

**METHOD DEVELOPMENT AND VALIDATION
FOR ANTIPSYCHOTIC DRUGS; APPLICATION TO
PHARMACEUTICAL PREPARATIONS AND
BIOLOGICAL SAMPLES**



Ph. D Thesis

By

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**INSTITUTE OF CHEMICAL SCIENCES
UNIVERSITY OF PESHAWAR
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**INSTITUTE OF CHEMICAL SCIENCES
UNIVERSITY OF PESHAWAR
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MARCH 2013**

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Certified that **Mr. Muhammad Naeem Khan** S/O Ajar Muhammad has carried out his research and experimental work on the topic entitled as “**Method Development and Validation for Antipsychotic Drugs; Application to Pharmaceutical Preparations and Biological Samples**” under our guidance and supervision. His research work is original and his dissertation is worthy of presentation to the University of Peshawar for the award of degree of **Doctor of Philosophy in Chemistry**.

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To my teachers & my mentors

Prof. Dr. Muhammad Rasul Jan and Prof. Dr. Jasmin Shah

Without whom this work would never have come into
being.

To those closest to me

My Parents, My Brothers, My Sisters & My Wife

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stay with me throughout my life.

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LIST OF ABBREVIATIONS

%	Percent
°C	Degree celsius
µg	Microgram
µgmL ⁻¹	Microgram per milliliter
AFR	Alternating fitting residue
AgNPs	Silver nanoparticles
CL	Chemiluminescence
CYP	Cytochrome P450
CIT	Citalopram
CZE	Capillary zone electrophoresis
CD	Cyclodextrin
cm	Centimeter
DI	Deionized water
DM	Diabetes mellitus
EEM	Excitation-emission matrix
EJCR	Elliptical joint confidence region
FDA	Food and drug administration
FIA	Flow injection analysis
g	Gram
g/mol	Gram per mole
HPLC	High performance liquid chromatography
HPTLC	High performance thin layer chromatography
ICH	International conference on harmonization
kV	Kilo volt
Ltd	Limited
LOD	Limit of detection
LOQ	Limit of quantification

$\text{L mol}^{-1} \text{ cm}^{-1}$	Liter per mol per centimeter
mg	Milligram
mgmL^{-1}	Milligram per milliliter
MEB	Mebeverine hydrochloride
mgL^{-1}	Milligram per liter
mL	Milliliter
MS	Mass spectrometry
min	Minute
M	Molarity
molL^{-1}	Mole per liter
mm	Millimeter
mV	Milli volt
mmolL^{-1}	Milli mole per liter
mLmin^{-1}	Milliliter per minute
mgtab^{-1}	Milligram per tablet
μM	Micromolar
μgL^{-1}	Microgram per liter
μm	Micrometer
$\mu\text{g cm}^{-2}$	Microgram per centimeter square
nm	nanometer
ngmL^{-1}	Nanogram per milliliter
nmolL^{-1}	Nano mole per liter
Pvt	Private
pgmL^{-1}	Pico gram per milliliter
r^2	Correlation coefficient
RE	Relative error
RSD	Relative standard deviation
RP	Reversed Phase
S	Standard deviation
SDS	Sodium dodecyl sulfate

SLP	Sulpiride
SWATLD	Self-weighted alternating trilinear decomposition
TD	Tardive dyskinesia
TLC	Thin layer chromatography
TEM	Transmission electron microscopy
UV	Ultra violet
USA	United states America
V	Volt
W	Watt

SUMMARY

Quantification of drugs in pharmaceutical formulations and biological fluids is enormously important from the health point of view. A variety of chromatographic methods have been reported for the determination of antipsychotic drugs in pharmaceutical formulations and biological fluids. However most of these methods require sophisticated and expensive instruments, lengthy operation, large quantity of solvents and highly skilled manpower. Therefore, there is always a need of analytical methods involving simple and inexpensive instrumentation but at the same time possessing high sensitivity, selectivity, accuracy and repeatability for the quantification of active ingredients of the drugs. The present work is an effort to achieve the same objectives using spectrophotometer and spectrofluorimeter.

The first chapter of this thesis involved introduction of antipsychotics drugs. It includes classification, pharmacological and pharmacokinetics of antipsychotics and knowledge concerning physical and chemical features of antipsychotics (citalopram, sulpiride and fluoxetine) selected for this research.

The second chapter comprises a review of the relevant reported methods for the determination of selected drugs. These include chromatographic, capillary electrophoresis, spectrophotometric and spectrofluorimetric methods for determination of selected drugs in commercial formulations and biological fluids. These reported methods have been discussed in the light of their scope, sample preparation, reagents used, linearity range, limits of detection and quantification and percent recovery.

The third and last chapter deals with experimental work with the results and discussion of different methods investigated for the determination of active ingredients of the selected drugs in numerous pharmaceutical formulations and its validation for spiked biological samples. This chapter is divided into three parts. The first part defines spectrofluorimetric and chemiluminescence methods for determination of citalopram; second part describes spectrofluorimetric and chemiluminescence methods for determination of sulpiride and third part is concerned with spectrophotometric determination of fluoxetine.

Section 3.1 describes a spectrofluorimetric method for the quantification of citalopram in pure form, in the pharmaceutical preparations and in human blood plasma and urine. The proposed method is based on the oxidation of citalopram by Ce (IV) in acidic media to produce fluorescent Ce (III), and was measured spectrofluorimetrically at 353 nm after excitation at 252 nm. All experimental parameters affecting the oxidation of citalopram such as Ce (IV) concentration, type and concentration of acid, temperature and heating time were studied and optimized. Under the optimized experimental conditions a linear range of concentration versus fluorescence intensity was investigated and was found to range from 0.02 to 1.4 $\mu\text{g mL}^{-1}$. The LOD and LOQ were found to be $6.9 \times 10^{-3} \mu\text{g mL}^{-1}$ and $2.3 \times 10^{-2} \mu\text{g mL}^{-1}$ respectively. The effect of commonly used excipients in the quantification of citalopram has been studied and no effect was found. The proposed method was applied successfully for citalopram determination in pure form, pharmaceutical preparations and in biological fluids. Good recoveries in the range of 95.31–101.67%, 88.50–96.67% and 90.0–96.67% were obtained for pharmaceutical preparations (tablets), blood plasma and human urine respectively.

Section 3.2 deals with the development and validation of spectrofluorimetric method for the determination of citalopram in bulk and pharmaceutical preparations. The method is based on the enhancement of the weak fluorescence signal of the Tb (III)-citalopram system by the addition of silver nanoparticles. Fluorescence intensities were measured at 555 nm after excitation at 281 nm. The prepared silver nanoparticles (AgNPs) were characterized by UV-Visible spectra and transmission electron microscopy. Various factors affecting the formation of citalopram-Tb (III)-AgNPs complexes were optimized. Fluorescence intensity versus concentration plot was linear over the range of 0.02–14 $\mu\text{g mL}^{-1}$, with good correlation coefficient of 0.9978. The LOD and LOQ were found to be $7.15 \times 10^{-6} \mu\text{g mL}^{-1}$ and $2.38 \times 10^{-5} \mu\text{g mL}^{-1}$ respectively. The proposed method was found to have good reproducibility with a RSD of 3.66% (n=6). The interference effects of common excipients found in pharmaceutical preparations were studied. The developed method was validated statistically by performing recoveries studies and successfully applied for the assay of citalopram in bulk powder and pharmaceutical preparations.

Percent recoveries were found to range from 98.98 to 100.97% for bulk powder and from 96.57 to 101.77% for pharmaceutical preparations.

Section 3.3 is concerned with a silver nanoparticle enhanced chemiluminescence method for citalopram determination in pharmaceutical formulations and human plasma. The method is based on the enhancement of the weak CL signal of the luminol-H₂O₂ system. It was found that the CL signals arising from the reaction between alkaline luminol and H₂O₂ substantially increased by the addition of citalopram in the presence of silver nanoparticles. Various experimental parameters affecting CL intensity were optimized for the determination of citalopram. Under the optimum conditions, CL intensity was found to be rectilinear to the concentration of citalopram in the range 40–2500 ngmL⁻¹, with a correlation coefficient of 0.9997. The LOD and LOQ of the proposed method were 3.78 ngmL⁻¹ and 12.62 ngmL⁻¹ respectively. Furthermore the proposed method was found to have excellent reproducibility with a RSD value of 3.65% (n=7). Potential interferences by common excipients were also studied. The developed method was validated statistically using recovery studies and was successfully applied for the determination of citalopram in pure form, in pharmaceutical preparations and in spiked human plasma samples. Percent recoveries were found to range from 97.71 to 101.99% for the pure form and 97.84 to 102.78% for pharmaceutical preparations and from 95.65 to 100.35% for spiked human plasma.

Section 3.4 of thesis deals with a validated spectrofluorimetric method for determination of sulpiride in commercial formulations using Hantzsch condensation reaction. The method is based on the condensation of the primary amino group of alkaline hydrolytic product of sulpiride with acetyl acetone and formaldehyde in acidic medium to form a fluorescent product. The excitation and emission wavelength are 431 nm and 483 nm respectively. Reaction conditions affecting the condensation reaction were sensibly optimized and a linear range of 0.1–3.5 µgmL⁻¹ with good correlation coefficient between florescent intensity and concentration of sulpiride was found at optimum parameters. The LOD and LOQ were found to be 1.1×10^{-2} and 3.9×10^{-2} µgmL⁻¹ respectively. The proposed method was successfully applied for the quantification of sulpiride in bulk powder and commercial formulations. The effect of common pharmaceutical excipients

and co-administered drugs were also studied and no interferences were observed. The validity of the method was tested by analyzing sulpiride in bulk powder and pharmaceutical formulations through recovery studies. Percent recoveries were found to be in the range of 98.62 to 98.34% for bulk powder and 97.09 to 100.57% for commercial formulations. The results were validated statistically with those obtained by reference literature HPLC method.

Section 3.5 of the thesis deals with the spectrofluorimetric determination of sulpiride in pharmaceutical preparations and human plasma through derivatization with 2-cyanoacetamide. The developed method is based on the derivatization reaction of 2-cyanoacetamide with sulpiride in the presence of ammonia. The derivatized reaction product exhibited maximum fluorescence intensity at 379 nm after excitation at 330 nm. All variables affecting the reactions were carefully studied and incorporated into the procedure. The concentration plot of fluorescence intensity was linear over the concentration range of 0.2 to 20 $\mu\text{g mL}^{-1}$ with correlation coefficient of 0.9985. The LOD and LOQ were found to be 0.82 ng mL^{-1} and 2.73 ng mL^{-1} respectively. The proposed method was validated according to ICH guidelines. The effect of common excipients and co-administered drugs were also studied. The accuracy of the method was checked using the standard addition method and percent recoveries were found ranged from 99.00 to 101.25% for pharmaceutical preparations and 97.00 to 97.80% for spiked human plasma. The method was successfully applied to commercial formulations and the results obtained for the proposed methods were compared with a HPLC reference method and statistically evaluated using the student's t-test for accuracy and the variance ratio F-test for precision.

Section 3.6 describes a validated micellar-enhanced spectrofluorimetric method for the quantification of sulpiride in pure form, commercial formulations, and human plasma and urine samples in the presence of sodium dodecyl sulphate (SDS) surfactant micelle. Experimental parameters like buffer, surfactants type and volume were studied. A linear relationship was found between sulpiride concentration and fluorescence intensity in the range of 0.02–7.0 $\mu\text{g mL}^{-1}$ containing 0.2 M SDS with λ_{ex} 292 nm and λ_{em} 343 nm with a correlation coefficient of 0.9987. The LOD and LOQ were found to be 1.9×10^{-2} $\mu\text{g mL}^{-1}$

and $5.9 \times 10^{-2} \mu\text{g mL}^{-1}$ respectively. The proposed method was successfully applied for the determination of sulpiride in pure and dosage form; the percentage recoveries agreed well with the label value. The method was successfully used for the determination of sulpiride in spiked human plasma and urine samples with percentage recoveries varying from 98.33–99.75% and 95.67–101.67% respectively. The results of the proposed method have been compared with the literature HPLC method and no significant difference was found between them.

Section 3.7 deals with the determination of sulpiride in pharmaceutical formulations and biological fluids using a Cr (III) enhanced chemiluminescence method. The method is based on increased CL intensity of a luminol- H_2O_2 system in response to the addition of Cr (III) under alkaline conditions. The CL intensity of the luminol- H_2O_2 -Cr (III) system was greatly enhanced by the addition of sulpiride and the enhanced CL intensity was proportional to the concentration of sulpiride in a sample solution. Various parameters affecting the CL intensity were systematically investigated and optimized for determination of the sulpiride in a sample. Under the optimum conditions, the CL intensity was proportional to sulpiride concentration in the range of 0.068–4.0 $\mu\text{g mL}^{-1}$, with a good correlation coefficient of 0.997. The detection and quantification limits were found to be $8.50 \times 10^{-6} \mu\text{g mL}^{-1}$ and $2.83 \times 10^{-5} \mu\text{g mL}^{-1}$ respectively. The proposed method has good reproducibility with a RSD value of 2.70% (n=7). The effects of common excipients and metal ions were studied for their interference effect. The proposed method was statistically validated through recovery studies and was successfully applied for the determination of sulpiride in pure form, pharmaceutical preparations and biological samples. The percentage recoveries were found to range from 99.10–100.05% for pure form, 98.10–100.20% for pharmaceutical preparations and 97.90–101.40% for spiked human plasma.

Section 3.8 of thesis deals with the quantitative determination of fluoxetine in pharmaceuticals and plasma samples using bromatometric method. The method is based on the addition of measured excess amount of bromate-bromide mixture to fluoxetine in hydrochloric acid medium. The residual bromine was determined by reacting with methyl orange and absorbance was measured at 505 nm. Linear relationship between absorbance

Summary

and fluoxetine concentration was found and Beer's law was obeyed in the concentration range of 0.4–12 $\mu\text{g mL}^{-1}$. The molar absorptivity was found to be $3.8 \times 10^4 \text{ L mol}^{-1} \text{ cm}^{-1}$. The LOD and LOQ was calculated and found to be 0.32 $\mu\text{g mL}^{-1}$ and 1.0 $\mu\text{g mL}^{-1}$ respectively. The effects of common excipients were investigated for their interferences in the assay. The validity of the proposed method was checked through recoveries studies and was successfully applied for the determination of fluoxetine in pharmaceutical formulations and biological samples. Percent recoveries were found to be in the range of 97.00–101.00% for pharmaceutical formulations and from 97.00–99.00% for spiked human plasma.

INTRODUCTION

1.0 Introduction^[1-30]

A drug is defined as, any constituent that when absorbed by a living organism, modifies normal body function. In pharmacology, a drug is a chemical substance applied in the handling, treatment, and diagnosis of disease or used to develop physical or mental safety.

Classification

Pharmaceutical drugs can be classified in different ways on the basis of chemical properties, mode of administration and biological system exaggerated. Simple classes of medicine comprise:

1. Antipyretics: These are drugs meant to prevent or reduce fever.
2. Analgesics: Drugs meant for control of pain.
3. Antimalarial drugs: Medicines that are used for the treatment of malaria.
4. Antiseptic: Drugs that prevents the production of infectious microorganisms.
5. Antibiotic: Drugs which is used to kill or slows down the growth of bacteria.
6. Antipsychotics: Drugs used to manage mental illness.

Antipsychotics

A class of medicines used for the treatment of “psychosis” and others mental and emotional disorders. “Psychosis” is a Greek word “psyche” means mind and “osis” means abnormal conditions, refers to an abnormal condition of the mind. Antipsychotic drugs are generally used for the treatment of psychosis, schizophrenia, nervousness, anxiety, mania and for violent impulsive behavior.

History of antipsychotics

Antipsychotic has an old history. The first antipsychotic drugs were introduced in 1950. Chlorpromazine was developed for the first time as a clinical anesthetic and was applied on patients due to its powerful relaxing result. At that time it was considered as a non-permanent “pharmacological lobotomy”. At that time lobotomy was used for the treatment of much behavior disorders including psychosis. However, chlorpromazine was

proved to be more effective to reduce psychosis effect in a more specific mode than lobotomy. Older antipsychotics were developed for the first time in 1950s and acts predominantly to reduce the effect of dopamine in the brain. Newer antipsychotics were developed from the 1970s onwards. The newer antipsychotics are rarely used as compared to older antipsychotics as they cause movement disorders as a side effect. Antipsychotics drugs are the top selling class of pharmaceutical now a day in USA, producing yearly income of about 14.6 billion dollars.

Classification of antipsychotics drugs

Antipsychotics drugs can be classified on the basis of clinical action and mechanism of action.

- a. Typical or first-generation antipsychotics; decrease the levels of neurotransmitter called dopamine.
- b. Atypical or second-generation antipsychotics; lower dopamine and also acts on other neurotransmitters, primarily serotonin.
- c. Third generation antipsychotics

The classification of typical/first generation antipsychotics is based on their chemical structure while the atypical/second generation antipsychotics are classified on the basis of their pharmacological properties.

I. First generation/typical antipsychotics

First generation antipsychotics are older class of drugs that have been used for the treatment of psychotic disorders for many decades. It can be used to handle psychosis and improve thinking, mood and behavior. These drugs may enable a patient to return to a more normal daily life while living with schizophrenia. Chlorpromazine, fluphenazine, perphenazine, trifluoperazine and haloperidol are the members of first generation. The side effects of first-generation antipsychotics include tremors, tardive dyskinesia, allergic reactions, lower white blood cell count, abnormal heart beat, low blood sugars, loss of appetite, and frequent urination.

I. Second generation/atypical antipsychotics

The second generation antipsychotics also known as atypical antipsychotics are the most important drugs developed in the past decades for the treatment of severe psychological syndromes such as schizophrenia, schizoaffective disorders and mania. Both first and second-generation antipsychotics drugs have a tendency to block the brain's dopamine pathway receptors; however the second generation's antipsychotics are less likely to cause extrapyramidal side effects. Due to fewer side effects of second generation's antipsychotics, they are used first when a person is first identified with schizophrenia. Amisulpiride, clozapine, aripiprazole, olanzapine, quetiapine, risperidone, and ziprasidone are the members of second generations. Second generation antipsychotics drugs have higher rate of responders, efficiency in patients with refractory disease, minor risk of suicides, efficient ability and a better quality of life.

Third generation antipsychotics

Aripiprazole is the third generation antipsychotic. Its mechanism of action is different from first generation and second generation antipsychotics. Both typical and atypical antipsychotics produce their effect by blocking D₂ receptor. Aripiprazole have D₂ partial agonism properties and stabilizing the dopamine system.

Mechanism of action

Antipsychotics drugs have a tendency to block the D₂ receptors in the dopamine pathways of the brain as a result the dopamine liberated in these pathways has minimum effect. Surplus release of dopamine in the mesolimbic pathway has been related to psychotic experiences. The blockade of dopamine receptors in this pathway control psychotic experiences.

It is known that the clinical efficiency of antipsychotics is accompanying with a blockade of 60% to 80% of D₂ receptor in the brain. Atypical antipsychotics drugs also block the D₂ receptor and some particularly block serotonin receptors (5HT_{2A}, C and 5HT_{1A} receptors).

Pharmacokinetics ^[31-33]

The process by which a drug is absorbed into systemic circulation, spread through the tissues, metabolized and excreted is called pharmacokinetics. Cytochrome P450 (CYP) system of enzymes is important to the pharmacokinetics of psychotropic drugs. These enzymes have higher concentration in liver but are also present in kidney and lungs. The antipsychotic drugs are most commonly administered orally. They can also be injected in some cases but this method is rare. Antipsychotic are highly protein and tissue-bound compound with large volume of distribution. After oral administration, the antipsychotics are easily absorbed from the gastrointestinal tract and can readily pass the blood brain barrier and placental wall. After reaching the brain, the antipsychotic make their way to the nerve cells and start its action by binding to the receptors. The antipsychotics are completely denatured during the metabolism and are eliminated through urine. Antipsychotics drugs have long half-lives. The half-life for each drug is different but the occupancy of the D₂ receptor falls off within 24 hours with second generation antipsychotics while for first generation, it lasting over 24 hours. Therefore, degeneration into psychosis occurs faster with second generation antipsychotics as compared to first generation antipsychotics, as the excretion of drug is increased and no longer functioning in the brain.

Therapeutic uses

Common disorders for which antipsychotics may be used are schizophrenia, bipolar disorders and delusional disorders. Antipsychotics are also used to balance certain brain chemicals called neurotransmitters. Atypical/second generation antipsychotics drugs are used for elderly patients having symptoms of psychosis and behavioral disturbances. These kinds of symptoms appear in Alzheimer's disease and Parkinson's disease. Risperidone and olanzapine are used for the psychosis treatment in patients with Alzheimer's disease and quetiapine and clozapine are used for the treatment of psychosis in patients with Parkinson's disease. With the increase in life expectancy in rich countries, the use of atypical antipsychotic drugs increases. According to the National

Institute of Health estimation, the number of American people having Alzheimer's disease will be 8.5 million up to 2030.

Typical/first generation antipsychotic drugs such as haloperidol have been used usually for the treatment of psychotic and behavior disturbances in aged patients, but they have some concerning side effects. The newer atypical antipsychotic drugs are better than the older drugs due to fewer side effects and improved efficiency in the treatment of the negative symptoms of psychosis.

Side effects of antipsychotics ^[34, 35]

Like all other medications, antipsychotic have also some side effects along with their beneficial effects. Side effects of antipsychotic drugs can be treated by changing the dose of the drug, moving to a different drugs or taking a surplus medications. In the initial stage of drug treatment, patients may be concerned by side effects like, restlessness, drowsiness, muscle spasms, tremor, dry mouth, or blurring of vision. Tardive dyskinesia (TD) is a condition characterized by involuntary movements affecting the mouth, tongue and lips and sometimes the trunks, arms and legs. About, 15-20% of patients who have been taking the first generation antipsychotic drugs for long time facing these problems but TD can also occur in patients who are receiving these drugs for shorter period of time.

The atypical antipsychotic drugs have fewer side effects as compared to typical. The atypical /second generation antipsychotic may cause weight gain, diabetes mellitus (DM). High dose of these drugs may also cause social withdrawal and symptoms like Parkinson's disease a condition that disturbs movements. The second generation antipsychotics (particularly olanzapine and clozapine) appear to cause weight gain more frequently than the first generation antipsychotics.

Brief introduction of drugs selected for this thesis ^[36-52]

1.1 Citalopram

Citalopram (CIT) is an orally administered second generation antidepressants, also referred to as a selective serotonin reuptake inhibitor. CIT is used for the treatment of

depression, social anxiety disorder, panic disorder, and obsessive compulsive disorder. It is a bicyclic phthalate derivative and is not related to the tricyclic or tetracyclic antidepressants. CIT has clinical efficacies comparable with those of classical tricyclic antidepressants, but lacks the adverse anticholinergic and cardiovascular effects commonly associated with these drugs. Furthermore, CIT prevents the transport of the neurotransmitter serotonin to nerve terminals and to other cells that accumulate serotonin. CIT is metabolized by cytochrome P enzyme to demethylated metabolites, desmethylcitalopram and didesmethylcitalopram. Desmethylcitalopram is its main metabolite and like CIT it also inhibits 5-HT reuptake, but its potency is considerably weaker.

1.1.1 Physical characteristics

Molecular formula $C_{20}H_{21}FN_2O$

Molecular mass 324.392 g/mol

Structure formula: The structural formula of citalopram is shown in figure 1.1

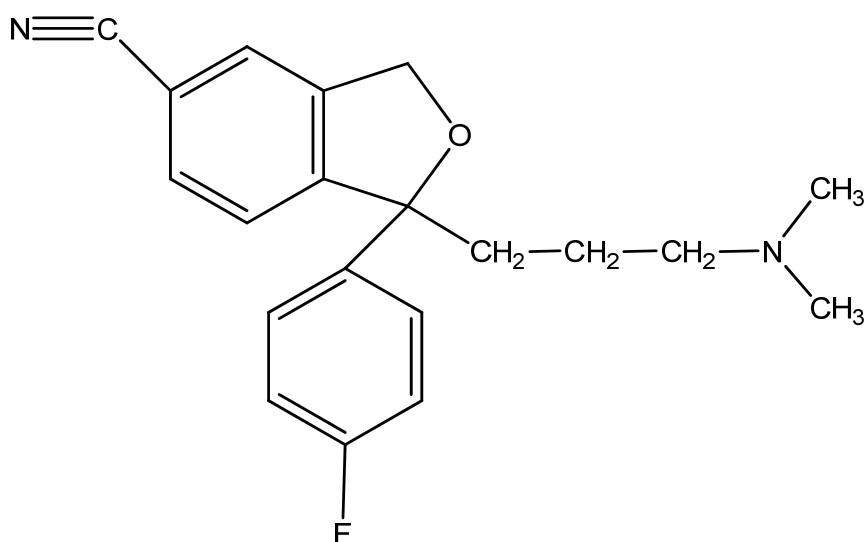


Fig 1.1 Structural formula of citalopram

Introduction

IUPAC name: 1-(3-dimethylaminopropyl)-1-(4-fluorophenyl)-1, 3-dihydroisobezofuran-5-carbonitrile

Appearance: Citalopram is white to off-white powder.

Melting point: 182-183 °C.

