig. 4.45 Relation between absorbance of levofloxacin–CTAB with varying concentrations of CTAB

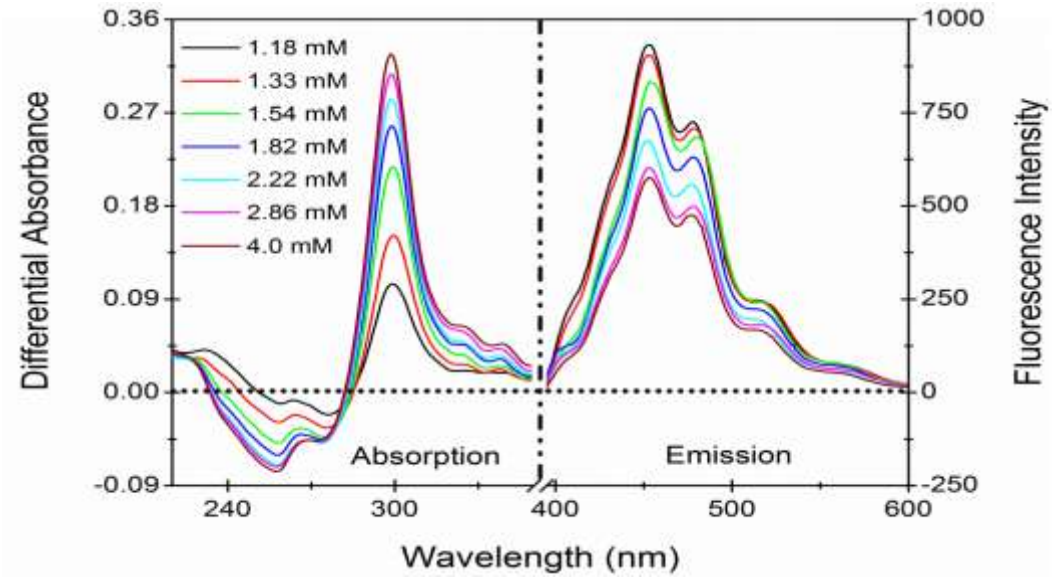


Fig. 4.46 Differential absorption and fluorescence emission spectra of levofloxacin-CTAB with varying concentrations of CTAB at pH 7.4

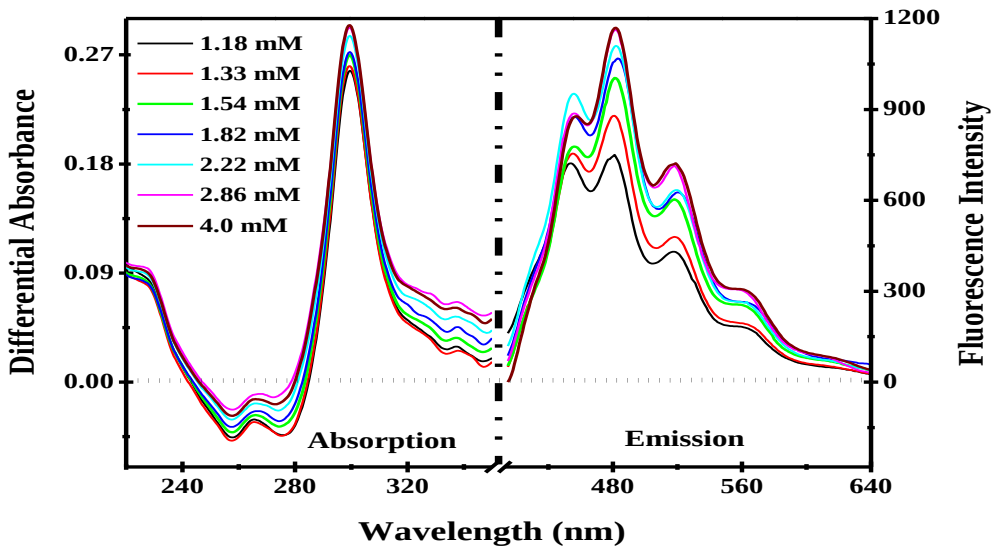


Fig. 4.47 Differential absorption and fluorescence emission spectra of levofloxacin-CTAB with varying concentrations of CTAB at pH 5.5