Solubility: sparingly soluble in water and highly soluble in ethanol.

1.1.2 Dosage and mode of administration

CIT is normally administered one dose of 20 mg with or without food. After one week the dose may be increased to 40 mg as 60 mg dose has not been proved to be more effective than 40 mg. For patients who are more than 60 years old, 20 mg once a day is the maximum dose advised. Children up to 11 years of age are initially given 5 to 10 mg per day which is increased gradually by 10 mg per day every two weeks. For 12 to 18 years, 10 to 20 mg per day given once a day is increased slowly by 10 mg per day every two weeks. In case of oral drop, the correct number of drops should be counted added into water or apple or orange juice, mixed well and then drink immediately. If a dose is missed, it should be taken as soon as remember and taking double dose for the missing one should always be avoided. Taking CIT should not be suddenly stopped, by doing this it can cause withdrawal symptoms like dizziness, sleep disturbances, headache, nausea, and feeling of weakness. Withdrawal symptoms can be avoided by stopping the drugs slowly over a period of weeks or months. CIT is available in 10, 20 and 40 mg of tablets and in oral drops (10 mgmL⁻¹).

1.1.3 Overdosage

Overdosage of CIT may cause vomiting, sweating, nausea, rapid heartbeat, sedation, confusion, dizziness, tremor, and coma. With overdosage, the blood or plasma citalopram concentration increases. Patients who are taking therapeutic dose of drug, their plasma or blood citalopram concentrations is in the range from 50 to 400 µgL⁻¹, with overdosage the concentration is 1000 to 3000 µgL⁻¹, and 3.0 to 30 mgL⁻¹ in those who do not survive.

1.1.4 Side effects

Nausea, dry mouth, vomiting, weight changes, drowsiness, numerous urination, diarrhea, excess sweating, fatigue, and trembling are the common side effects of citalopram. Less common side effects comprise changes in blood pressure, anxiety, sleep problem, headache, and dizziness. In case of sedation, the dose may be recommended at sleep time rather than in the morning.

1.2 Sulpiride

Sulpiride is the second generation antipsychotic drugs of the benzamide class commonly used in the handling of schizophrenia and foremost depressive syndrome. It is a selective dopamine D₂ antagonist with antipsychotic and antidepressant activity. Sulpiride has been widely applied in the treatment of depression, schizophrenia and psychopathology of senescence, gastric or duodenal ulcers, and various dizziness disorders. Dolmatil and Sulpor are brand name of sulpiride.

1.2.1 Physical characteristics

Molecular formula C₁₅H₂₃N₃O₄S

Molecular mass 341.427 g mol⁻¹

Structure formula; The structural formula of sulpiride is shown in figure 1.2

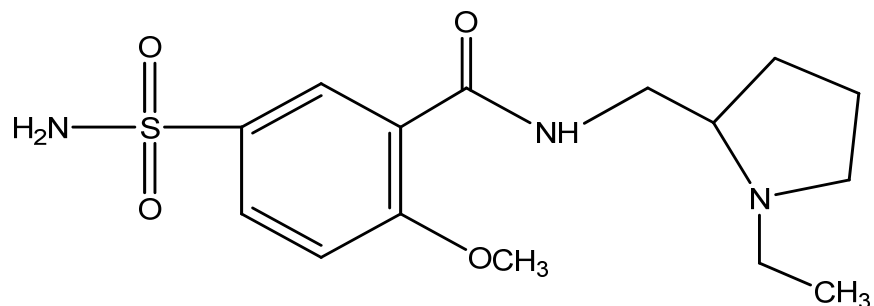


Fig 1.2 Structural formula of sulpiride

IUPAC name: 5-(aminosulfonyl)-N-[(1-ethylpyrrolidin-2-yl)methyl]-2-methoxybenzamide

Appearance; white crystalline powder

Melting point; 178 °C

Solubility; Sulpiride is insoluble in water, hardly soluble in ethanol, methanol, and acetone, easily soluble in acids.

1.2.2 Dosage and mode of administration

The recommended dose of sulpiride for schizophrenia is 200–400 mg orally twice a day. The dose may be slowly increased to a maximum of 1200 mg per day based on the clinical response. In severe schizophrenia, intramuscular doses of 600 to 800 mg have been recommended. For normal patient lower doses of sulpiride may be sufficient, however for chronic schizophrenia patients higher doses may be needed. Sulpiride is excreted renally, and in renal deficiency dose adjustment is recommended. Sulpiride is not recommended for children of under 14 years of age. The drugs are available in 50, 200 and 400 mg tablets.

1.2.3 Pharmacokinetics

Sulpiride can be gradually absorbed from the gastrointestinal tract. The peak serum level reaching in 2 to 6 hours. Sulpiride does not appear to digest entirely, designated by 70-90% of intravenous doses and 15-25% of oral doses being excreted unchanged in the urine. Excess amounts of sulpiride have also been excreted in feces. The usual half-life of sulpiride is 6 to 8 hours.

1.2.4 Overdosage

The interval of intoxication caused by the overdoses of sulpiride is short, and the symptoms disappearing within a few hours. Comas caused by the excess doses have lasted up to four days. The range of toxic dose of sulpiride is 1.0-16 g but no deaths have occurred even at a dose of 20 g. The toxicity caused by the overdosage of sulpiride may be varying depending upon the size of the dose taken. A single dose of 1.0-3.0 g causes restlessness and confusing of awareness, 3.0 - 7.0 g doses may cause anxiety, misperception and extrapyramidal symptoms. Dose of more than 7.0 g can cause coma and low blood pressure.

1.2.5 Side effect

Sulpiride is well tolerated and only minor side-effects are reported at the recommended doses. These include TD, hypertension, hypotension, restlessness, dry mouth, weight gain, sweating, nausea, blurred vision, and difficulties with micturition and constipation.

1.3 Fluoxetine

Fluoxetine is an oral antidepressant drug belongs to the class of selective serotonin reuptake inhibitor. Fluoxetine was first developed by researchers at Eli Lilly and company in 1974. Eli Lilly researchers found that fluoxetine acts as selective serotonin reuptake inhibitor and were effective as antidepressants. In 1977, it was presented to the U.S. FDA for approval. In 1987, the company received the final approval and marketed the drug with the brand name "Prozac" in the United States. In August 2001, fluoxetine went off-patent.

Fluoxetine is used for the treatment of major depressive disorder, bulimia nervosa, panic disorder, and obsessive-compulsive disorder.

1.3.1 Physical characteristics of Fluoxetine

Molecular formula $C_{17}H_{18}F_3NO$

Molecular mass $309.33 \text{ g mol}^{-1}$

Structure formula; The structural formula of fluoxetine is shown in figure 1.3

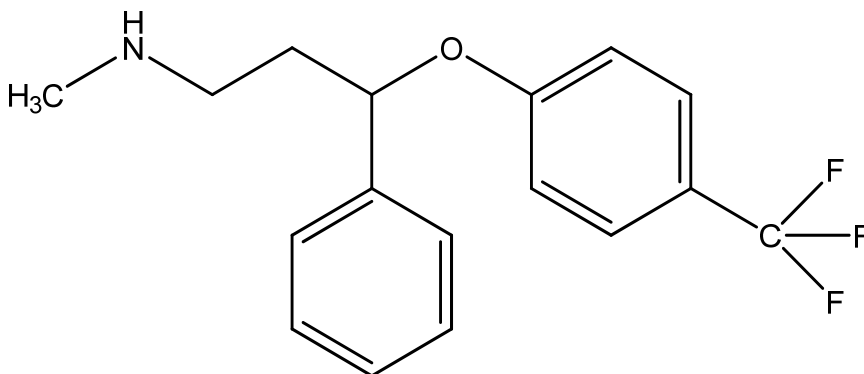


Fig 1.3 Structural formula of fluoxetine

IUPAC name: N-methyl-3-phenyl-3-[4-(trifluoromethyl) phenoxy] propane-1-amine

Appearance; white to off white crystalline powder

Melting point; $157.5\text{--}158.7^\circ\text{C}$

Solubility; Fluoxetine is soluble in water, methanol and chloroform, insoluble in hexane, ethyl acetate and benzene.

1.3.2 Dosage and mode of administration

Fluoxetine should be taken orally. The recommended initial dose of fluoxetine is 20 mg once a day in the morning or two times a day in the morning and at the noon. For adults the dose is 20–80 mg daily. In case of depression in children the recommended dose is

10–20 mg per day, while in bulimia the dose is 60 mg/day. In case of panic disorder it is 10 to 60 mg/day.

1.3.3 Pharmacokinetics

Fluoxetine is freely absorbed from the gastrointestinal tract with maximum plasma concentration happening from 6–8 hours after oral administration. Fluoxetine is broadly distributed throughout the body. Fluoxetine is absorbed in the liver by the cytochrome P450 and desmethyl metabolites norfluoxetine is produced. Norfluoxetine has similar activity as fluoxetine. Fluoxetine has half-life ranging from 1 to 4 days after a single dose while norfluoxetine has a half-life of 7 to 9 days.

1.3.4 Overdosage

The overdoses of fluoxetine may cause confusion, breathing problems, dizziness, hypertension, hypotension, fever, coma, irregular heart beat and nervousness.

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LITERATURE REVIEW

Mandrioli et al. (1) reported a spectrofluorimetric and a capillary zone electrophoretic method for the quantification of fluoxetine in commercial formulations. The excitation and emission wavelengths used for fluorimetric method were 230 nm and 293 nm respectively. An uncoated fused-silica capillary and phosphate buffer of pH 2.5 was used as the background electrolyte. Fluoxetine was extracted from the marketed capsules by single step dissolution with methanol/water followed by filtration and dilution. The results obtained by both methods were suitable in terms of precision. The electrophoretic method gave good results with RSD value less than 2.0%. The proposed methods were fast and accurate with percent recoveries between 97.50 and 102.6%.

Green et al. (2) described a precise and sensitive method for the quantification of fluoxetine and norfluoxetine in human plasma samples. Oasis HLB cartridges was used for the extraction of analyte from human plasma. Analysis was carried out on an Xterra MS C₁₈ column using a fast gradient by reversed phase liquid chromatography. Ionization of fluoxetine, norfluoxetine and the internal standard fluvoxamine were carried out by the Turbolonspray interface operating in positive mode. Detection was carried out through multiple reactions monitoring of the characteristic ion dissociation transition m/z 310.3→44.1 for fluoxetine, 296.2→134.3 for norfluoxetine and 319.2→71.1 for fluvoxamine. Using a sample volume of 0.5 mL, the calibration curve was linear in the concentration range of 0.5 to 50 ngmL⁻¹. The developed method is accurate and precise with intra-batch and inters batch precision (%CV) less than 15% and accuracy (%RE) less than ±15 for both fluoxetine and norfluoxetine. The developed method has been successfully applied for the clinical study.

El-dawy et al. (3) described an accurate, easy and sensitive HPLC method for the analysis of fluoxetine in pharmaceutical formulations, human plasma and in biological fluid. A reversed phase-C₁₈ column using a mixture of acetonitrile and triethylamine buffer (48:52 v/v) as a mobile phase with UV detection at 228 nm was used for the analysis. Pyridoxine was used as internal standard and a linear calibration plot was observed. The retention time for fluoxetine and pyridoxine were 3.20 and 2.10 min respectively. The method was used for the analysis of fluoxetine in spiked human plasma. A linear behavior between absorbance with fluoxetine concentration was observed in the range of

10 to 300 μgL^{-1} with limit of detection 5.0 μgL^{-1} . The percentage recovery for fluoxetine was found to be 97.99% with standard deviation of 2.39. The method was successfully used for the fluoxetine determination in human plasma sample after taking a single dose of one Prozac capsule.

Chiba et al. (4) described a column-switching HPLC technique with fluorescence detection for the simultaneous analysis of four benzamide type antipsychotic drugs: sulpiride, tiapride, sultopride and metoclopramide in human serum. The method involves the use of a TSKgel Super-OSD as an analytical column and a TSKgel G 2000SW as a pretreatment column. At the optimized experimental conditions, the four drugs were eluted at 18 minutes. The limits of detection ($S/N=3$) were found to be 1.0 ngmL^{-1} , 4.0 ngmL^{-1} , 2.0 ngmL^{-1} and 0.5 ngmL^{-1} for sulpiride, tiapride, sultopride and metoclopramide respectively. The proposed method was successfully used for the analysis of sulpiride in human serum samples.

Cho and Lee (5) developed a fast, selective and extremely sensitive reverse-phase HPLC technique for the analysis of levosulpiride in human urine and serum. In this method extraction with dichloromethane was carried out followed back extraction into 0.025 M sulfuric acid. A reversed-phase isocratic elution with a Luna C₁₈ (2) 5 μm column was utilized for HPLC analysis. A mixture of acetonitrile and 0.01M potassium hydrogen phosphate (30:70, v/v) was used as a mobile phase. The pH of mobile phase was adjusted to 8.5 with triethylamine. Excitation and emission wavelength are 300 nm and 365 nm respectively. The chromatogram obtained by the proposed method showed good sensitivity and resolution and there were no interferences of human serum and urine. For serum the calibration plot was rectilinear in the range of 0.25–200 ngmL^{-1} and for urine it was 0.2–20 ngmL^{-1} with good correlation coefficient greater than 0.997. The precision and accuracy of intraday and interday reading were agreed with the international requirements. The percentage recoveries obtained by the proposed method for human serum was $89.8 \pm 3.7\%$. The limit of quantification for human serum and urine were 0.25 ngmL^{-1} and 0.2 $\mu\text{g mL}^{-1}$ respectively. This lower limit of quantification was sensitive for pharmacokinetic study. The proposed method was used for the pharmacokinetic study of

levosulpiride in human volunteers after a single oral dose of 25 mg tablet of levosulpiride.

Jin et al. (6) described a fast and simple HPLC method for the assay of levosulpiride in human plasma samples. Levosulpiride was determined in human plasma after extraction with ethyl acetate/methylene chloride (5:1, v/v). A RP C₁₈ column with fluorescence detector (excitation wavelength 300 nm and emission wavelength 365 nm) was used for separation and quantification. The mobile phase used was consisting of methanol and 20 mM phosphate buffer pH 3.5 (16:84 v/v). The proposed method was found to be specific and sensitive with limit of quantification 5.0 ngmL⁻¹. The proposed HPLC method was evaluated by determining the precision and accuracy in the concentration range of 5.0–150 ngmL⁻¹. The RSD in inter and intra-day corroboration were found to be 8.16–19.75 and 3.90–11.69% respectively. The proposed method has been successfully used to the bioequivalence scrutiny of two levosulpiride in commercial formulations.

Paek et al. (7) described a fast, perceptive and selective hydrophilic interaction liquid chromatography-tandem mass spectrophotometric method for the analysis of levosulpiride in human plasma sample. An internal standard tiapiride and levosulpiride were extracted from human plasma with ethyl acetate (pH 11). The analysis was carried on an Atlantic HILICS silica column using acetonitrile-ammonium-formate (190 mM, pH 3) (94:6, v/v) the mobile phase. The calibration curve was linear in the range of 1.0–200 ngmL⁻¹ ($r = 0.999$). Using 100 μ L plasma samples, the lower quantification limit for levosulpiride was 1.0 ngmL⁻¹. The coefficient of variation and the relative error for intraday and interday analysis were -2.9 to -0.1% and 3.8 – 9.1% respectively. The percentage recoveries for levosulpiride ranged from 80.5 to 87.4%, with that of tiapiride (internal standard) being 84.6%. The developed method has been successfully used for the pharmacokinetic study of levosulpiride in humans.

Jin et al. (8) presented a validated HPLC method for the assay of levosulpiride in human plasma. Levosulpiride was extracted from plasma samples with ethylacetate and methyl chloride in the ratio of 5:1 v/v. Analysis was carried out on a RP C₁₈ column with fluorimetric detector (λ_{ex} 300 nm and λ_{em} 365 nm). Mobile phase used consisted of a mixture of methanol and 20 mM phosphate buffer of pH 3.5 (16:84 v/v). The

quantification limit was 5.0 ngmL^{-1} and the method was found to be specific and sensitive. The validity of the method was checked by the inter and intra-day determination of the drug in the concentration range of 5.0 to 150 ngmL^{-1} . The RSD values were found to be 8.16 to 19.75 and 3.90 to 11.69% for inter and intra-day analysis respectively. The method was used for the determination of two 25 mg levosulpiride tablet after a single oral dose.

Skibinski et al. (9) presented three simple, fast and accurate analytical methods for the determination of citalopram in commercial formulations. The first capillary zone electrophoresis (CZE) method was followed using fused silica capillary with UV detection at 239 nm, with 67 mM pH 7.0 phosphate buffer as the background electrolyte and 30 kV of separation voltage. In second direct spectrophotometric method the absorbance of the analyte was measured at 239 nm, and the third derivative spectrophotometric method involved the measuring of the value of the second derivative of the spectrum at 210 nm. The calibration curve was rectilinear over the concentration range of $5.0\text{--}50 \text{ }\mu\text{gmL}^{-1}$ for CZE method and $2.0\text{--}12 \text{ }\mu\text{gmL}^{-1}$ for spectrophotometric methods. The percent recovery for CZE was from 98.6% to 102.4% with RSD value from 1.36 to 2.30%. For direct spectrophotometry and in derivative spectrophotometry the values were found to be in the range of 95% to 98% and 97.6% to 103.3% with RSD from 1.80 to 2.70% and 1.52 to 2.68% respectively.

Valse et al. (10) presented a fast and HPLC technique for the separation of fluoxetine and norfluoxetine in human plasma samples. The paroxetine is used as an internal standard. The separation of these mixtures was carried out on C18 column. A mixture of acetonitrile and 40 mM KH_2PO_4 buffer of pH 2.3 (31:69 v/v) were used as a mobile phase. The fluoxetine and norfluoxetine were separated by fluorimetric detection at 230 nm as λ_{ex} and 312 nm as λ_{em} . The calibration curve was linear in the concentration range of 1.0 to 39 ngmL^{-1} for fluoxetine and $0.9\text{--}36 \text{ ngmL}^{-1}$ for norfluoxetine. The intraday and interday accuracy and precision ranges between 7.9 to 12.4 for fluoxetine and 0.7 to 14.7% for norfluoxetine. The method has been successfully used for the determination of these drugs in plasma samples got of healthy volunteers given a single oral dose of 40 mg fluoxetine.

LLerena et al. (11) reported a HPLC method for the analysis of most frequently used antidepressant drugs fluoxetine and norfluoxetine in human plasma. The fluoxetine and norfluoxetine were separated in a reversed phase column after liquid-liquid extraction. The ultraviolet detection at 226 nm was used for analysis. The effect of different interferences found in the psychoactive drugs was also investigated. The percent recoveries obtained by the proposed method for fluoxetine was 87% and for norfluoxetine it was 93%. The quantification limit obtained for both the drugs under the optimized condition was 14 nmolL^{-1} . Less than 10% coefficient of variation indicates reproducibility of the method. The plasma samples of 29 patients were analyzed and great variability in plasma concentration of fluoxetine and norfluoxetine as well as in fluoxetine/norfluoxetine ratio was found. The results obtained by the proposed method shows the implication of hereditarily polymeric enzymes, probably CYP2D6, CYP2C9 and CYP2C19 in the absorption of fluoxetine to norfluoxetine. Therapeutic drug observing should thus be beneficial in patients treated with fixed doses.

Darwish (12) reported three simple, sensitive and validated spectrophotometric methods for the assay of fluoxetine, sertraline, and paroxetine in marketed formulations. These methods involve the interaction of the free secondary amino group of the studied drugs with acetaldehyde to form N-alkylvinylamine. The N-alkylvinylamine then on reaction with chloranil, bromanil, and 2, 3-dichloronaphthoquinone form colored vinylamino-substituted quinones with λ_{max} at 665, 655, and 580 nm respectively. Various experimental parameters influencing the reaction were thoroughly studied and optimized. At optimized conditions, the calibration curves between the concentration and absorbance of the studied drugs were linear in the range of 4.0 to $120 \text{ } \mu\text{g mL}^{-1}$ with good correlation coefficients (0.9986 to 0.9999). The detection limits were found to be in the range of 1.19 to $2.98 \text{ } \mu\text{g mL}^{-1}$. The methods were precise and the RSD values were 0.56–1.24%. The developed methods were effectively used for the quantifications of the three drugs in pure form and in commercial formulations. The methods were accurate and the percentage recoveries obtained were in the range of 99.10 to 101.30% with standard deviation of 1.15 to 1.92%. The results of the proposed methods were satisfactorily compared with the reported methods.

Zayed (13) reported two methods for the simultaneous determination of sulpiride and mebeverine hydrochloride in their mixtures. The first method is based on the first derivative of the ratio spectra by evaluating the amplitude at 234.9 and 263.7 nm for sulpiride and mebeverine hydrochloride respectively. The limits of detection were found to be 0.34 and 0.72 $\mu\text{g mL}^{-1}$ for sulpiride and mebeverine hydrochloride respectively. The calibration curves are linear in the concentration range of 1.0 to 10 $\mu\text{g mL}^{-1}$ for sulpiride and 4.0 to 40 $\mu\text{g mL}^{-1}$ for mebeverine hydrochloride. The second method is based on a chemometric method in which the concentration of pure drugs was obtained in 0.1 M hydrochloric acid. The absorbance data was obtained by evaluating the absorbance in the range of 200 to 300 nm in their zero order spectra. The calibration curve was plotted by using the absorbance data and the concentration data for the calculation of the unknown concentration of sulpiride and mebeverine hydrochloride in their mixtures. For calculation of numerical values, Matlab R 12 version 6 and Origin 5 software were used. No separation steps are required in this procedure. The proposed methods were effectively used for the analysis of pharmaceutical formulation of Colona tablets.

Juan et al. (14) developed a novel, fast and sensitive HPLC-electrospray ionization mass spectrometry technique for the simultaneous determination and screening of four most frequently recommended nontricyclic antidepressants: citalopram, fluoxetine, paroxetine and venlafaxine in human plasma. The samples were first made alkaline and then the analytes were extracted by solid-phase-extraction column. The HPLC separation of the analytes was carried out on a MACHEREY-NAGEL C₁₈ (250 mm $\times$ 4.6 mm, 5 μm , Germany) column. The mobile phase used consisted of water (formic acid 0.6%, ammonium acetate: 30 mmolL⁻¹)-acetonitrile (35:65 v/v). The flow rate was 0.85 mL min⁻¹. The electrospray ionization ion source of the mass spectrometer was used for the ionization of the compounds and detection was done in the selected ion recording mode. The calibration plots were linear in the range of 5.0 to 1000 ngmL⁻¹ for all the analytes. The correlation coefficient for all the analytes were found to be higher than 0.990. The average extraction recovery for all the analytes was higher than 73.2%. The recoveries obtained by the proposed method were greater than 95%. The detection limits for citalopram, fluoxetine, paroxetine and venlafaxine were found to be 0.3, 0.5, 0.3 and 0.1 ngmL⁻¹ respectively. The intra and inter-day variation coefficients were less than 15%.

The method was found to be accurate, sensitive, and simple and can be used for routine analysis of drugs as toxicological screening for the pharmacokinetics and metabolism of the studied drugs.

Koo et al. (15) presented a validated analytical method for the quantification of levosulpiride in human plasma. The levosulpiride was extracted by a two-step procedure from plasma sample. An aliquot of the aqueous phase was then injected into a HPLC system connected with a fluorimetric detector. The detector response was linear in the concentration range of 2.5–500 ngmL⁻¹. The intra and inter day precision was found to be less than 15.4 and 10.1% respectively. The accuracy of the method was in the range of 89.7– 109.4%. The method was applied for the pharmacokinetic characterization of levosulpiride after a 75 mg oral dose in humans.

Rao et al. (16) described a simple, fast and robust liquid chromatographic technique for enantiospecific separation and determination of citalopram in pure form and commercial formulations. UV and polarimetric detectors coupled in series were used. Baseline separation with resolution ≥ 3.0 was attained within 20 minutes on Chiralcel OD-H (250 mm $\times$ 4.6 mm) 5 μ m column. The mobile phase used consisted of n-hexane: 2-propanol: triethylamine (95: 05: 0.1 v/v/v) with a flow rate of 1 mL min⁻¹ at 25 °C. The effect of 2-propanol, triethylamine and temperature on enantioselectivity and enantiomers resolution was studied. For quantitative determination of citalopram, clopidogrel hydrogen sulphate was used as an internal standard with UV detector at 240 nm. For enantiomer identification, polarimetric detector was used. The detection and quantification limits for both the enantiomers were attained to be 0.5 and 1.3 μ gmL⁻¹ respectively. A linear behavior was observed in the range of 50 to 600 μ gmL⁻¹ with correlation coefficient greater than 0.9999. The precision of the method was checked by inter and intra-day tests. The percent recoveries were found to be 99.38 to 100.41% with RSD value less than 0.63%. The method was validated statistically and found to be fit for determination of enantiomeric purity of citalopram in bulk powder and pharmaceutical preparations.

Afkhami et al. (17) proposed a simple, fast and accurate batch and flow injection spectrophotometric methods for the analysis of fluoxetine. Fluoxetine through

competitive complexation reaction form a colored complex with phenolphthalein- β -cyclodextrin inclusion complex. The absorbance of the complex increased with increase in fluoxetine concentration and was measured at 554 nm. By non-linear least square fitting, the formation constant for fluoxetine- β -CD was calculated. The calibration curves were linear in the concentration range of 7.0×10^{-6} to 2.4×10^{-4} molL⁻¹ and 5.0×10^{-5} to 1.0×10^{-2} molL⁻¹ for batch and flow method respectively. The detection and quantification limit for batch method was found to be 4.13×10^{-6} molL⁻¹ and 1.38×10^{-5} molL⁻¹ respectively, while for flow method it was found to be 2.46×10^{-5} molL⁻¹ and 8.22×10^{-5} molL⁻¹ respectively. The sampling rate in FIA method was 80 ± 5 samples per hour. The method was employed for fluoxetine analysis in pharmaceutical formulations and human urine samples.

El-Sherbiny DT (18) reported a micellar enhanced spectrofluorimetric method for the determination of citalopram in commercial formulations and spiked human plasma. The citalopram is native fluorescent and its fluorescence intensity was measured in citrate buffer (pH 6.5) alone and with micellar system using beta cyclodextrin and sodium dodecyl sulfate (SDS) as fluorescence enhancers at λ_{em} 305 nm and λ_{ex} 242 nm. The effects of ionic and nonionic surfactants were studied by evaluating the fluorescence intensity of analyte-surfactants system. Citalopram shows maximum fluorescence intensity in CDs and SDS micelles in slightly acidic aqueous solution (pH 6.5). The fluorescence intensity of citalopram was greatly enhanced with SDS. The fluorescence intensity was enhanced 125% with CDs medium and 250% in SDS medium as compared to slightly acidic aqueous solution. The calibration curves were rectilinear over the concentration range of 0.06–0.64, 0.04–0.40, and 0.02–0.26 μ gmL⁻¹ in citrate buffer only, for beta-CDs and SDS respectively. The limit of detection for citrate buffer, for β -CDs and SDS were found to be 0.02, 0.01 and 0.007 μ gmL⁻¹ respectively. The high sensitivity of the method using SDS as organized medium allowed it for the analysis of citalopram in spiked human plasma. Interferences effect from endogenous amino acids was studied and has been eliminated by solid-phase extraction procedure. The mean percent recovery was found to be $100.1 \pm 0.8\%$ ($n = 5$).

Satana et al. (19) reported a capillary electrophoresis method for the analysis of citalopram. Separation of compounds were carried out at 28 kV in 75 μ m i.d. fused silica capillary tubing total length 85 cm and effective length 65 cm. Borate buffer (10 mM) of pH 8.5, comprising 10% v/v methanol was used as running buffer. Propylparaben was used as an internal standard. The retention time for citalopram and propylparaben were 3.5 and 5.5 minutes respectively. The results were linear and reproducible. The LOD and LOQ were found to be 5.73×10^{-6} and 1.72×10^{-5} M respectively. Citalopram was also determined by a UV-spectrophotometric method. There were no significant differences between the results of the two methods. The percentage recovery obtained for citalopram content in pharmaceutical preparation was $100.8 \pm 2.95\%$. The method was also applied for the quantification of citalopram in serum and $26.7 \pm 0.1\%$ of the free drug was found showing that 73.3% of the drug was bound to protein.

Li et al. (20) presented a simple and sensitive method for the simultaneous determination of sulpiride and tiapride in human urine samples. The method is based on capillary electrophoresis connected with electrochemiluminescence detection by end column approach. β -cyclodextrin was added as a functioning buffer for complete separation of sulpiride and tiapride. All the experimental condition were studied and optimized. The method showed a good linear range from 1.0×10^{-7} to 1.0×10^{-4} for both sulpiride and tiapride with correlation coefficient greater than 0.995 ($n=6$). The limit of detection for sulpiride and tiapride were 1.0×10^{-8} M and 1.5×10^{-8} M at a signal to noise ratio of 3 respectively. For six replicate of 2.0 μ M sulpiride and 3.0 μ M tiapride the relative standard deviation were 1.8 and 2.5% respectively. To get rid of the effect of ionic strength in sample, an extraction step was achieved with ethyl acetate at pH 11. The percentage recoveries of sulpiride and tiapride in human urine were found between 84 and 95%. The recoveries result shows that the developed method was suitable for the determination of sulpiride and tiapride in clinical as well as biochemical laboratories.

Raza (21) presented two fast, simple and sensitive spectrophotometric techniques for the quantification of citalopram hydrobromide in pharmaceutical preparations. Method A is based on the direct UV spectrophotometric determination of citalopram hydrobromide in tablets in methanol solvent at 240 nm. A linear range of concentration of citalopram

versus absorbance was found from 5.0–40 $\mu\text{g mL}^{-1}$. The molar absorptivity was $1.4 \times 10^4 \text{ L mol}^{-1} \text{ cm}^{-1}$. In method B, the citalopram react as n-electron donor with 2, 3-dichloro-5, 6-dicyano-1, 4-benzoquinone as π -acceptor to form a highly colored complex which gives maximum absorbance at 540 nm. Beer's law range was 10–250 $\mu\text{g mL}^{-1}$ with molar absorptivity of $3.3 \times 10^3 \text{ L mol}^{-1} \text{ cm}^{-1}$. The detection and quantification limit was found to be 5.2 $\mu\text{g mL}^{-1}$ and 17.4 $\mu\text{g mL}^{-1}$ respectively. The reported methods can be applied for the measurement of citalopram hydrobromide in pharmaceutical dosage form and were free from interferences.

Greiner et al. (22) developed a column switching HPLC method for enumerative determination of (es-) citalopram and desmethyl (es-) citalopram in serum. The serum was inoculated on the top of a LiChrospher CN 20 μm precolumn for sample clean up with a solvent of 8% acetonitrile using deionized water. Drugs were eluted on LiChrospher CN 5 μm column by back flush stream. Phosphate buffer (8 mmol/l) having pH 6.4 with acetonitrile (50/50, v/v) were used at a flow rate of 1.5 mL min^{-1} . The internal standard used was Haloperidol. Ultraviolet spectrophotometry at 210 nm was used for determination of analyte. The limit of detection for (es-) citalopram was 6.0 ng mL^{-1} . The developed method was found to be fit for therapeutic drug examining in patients treated with citalopram or escitalopram.

Satana et al. (23) reported a fluorimetric method for the direct determination of citalopram in pharmaceutical preparations. The method has the advantage of simplicity, selectivity and sensitivity. Standard citalopram and commercial formulations were dissolved in distilled water and fluorescence emission intensity was measured at 300 nm after excitation at 240 nm. The effect of concentration on fluorescence intensity was found to be linear from 5.0×10^{-7} to $2.5 \times 10^{-6} \text{ M}$. LOD and LOQ were found to be 8.0×10^{-9} and $2.6 \times 10^{-8} \text{ M}$ respectively. The method was effectively applied for the determination of citalopram in dosage form. The percent recoveries ranged from 100.50–101.30%. No interference effect was observed from common pharmaceutical excipients. The results of the proposed method were compared with established UV-VIS spectrophotometric method and no significance difference was observed between the two in terms of precision and accuracy.

Satana et al. (24) reported a flow injection-solid phase extraction method using spectrofluorimetric detection for the determination of citalopram in commercial formulations and spiked human serum. The solid phase extraction cartridges contain 200 mg C18 with a particle size of 45 μm . The most suitable solvent for elution was ethanol and 0.1 M acetic acid (40:60, v/v). The optimized injection volume was 1.0 mL with a flow rate of 2.5 mL min⁻¹. The excitation and emission wavelengths were 240 and 300 nm. Under the optimized condition, the calibration curves were linear in the concentration range of 1.0×10^{-7} to 1.5×10^{-6} M in aqueous solution and 2.5×10^{-7} to 1.2×10^{-6} M in serum. The limit of detection and quantification values for citalopram in tablets were 1.6×10^{-8} and 5.2×10^{-8} M respectively and in serum were 1.7×10^{-8} and 5.5×10^{-8} M respectively. The developed method has been effectively used for the quantification of citalopram HBr in pharmaceutical tablets and in spiked human serum with percentage recoveries of 99.5–100.6% and 98.8–100.9% respectively.

Nie et al. (25) described a novel but applicable method for the determination of antipsychotic drug sulpiride in human urine. The method is based on the assimilation of excitation-emission matrix fluorescence and second order calibration method based on the AFR and SWATLD algorithms. The proposed method can be used for the direct analysis of sulpiride using a pretreatment step. There is no effect of the natural fluorescence interferences. By using AFR and SWATLD the average percentage recoveries for triplicate were found 101.2 ± 2.1 and $94.4 \pm 0.7\%$ respectively. Furthermore the accuracy of the two algorithms was checked by elliptical joint confidence region tests and analytical figure of merit such as selectivity, sensitivity and detection limit. The results of the proposed method showed that the method could be successfully applied for the determination of sulpiride in human urine sample, but AFR was slightly better than SWATLD.

Vasantharaju et al. (26) presented a spectrofluorimetric method for the determination of citalopram in pure form and pharmaceutical formulations. The method is simple, accurate, sensitive, reproducible and based on native fluorescence of citalopram in the presence of 0.05 M sulfuric acid. The λ_{ex} and λ_{em} are 239 nm and 300 nm respectively. All experimental conditions such as the effect of pH, temperature, reaction time,

dilutions, different solvent, and the effect of commonly found excipients were studied. Under the optimized conditions, the calibration of concentration of citalopram verses fluorescence intensity was linear in the range of 0.10 to 0.90 $\mu\text{g mL}^{-1}$. The method was validated and applied for the determination of citalopram in commercial dosage form, with percentage recovery of 99.08% to 99.28%.

In another paper Raza et al. (27) presented a spectrophotometric determination of citalopram hydrobromide in pure form and pharmaceutical formulations. The citalopram was extracted from commercial formulations with chloroform in alkaline medium. The extracted citalopram react with 7, 7, 8, 8-tetracyanoquinodimethane (TCNQ) as π -acceptor and as a result a colored complex was formed which shows maximum absorbance at 570 nm. Beer's law range was 8.0–240 $\mu\text{g mL}^{-1}$ with molar absorptivity of $4.2 \times 10^3 \text{ L mol}^{-1} \text{ cm}^{-1}$. Different experimental conditions were optimized. The results of the proposed method were statistically validated. The method is fast, sensitive, precise and accurate for the determination of citalopram hydrobromide in dosage forms. The effect of commonly used excipient was also studied and no interferences effect was observed.

Musenga et al. (28) investigated a capillary electrophoretic method for the enantioselective determination of amisulpiride in pharmaceutical preparations using β -cyclodextrin sulfate as a chiral selector. Different experimental conditions such as pH and concentration of buffer, type and concentration of cyclodextrin and capillary temperature were studied and optimized. Using a fused silica capillary (50 μm i.d. and 33, 8.5 cm, total and effective length respectively) a baseline enantioseparation of the racemic compound was obtained in less than 10 minutes. The background electrolyte used consist of 10 mM citrate buffer at pH 3.5 along with 0.22% (w/v) β -cyclodextrin sulfate at 20 °C and the applied voltage is +15 kV. The commercial formulations of amisulpiride were determined after extraction of analyte with methanol. The developed method was statistically validated. The results of the proposed method shows good precision, accuracy, selectivity and the quantity of drugs found shows good agreement with the label value. The proposed method has been successfully applied for the enantiomeric determination of amisulpiride in pharmaceutical preparations.

Fernandes et al. (29) presented a technique using stir bar sorptive extraction with in situ derivatization, in design with either thermal or liquid desorption online coupled to gas chromatography-mass spectrometry for the analysis of fluoxetine in plasma samples. Derivatizing agent used was ethylchloro formate generating symmetrical peaks. Different experimental conditions such as extraction time, polarity of solvent and time for analyte desorption were investigated. The proposed method was validated and shows specificity with linear correlation coefficient greater than 0.99. The method is precise with RSD value less than 15% and quantification limit of 30 and 1.37 pgmL⁻¹ for liquid and thermal desorption respectively. The developed method is simple, sensitive and should be used for the determination of fluoxetine in typical and trace concentration levels.

Shah et al. (30) described a flow injection spectrophotometric method for the analysis of fluoxetine in pharmaceutical preparations. Fluoxetine was hydrolyzed in basic medium and the resultant methylamine product was reacted with sodium nitroprusside. The colored product formed was monitored at 510 nm by flow injection method using UV/visible detector. Linearity was observed in the range of 0.5 to 25 µgmL⁻¹ with molar absorptivity of 2.19×10^4 . The percentage recoveries obtained for the analysis of fluoxetine in commercial formulation was calculated to be 99.70 ± 0.18 to 99.40 ± 0.22 . The detection and quantification limit was found to be 0.15 ± 0.01 mgmL⁻¹ and 0.29 ± 0.03 mgmL⁻¹ respectively. The method was effectively used for the determination of fluoxetine in pharmaceutical capsules and tablets.

Flores et al. (31) reported a novel micellar electrokinetic chromatographic technique for the analysis of mixture of breast cancer handling drug letrozole and antidepressant drug citalopram and their chief metabolites in human urine samples. Borate buffer (15 mM) of pH 9.2 comprising 20 mM SDS were used to achieve good results with 12% v/v 2-propanol as a background electrolyte. The drugs were separated through a fused silica capillary at 40 °C by using 6s (3.45 kPa) of hydrodynamic injection and 30 kV of separation voltage. Under these conditions, the migration times for the studied compounds were found to be between 3.8 and 8 minute. The curve was linear in the range of 0.4–5.0 µgmL⁻¹ for all compounds. Limit of detection for urine samples were found between 12.5 and 25 ngmL⁻¹. The method was validated and found to be accurate,

precise, specific, sensitive, rugged and robust. The developed method has been successfully applied for the analysis of letrozole, citalopram and their metabolites in human urine at clinical laboratories. Before analysis of these compounds, an extraction-preconcentration step was used for sample purification and enrichment. For this purpose, a preconditioned C₁₈ cartridge and methanol was used as an elution. The proposed method was used for the analysis of these drugs in three urine samples taken from patients treated with letrozole or citalopram.

Sartori et al. (32) presented a simple conductometric titration of fluoxetine hydrochloride in commercial formulations using silver nitrate as titrant. The chloride of the HCl of the fluoxetine molecule form an insoluble silver chloride salt with silver nitrate. The method is fast, precise and economical. Three samples were analyzed by the proposed method and the percent recoveries ranged from 95.30 to 105%. The proposed method has been compared with an approved method and the t-test revealed that the results of the two methods were found in good agreement at the 95% confidence level.

Darwish et al. (33) presented novel, easy and sensitive spectrophotometric and spectrofluorimetric techniques for the analysis of fluoxetine hydrochloride in commercial formulations. In method A, fluoxetine react with 1, 2-naphthaquinone-4-sulphonoate in basic medium of pH 11 giving a colored product which is monitored spectrophotometrically at 490 nm. In method B, fluoxetine react with 4-chloro-7-nitrobenzo-2-oxa-1, 3-diazole in basic medium (pH 8.0) and form a fluorescent product which measured spectrofluorimetrically at 545 nm after excitation at 490 nm. Different experimental parameters influencing the reaction of fluoxetine with 4-chloro-7-nitrobenzo-2-oxa-1,3-diazole and 1, 2-naphthaquinone-4-sulphonoate were thoroughly investigated. Under the optimized experimental parameters, a good linearity was found between the reading and concentration of fluoxetine in the range of 0.3 to 6.0 µg mL⁻¹ for method A and 0.035 to 0.5 µg mL⁻¹ for method B. The detection limit for method A and method B were found to be 0.1 and 0.01 µg mL⁻¹ respectively. Both the methods have been effectively used for the analysis of fluoxetine in pharmaceutical preparations.

Franceschi et al. (34) presented a fast, sensitive and selective method for the simultaneous determination of fluoxetine and its principal active metabolites in human

serum samples. The method is based on RP-HPLC linked with MS/MS detection. For analytes extraction, a simple three step liquid-liquid procedure was followed. Analytes were separated by using C18 column. Due to sensitivity and low quantification limit, the proposed HPLC/MS/MS technique was used for the routine analysis of drug and for the studies of pharmacokinetic.

Zayed (35) reported two easy, precise and sensitive spectrophotometric methods for the analysis of sulpiride in commercial formulations. These techniques are based on the charge transfer complexation reaction between sulpiride and p-chloranilic acid as π acceptor or iodine as σ -acceptor. The colored complexes formed give maximum absorbance at 518 nm for p-chloranilic acid and 363, 294 nm for iodine method. The calibration plots were linear in the concentration range of 13.7 to 341.4 $\mu\text{g mL}^{-1}$ for p-chloranilic acid and 1.7 to 20.5 $\mu\text{g mL}^{-1}$ for iodine method. The proposed method has been used for the analysis of sulpiride in commercial formulations (Dogmatil Fort tablets). The results of the proposed method were compared with the official method. By using Benesi-Hildebrand plots, the complex association constant and standard free energy changes were calculated.

Abdelal et al. (36) reported a fast, simple and perceptive synchronous fluorimetric and second derivative synchronous fluorimetric method for the simultaneous determination of sulpiride (SLP) and its alkaline degradation product (DSLP). The method is based on the assay of the synchronous fluorescence of sulpiride and its degradation product using borate buffer of pH 8 at $\Delta\lambda$ of 45 nm. The peak amplitude (^2D) was measured at 295.5 and 342 nm respectively. Different experimental condition influencing the synchronous fluorescence intensity of SLP and DSLP were examined and optimized. The calibration curves for SLP and DSLP were linear over the concentration range of 0.05–1.0 and 2.0–10 $\mu\text{g mL}^{-1}$ respectively. The detection limit for SLP and DSLP were 0.02 and 0.40 $\mu\text{g mL}^{-1}$ and limit of quantification were 0.05 and 1.2 $\mu\text{g mL}^{-1}$ respectively. The developed method has been used for the determination of SLP and DSLP in pharmaceutical capsules and tablets. The results of the proposed method were statistically compared with those obtained by the official method and good agreement was found between them. The method was employed for the kinetic study of the alkaline induced degradation of the

drug. The method was also used for the assay of sulpiride in vivo and in vitro. The percentage recoveries for spiked and real human plasma for triplicate analysis were found to be 100 ± 2.04 and 92 ± 3.0 respectively.

Taha et al. (37) reported two sensitive methods for the assay of a racemic mixture of citalopram and its enantiomer S-(+) escitalopram. In first method, direct intrinsic fluorescence was measured by using sodium dodecyl sulfate as micelle enhancer. The proposed method was also used for the determination of escitalopram in spiked human plasma. The effect of common co-administered drugs was also studied. The second TLC densitometric method developed was based on numerous chiral selectors. Under the optimum conditions, the TLC method was found to be sensitive and selective for identification and quantitative determination of enantiomeric purity of escitalopram in drugs and drug products. The method can be used to analyze contamination of pure isomers with the cheap racemic form.

Naryana et al. (38) described two fast and sensitive spectrophotometric methods for the quantitative determination of citalopram in pure form and commercial formulations. In method A, citalopram is oxidized by excess of bromate bromide mixture in acidic medium. Known amount of Fe (II) reduces the residual oxidant. The Iron (III) then forms an iron (III)-thiocyanate-complex which measured at 480 nm. Beer's law range was 1.0–7.0 $\mu\text{g mL}^{-1}$ for citalopram. The molar absorptivity and sandell's sensitivity was found to be $2.10 \times 10^4 \text{ L mol}^{-1} \text{ cm}^{-1}$ and $0.019 \mu\text{g cm}^{-2}$ respectively. In method B, a complexing agent 1, 10-phenanthroline was used which results in the formation of Fe (II)-1, 10-phenanthroline complex having λ_{max} at 510 nm. Beer's law range was observed to be 0.6 to 6.2 $\mu\text{g mL}^{-1}$ with molar absorptivity $7.30 \times 10^4 \text{ L mol}^{-1} \text{ cm}^{-1}$ and sandell's sensitivity is $5.5 \times 10^{-3} \mu\text{g cm}^{-2}$. The effect of commonly found excipients was also studied for both methods and no interferences were observed from these excipients. The relative standard deviation value is less than 2.0% which shows good precision of the two methods. Both methods have been successfully used for the measurement of citalopram hydrobromide in pure form and in pharmaceutical preparations.

Walash et al. (39) described a simple and very sensitive first derivative synchronous spectrofluorimetric method for the simultaneous determination of mixture of sulpiride

(SUL) and mebeverine hydrochloride (MEB). The method is based on the measurement of the synchronous fluorescence intensity of SUL and MEB in water at $\Delta\lambda = 100$ nm. Different experimental conditions altering the fluorescence intensity of SUL and MEB were investigated. The calibration curves were linear at optimum conditions over the concentration range of $0.05\text{--}1.0\ \mu\text{g mL}^{-1}$ for SUL and $0.2\text{--}3.2\ \mu\text{g mL}^{-1}$ for MEB. The LOD and LOQ for sulpiride were found to be 0.006 and $0.02\ \mu\text{g mL}^{-1}$ respectively and for that of mebeverine hydrochloride is 0.01 and $0.05\ \mu\text{g mL}^{-1}$ respectively. The method has been effectively used for the analysis of the two drugs in synthetic mixtures and in pharmaceutical tablets. The method has also been used for the investigation of both SUL and MEB in real human plasma due to its high sensitivity and the percentage recoveries for replicate were found to be 91.36 ± 8.46 and 91.49 ± 4.25 respectively. For spiked human plasma the percentage recoveries for both MEB metabolite and SUL were 99.82 ± 2.53 and 98.84 ± 6.20 respectively.

Naguib et al. (40) developed a highly selective and validated sensitive stability signifying technique for simultaneous analysis of SULP and MEB. The method is also used to determine these compounds even in the presence of their described impurities and hydrolytic degradation in pure form and in commercial formulations. The first method used for the analysis of these drugs is HPLC with UV detection at 221 nm, by which the SULP and MEB mixtures are separated. Metopimazine is used as an internal standard. The mobile phase consists of acetonitrile and water (70:30 v/v) adjusted to pH 7.0. The method shows a rectilinear range of $5.0\text{--}40\ \mu\text{g mL}^{-1}$ for sulpiride and $5.0\text{--}60\ \mu\text{g mL}^{-1}$ for mebeverine hydrochloride. The mean percentage recoveries obtained by the HPLC method for sulpiride and mebeverine hydrochloride were 99.75% (SD 0.910) and 99.99% (SD 0.450) respectively. The second method is HPTLC where silica gel HPTLC F₂₅₄ plates were used for the separation of sulpiride and mebeverine hydrochloride with mobile phase consisting of absolute ethanol : methylene chloride: triethylamine (7:3:0.2 by volume) and separated bands are scanned at 221 nm. The calibration curves are linear over the concentration range of $0.4\text{--}1.4$ and $0.2\text{--}1.6\ \mu\text{g mL}^{-1}$ for SULP and MEB respectively. The average percentage recoveries obtained for sulpiride and mebeverine hydrochloride were 101.01 % (SD 1.991) and 100.44% (SD 1.868) respectively.

Lin et al. (41) described a sensitive and selective liquid chromatography-mass spectrometry technique for sulpiride determination in rabbit plasma. The analyte drug and internal standard from plasma were extracted by liquid-liquid extraction with ethyl acetate. The mobile phase consists of 0.2% formic acid in water and acetonitrile (60:40, v/v). Positive ion mode electrospray ionization was used for detection. The calibration curve was linear over the range of 10–2000 ngmL⁻¹ for sulpiride. The quantification limit was found to be 10 ngmL⁻¹. The inter and intra-day precisions were also checked and were found less than 12%. The method was accurate with percent recoveries from 94.1 to 108.7% for sulpiride. The proposed method was effectively used for the analysis of sulpiride in rabbit plasma for pharmacokinetic study.

Samanta et al. (42) described a RP-HPLC approach for the determination of Escitalopram in bulk and in pharmaceutical preparations. The method is easy, accurate and precise. The mobile phase consists of phosphate buffer of pH 7 and a mixture of organic solvent (methanol and acetonitrile in 1:1 v/v). The mobile phase was made by mixing buffer solution and mixture of organic solvent in the ratio of 55:45 v/v respectively. Analysis was carried out on 150 mm × 4.6 mm Xterra RP 18, 5µm keeping flow rate of 1.2 mL min⁻¹ with UV detection at 238 nm. 10 µL volumes were used for injection with run time of 10 minutes. The proposed method is an attempt to develop a HPLC method for the determination of Escitalopram in bulk and in pharmaceutical preparations.

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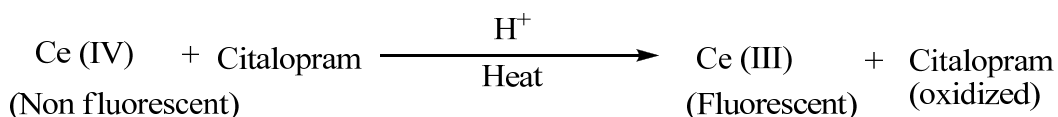
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EXPERIMENTAL
RESULTS and DISCUSSION

3.1 SPECTROFLUORIMETRIC METHOD FOR QUANTIFICATION OF CITALOPRAM IN PHARMACEUTICAL PREPARATIONS AND BIOLOGICAL FLUIDS THROUGH OXIDATION WITH Ce (IV)

3.1.1 Method development strategy for the determination of citalopram by spectrofluorimetric method

Redox reactions have been widely used as a base for the development of a simple and sensitive spectrofluorimetric method for the determination of many therapeutic drugs. Ce (IV) has high oxidation potential and has been commonly used as an efficient oxidizing reagent for the analysis of pharmaceutical drugs. Ce (IV) solution in acidic medium is non-fluorescent while its reduced form Ce (III) is fluorescent. Citalopram is non-fluorescent and could not be determined directly by spectrofluorimetry. Citalopram on reaction with Ce (IV) in acidic medium oxidized and at the same time reduces Ce (IV) to Ce (III). This reaction could be used for the indirect fluorimetric determination of citalopram in pharmaceutical preparation and biological fluids. The probable redox reaction of citalopram with Ce (IV) is presented below.