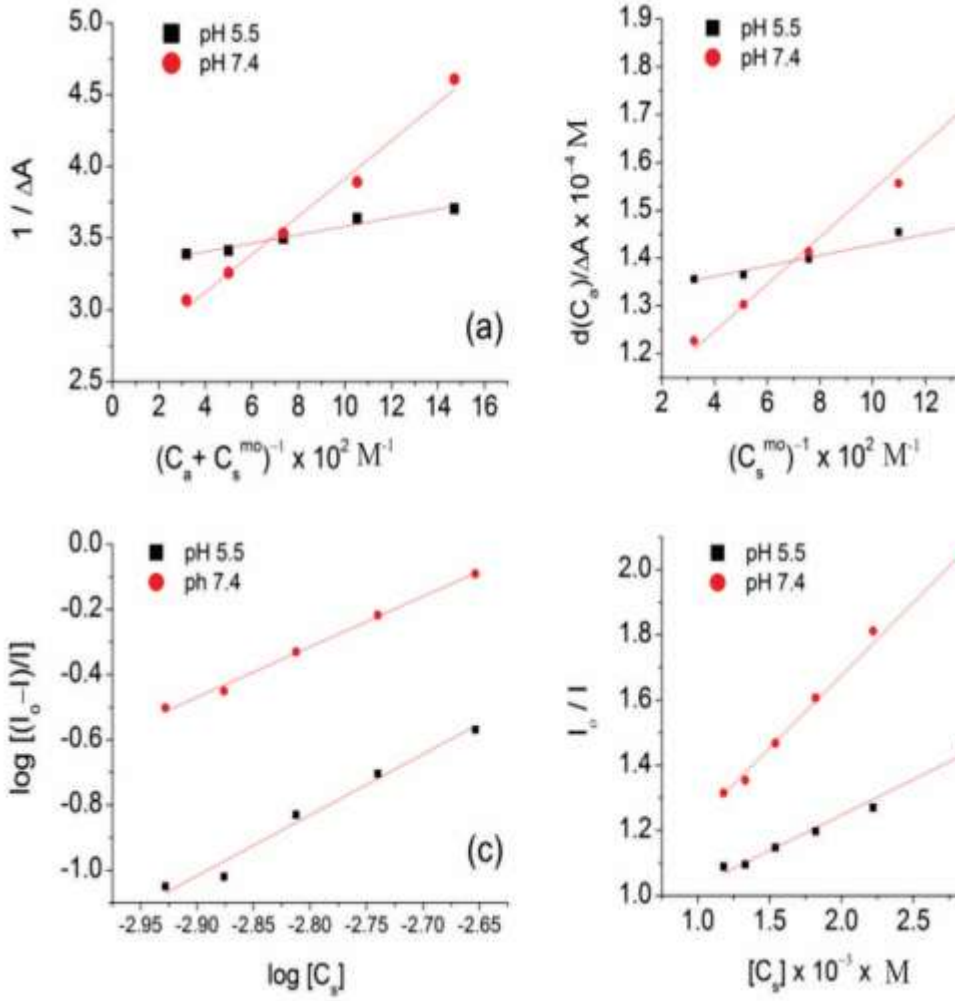


Fig. 4.48 Plot based on (a) Kawamura relation (b) Benesi–Hildebrand relation (c) Lineweaver–Burk relation (d) Stern–Volmer relation for analysis of evofloxacin–CTAB association

Table 4.4: Values of K_c, K_x, K_b, K_{sv}, ΔG^o_p and ΔG^o_b oflevofloxacin-CTAB under

different pH conditions at 25±0.1°C

pH	UV-Visible measurements					Fluorescence measurements			HPLC measurements	
	$K_c(M^{-1})$ (± RSD %)	$K_x(M^{-1})$ (± RSD %)	ΔG^o_p (kJ mol^{-1}) (± RSD %)	$K_b(M^{-1})$ (± RSD %)	ΔG^o_b (kJ mol^{-1}) (± RSD %)	$K_b(M^{-1})$ (± RSD %)	ΔG^o_b (kJ mol^{-1}) (± RSD %)	$K_{sv}(M^{-1})$ (± RSD %)	$K_2(M^{-1})$ (± RSD %)	ΔG^o_b (kJ mol^{-1}) (± RSD %)
5.5	16,795 ±3.2	932,962 ±4.3	-34.06 ±2.5	14,286 ±1.3	-23.70 ±2.1	14,454 ±1.7	-23.73 ±1.9	218 ±4.3	-	-
7.4	801 ±3.0	44,495 ±2.6	-26.52 ±1.3	1000 ±2.2	-17.11 ±2.2	871 ±1.3	-16.77 ±0.7	445 ±1.4	972 ±3.1	-13.91 ±0.7

The value of levofloxacin-CTAB binding constant $K_2 = 9.72 \times 10^2\ M^{-1}$ obtained from MLC measurements at pH 7.4 was in good agreement with binding constant obtained from photo-luminescent measurement. Likewise the Gibb's free energy change (ΔG^o_b) value for levofloxacin drug at pH 7.4 when it moved from aqueous phase to the micellar interior region was found to be ΔG^o_b = -13.91kJ mol^{-1} which corresponds to values obtained from photo-luminescent measurements.