3.1.2 Preliminary investigation of the probability of redox reaction of citalopram with Ce (IV)

Preliminary studies were performed to investigate the probable oxidation of citalopram by Ce (IV) in acidic medium which led to the docolourization of the reagent solution. Initially high concentration of citalopram (10 µgmL⁻¹), Ce (IV) (100 µgmL⁻¹) and high concentration of sulfuric acid (4.0 M) were mixed in a conical flask and heated on a boiling water bath for 30 minutes to confirm the redox reaction. Yellow color of the Ce (IV) solution vanished representing the reduction of Ce (IV) to Ce (III) and the possibility of its consequent indirect spectrofluorimetric determination of citalopram. Therefore, further studies were focused on optimization of various parameters proceeding the oxidation of citalopram and are presented below.

3.1.3 Optimization of experimental parameters for spectrofluorimetric determination of citalopram using Ce (IV)

After the preliminary investigation, it was obvious that citalopram could be oxidized by Ce (IV) and reduced to Ce (III) which is fluorescent active and its fluorescent intensity could be measured with spectrofluorometer. For quantitative determination of citalopram different experimental parameters of the method were optimized.

Instruments

Fluorescence intensities were measured on a RF-5301 PC Spectrofluorophotometer (Shimadzu Japan), equipped with 150 W Xenon lamp, excitation, emission grating monochromators and 1×1 cm quartz cell. Digital analytical balance (Germany), electric thermostatic water bath (Yu Jia China) and a clinical centrifuge (Model 800, China) for centrifugation of plasma and urine samples.

Reagents

Analytical grade purity reagents were used. Ammonium cerium (IV) sulfate dihydrate (reagent grade ACS, Scharlau Barcelona Spain), sulfuric acid (95-98% Merck Darmstadt, Germany). Standard reference citalopram was provided by Z-Jan's Pharmaceutical Industry, Industrial Estate Hayatabad Peshawar, Pakistan. Commercial formulations of citalopram, Citalo, Promcit, Lopram (all as 20 mg tablets) were purchased locally.

Reagents solution preparation

100 $\mu\text{g mL}^{-1}$ of Ce (IV) fresh solution was prepared daily by dissolving 0.0225 g of ammonium cerium (IV) sulfate dihydrate in 4.0 M sulfuric acid with constant shaking and diluted to 50 mL with the same solvent. Sulfuric acid solution (4.0 M) was prepared by diluting 21.792 mL of concentrated sulfuric acid (95–98%) to 100 mL with distilled water.

Standard citalopram solution

Standard citalopram stock solution ($100\ \mu\text{g mL}^{-1}$) was prepared by dissolving 0.01 g of reliable standard citalopram in distilled water and made the volume up to 100 mL with distilled water. Working standard solutions of $10\ \mu\text{g mL}^{-1}$ and $1.0\ \mu\text{g mL}^{-1}$ were prepared daily by diluting appropriate amounts of stock solution with distilled water.

Sample preparation

Contents of the five tablets of each sample (Lopram 20 mg tablets, manufactured by Hansel Pharmaceuticals (Pvt) Ltd., Lahore, Pakistan, Citalo 20 mg tablets manufactured by Platinum Pharmaceuticals (Pvt) Ltd., Karachi, Pakistan and Pramcit 20 mg tablets manufactured by Nabiqasim Industries (Pvt) Ltd., Karachi, Pakistan were weighted individually to find average weight of one tablet. The tablets form of each brands were ground separately in a mortar. An amount of 0.01 g powder tablets of citalopram was then dissolved in small amount of distilled water with vigorous shaking. The solution was filtered in a 100 mL volumetric flask and made the volume up to the mark with distilled water. An appropriate volume of this solution was then diluted with distilled water to get a citalopram concentration within the working range.

Procedure

Working standard citalopram solution ($2.0\ \text{mL}$ of $20\ \mu\text{g mL}^{-1}$) was taken in a 100 mL conical flask and $2.0\ \text{mL}$ of Ce (IV) solution ($100\ \mu\text{g mL}^{-1}$) was added to it. The flasks were heated for 40 minutes on a boiling water bath. After cooling, the solutions were transferred to 25 mL volumetric flask and the volume was made up to the mark with distilled water. Maximum excitation (λ_{ex}) and emission (λ_{em}) wavelengths for the fluorimetric behavior of Ce (IV) were investigated. For excitation spectrum the analyte was scanned in the range of 220–285 nm and for emission spectrum the analyte was scanned in the range of 320–400 nm after excitation at 252 nm using spectrofluorophotometer (figure 3.1.1).

To optimize the Ce (IV) concentration, $2.0\ \text{mL}$ of $20\ \mu\text{g mL}^{-1}$ standard citalopram solution was taken in six reaction flasks. To each reaction flask, $2.0\ \text{mL}$ of Ce (IV) solution in the

concentration range of 50–600 $\mu\text{g mL}^{-1}$ was added and preceded the same way as described before and fluorescence intensity was measured at λ_{ex} 252 nm and λ_{em} 353 nm (figure 3.1.2).

To optimize the volume of Ce (IV) solution, different volume (1.0–4.0 mL) of the 100 $\mu\text{g mL}^{-1}$ of the reagent was used maintaining rest of the procedure the same and fluorescence intensity was measured (figure 3.1.3).

To optimize the sulfuric acid concentration, 2.0 mL of 100 $\mu\text{g mL}^{-1}$ Ce (IV) solution prepared in different concentration of sulfuric acid in the range of 1.0–6.0 M were taken in six reaction flasks. 2.0 mL of 20 $\mu\text{g mL}^{-1}$ standard citalopram solutions were added to each flask keeping rest of the procedure the same. Fluorescence intensity was measured at optimum excitation and emission wavelengths (figure 3.1.4).

To optimize the heating temperature, optimized amount of the reagent's solutions were taken in six reaction flasks and 2.0 mL of citalopram (20 $\mu\text{g mL}^{-1}$) standard solution was added pursued by heating in the temperature range of 50–100 $^{\circ}\text{C}$ for 15 minutes. After cooling diluted to 25 mL with distilled water in volumetric flasks and fluorescence intensity was measured at optimum wavelengths (figure 3.1.5). Heating time was optimized by varying only the heating time from 10–60 minutes keeping rest of the conditions the same. The results are shown in figure 3.1.6.

Stability of the reaction product was also studied by evaluating the fluorescence intensity for up to two hours with 10 minutes interval. The results are shown in figure 3.1.7.

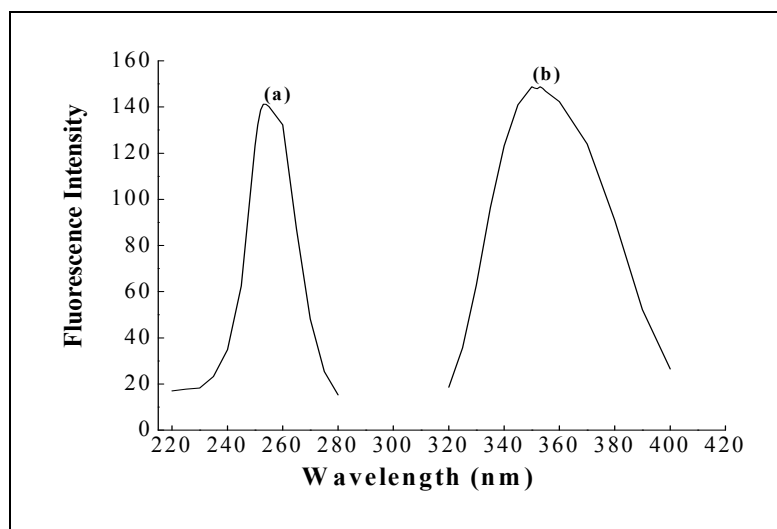


Figure 3.1.1 Excitation (a) and emission (b) spectra of cerium (III) formed after reaction with citalopram

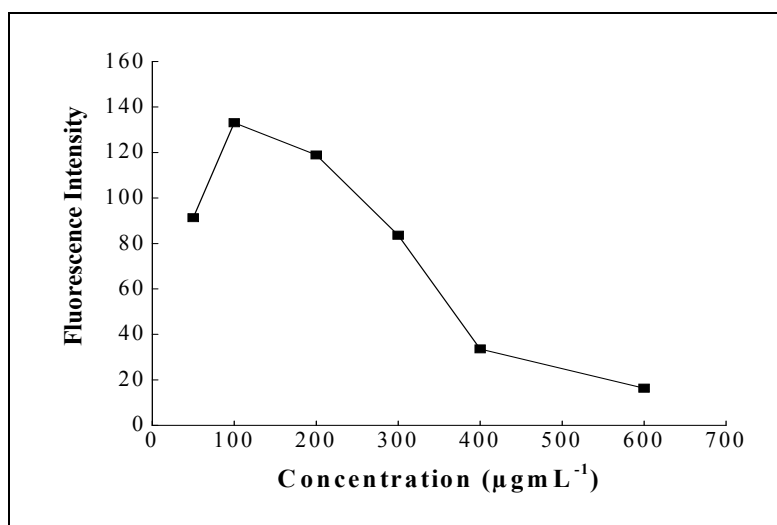


Figure 3.1.2 Effect of concentration of Ce (IV) on the spectrofluorimetric determination of citalopram

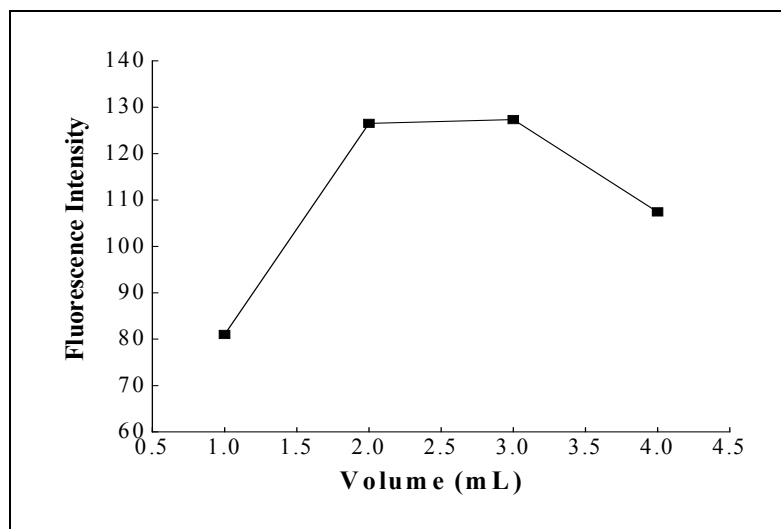


Figure 3.1.3 Effect of volume of Ce (IV) solution on the spectrofluorimetric determination of citalopram

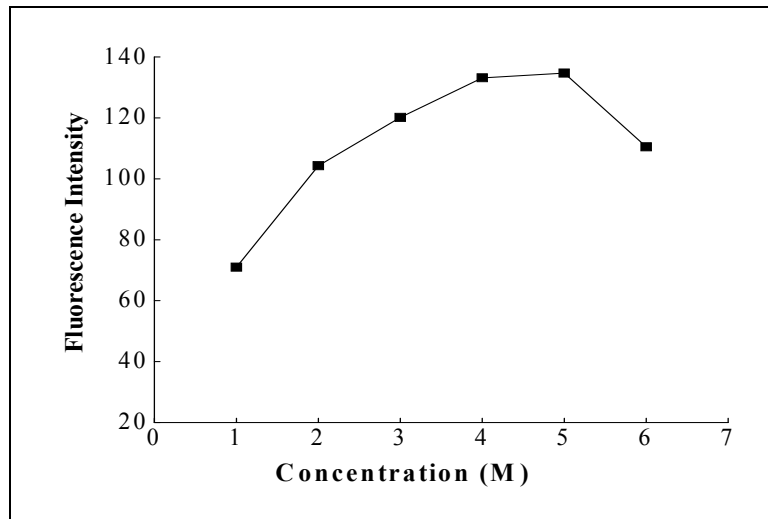


Figure 3.1.4 Effect of sulfuric acid concentration on the spectrofluorimetric determination of citalopram

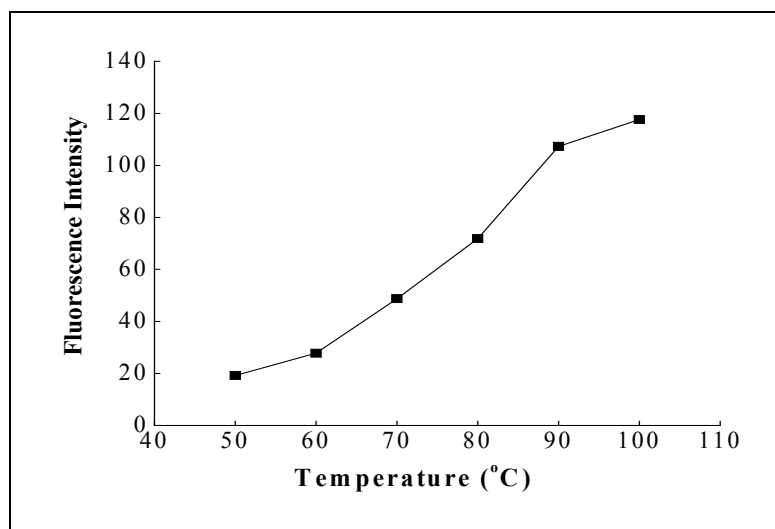


Figure 3.1.5 Effect of temperature on the spectrofluorimetric determination of citalopram

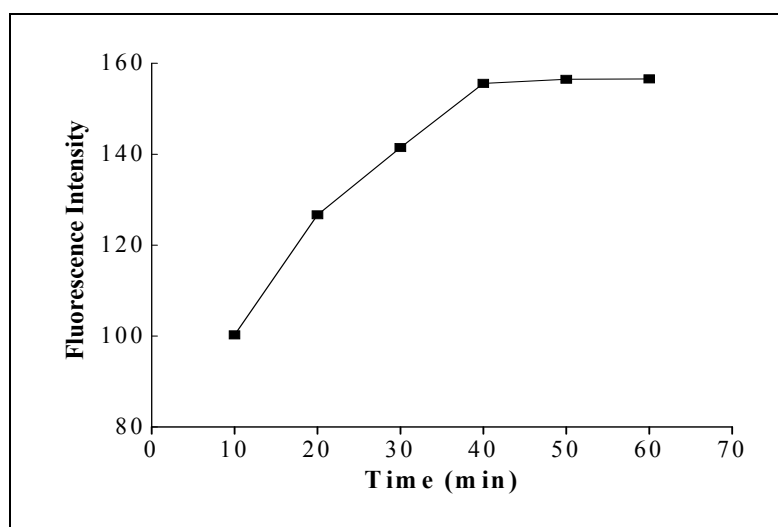


Figure 3.1.6 Effect of heating time on the spectrofluorimetric determination of citalopram

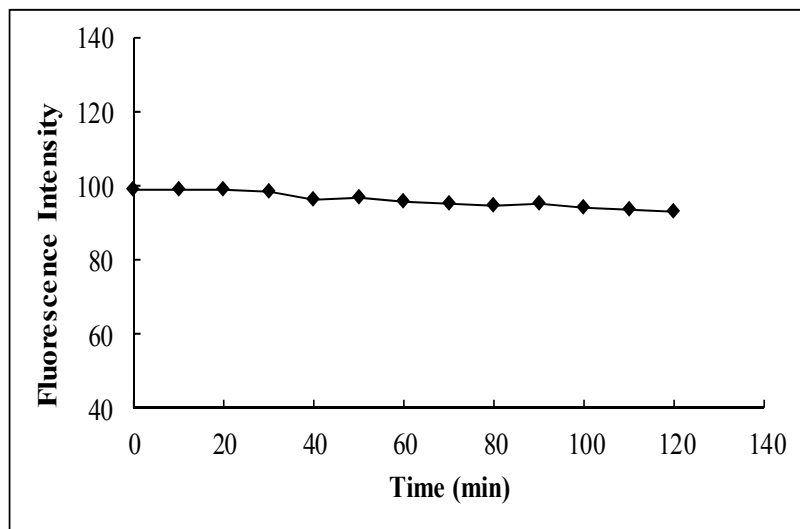


Figure 3.1.7 The effect of time on fluorescene intensity of Ce (III)

3.1.4 The Effect of citalopram concentration on fluorescence intensity of Ce (III)

Same instrument, reagent and solutions were used as mentioned before.

Procedure

Proper volumes of citalopram standard stock solution to produce a final concentration of $0.02\text{--}0.1\ \mu\text{g mL}^{-1}$ were taken in five separate flasks. $2.0\ \text{mL}$ of $100\ \mu\text{g mL}^{-1}$ acidic cerium (IV) solution was added to each flask. The flasks were heated for 40 minutes on a boiling water bath and after cooling diluted to $25\ \text{mL}$ with distilled water. Fluorescence intensity was measured at $\lambda_{\text{em}}\ 353\ \text{nm}$ and $\lambda_{\text{ex}}\ 252\ \text{nm}$ against a reagent blank prepared in the same way without adding citalopram (table 3.1.1 and figure 3.1.8).

Table 3.1.1 Effect of concentration of citalopram on the fluorescence behavior of Ce (III)

Concentration ($\mu\text{g mL}^{-1}$)	Fluorescence Intensity
0.00	0.00
0.02	5.44
0.04	11.624
0.06	16.512
0.08	21.029
0.10	26.401

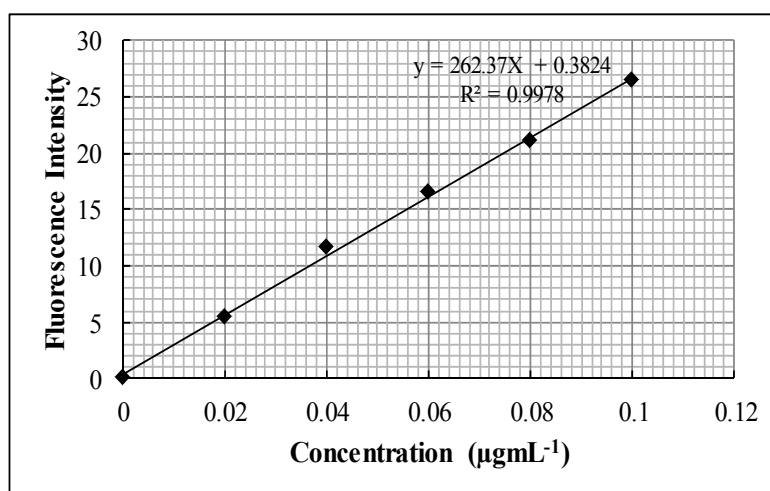


Figure 3.1.8 Effect of concentration of citalopram on the fluorescence behavior of Ce (III)

Standard deviation and relative standard deviation (RSD %) were calculated by analyzing six replicate of analytes having lowest measurable concentration. LOD and LOQ were calculated using the following equations.

$$\text{LOD} = 3S$$

$$\text{LOQ} = 10S$$

Where S is the standard deviation of the method

All the analytical parameters calculated for the proposed method are given in table 3.1.2.

Table 3.1.2 Analytical parameters for the spectrofluorimetric determination of citalopram

Parameters	Value
λ_{ex} (nm)	252
λ_{em} (nm)	353
Concentration range ($\mu\text{g mL}^{-1}$)	0.02–1.4
Limit of detection ($\mu\text{g mL}^{-1}$)	0.006
Limit of quantification ($\mu\text{g mL}^{-1}$)	0.023
Regression equation (y)	$Y=262.35X + 0.384$
Slope (b)	262.35
Intercept (a)	0.384
Correlation coefficient (r^2)	0.9978
Relative standard deviation (%)	5.91

3.1.5 Application of the investigated method for the citalopram determination in pharmaceutical formulations

A. Effect of common excipients on determination of citalopram by the proposed method

Same instrument, reagent and standard solution were used as mentioned before.

Procedure

The selectivity of the proposed method for the determination of citalopram was calculated by studying the interferences effect of common excipient that might be added during preparations of the tablets. For this purpose a known concentration of standard citalopram ($0.02 \mu\text{g mL}^{-1}$) was evaluated by the same procedure as explained before. Then standard citalopram ($0.02 \mu\text{g mL}^{-1}$) and different concentrations of excipients maintaining drug: excipients ratio of 1:1, 1:2 and 1:4 were mixed in separate reaction flasks pursued by the addition of 2.0 mL of $100 \mu\text{g mL}^{-1}$ Ce (IV) solutions. Fluorescence intensity was measured at 353 nm. The analysis were performed in triplicates and mean fluorescence intensity was calculated (table 3.1.3 and figure 3.1.9).

Table 3.1.3 Interferences effect of common excipients on determination of citalopram

Excipients added	Percent recovery		
	1:01	1:02	1:04
Lactose	96.66	101.66	96.66
Glucose	100.00	101.66	96.66
Starch	96.11	101.51	98.48
Talc	100.00	95.00	100.00
Mag. Stearate	101.75	101.75	99.99
Sorbitol	101.58	93.64	101.59
Sucrose	95.65	92.00	92.74

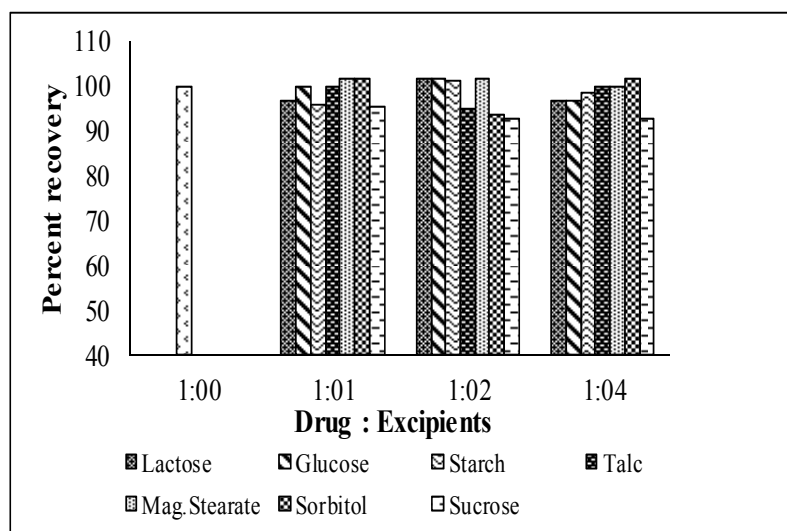


Figure 3.1.9 Interferences effect of common excipients on determination of citalopram by the proposed method

B. Determination of precision and accuracy of the proposed method

Same instrument, reagent, standard solution and sample solution were used as mentioned before.

Procedure

Precision and accuracy of the method was determined by analyzing three separate sample solution of pharmaceutical preparations (Citalo, 20 mg tablets, Pramcit, 20 mg tablets, and Lopram, 20 mg tablets) at three different concentration levels (0.02, 0.04, and 0.06

$\mu\text{g mL}^{-1}$) in triplicate. Calibration curve was plotted using the same procedure as already described and known concentration of these sample solutions were analyzed by the proposed method. The results are given in table 3.1.4.

Table 3.1.4 Evaluation of precision and accuracy of the proposed method for citalopram determination in pharmaceutical preparations

Sample	($\mu\text{g mL}^{-1}$) taken	($\mu\text{g mL}^{-1}$) found	RE (%)	% Recover $\pm$ RSD
Citalo tablets 20 mg	0.02	0.020	1.67	98.33 ± 2.75
	0.04	0.041	-3.33	103.33 ± 1.39
	0.06	0.061	-2.22	102.22 ± 1.88
Pramcit tablets 20 mg	0.02	0.020	0.00	100.00 ± 3.52
	0.04	0.038	4.17	95.83 ± 3.97
	0.06	0.060	0.00	100.00 ± 2.88
Lopram tablets 20 mg	0.02	0.020	1.67	98.33 ± 2.92
	0.04	0.041	-2.50	102.50 ± 4.30
	0.06	0.058	2.78	97.22 ± 3.56

C. Percent recovery of citalopram from commercial formulations (Standard addition method)

Same reagent, instrument, standard solution and sample solution were used as mentioned before.

Procedure

0.02, 0.04, and 0.06 $\mu\text{g mL}^{-1}$ standard citalopram solutions was added to the pre-analyzed 0.02 $\mu\text{g mL}^{-1}$ commercial sample of citalopram and fluorescence intensity was measured pursuing the same procedure as described before. Triplicate readings were taken and the mean percent recovery was calculated by the following formula (table 3.1.5).

$$\% \text{ Recovery} = \frac{\mu\text{g mL}^{-1} \text{ found in the fortified sample} - \mu\text{g mL}^{-1} \text{ present in the sample}}{\mu\text{g mL}^{-1} \text{ of standard added}} \times 100$$

Table 3.1.5 Evaluation of recovery (%) of citalopram from commercial formulations by the proposed method

Samples	Sample (μgmL^{-1})	(μgmL^{-1}) Added	(μgmL^{-1}) Found	%Recovery $\pm$ RSD
Citalo tablets 20 mg	0.0200	0.0200	0.0380	95.00 $\pm$ 3.11
		0.0400	0.0570	95.00 $\pm$ 4.56
		0.0600	0.0773	96.67 $\pm$ 3.45
Pramcit tablets 20 mg	0.0200	0.0200	0.0420	105.00 $\pm$ 5.67
		0.0400	0.0585	97.50 $\pm$ 2.97
		0.0600	0.0813	101.67 $\pm$ 2.48
Lopram tablets 20 mg	0.0200	0.0200	0.0400	100.00 $\pm$ 4.76
		0.0400	0.0594	99.00 $\pm$ 2.84
		0.0600	0.0788	98.50 $\pm$ 2.55

D. Determination of active ingredients in the commercial formulations

Same reagent, instrument, standard solution and sample solution were used as mentioned before.

Procedure

Solutions of the commercial formulations were prepared by the same procedure as mentioned before. Working sample solution of $1.0 \mu\text{gmL}^{-1}$ was prepared by diluting suitable amount of the stock sample solution with distilled water. Known volumes of this sample solution ($1.0 \mu\text{gmL}^{-1}$) were then evaluated by the proposed method in triplicate (table 3.1.6).

Table 3.1.6 Determination of active ingredients in the commercial formulations

Brand name	Active ingredient (mgtab ⁻¹)		t-test value (4.303)
	Labeled value	Found value	
Citalo	20	20.28 $\pm$ 0.25	1.93
Pramcit	20	19.67 $\pm$ 0.58	0.98
Lopram	20	19.94 $\pm$ 0.58	0.18

3.1.6 Validation of the proposed method in spiked biological samples

To investigate the applicability of the proposed method in different matrices, recovery test was performed on spiked sample of urine and human plasma.

A. Spiked plasma and urine sample

Procedure

Blood plasma was kindly granted by healthy volunteers. Known volume of plasma (1.0 mL) was spiked with proper volume of the standard citalopram solution to give final drug concentration of 0.02–0.06 $\mu\text{g mL}^{-1}$. To each of these solution 6.0 mL ethylacetate: n-hexane (3:7) was added and was centrifuged at 1000 rpm for 15 minutes. The organic layer was separated and evaporated to dryness at room temperature. The residue was dissolved in 2.0 mL distilled water and 2.0 mL of 100 $\mu\text{g mL}^{-1}$ Ce (IV) solution was added to each solution. Fluorescence intensity was measured by the same procedure as mentioned before against a reagent blank prepared in the same way without the addition of citalopram (table 3.1.7).

Table 3.1.7 Spectrofluorimetric determination of citalopram in spiked plasma by the proposed method

Sample	Spiked plasma		
	($\mu\text{g mL}^{-1}$) taken	($\mu\text{g mL}^{-1}$) found	%Recovery $\pm$ RSD
Plasma	0.02	0.017	88.50 $\pm$ 4.63
	0.04	0.037	92.50 $\pm$ 2.70
	0.06	0.058	96.67 $\pm$ 3.61

Similarly urine sample from healthy volunteer at normal condition was taken and 1.0 mL of urine was spiked with suitable volume of the standard citalopram solution to give final citalopram concentration of 0.02–0.06 $\mu\text{g mL}^{-1}$ and the rest of the procedure was kept the same as for plasma sample (table 3.1.8).

Table 3.1.8 Spectrofluorimetric determination of citalopram in spiked urine by the proposed method

Sample	Spiked urine		
	($\mu\text{g mL}^{-1}$) taken	($\mu\text{g mL}^{-1}$) found	%Recovery $\pm$ RSD
Urine	0.02	0.018	90.00 $\pm$ 4.00
	0.04	0.038	95.00 $\pm$ 2.50
	0.06	0.058	96.67 $\pm$ 3.47

3.1.7 Results and discussion

A simple, fast and sensitive spectrofluorimetric method has been developed for determination of citalopram in bulk, commercial formulations and biological samples. The method is based on reduction of cerium (IV) by citalopram to fluorescent active cerium (III). Citalopram can be quantified by evaluating the fluorescence intensity of the product Ce (III) with spectrofluorimeter. The excitation and emission spectrum of the product Ce (III) shows optimum excitation wavelength at 252 nm and emission wavelength at 353 nm (figure 3.1.1).

The effect of concentration of Ce (IV) on its reaction with citalopram was studied in the range of 50–600 $\mu\text{g mL}^{-1}$ (figure 3.1.2). It was noted that fluorescence intensity increased with increase in concentration of Ce (IV) up to 100 $\mu\text{g mL}^{-1}$, and after that it decreases. A stable, maximum and reproducible signal is produced with 100 $\mu\text{g mL}^{-1}$ Ce (IV). Therefore, this concentration was selected for further analysis. The effect of the volume of Ce (IV) solution was also studied in the range of 1.0–4.0 mL (figure 3.1.3) and 2.0 mL of 100 $\mu\text{g mL}^{-1}$ Ce (IV) solution produced maximum fluorescence intensity. Therefore, 2.0 mL of Ce (IV) solution was selected for further study.

Ce (IV) acts as an oxidizing reagent in acidic media. The effect of acid concentration was also investigated in the range of 1.0–6.0 M of sulfuric acid solution (figure 3.1.4). Maximum fluorescence intensity of Ce (III) was observed at 4.0 M of sulfuric acid. Therefore, 4.0 M of sulfuric acid was preferred as optimum concentration for further study.

The effect of heating temperature on the reaction was investigated in the range of 50–100°C using boiling water bath. Maximum signal of Ce (III) was observed at 100°C (figure 3.1.5). Heating time for the reaction was also optimized (figure 3.1.6) and it was found that the reaction could be completed by heating for 40 minutes at boiling water temperature.

Stability of the reaction product was also studied and no change in fluorescence intensity was observed up to 120 minutes (figure 3.1.7).

Analytical characteristics

Under optimal experimental conditions, a linear range of concentration of citalopram versus fluorescence intensity was observed in the concentration range of 0.02–0.10 $\mu\text{g mL}^{-1}$ with good correlation coefficient ($r^2 = 0.9978$) (figure 3.3.8). The LOD was calculated by using the minimum level of concentration at which citalopram can be detected reliably (3S) using five replicates determination and the LOQ was similarly calculated by using the lowest concentration of citalopram that can be measured with satisfactory precision and accuracy (10S). Precision of the method was calculated from the value of standard deviation and relative standard deviation. The small values of standard deviation and relative standard deviation show good precision of the method (table 3.1.2).

Applications

To evaluate the selectivity of the method toward its applicability for the analysis of pharmaceutical formulations containing citalopram, the interferences effect of common excipients generally present in the pharmaceutical preparations were explored. Solutions comprising citalopram and one of the excipients taken individually in concentration one, two and four times greater than that of citalopram were investigated by the proposed method. A 5% error criterion was adopted. None of these common excipients was found to cause any interference (Table 3.1.3 and figure 3.1.9). The recovery of citalopram was found to be in the range of 92.0 ± 4.28 – $101.75 \pm 2.50\%$.

The validity of the method was confirmed by standard addition method to different pharmaceutical preparations of citalopram (table 3.1.5). The recovery was in the range of $95.0 \pm 3.11\%$ to $96.67 \pm 3.45\%$, $97.50 \pm 2.97\%$ to $105.0 \pm 5.67\%$ and $98.50 \pm 2.55\%$ to $100.0 \pm 4.76\%$ for Citalo, Pramcit and Lopram respectively.

The proposed method was successfully applied for analysis of citalopram in various pharmaceutical preparations (table 3.1.4 and 3.1.6). For all the formulations analyzed, the results of the proposed method were in good agreement with the labeled contents.

Good sensitivity, high precision and accuracy obtained by the proposed method suggest its applicability for the quantification of citalopram in plasma and urine samples. In case of plasma, the percent recovery was found to be $92.56 \pm 3.61\%$ while in case of urine, it was found $93.89 \pm 3.47\%$ (table 3.1.7 and 3.1.8). The method can be successfully applied for the determination of citalopram in biological samples and in clinical analysis.

3.2 SPECTROFLUORIMETRIC METHOD FOR THE DETERMINATION OF CITALOPRAM IN BULK AND PHARMACEUTICAL PREPARATIONS BASED ON THE MEASUREMENT OF THE SILVER NANOPARTICLES-ENHANCED FLUORESCENCE OF CITALOPRAM/TERBIUM COMPLEXES

3.2.1 Method development strategy for the spectrofluorimetric determination of citalopram

Lanthanides ions perform a significant role as luminescence probes in bioanalytical applications due to their inherent sensitivities and selectivity's. Metallic nanostructures can change the spectral properties of fluorophores. The interaction of metal with fluorophore can significantly enhance luminescence intensity. Colloidal silver nanoparticles (Ag NPs) strongly enhanced the fluorescence intensity of the complex formed between the citalopram and Tb (III) ion. The Ag NPs transfer the resonance energy to the fluorophore through the interaction of the excited-state fluorophores and surface plasmon electrons in the Ag NPs. This phenomenon could be used for the determination of citalopram in pharmaceutical preparations. The possible mechanism of Ag NPs enhanced luminescence of Tb (III)-citalopram complex is presented below.

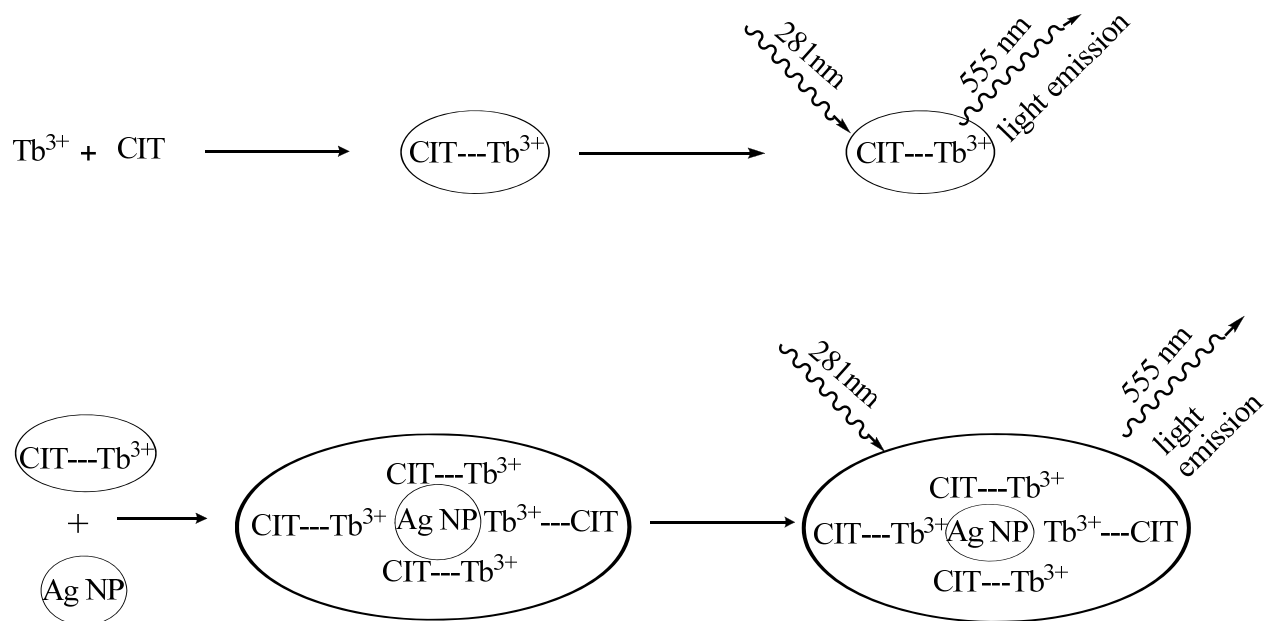


Figure 3.2.1 Schematic diagram of possible mechanism of AgNPs enhanced luminescence of Tb³⁺-citalopram complex

3.2.2 Preliminary investigation of the possibility of silver nanoparticles-enhanced fluorescence of citalopram/terbium complexes

Preliminary studies were conducted to investigate the possible formation of Ag NP-CIT-Tb³⁺ complex. Initially high concentration of citalopram (14 µgmL⁻¹), Tb³⁺ ion (2.0 × 10⁻⁴ molL⁻¹) and Ag NPs (1.4 × 10⁻⁴ molL⁻¹) were mixed in a volumetric flask and fluorescence intensity was measured. Further studies were focused on optimization of different parameters preceding to the possible enhancement of fluorescence intensity of citalopram and are given below.

3.2.3 Preparation and characterization of silver nanoparticles

Colloidal solutions of silver nanoparticles were prepared as per previously described method [1]. Briefly, 25 mL of AgNO₃ (1.0 × 10⁻³M) was dropwise added to 75 mL of a freshly prepared aqueous solution of NaBH₄ (2.0 × 10⁻³M) with vigorous stirring. After 10 minutes, 5.0 mL of sodium citrate solution (1.0% w/w) was added to stabilize the AgNPs formed. The yellow colloidal solution of Ag NPs was then stirred for another 20

minutes and aged for 2 days at 4 °C before use. The prepared AgNPs were characterized by UV-visible spectroscopy (figure 3.2.2) and transmission electron microscopy (TEM) (figure 3.2.3) at an accelerating voltage of 100 kV. The concentration of the prepared AgNPs was $2.4 \times 10^{-4} \text{ molL}^{-1}$, based on the concentration of AgNO_3 solution used for their preparation.

3.2.4 Optimization studies for the determination of silver nanoparticles-enhanced fluorescence of citalopram/terbium complexes

Instruments

A F-4500 spectrofluorometer Hitachi (Japan) equipped with 150-watt xenon lamp and photomultiplier tube and a 1×1 cm quartz cell were used for measurement of fluorescence intensities. The instrument was operated with excitation and emission slit widths of 5 nm. A UV-1800 Shimadzu UV-Vis spectrophotometer was used for absorption measurement. A pH meter (Mettler-Toledo MP 220, US) was used to adjust the pH value of the buffer.

Reagents

Analytical grade reagents and doubly deionized water (DI) was used throughout. Terbium nitrate ($\text{Tb}(\text{NO}_3)_3 \cdot 5\text{H}_2\text{O}$), Tris (hydroxyl methyl amino methane), and silver nitrate were obtained from Sigma-Aldrich, and sodiumborohydride (NaBH_4) and sodium citrate from Merck (Germany). Standard reference citalopram was provided by Z-Jan's Pharmaceutical Industry (Hayatabad Peshawar, Pakistan). Commercial formulations of citalopram, that is, Lopram, Citalo, and Pramcit (all as 20 mg tablets) were manufactured by Hansel Pharmaceuticals (Lahore, Pakistan), Platinum Pharmaceuticals (Karachi), and Nabiqasim Industries (Karachi), respectively.

Solution preparation

Terbium nitrate ($1.0 \times 10^{-3} \text{ M}$) was prepared by dissolving 0.02175 g of $\text{Tb}(\text{NO}_3)_3 \cdot 5\text{H}_2\text{O}$ in DI water and diluted to 50 mL with the same solvent. Tris-HCl buffer solution (0.1 M) was prepared by dissolving 0.605 g of tris (hydroxyl methyl amino methane) in 50 mL

deionized water and adjusted its pH with 0.1 molL^{-1} HCl. Working solutions were freshly prepared before use from stock solution by dilution with DI water.

Preparation of Standard Solution

A standard stock solution of citalopram ($250 \text{ }\mu\text{g mL}^{-1}$) was prepared by dissolving 0.0125 g of citalopram standard in 2.0 mL of distilled ethanol with shaking and diluting to 50 mL with DI water. Working solutions of 10 and $5.0 \text{ }\mu\text{g mL}^{-1}$ were prepared fresh daily by dilution with DI water.

Preparation of sample solution ($250 \text{ }\mu\text{g mL}^{-1}$)

The contents of five tablets of each sample i.e. Lopram, Citalo and Pramcit (all as 20 mg tablets) were weighed separately to get average weight of one tablet. The tablets from each brand were ground separately in a mortar. Powder equivalent to 0.0125 g of citalopram were then dissolved in 2.0 mL of ethanol, DI water was added, and the mixtures were sonicated for 10 min. The resultant solutions were then filtered and made the volume up to 50 mL with DI water. An appropriate volume of this solution was then diluted with DI water to obtain a citalopram concentration within the working range.

Procedure

An appropriate volume of citalopram stock solution, diluted to give a final concentration of $0.02\text{--}14 \text{ }\mu\text{g mL}^{-1}$, was added to 10 mL volumetric flasks. Tris-HCl buffer solution (0.5 mL; pH = 8), 1.0 mL of $2.0 \times 10^{-4} \text{ molL}^{-1}$ Tb (III) ion solution, and 1.0 mL of $1.4 \times 10^{-4} \text{ molL}^{-1}$ Ag NP solution were then added. These solutions were then diluted to 10 mL with DI water, mixed thoroughly, and let to stand for 10 minutes for complex formation. It was then placed in $1 \times 1 \text{ cm}$ quartz cells one by one and maximum excitation (λ_{ex}) and emission wavelength (λ_{em}) were recorded. For excitation spectrum the analyte was scanned in the range of 250–350 nm and for emission spectrum the analyte was scanned in the range of 450–650 nm using spectrofluorometer (figure 3.2.4).

For investigation the effect of different buffer solutions, appropriate volume of citalopram stock solution to give final drug concentration of $15 \text{ }\mu\text{g mL}^{-1}$ was taken in 10 mL volumetric flasks. Then 1.0 mL of different buffers i.e. carbonates, borate, phosphate,

and Tris-HCl buffer solutions of the same pH were added. This was pursued by addition of 1.0 mL of 2.0×10^{-4} molL⁻¹ Tb (III) ion solution and 1.0 mL of 1.4×10^{-4} molL⁻¹ Ag NP solution. Each solution was then diluted to 10 mL with DI water, mixed thoroughly, and let to stand for 10 minutes for complex formation. The fluorescence intensity of each solution was then measured at 555 nm after excitation at 281 nm (figure 3.2.5).

To optimize the pH, 1.0 mL of Tris-HCl buffer solution of pH 7.0–10 was added in triplicate to 15 µgmL⁻¹ citalopram solution pursued by the same procedure as described above and fluorescence intensity was measured (figure 3.2.6).

For volume optimization of Tris-HCl buffer (pH 8.0), only solution volume was varied in the range of 0.2–2.5 mL keeping rest of the procedure the same (figure 3.2.7).

To optimize the concentration of Tb (III) ion on CIT-Tb (III)-AgNPs fluorescence intensities, only the concentration of Tb (III) ion was varied in the range of $1-8 \times 10^{-4}$ molL⁻¹. The reaction was preceded in the same way as stated before and fluorescence intensity was measured (figure 3.2.8).

For volume optimization of Tb (III) ion (2.0×10^{-4} molL⁻¹) only volume of the solution was varied in the range of 0.5–2.5 mL keeping rest of the procedure the same (figure 3.2.9).

For concentration of silver nanoparticles optimization, only the concentration of silver nanoparticles was varied in the range of 6.0×10^{-5} to 2.2×10^{-4} molL⁻¹ while rest of the procedure was the same (figure 3.2.10).

Similarly for finding optimum volume of silver nanoparticles (1.4×10^{-4} molL⁻¹), volume was varied in the range of 0.5–2.5 mL. The fluorescence intensity was measured using the same procedure. The results are shown in figure 3.2.11.

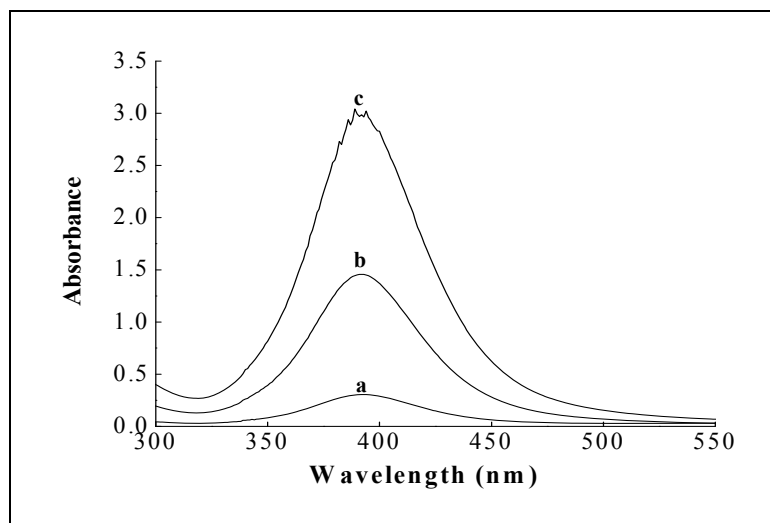


Figure 3.2.2 UV-visible spectrum of the Ag NPs

Conditions; Ag NPs: $2.4 \times 10^{-5} \text{ molL}^{-1}$ (a); $1.2 \times 10^{-4} \text{ molL}^{-1}$ (b); $2.4 \times 10^{-4} \text{ molL}^{-1}$ (c)

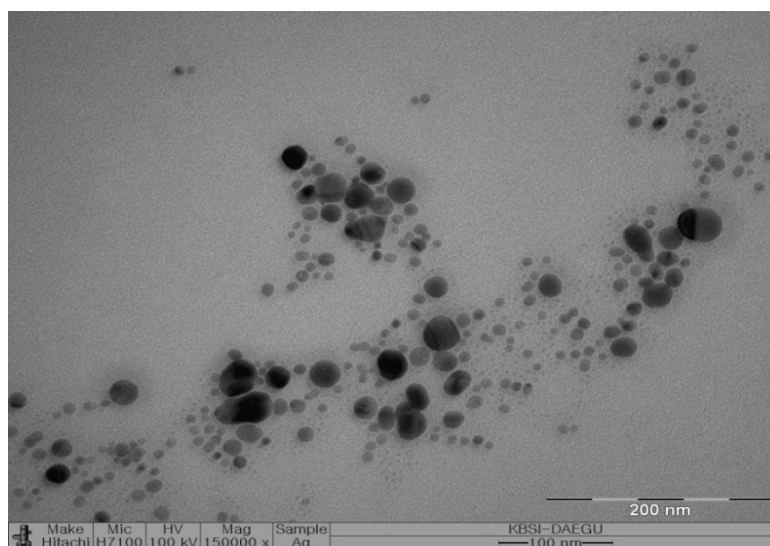


Figure 3.2.3 TEM image of the Ag NPs

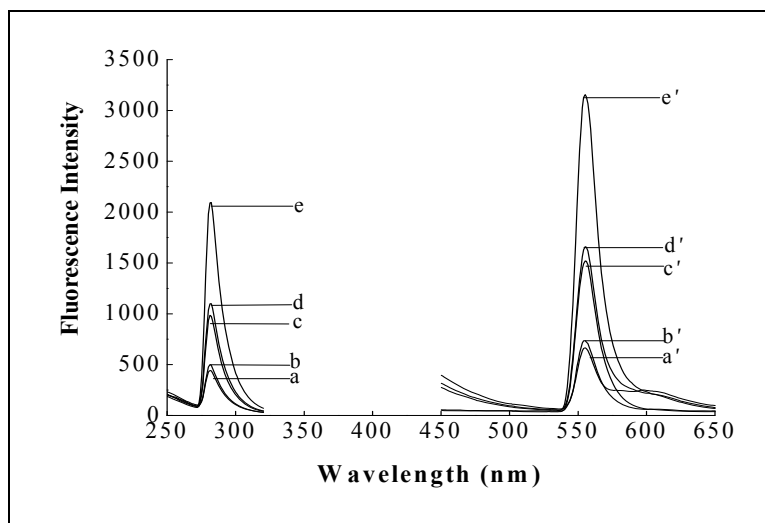


Figure 3.2.4 Excitation and emission spectra of Tb (III) (a, a'); Citalopram-Ag NPs (b, b'); Tb (III)-Ag NPs (c, c'); Tb (III)-citalopram (d, d'); Tb (III)-citalopram-Ag NPs (e, e')

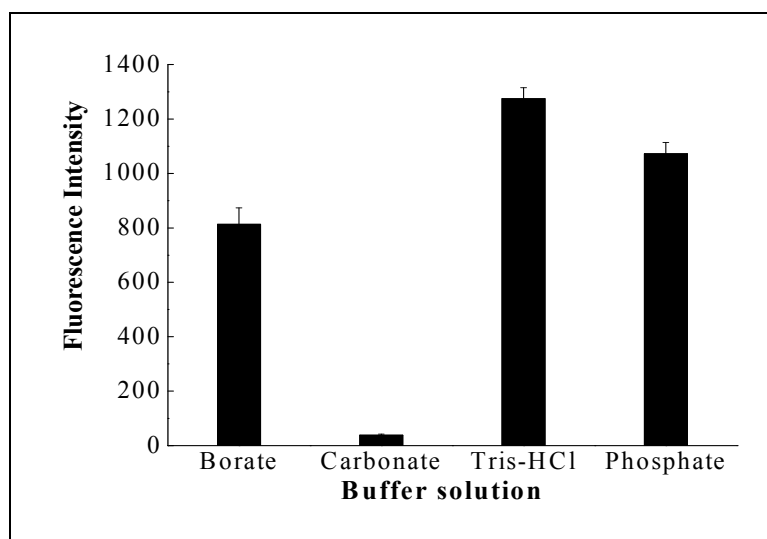


Figure 3.2.5 Effect of buffer on the spectrofluorimetric determination of citalopram