4.5 Sparfloxacin

4.5.1 Development and optimization of micellar HPLC procedure.

Sparfloxacin is a newly developed potent antibacterial drug that belongs to fluoroquinolone group of antibiotics. The substance has been reported to be highly active against a wide range of pathogenic bacteria specially gram positive bacteria comparatively with other fluoroquinolones. Sparfloxacin possess two pKa values, corresponding to pH 6 and pH 9 [194-195]. There was no micellar liquid chromatographic procedure reported in the literature for the determination of sparfloxacin, instead a conventional HPLC method was reported [196]. For the optimization of micellar liquid chromatographic method different stationary phases, organic solvents, phosphate buffers and various CTAB concentration in different phosphate buffers were used to finalize the mobile phase at pH 7.4. Various suitable phosphate buffers e.g. KH_2PO_4, K_2HPO_4, NaH_2PO_4, Na_2HPO_4 and their concentration (1M, 0.5M, 0.1M, 0.05M, 0.01M) were optimized with pH 7.4. Finally 0.05M KH_2PO_4 buffer was found suitable.

Different surfactant concentration (in phosphate buffer solution pH 7.4) and methanol were tried in different proportion (85:15, 95:05, 90:10, 100:10, 100:15 and 100:13). Various columns were used like Hypersil100 C18, Purospher STAR RP-18, Hypersil-ODS, Hypersil-BDS C18. Peak broadening and shoulder formation was observed. This issue might be due to the interaction of oxygen atoms of the carboxylic group in drug with the ionic head group of the monomers of CTAB adsorbed on the stationary phase. To increase the polarity of mobile phase methanol was replaced by acetonitrile. However very symmetrical peak of sparfloxacin was obtained with 0.05M KH_2PO_4 as buffer and acetonitrile as organic modifier in a ratio (100:13 v/v) on Purospher STAR RP-18 column at wavelength 290nm.

4.5.2 Determination of the binding constant by MLC

In present study, MLC determination for the sparfloxacin has been investigated. The findings revealed that the surfactant head groups support the drug to solublize in the external part of the micelle, which may probably be more supportive in the drug controlled release. This is very useful to understand physiochemical processes at

molecular level. The chromatographic behavior of sparfloxacin was determined on Purospher STAR RP-18 column (250 x 4.6mm, 5µm) and presented in Fig.4.49. The binding constant (K_2) value of the drug sparfloxacin with the CTAB was estimated by Arunyanart and Cline-Love equation 1.

Binding constant value (K_2) for the sparfloxacin-CTAB obtained from slope/intercept ratio of the plot $1/k^{/}$ against [M_m] (Fig. 4.50) was 1.46 x $10^2 M^{-1}$. Generally, this value shows the ability of the drug molecules under probe to sense electrostatic environment in phospholipid membranes and associated structures. Likewisethe Gibb's free energy change (ΔG^o_b) value for sparfloxacin molecules when they moved from aqueous phase to the micellar interior region was ΔG^o_b = -12.36 KJ mol^{-1}. Overall, these results indicated the ability of probe molecule as a reporter for sensing electrostatic environment in biomembrane and related organized assemblies. Electrostatic forces are driving forces in drug-membrane binding. The charged phospholipid groups of biomembrane have significant contribution in these interactions. Owing to these charged groups, surface of the biomembrane presented a net charge in aqueous medium giving rise to different binding properties of charged and uncharged form of molecule such as drug which consequently resulted in the electrostatic interaction between amphiphilic drug and biomembrane.