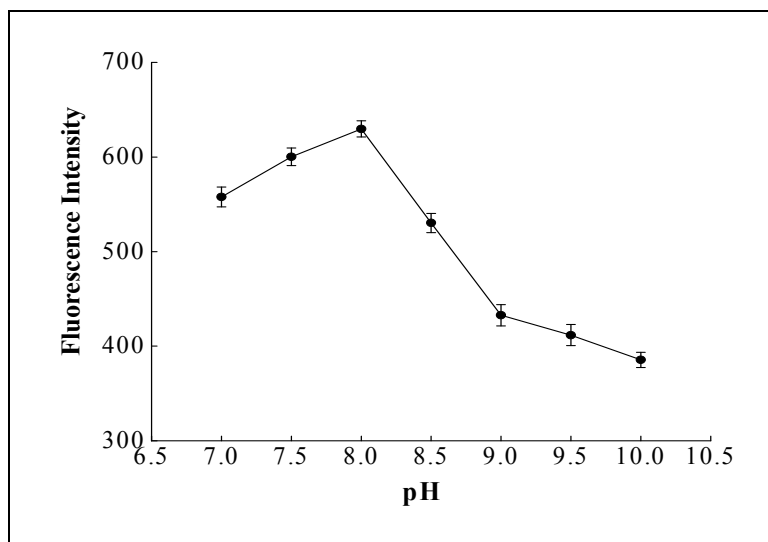


Figure 3.2.6 Effect of pH on the spectrofluorimetric determination of citalopram

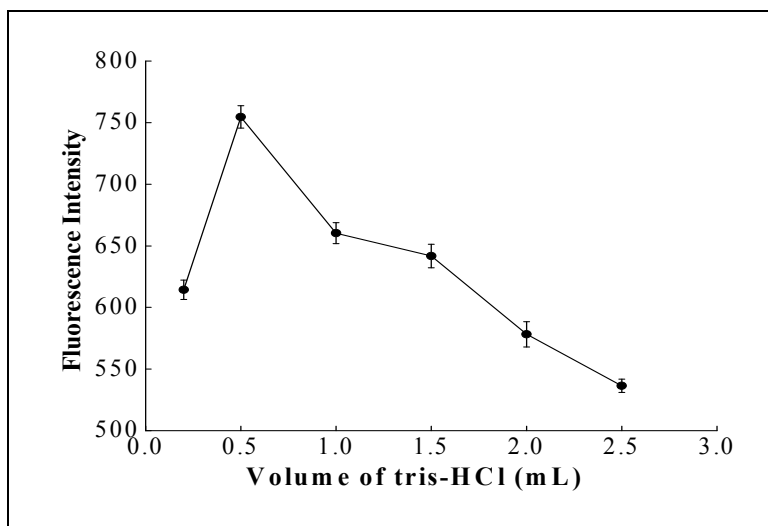


Figure 3.2.7 Effect of volume of Tris-HCl on the spectrofluorimetric determination of citalopram

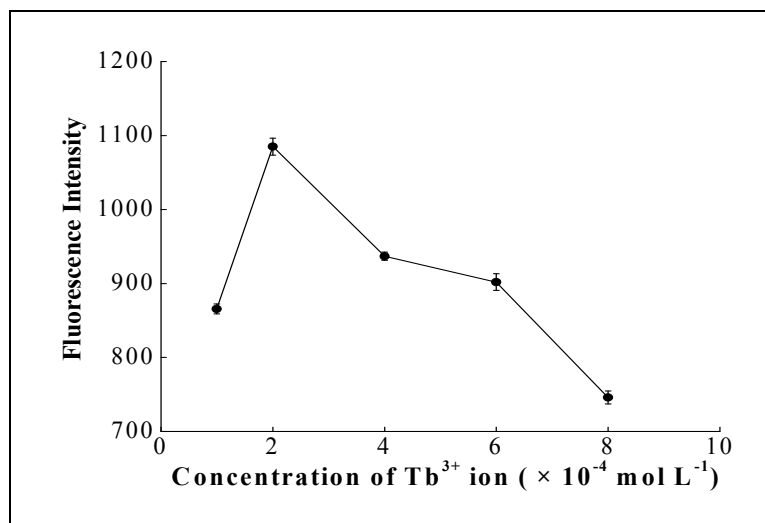


Figure 3.2.8 Effect of concentration of Tb^{3+} ion on the spectrofluorimetric determination of citalopram

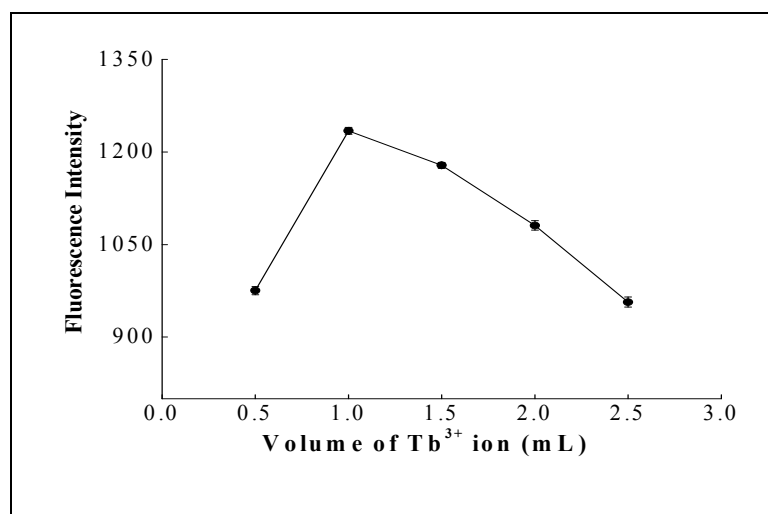


Figure 3.2.9 Effect of volume of Tb^{3+} ion on the spectrofluorimetric determination of citalopram

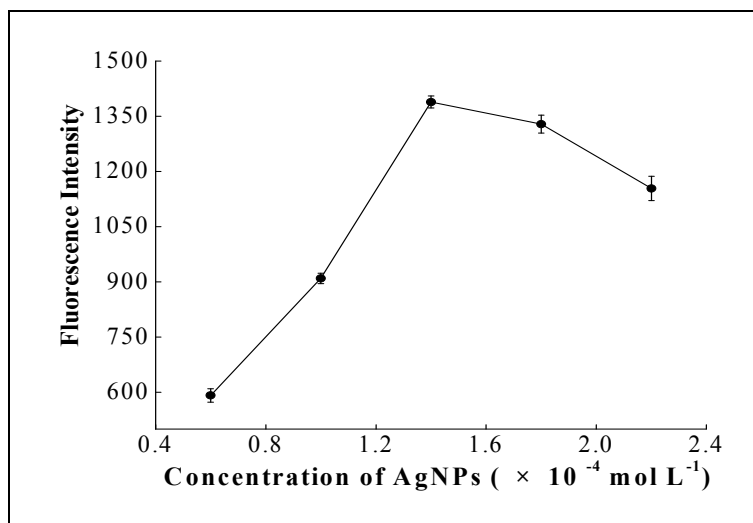


Figure 3.2.10 Effect of concentration of silver nanoparticles on the spectrofluorimetric determination of citalopram

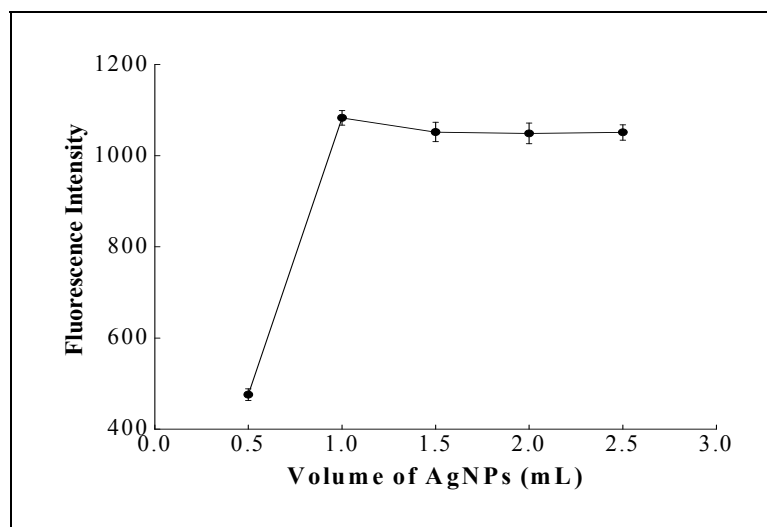


Figure 3.2.11 Effect of volume of Ag NPs on the spectrofluorimetric determination of citalopram

3.2.5 Effect of concentration of citalopram on fluorescence intensity

Instrument, reagents and solutions were the same as mentioned before.

Procedure

An appropriate volume of citalopram stock solution, diluted to give a final concentration of 0.02–14 $\mu\text{g mL}^{-1}$, was added to 10 mL volumetric flasks. Tris-HCl buffer solution (0.5 mL; pH = 8.0), 1.0 mL of $2 \times 10^{-4} \text{ mol L}^{-1}$ Tb^{3+} ion solution, and 1.0 mL of $1.4 \times 10^{-4} \text{ mol L}^{-1}$ Ag NP solution were then added to it. The resulting solutions were then diluted to 10 mL with DI water, mixed thoroughly, and allowed to stand for 10 minutes for complex formation. The fluorescence intensity was measured against a reagent blank at 555 nm after excitation at 281 nm (table 3.2.1 and figure 3.2.12). For determination of citalopram in pharmaceutical samples, the calibration plot of fluorescence intensity versus concentration of citalopram was constructed for lower concentration (figure 3.2.13).

Table 3.2.1 Effect of concentration of citalopram on fluorescence intensity

Concentration ($\mu\text{g mL}^{-1}$)	Fluorescence Intensity
0.00	0
0.02	7.0
0.04	13.0
0.06	21.0
0.08	26.0
0.10	32.0
0.50	80.32
1.00	164.0
2.00	283.0
4.00	530.0
6.00	756.0
8.00	943.0
10.0	1170
12.0	1398
14.0	1578

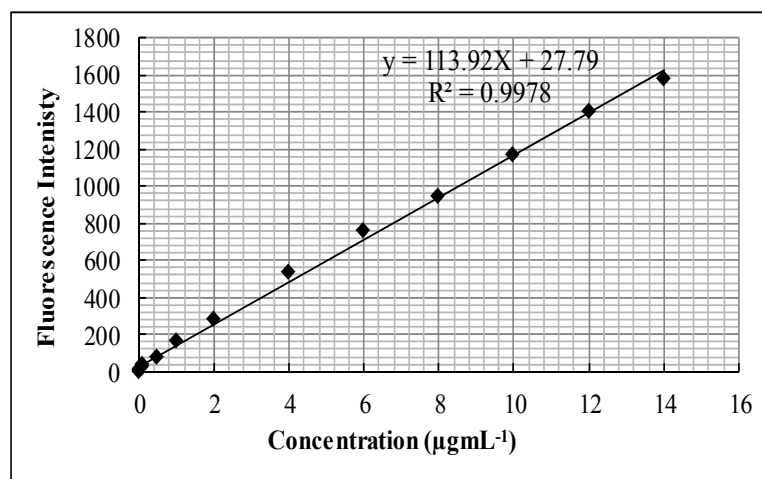


Figure 3.2.12 Effect of concentration of citalopram on fluorescence intensity at higher concentration

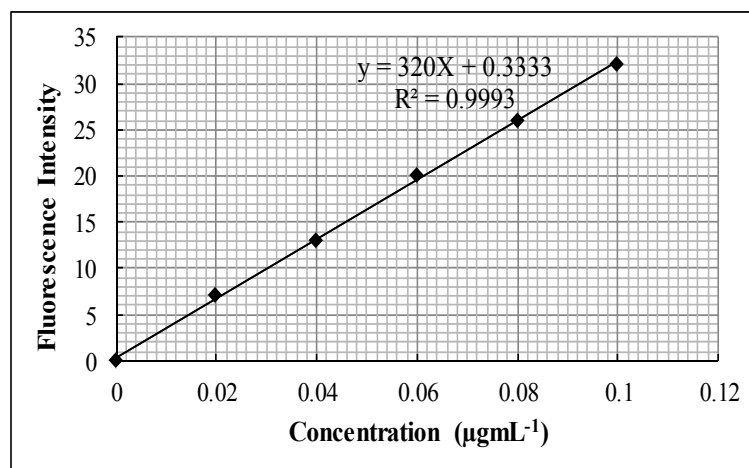


Figure 3.2.13 Effect of concentration of citalopram on fluorescence intensity at lower concentration

Determination of LOD, LOQ and other analytical characteristics for determination of citalopram by the proposed spectrofluorimetric method**Procedure**

Fluorescence reading for six replicates with lowest measurable concentration of citalopram ($0.02 \mu\text{g mL}^{-1}$) was taken using the proposed method pursuing the same optimized procedure as stated before. From the results the LOD, LOQ and other analytical characteristics was calculated (table 3.2.2).

Table 3.2.2 Analytical characteristics for the spectrofluorimetric determination of citalopram

Parameter	Value
λ_{ex} (nm)	281
λ_{em} (nm)	555
Linear range ($\mu\text{g mL}^{-1}$)	0.02–14
Limit of detection ($\mu\text{g mL}^{-1}$)	7.15×10^{-6}
Limit of quantification ($\mu\text{g mL}^{-1}$)	2.38×10^{-5}
Regression equation (y)	$Y = 320X + 0.333$
Slope (b)	320
Intercept (a)	0.333
Correlation coefficient (r^2)	0.9993
Standard deviation ($\mu\text{g mL}^{-1}$)	7.630×10^{-4}
Relative standard deviation (%)	3.66

3.2.6 Investigation of interferences on determination of citalopram by the proposed method

The selectivity of the proposed method for the analysis of citalopram containing pharmaceutical preparation was evaluated by studying the interferences effect of common excipient that are generally added during preparations of the tablets. For this purpose a known concentration of standard citalopram ($0.02 \mu\text{g mL}^{-1}$) was evaluated by the same method as illustrated before. Then standard citalopram ($0.02 \mu\text{g mL}^{-1}$) and different concentrations of excipients maintaining drug: excipients ratio of 1:5, 1:10 and 1:20 were mixed in 10 mL volumetric flasks. To these the optimized amounts of all reagents were added and fluorescence intensity was measured. The analysis were performed in triplicates and mean fluorescence intensity was calculated (table 3.2.3 and figure 3.2.14).

Table 3.2.3 Interferences effect of common excipients on determination of citalopram ($0.02 \mu\text{g mL}^{-1}$) using the proposed method

Excipients	Excipients added ($\mu\text{g mL}^{-1}$)	Drug : Excipients	%Recovery $\pm$ RSD
Glucose	0.1	1:05	98.87 ± 2.23
	0.2	1:10	99.12 ± 1.65
	0.4	1:20	98.45 ± 2.22
Fructose	0.1	1:05	99.21 ± 3.31
	0.2	1:10	97.77 ± 3.08
	0.4	1:20	98.22 ± 4.21
Starch	0.1	1:05	100.12 ± 1.23
	0.2	1:10	100.56 ± 3.21
	0.4	1:20	101.34 ± 2.78
Lactose	0.1	1:05	98.88 ± 4.31
	0.2	1:10	99.94 ± 2.11
	0.4	1:20	97.97 ± 1.73
Talc	0.1	1:05	100.32 ± 3.21
	0.2	1:10	99.56 ± 4.11
	0.4	1:20	98.65 ± 2.35
Mg. Stearate	0.1	1:05	99.16 ± 1.56
	0.2	1:10	100.54 ± 2.81
	0.4	1:20	98.55 ± 3.32

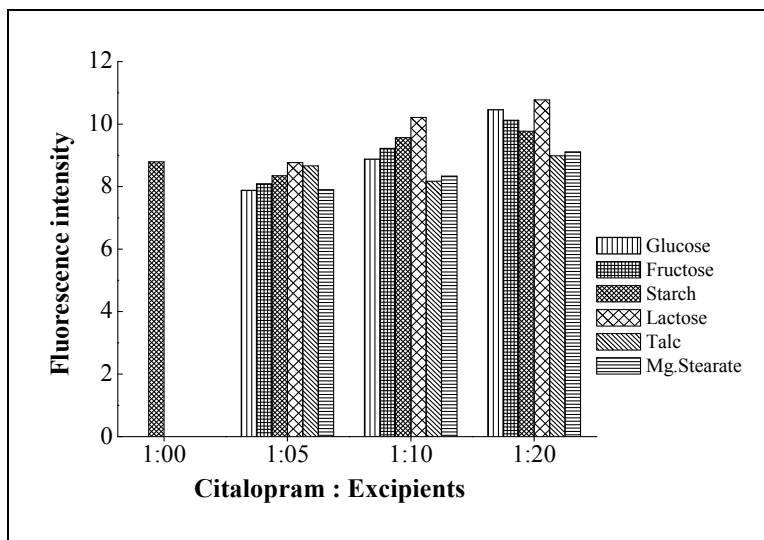


Figure 3.2.14 Effect of common excipients on determination of citalopram

3.2.7 Application of the investigated method for the citalopram determination in pharmaceutical preparations

A. Determination of precision and accuracy of the investigated method

Instrument, reagents and standard solution used were the same as mentioned before.

Procedure

The precision and accuracy of the developed method was evaluated by determining citalopram in pure form and pharmaceutical preparations using three different pharmaceutical preparations (each containing 20 mg of the active ingredients of citalopram) in three different concentrations within the calibration curve range. All readings were taken in triplicate. Calibration curve was plotted by the same procedure as mentioned before and known concentrations (0.02, 0.04 and 0.06 $\mu\text{g mL}^{-1}$) of these sample solution were then analyzed by the proposed method. The results are given in table 3.2.4 for the standard and in table 3.2.5 for pharmaceutical preparations.

Table 3.2.4 Evaluation of accuracy and precision of the proposed method using solutions of pure citalopram

($\mu\text{g mL}^{-1}$) taken	($\mu\text{g mL}^{-1}$) found	%Recovery $\pm$ RSD
0.02	0.0197	98.98 $\pm$ 1.40
0.04	0.0403	100.97 $\pm$ 2.38
0.06	0.0605	100.97 $\pm$ 1.63
Mean		100.30
$\pm$ SD		1.148
t-test		0.452 (4.303)

Table 3.2.5 Evaluation of accuracy and precision of the proposed method for citalopram determination in pharmaceutical preparations

Pharmaceutical formulations	($\mu\text{g mL}^{-1}$) taken	($\mu\text{g mL}^{-1}$) found	%RE	%Recovery $\pm$ RSD
Pramcit, 20 mg tablet	0.02	0.0204	-2.14	102.14 $\pm$ 4.29
	0.04	0.0403	-0.90	100.91 $\pm$ 2.81
	0.06	0.0598	0.28	99.72 $\pm$ 3.22
Lopram, 20 mg tablet	0.02	0.0201	-0.80	100.80 $\pm$ 3.81
	0.04	0.0388	2.91	97.09 $\pm$ 4.74
	0.06	0.0599	0.14	99.86 $\pm$ 3.25
Citalo, 20 mg tablet	0.02	0.0203	-1.95	101.95 $\pm$ 4.60
	0.04	0.0385	3.71	96.29 $\pm$ 4.25
	0.06	0.0602	-0.39	100.39 $\pm$ 2.91

B. Percent recovery of citalopram from commercial formulations (Standard addition method)

Same instrument, reagents, standard solution and sample solutions were used as stated before.

Procedure

For determination of percent recovery of citalopram from pharmaceutical preparations, 0.02, 0.04 and 0.06 $\mu\text{g mL}^{-1}$ standard citalopram solution was mixed with pre-analyzed

commercial sample of citalopram ($0.02 \mu\text{g mL}^{-1}$). The fluorescent intensity was measured at 555 nm using excitation wavelength of 281 nm. The total amount of citalopram in each prepared sample was calculated using calibration curve. The percent recovery was calculated using the formula given below. The results are given in table 3.2.6.

$$\% \text{ Recovery} = \frac{\mu\text{g mL}^{-1} \text{ found in the fortified sample} - \mu\text{g mL}^{-1} \text{ present in the sample}}{\mu\text{g mL}^{-1} \text{ of standard added}} \times 100$$

Table 3.2.6 Evaluation of citalopram percent recovery in commercial formulations (tablets) using the standard addition method

Pharmaceutical preparations	Sample ($\mu\text{g mL}^{-1}$)	($\mu\text{g mL}^{-1}$) added	($\mu\text{g mL}^{-1}$) found	%Recovery $\pm$ RSD
Pramcit, 20 mg tablet	0.02	0.01979	0.02015	101.77 ± 5.47
		0.04038	0.04007	99.19 ± 4.55
		0.06058	0.05975	98.63 ± 2.10
Lopram, 20 mg tablet	0.02	0.01979	0.01998	100.96 ± 4.22
		0.04038	0.04039	100.02 ± 3.25
		0.06058	0.06073	100.25 ± 0.65
Citalo, 20 mg tablet	0.02	0.01979	0.01996	100.85 ± 3.48
		0.04038	0.03900	96.57 ± 2.82
		0.06058	0.05955	98.30 ± 2.59

C. Determination of active ingredients in the commercial formulations

Same instrument, reagents, standard solution and sample solutions were used as stated before.

Procedure

Solutions of the commercial formulation of known concentration were prepared by the same way as mentioned before. Appropriate volumes of these solutions were then diluted with DI water to confirm that concentrations of citalopram in final sample solutions were within the working range. Aliquots of these solutions were then analyzed using the

procedure described above and actual citalopram contents of tablets were calculated using the calibration equation ($Y=320X + 0.333$). Triplicate analyses were performed in each case. The results are given in table 3.2.7.

Table 3.2.7 Determination of active ingredients of citalopram in the commercial formulations

Brand name	Active ingredient (mg/tablet)		
	Label value	Found value $\pm$ SD	% Recovery $\pm$ RSD
Pramcit, 20 mg tablet	20	20.16 $\pm$ 0.235	100.80 $\pm$ 1.17
Lopram, 20 mg tablet	20	19.82 $\pm$ 0.372	99.12 $\pm$ 1.86
Citalo, 20 mg tablet	20	19.87 $\pm$ 0.551	99.36 $\pm$ 2.75

3.2.8 Results and discussion

A fast, simple, and accurate method was developed for the determination of citalopram using silver nanoparticle-enhanced terbium-sensitized fluorescence. For enhancing the fluorescence signal, intermolecular energy from Tb^{3+} to citalopram as a ligand has been explored. As shown in figure 3.2.4 a', the Tb^{3+} ion has a very weak emission spectrum, but in the presence of citalopram this fluorescence intensity is increased (figure 3.2.4.d') which shows that a complex of Tb^{3+} and citalopram has been formed and intramolecular energy transfer has occurred. Furthermore, the addition of colloidal AgNPs to the CIT- Tb^{3+} system greatly enhances fluorescence intensities (figure 3.2.4 e').

To establish the most favorable conditions for CIT- Tb^{3+} -AgNPs system, all the parameters affecting the reaction like pH, volume and concentration of Tb^{3+} solution and silver nanoparticles were thoroughly studied and optimized.

The fluorescence intensity of CIT- Tb^{3+} -Ag NPs system is highly pH dependent. The effect of pH was investigated in the range of 7.0–10. The results obtained showed that maximum fluorescence intensity was observed at pH 8.0 (figure 3.2.6), after which the intensity decreased possibly because of the precipitation of terbium as hydroxide. The effects of carbonate, borate, phosphate, and Tris-HCl buffer solutions of the same pH

were also studied. Maximum fluorescence intensity was observed for the Tris-HCl buffer solution (figure 3.2.5). The effect of volume of Tris-HCl solution was also studied in the range of 0.2–2.5 mL (figure 3.2.7). It was observed that 0.5 mL of pH 8.0 Tris-HCl buffer produced maximum fluorescence intensities.

The effects of Tb^{3+} concentration on CIT- Tb^{3+} -AgNP fluorescence intensities were investigated in the range $1\text{--}8 \times 10^{-4} \text{ molL}^{-1}$. Maximum fluorescence intensity was observed at $2.0 \times 10^{-4} \text{ molL}^{-1}$ of Tb^{3+} (figure 3.2.8). The volume of Tb^{3+} was also optimized and 1.0 mL of $2.0 \times 10^{-4} \text{ molL}^{-1}$ Tb^{3+} produced maximum fluorescence intensity (figure 3.2.9).

The effect of AgNP concentration was investigated in the range of 6.0×10^{-5} to $2.2 \times 10^{-4} \text{ molL}^{-1}$. CIT- Tb^{3+} -AgNP fluorescence intensities increased up to AgNP concentration of $1.4 \times 10^{-4} \text{ molL}^{-1}$ and then decreased (figure 3.2.10). The effect of AgNP solution volume was also investigated in the range of 0.5–2.5 mL (figure 3.2.11) and it was found that 1.0 mL of $1.4 \times 10^{-4} \text{ molL}^{-1}$ AgNPs produced maximum signal, and thus, this concentration was used for further analyses.