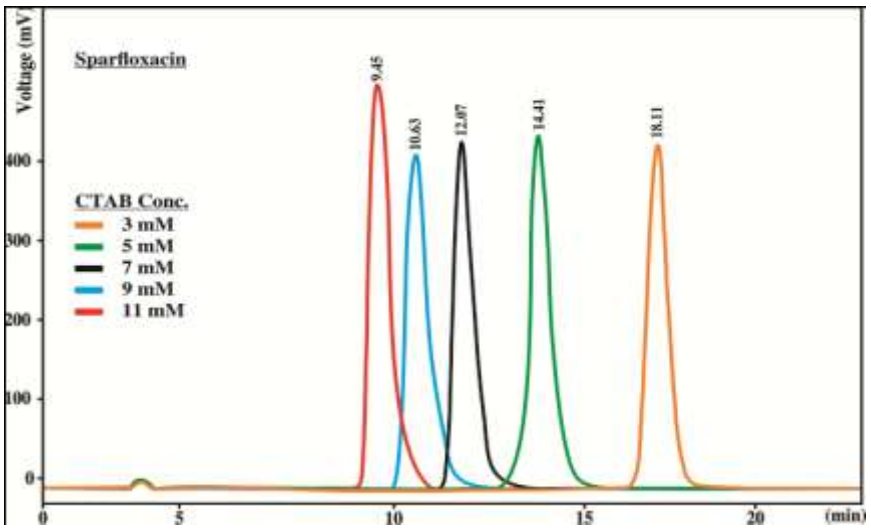


Fig. 4.49 Chromatogramof sparfloxacin-CTAB with varying concentrations of CTAB at physiological pH 7.4 determined on Purospher STAR RP-18 column (250 × 4.6mm, 5µm)

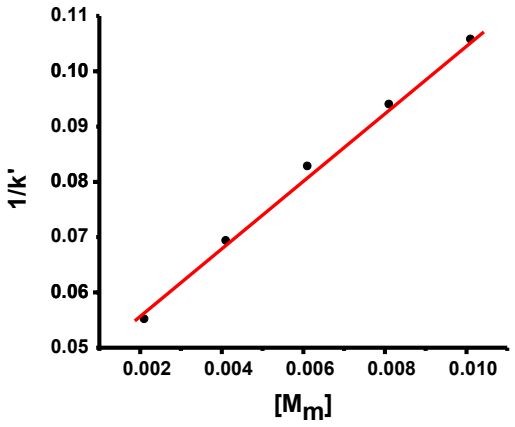


Fig. 4.50 Plot based on Arunyanart and Cline-Love relation for binding analysis of sparfloxacin–CTAB association

4.6 Conclusion

The present research illustrates the molecular interaction of the potent antibiotic drugs (single drugs as well as fixed dose combinations) integrated into surfactant micellar self-assemblies in premicellar and postmicellar concentration. Micellar liquid chromatography (MLC), differential absorption and emission spectrometry were performed to probe the drug–CTAB association, whereas interaction modes of drug–surfactant were quantified by determining binding capacities and related Gibb's free energies at various pH conditions.The binding values of drug–CTAB obtained from micellar liquid chromatography measurements are found to be in good agreement with as measured by electronic spectroscopy. Experimental datarevealed that below the critical micellar concentration of surfactant, no molecular interactions were observed for drugs and surfactant. The probable reason is the electrostatic repulsive forces among positively charged ammonium group of the surfactant monomers and positively charged nitrogen atom on the drug molecule [75]. However, in micellar and postmicellar concentration the changes in spectral values can be depicted in terms of binding of drugs inside the micellar assemblies which corresponds to the inclusion of drug molecules inside the micellar assemblies. The association of drugs with cationic surfactant CTAB is in good agreement as quantified by the chromatographic and spectral-luminescent measurements. The chromatographic technique was specifically utilized to investigate the mutual interaction of FDC drugs with themselves while interacting with the model membranes. In case of the fixed dose combinations, norfloxacin/metronidazole and ofloxacin/ornidazole, nearly the same values of binding constant and Gibb's energy changes for free drug and when the drugs in combination revealed that in fixed dose combination both the drugs do not interact with each other and show no influential behavior on each other. Both drugs independently bind with the surfactant micelles. However to investigate the functional and structural aspects of biological membrane, understanding of the drugs integrated into the micelle is quite helpful. Due to hydrophobic and substantial electrostatic inputs, drugs initially adsorb onto the surface of the micelle and are subsequently integrated into the peripheral part of palisade layer of the micelle. The micellar interior is a non-polar region in contrast to the polar aqueous bulk solution; therefore amphiphilic drug molecules tend to reside inside the micelles in the vicinity of the

polar surface [123]. This phenomenon actually facilitates penetration binding [128]. This host–guest interaction may be helpful for elucidating the drug delivery system across plasma membrane mediated by the micelles.

CHAPTER NO. 5

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