Analytical Characteristics

The fluorescence intensity of the CIT- Tb^{3+} -AgNPs system increases linearly with citalopram concentration. Under optimum experimental conditions, the calibration curve of fluorescence intensity versus citalopram concentration was linear in the range of 0.02–14 $\mu\text{g mL}^{-1}$, with an excellent correlation coefficient of 0.997–0.9993 (figure 3.2.12 and figure 3.2.13). The LOD as per criteria of IUPAC was found to be $7.15 \times 10^{-6} \mu\text{g mL}^{-1}$. Similarly the LOQ was also calculated and was found to be $2.38 \times 10^{-5} \mu\text{g mL}^{-1}$. The linear regression equation, intercept, slope, correlation coefficient, and relative standard deviation were interpreted and are given in table 3.2.2.

Application

To evaluate the selectivity of the method for analysis of the pharmaceutical formulations containing citalopram, the interferences effect of different excipients were studied. The effects of commonly used excipients like glucose, fructose, starch, lactose, talc, and

magnesium stearate was studied. The interference study was carried out by preparing samples containing a fixed amount of citalopram ($0.02 \mu\text{g mL}^{-1}$) and 5.0, 10, and 20 fold concentrations ladders of excipients. A 5% error criterion was adopted. None of these common excipients was found to cause any interference. Average recoveries attained were in the range 97.77% to 101.34% (figure 3.2.14 and table 3.2.3).

The precision of the developed method was studied by ascertaining citalopram in pure form and pharmaceuticals formulations using three different concentrations within the calibration curve range in triplicate. The results are listed in table 3.2.4 for the standard and in table 3.2.5 for pharmaceutical preparations. The percent recoveries obtained ranged from 98.98–100.97% for the standard and 96.29–102.14% for pharmaceutical preparations with narrow relative standard deviations, indicating that proposed method has good reproducibility.

The validity of the present method was evaluated by standard addition method using three different labels of tablets Pramcit, Lopram, and Citalo (all contain 20 mg of citalopram). Known amounts of standard citalopram solution were added to the tablet solutions and analyzed as described above. Percent recoveries were calculated and ranged from 96.57–101.77% (table 3.2.6). The proposed method was successfully applied to the determination of citalopram in the three pharmaceutical preparations. The results obtained were in close agreement with label quantities (table 3.2.7), which shows that the proposed method can be used for determination of citalopram contents in pharmaceutical formulations.

References

1. J.Z. Guo, H. Cui, W. Zhou, W. Wang, “Ag nanoparticles sensitized chemiluminescent reaction between luminal and hydrogen peroxide”, *Journal of Photochemistry and Photobiology Chemistry*, 193, 89–96, (2008).

3.3 SILVER NANOPARTICLE ENHANCED CHEMILUMINESCENCE (CL) METHOD FOR THE DETERMINATION OF CITALOPRAM IN PHARMACEUTICAL PREPARATIONS AND HUMAN PLASMA

3.3.1 Theoretical aspect of reaction mechanism

The products of the CL reaction were 3-aminophalate ions, that is, the oxidation product of luminol. H_2O_2 could oxidize citalopram and produce energy easily when catalyzed by AgNPs, which facilitate the transition of oxidized citalopram from its ground state to the excited state. Thus, when this excited state returns to the ground state, it could transfer energy to ground state 3-aminophalate ions, and increase the CL phenomena. The proposed CL reaction mechanism for the luminol- H_2O_2 -citalopram-AgNPs system is shown in figure 3.3.1.

Preparation and characterization of silver nanoparticles

Colloidal solutions of silver nanoparticles were prepared as per previously described method. The prepared AgNPs were characterized by UV-Visible spectroscopy (figure 3.3.2) and transmission electron microscopy (TEM) (figure 3.3.3) at an accelerating voltage of 100 kV.

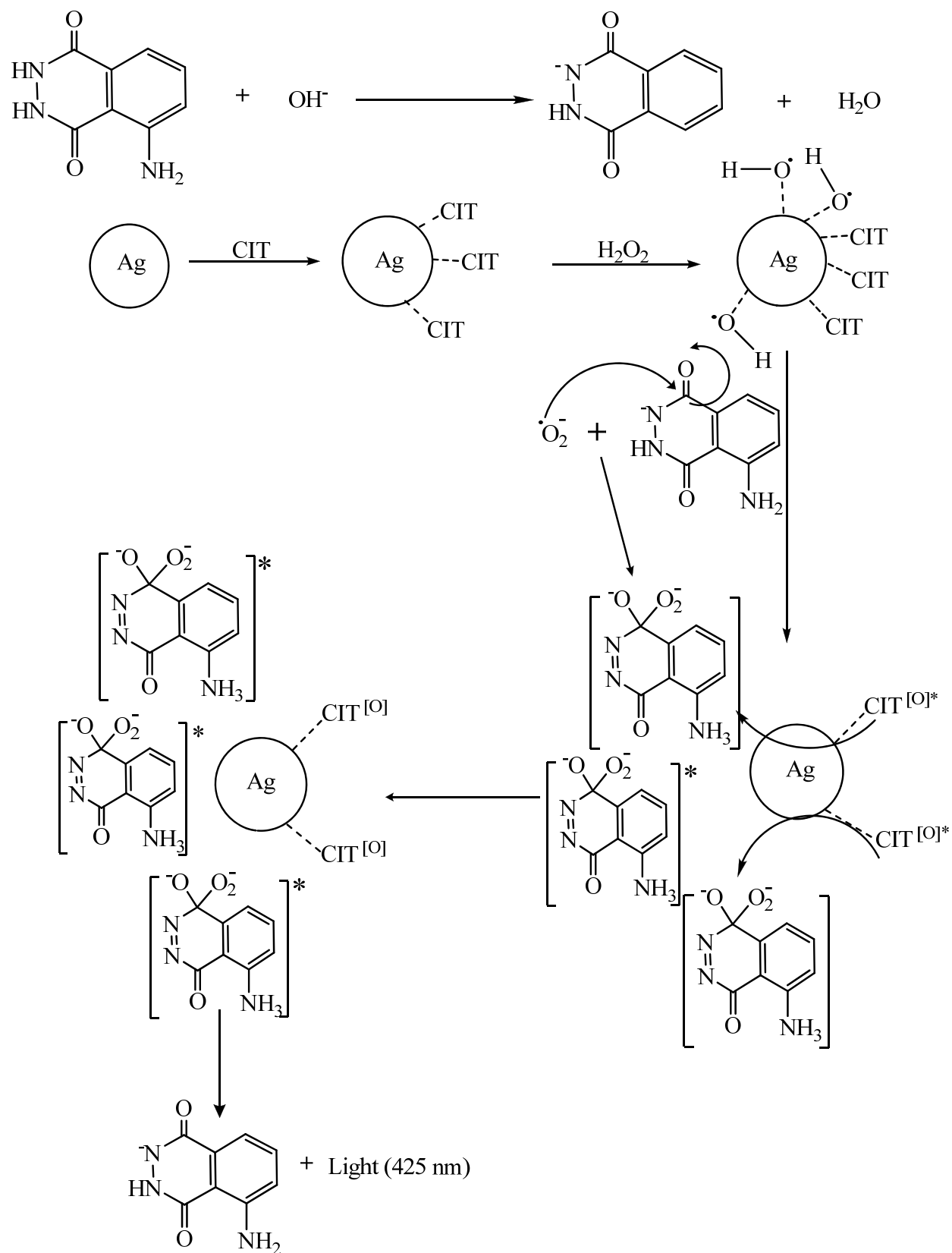


Figure 3.3.1 Scheme of proposed CL reaction mechanism

3.3.2 Preliminary investigation of the possibility of silver nanoparticles enhanced CL method for the determination of citalopram

Preliminary studies were conducted to investigate the possible enhancement of the chemiluminescence intensities of luminol-H₂O₂ system by AgNPs. Initially high concentration of citalopram (2500 ngmL⁻¹), different buffer solutions at different pH (12-14), Ag NPs (1.0×10^{-4} molL⁻¹), luminol solution (2.0×10^{-3} molL⁻¹) and H₂O₂ (4.0×10^{-2} molL⁻¹) were injected in the reaction cell and CL intensity was measured. The CL spectrum of luminol and H₂O₂ catalyzed by AgNPs in the presence of citalopram is shown in figure 3.3.4. The curve 'a' shows the CL spectrum of luminol-H₂O₂ with maximum intensity at 428 nm. CL intensity increased when AgNPs were injected into the luminol-H₂O₂ system (curve c), and when citalopram was introduced into the luminol-H₂O₂-AgNP catalyzed system, CL intensity increased strongly (curve d).

3.3.3 Optimization studies for the determination of citalopram using silver nanoparticles enhanced CL method

Instruments

A Hitachi F-4500 spectrofluorometer was used to detect and record CL intensity of the luminol-H₂O₂-AgNPs system. For CL measurements, the light source of the instrument was turned off and using only the photomultiplier tube of the apparatus. The slit width of the emission monochromator was 5 nm and the power supply of the photomultiplier tube used to detect the CL signal was set at 700 V. Light producing reactions were carried out in a standard 1 × 1 cm quartz cell placed in the spectrofluorimeter cell holder. A UV-1800 Shimadzu UV-Vis spectrophotometer was used for absorption spectra. A pH meter (Mettler-Toledo MP 220, US) was used to adjust the buffer solution pH.

Materials and reagents

Analytical grade purity reagents and doubly deionized water (DI) was used throughout. Luminol (5-amino-2, 3-dihydro-1, 4-phthalazinedione), silver nitrate, Tris (hydroxyl methyl amino methane ((HOCH₂)₃CNH₂), NaH₂BO₃, KH₂PO₄, and NaHCO₃ (Sigma-Aldrich US), hydrogen peroxide and sodium hydroxide (Junsei Chemical Japan), and

sodiumborohydride (NaBH_4) and sodium citrate (Merck Germany) was used in this work. Standard reference citalopram was provided by Z-Jan's Pharmaceutical Industry (Hayatabad Peshawar, Pakistan). Commercial formulations of citalopram, that is, Lopram, Citalo and Pramcit (all as 20 mg tablets) were purchased locally.

Solution preparation

Luminol stock solution (4.0×10^{-3} M) was prepared by dissolving 0.035 g of luminol in 4.5 mL of 0.1 M NaOH, and diluting to 50 mL with deionized (DI) water. Stock solution was stored at 4 °C in the dark. Hydrogen peroxide stock solution (4.0 M) was prepared by diluting 10.21 mL of 30% H_2O_2 to 25 mL with DI water. Carbonate buffer solution (0.1 M) was prepared by dissolving 0.8401 g of NaHCO_3 in 100 mL DI water and the pH was adjusted with NaOH solution. All working solutions were prepared daily before use from stock solutions by appropriate dilution with DI water.

Preparation of standard solution

Standard stock solution of citalopram ($250 \mu\text{g mL}^{-1}$) was prepared by dissolving 0.0125 g of citalopram in 2.0 mL of distilled ethanol with shaking and made the volume up to 50 mL with DI water. Working solutions of citalopram at $10 \mu\text{g mL}^{-1}$ and $5.0 \mu\text{g mL}^{-1}$ were daily prepared by diluting proper volumes of stock solution with DI water.

Preparation of sample solution ($250 \mu\text{g mL}^{-1}$)

Five tablets containing nominally 20 mg of citalopram per tablet were weighed and the average weight of powder in one tablet was calculated. Tablets were ground to a fine powder in a mortar. Drug powder equivalent to 0.0125 g of citalopram was then dissolved in 2.0 mL of ethanol, DI water was added, and the mixture was sonicated for 10 minutes. The resultant solution was then filtered and diluted to 50 mL with DI water. An appropriate volume of this solution was then diluted with DI water to obtain a citalopram concentration in the final sample solution within the working range.

Procedure

CL intensity was measured by adding the following quantities of equilibrated solutions to a reaction cell using a micropipette; 300 μL of carbonate buffer solution (pH 13), 300 μL of AgNPs solution ($1.92 \times 10^{-4} \text{ molL}^{-1}$), 300 μL luminol ($3.0 \times 10^{-3} \text{ molL}^{-1}$) solution, and citalopram stock solution to give a final concentration of 40–2500 ngmL^{-1} . The CL emission spectrum was scanned in the range of 300–650 nm after injecting 300 μL ($8.0 \times 10^{-2} \text{ molL}^{-1}$) of H_2O_2 into the cell to initiate the reaction (Figure 3.3.4). CL intensity (ΔI) was calculated using $\Delta I = I_s - I_o$, where I_s and I_o are CL signals in the presence and absence of citalopram respectively.

For studying the effect of different buffer solutions, citalopram solution (2500 ngmL^{-1}) was injected in the reaction cell. Then borate, carbonate, phosphate, and Tris-HCl buffer solutions of the same pH were injected keeping rest of the procedure the same and CL intensity was measured (figure 3.3.5).

To optimize the pH, carbonate buffer in the pH range of 12–14 was injected to 2500 ngmL^{-1} citalopram standard solution pursued by the same procedure as described before and CL intensity was measured at the optimized wavelength (figure 3.3.6).

To optimize the concentration of luminol, only the concentration of luminol solution was varied in the range of $1.0\text{--}4.0 \times 10^{-3} \text{ molL}^{-1}$ keeping rest of the procedure the same and CL intensity was measured (figure 3.3.7).

To optimize the concentration of H_2O_2 , 2500 ngmL^{-1} citalopram solution was injected to a reaction cell and then H_2O_2 solution in the range of $2.0\text{--}20 \times 10^{-2} \text{ molL}^{-1}$ was injected keeping rest of the procedure the same and CL intensity was measured (figure 3.3.8).

For optimization of AgNPs concentration, only the concentration of AgNP was varied in the range of $1.2\text{--}2.4 \times 10^{-4} \text{ molL}^{-1}$ keeping rest of the procedure the same. CL intensity was measured and the results are shown in figure 3.3.9.

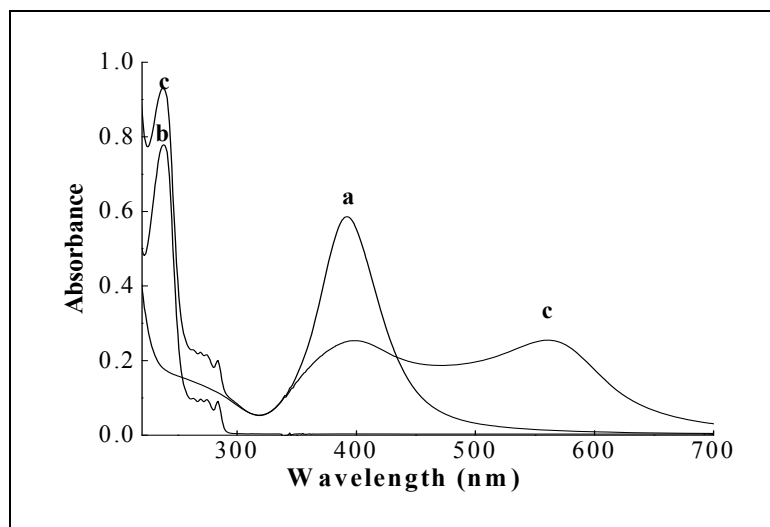


Figure 3.3.2 UV-visible spectra of (a) Ag NPs; (b) citalopram; (c) citalopram-Ag NPs
Conditions; citalopram, $20 \mu\text{g mL}^{-1}$; Ag NPs, $4.0 \times 10^{-5} \text{ mol L}^{-1}$

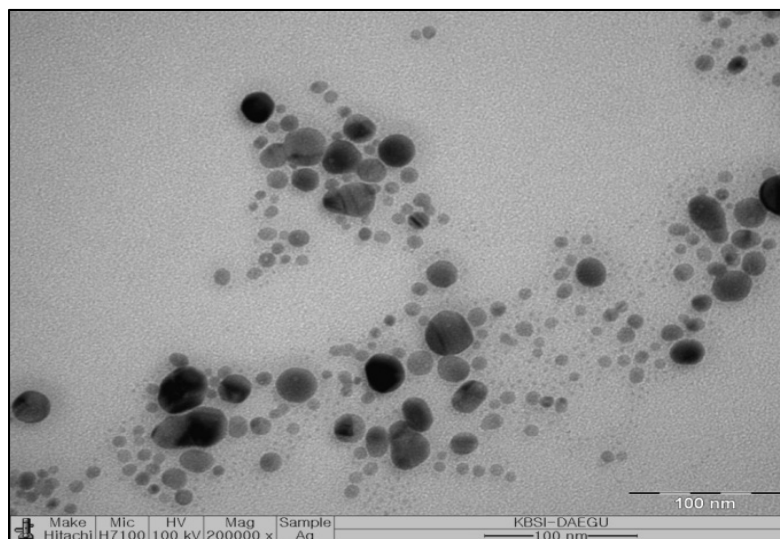


Figure 3.3.3 TEM image of the prepared AgNPs

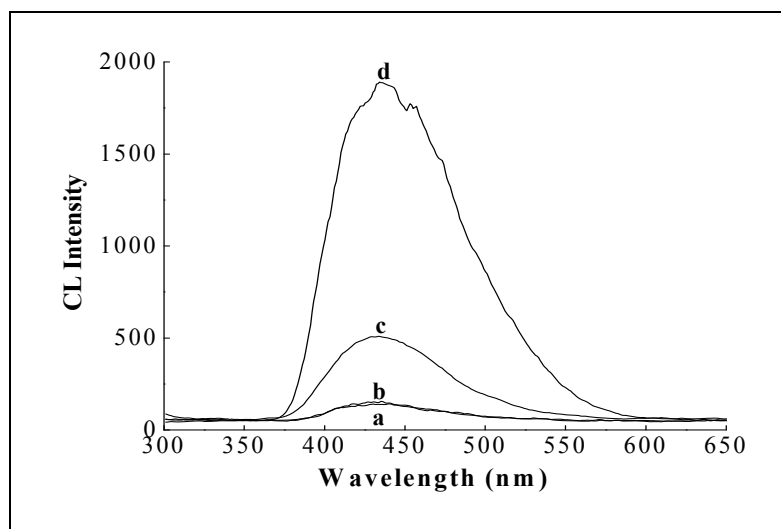


Figure 3.3.4 CL emission spectra (a) luminol-H₂O₂-buffer; (b) luminol-H₂O₂-buffer-citalopram; (c) luminol-H₂O₂-buffer-AgNPs; (d) luminol-H₂O₂-buffer-AgNPs-citalopram

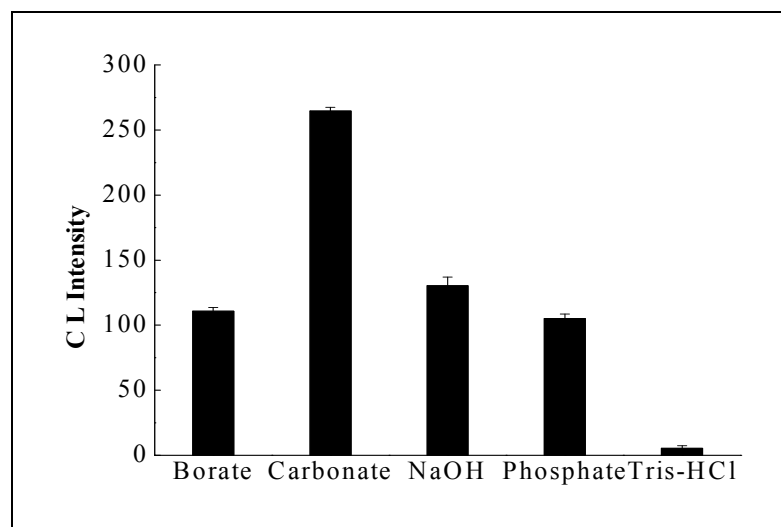


Figure 3.3.5 Effect of buffers on the CL determination of citalopram

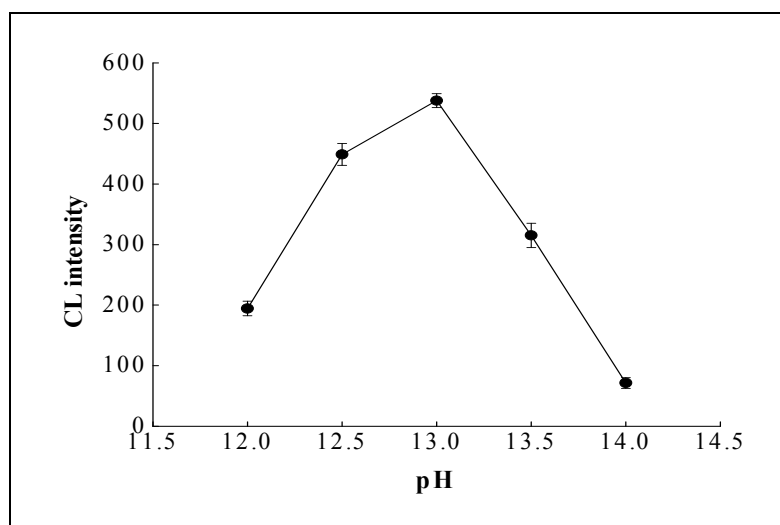


Figure 3.3.6 Effect of pH on the CL determination of citalopram

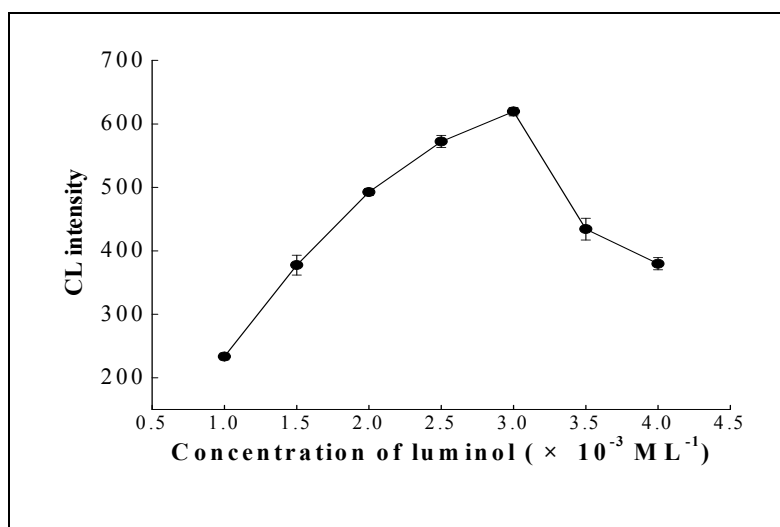


Figure 3.3.7 Effect of luminol concentration on the CL determination of citalopram

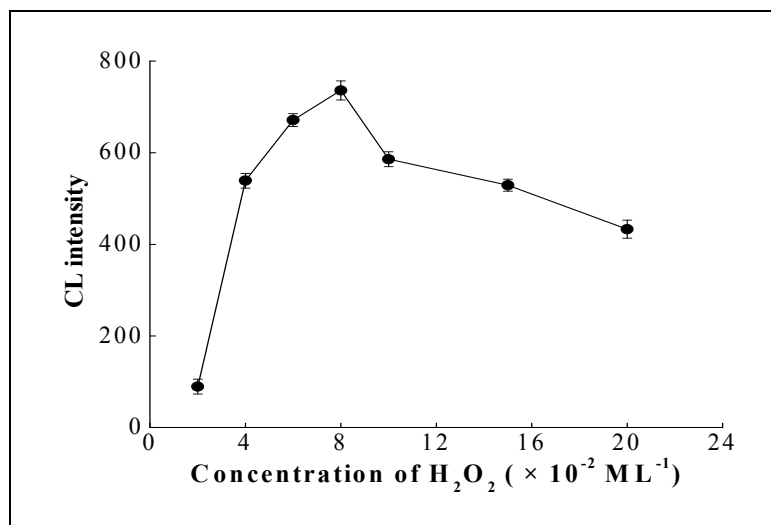


Figure 3.3.8 Effect of H_2O_2 concentration on the CL determination of citalopram

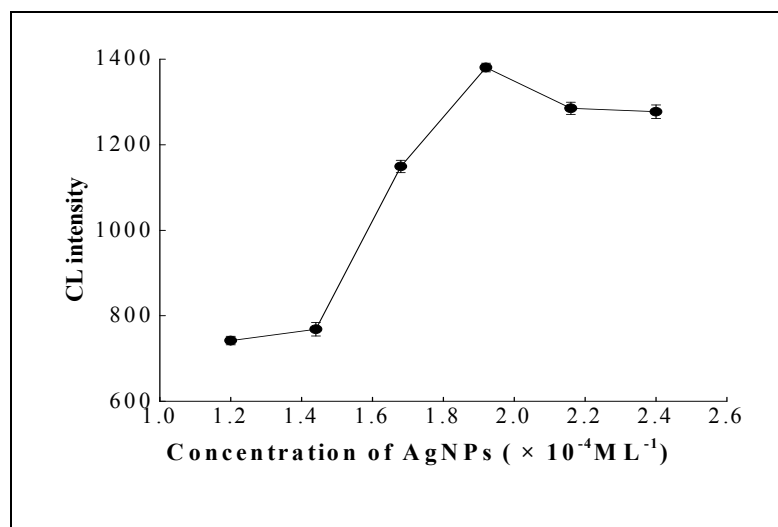


Figure 3.3.9 Effect of AgNPs concentration on the CL determination of citalopram

3.3.4 Effect of concentration of citalopram on CL intensity

Procedure

CL intensity was measured by adding the following quantities of solutions to a reaction cell using a micropipette; 300 μL of carbonate buffer solution (pH 13), 300 μL of AgNPs solution ($1.92 \times 10^{-4} \text{ molL}^{-1}$), 300 μL luminol ($3.0 \times 10^{-3} \text{ molL}^{-1}$) solution, and citalopram stock solution to give a final concentration of 40–2500 ngmL^{-1} . The kinetic-curve of the CL signal was measured at 432 nm after injecting 300 μL ($8.0 \times 10^{-2} \text{ molL}^{-1}$) of H_2O_2 into the cell to initiate the reaction. The average value of the measured CL intensity was obtained by averaging 11 points (from 15 to 25 sec post-injection). CL intensity (ΔI) was calculated using $\Delta I = I_s - I_o$, (table 3.3.1 and figure 3.3.10).

Table 3.3.1 Effect of concentration of citalopram on CL intensity

Concentration (ngmL^{-1})	CL Intensity
40	76.44
80	130.98
120	163.32
250	250.87
500	436.92
1000	857.54
2000	1621.27
2500	1943.23

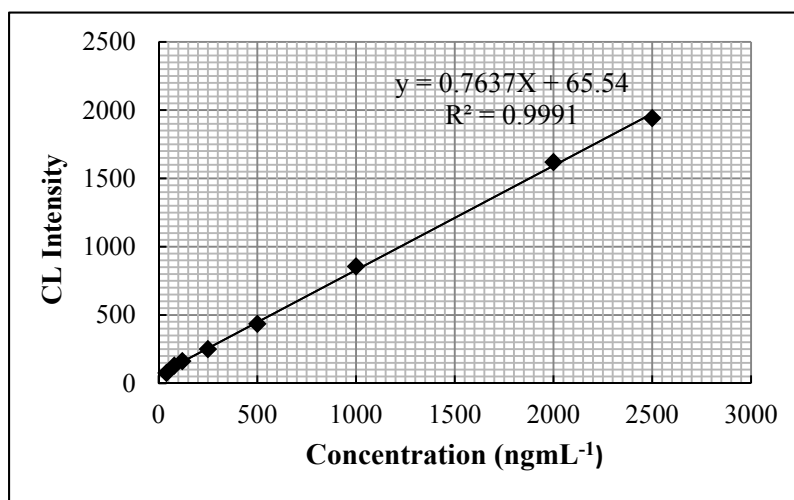


Figure 3.3.10 Effect of concentration of citalopram on CL intensity

Determination of LOD, LOQ and other analytical characteristics for determination of citalopram by the proposed CL method

Seven replicates solution of lowest measurable concentration of citalopram (40 ngmL⁻¹) were prepared and evaluated by the proposed method pursuing the same procedure as stated before. LOD, LOQ and other analytical characteristics were calculated and are given in table 3.3.2.

Table 3.3.2 Analytical parameters for silver nanoparticle enhanced chemiluminescence-based determination of citalopram

Parameter	Value
λ_{em} (nm)	432
Linear range (ngmL ⁻¹)	40–2500
Limit of detection (ngmL ⁻¹)	3.78
Limit of quantification (ngmL ⁻¹)	12.62
Regression equation (y)	Y= 305.15X + 68.06
Slope (b)	305.15
Intercept (a)	68.06
Correlation coefficient (r ²)	0.999
Standard deviation (ngmL ⁻¹)	1.41
Relative standard deviation (%)	3.65

3.3.5 Effect of common excipients on determination of citalopram by the proposed CL method

Procedure

The effects of common excipients like glucose, starch, fructose, sucrose, talc, lactose, and magnesium stearate used in pharmaceutical preparations of citalopram were studied for their interference effects. A series of solutions containing 40 ngmL⁻¹ of citalopram plus different amounts of these substances at 2, 5, and 10 fold the concentrations likely to be present were prepared. Then the optimized amount of all the reagents was added and CL intensity was measured by the procedure mentioned before (figure 3.3.11).

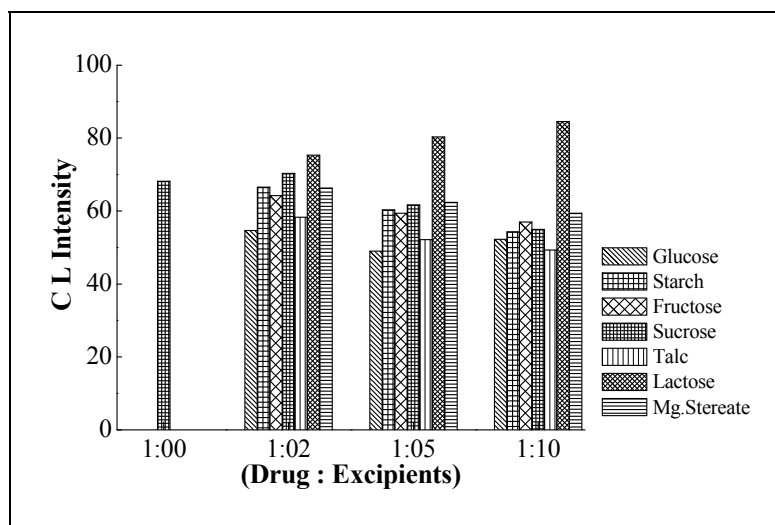


Figure 3.3.11 Effect of common excipients on determination of citalopram by the proposed CL method

3.3.6 Application of the investigated method for the analysis of citalopram in different pharmaceutical preparations

A. Determination of precision and accuracy of the investigated method

Same instrument, reagents and standard solution were used as mentioned before.

Procedure

The precision and accuracy of the proposed method was studied by analyzing solutions of pure citalopram, pharmaceutical formulations, and spiked plasma samples at three different concentrations within the calibration curve range. Calibration curve was plotted and known concentrations (40, 60 and 80 ngmL⁻¹) of these sample solution were then analyzed by the proposed method. The results are given in table 3.3.3 for the pure citalopram solution, in table 3.3.4 for pharmaceutical preparations and in table 3.3.5 for spiked plasma sample.

Table 3.3.3 Evaluation of accuracy and precision of the present method using solutions of pure citalopram

(ngmL ⁻¹) taken	(ngmL ⁻¹) Found	% Recovery ± RSD
40	39.298	98.25 ± 2.00
60	58.624	97.71 ± 4.21
80	81.594	101.99 ± 2.70
Mean		99.32
± SD		2.33
t-test		0.505 (4.303)

Table 3.3.4 Evaluation of accuracy and precision of the present method for citalopram determination in pharmaceutical preparations

Pharmaceutical preparations	(ngmL ⁻¹) taken	(ngmL ⁻¹) found	% Recovery ± RSD
Citalo, 20 mg tablet	40	39.19	97.96 ± 2.84
	60	60.38	100.63 ± 4.07
	80	79.45	99.31 ± 2.36
Lopram, 20 mg tablet	40	40.85	102.13 ± 3.86
	60	60.98	101.64 ± 4.65
	80	79.51	99.38 ± 3.30
Pramcit, 20 mg tablet	40	39.37	98.42 ± 4.60
	60	60.05	100.08 ± 4.25
	80	78.21	97.76 ± 2.39

Table 3.3.5 Evaluation of accuracy and precision of the present method for citalopram determination in spiked plasma

Sample	(ngmL ⁻¹) taken	(ngmL ⁻¹) Found	%Recovery ± RSD
Plasma	40	39.884	99.71 ± 4.40
	60	60.590	100.98 ± 3.46
	80	77.065	96.33 ± 2.68

B. Percent recovery of citalopram from commercial formulations (Standard addition method)

Instrument, reagents, standard solution and sample solutions used were the same as mentioned before.

Procedure

For determination of percent recovery of citalopram from pharmaceutical preparations, 40, 60 and 80 ngmL⁻¹ standard citalopram solution was mixed with pre-analyzed commercial sample of citalopram (40 ngmL⁻¹). The CL intensity was measured at 432 nm. The total amount of citalopram in each sample was calculated from calibration curve. Each experiment was performed in triplicates and the mean percent recovery was calculated by the following formula. The results are given in table 3.3.6.

$$\% \text{ Recovery} = \frac{\text{ngmL}^{-1} \text{ found in the fortified sample} - \text{ngmL}^{-1} \text{ present in the sample}}{\text{ngmL}^{-1} \text{ of standard added}} \times 100$$

Table 3.3.6 Evaluation of recovery test of citalopram in commercial formulation (tablets) by the present method

Pharmaceutical preparations	Amount added (ngmL ⁻¹)	Amount found (ngmL ⁻¹)	% Recovery ± RSD
Citalo, 20 mg tablet	39.298	39.776	101.21 ± 3.05
	58.624	60.259	102.78 ± 4.33
	81.594	82.609	101.24 ± 1.75
Lopram, 20 mg tablet	39.298	38.450	97.84 ± 3.74
	58.624	59.436	101.38 ± 0.77
	81.594	83.289	102.07 ± 3.51
Pramcit, 20 mg tablet	39.298	39.177	99.69 ± 2.50
	58.624	59.634	101.72 ± 1.90
	81.594	81.559	99.95 ± 2.54

C. Determination of active ingredients of citalopram in the commercial formulations

Instrument, reagents, standard solution and sample solutions used were the same as mentioned before.

Procedure

Sample solutions of pharmaceutical preparations were prepared by diluting the appropriate volume of the stock solution with distilled water. An appropriate volume of this solution was then diluted with DI water to obtain a citalopram concentration in the final sample solution within the working range. Aliquots of these solutions were then analyzed using the procedure described above and actual citalopram content per tablet was calculated. Triplicate analyses were performed in each case. The results are given in table 3.3.7.

Table 3.3.7 Determination of citalopram active ingredients in pharmaceutical preparations (tablets) by the present method

Brand name	Active ingredient (mg/tablet)		
	Label value	Found value $\pm$ SD	% Recovery $\pm$ RSD
Citalo, 20 mg tablet	20	19.86 $\pm$ 0.267	99.30 $\pm$ 1.33
Lopram, 20 mg tablet	20	20.21 $\pm$ 0.292	101.05 $\pm$ 1.46
Pramcit, 20 mg tablet	20	19.75 $\pm$ 0.240	98.76 $\pm$ 1.20

3.3.7 Validation of the proposed method in spiked human plasma

A 0.5 mL of a plasma sample was deproteinized by adding 5.0 mL of acetonitrile. The mixture was then centrifuged for 15 minutes at 3500 rpm, and the protein free supernatant obtained was diluted with DI water. Citalopram (250 $\mu\text{g mL}^{-1}$) was added and the volume was made up to 50 mL with DI water. Prior to analysis, the concentration of citalopram was adjusted into the working range with an appropriate addition of DI water. The analysis was then conducted as described above (table 3.3.5 and table 3.3.8).

Table 3.3.8 Recovery of citalopram in spiked plasma samples by the proposed method

Sample	(ngmL ⁻¹) added	(ngmL ⁻¹) found	% Recovery $\pm$ RSD
Plasma	39.298	38.709	98.50 $\pm$ 3.81
	58.624	58.833	100.35 $\pm$ 3.91
	81.594	78.051	95.65 $\pm$ 2.66

3.3.8 Results and discussion

A simple method has been developed for the determination of citalopram concentrations based on the enhancement of the CL of the luminol-H₂O₂-AgNPs system by citalopram. The CL intensity of the luminol-H₂O₂ reaction catalyzed by AgNPs was found to be remarkably increased in the presence of citalopram. The CL spectra of luminol and H₂O₂ catalyzed by AgNPs in the absence and presence of citalopram were recorded and are shown in figure 3.3.4. The curve 'a' shows the CL spectrum of luminol-H₂O₂ with maximum intensity at 428 nm. CL intensity increased when AgNPs were injected into the luminol-H₂O₂-system (curve c), and when citalopram was introduced into the luminol-H₂O₂-AgNP catalyzed system, CL intensity increased strongly (curve d). It was noted that the products of the CL reaction were 3-aminophalate ions, that is, the oxidation product of luminol. H₂O₂ could oxidize citalopram and produce energy easily when catalyzed by AgNPs, which facilitate the transition of oxidized citalopram from its ground state to the excited state. Thus, when this excited state returns to the ground state, it could transfer energy to ground state 3-aminophalate ions, and increase the CL phenomena (figure 3.3.1).

To establish the most favorable conditions for luminol-H₂O₂-citalopram-AgNPs system, all the parameters affecting the CL reaction like pH, concentration of luminol, H₂O₂, and silver nanoparticles were thoroughly studied and optimized.

The effect of pH was investigated using carbonate buffer in the pH range 12–14. The results obtained showed that maximum intensity was obtained at pH 13 (figure 3.3.6), and thus, pH 13 was selected for further analyses. In addition, the effects of some common buffer solutions like borate, carbonate, phosphate, and Tris-HCl buffers were

studied at pH 13 and the results were compared with unbuffered solution (0.1 M NaOH). Maximum CL intensity was observed using carbonate buffer (figure 3.3.5) and thus, carbonate buffer at pH 13 was used for further experiments.

The effects of luminol concentration were investigated in the range $1.0\text{--}4.0 \times 10^{-3} \text{ molL}^{-1}$. CL intensity was found to peak at a luminol concentration of $3.0 \times 10^{-3} \text{ molL}^{-1}$ (figure 3.3.7), and thus, this concentration was used for further analyses.

The effects of H_2O_2 concentration were studied in the range $2.0\text{--}20 \times 10^{-2} \text{ molL}^{-1}$. CL intensity was found to peak at a concentration of $8.0 \times 10^{-2} \text{ molL}^{-1}$ (figure 3.3.8), and thus, this concentration was used for further experiments.

Similarly the effect of AgNPs concentration was investigated in the range $1.2\text{--}2.4 \times 10^{-4} \text{ molL}^{-1}$. CL intensity increased up to a AgNPs concentration of $1.92 \times 10^{-4} \text{ molL}^{-1}$ and then stabilized (figure 3.3.9), and thus, this concentration was used for further experiments.

Analytical Figures of Merit

Under optimum experimental parameters, the plot of CL intensity versus citalopram concentration was linear in the range of $40\text{--}2500 \text{ ngmL}^{-1}$, with an excellent correlation coefficient of 0.999 (figure 3.3.10). LOD and LOQ values were calculated and found to be 3.78 ngmL^{-1} and 12.62 ngmL^{-1} respectively. The linear regression equation, intercept, slope, correlation coefficient, and RSD are given in table 3.3.2.

Application

To evaluate the selectivity of the method for quantification of pharmaceutical formulations containing citalopram, the interferences effects of different excipients like glucose, fructose, starch, lactose, talc, and magnesium stearate, which are commonly used in pharmaceutical tablets, were studied. The interference study was carried out by preparing samples containing a fixed amount of citalopram (40 ngmL^{-1}) and 2, 5 and 10 fold concentrations ladders of excipients. CL intensities of the prepared solutions (standard + excipients) were measured and compared with that attained for the standard

citalopram solution (40 ngmL^{-1}). No interference effects were observed from these common excipients in the determination of citalopram (figure 3.3.11).

The precision of the developed method was studied by analyzing solutions of pure citalopram, of commercial formulations, and by analyzing spiked plasma samples. The results are listed in table 3.3.3 for standard citalopram solutions, in table 3.3.4 for the pharmaceutical preparations, and in table 3.3.5 for the spiked plasma samples. Percent recoveries ranged from 97.71 to 101.99% for citalopram solutions, from 97.76 to 102.13% for pharmaceutical preparations, and from 96.33 to 100.98% for spiked plasma samples with good RSD value, signifying good reproducibility of the developed method.

The validity of the present method was investigated by the standard addition method using three different sorts of tablets (Lopram, Citalo, and Pramcit) and using spiked plasma samples. Specific amounts of standard citalopram solution were added to sample solutions and mixtures were analyzed as described above. Percent recoveries ranged from 97.84 to 102.78% for pharmaceutical preparations and from 95.65 to 100.35% for spiked plasma samples (table 3.3.6 and 3.3.8). The good percent recoveries in all cases demonstrate that the proposed method is suitable for the determination of citalopram concentration in commercial formulations and spiked plasma samples.

The present method was effectively used for the determination of citalopram active ingredients in three different types of pharmaceutical preparations. The results obtained of the proposed method were in close agreement with the label quantities (table 3.3.7). Due to high precision and accuracy of the proposed method and absence of interferences from common excipients, the method was successfully used for the determination of citalopram in spiked human plasma samples (table 3.3.5 and 3.3.8).

3.4 SPECTROFLUORIMETRIC METHOD FOR DETERMINATION OF SULPIRIDE IN COMMERCIAL FORMULATIONS USING HANTZSCH CONDENSATION REACTION

3.4.1 Proposed mechanism for condensation reaction of sulpiride with acetyl acetone and formaldehyde

Acetyl acetone together with formaldehyde reacts with aliphatic amines by Hantzsch condensation reaction forming a fluorescent product that can be measured by spectrofluorimeter. Sulpiride do not contain free amino group, while upon hydrolysis with alkali the cleavage of weak amide group occurs with production of free primary amine. This primary amine of sulpiride is used for determination through Hantzsch condensation reaction using acetyl acetone as β -diketone and formaldehyde as an aldehyde to form a fluorescent condensation product. In the first step the acetyl acetones on reaction with formaldehyde form 3, 5-diacetylheptane-2, 6 dione. In the second step the 3, 5-diacetylheptane-2, 6-dione undergoes condensation with amino group of the sulpiride and produces a fluorescent product which can be measured by spectrofluorimeter (figure 3.4.1).

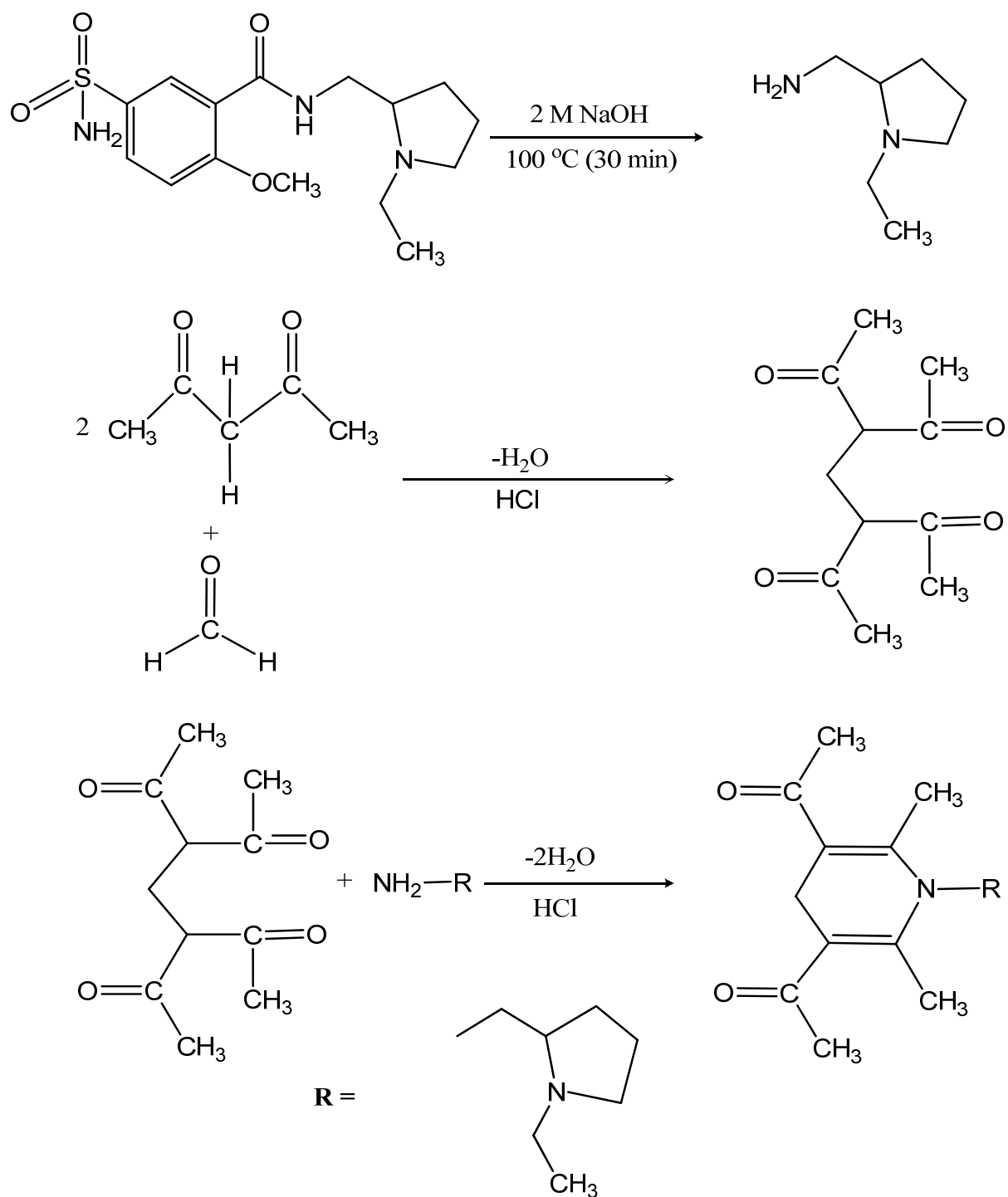


Figure 3.4.1 Proposed mechanism for condensation reaction of sulpiride with acetyl acetone and formaldehyde

3.4.2 Preliminary investigation of the possibility of condensation reaction between sulpiride with acetyl acetone and formaldehyde for its spectrofluorimetric determination

Preliminary studies were carried out to investigate the possible Hantzsch condensation reaction between sulpiride with acetyl acetone and formaldehyde in acidic medium. Initially high concentration of sulpiride ($4.0 \mu\text{g mL}^{-1}$), acetyl acetone (2.0 M), formaldehyde (25%) and hydrochloric acid (1.0 M) were mixed in a conical flask and heated for 20 minutes on a water bath at 100°C to ensure the condensation reaction. The reaction resulted in a fluorescence product, signifying successful condensation reaction of sulpiride with acetyl acetone and formaldehyde in acidic medium and subsequent spectrofluorimetric determination of sulpiride.

3.4.3 Optimization studies for spectrofluorimetric determination of sulpiride using Hantzsch condensation reaction

Instruments

All fluorescence intensities were measured on a RF-5301 PC spectrofluorophotometer. Digital analytical balance (Germany) and electric thermostatic water bath were used in this work.

Reagents

Analytical grade purity chemicals were used. Acetyl acetone (ACROS-01 Geel, Belgium), formaldehyde 37% and hydrochloric acid (Merck Darmstadt, Germany) were used in this work. Standard sulpiride powder was granted by Hang Zhou Pharma and Chem. Co China. Commercial formulations of sulpiride (sulpiride-50, 50 mg caps manufactured by Combitic Global D-2 industrial area Sonapat-131001 (Hr) India, Sulpiride-50, 50 mg caps manufactured by Xier kangtai Pharmaceutical Co. Ltd North zone, High-New Technology Industrial Zone, Pingxiang, Jiangxi, P.R.China, Sulpiride Capsules 50 mg manufactured by ARBRO pharmaceuticals Ltd 6/14 Kirti nagar,

Industrial Area, New Delhi (India), Sulpiride-50, 50 mg capsule manufactured by Unicare (India) Pvt. Ltd were purchased from local market.

Solutions preparation

Acetyl acetone solution (1.4 M)

Acetyl acetone solution (1.4 M) was prepared by mixing 7.19 mL of the reagent in distilled water and diluted to 50 mL in volumetric flask with the same solvent.

Formaldehyde solution (15%)

Formaldehyde solution (15%) was prepared by diluting 20.2 mL of 37% formaldehyde to 50 mL in volumetric flask with distilled water.

Hydrochloric acid solution (0.25 M)

Hydrochloric acid (0.25 M) solution was prepared by diluting 1.10 mL of (11.65 M) HCl to 50 mL with distilled water.

Standard sulpiride solution (100 $\mu\text{g mL}^{-1}$)

Standard stock solution of sulpiride (100 $\mu\text{g mL}^{-1}$) was prepared by dissolving 0.005 g of reliable standard in 5.0 mL of 2.0 M NaOH solution in a 50 mL conical flask and heated on a boiling water bath for 30 minutes. After cooling it was diluted to 50 mL with distilled water. Working standard solution (10 $\mu\text{g mL}^{-1}$) was freshly prepared by appropriate dilution of the stock solution with distilled water.

Sample solution (100 $\mu\text{g mL}^{-1}$)

Four capsules of each commercial formulation were weighed to get the average weight of one capsule. An accurately weighed amount of the powder equal to 0.005 g of sulpiride was dissolved in 5.0 mL of 2.0 M NaOH, heated on boiling water bath for 30 minutes and after cooling filtered, and made the volume up to 50 mL with distilled water. Sample solution (10 $\mu\text{g mL}^{-1}$) was prepared from stock solution by dilution with distilled water.

Procedure

1.0 mL of sulpiride standard solution ($100\ \mu\text{g mL}^{-1}$) was taken in a set of test tubes. 1.0 mL of 15% formaldehyde, 1.0 mL of 0.25 M hydrochloric acid and 2.5 mL of 1.4 M acetyl acetone was added to each test tube. The components of mixture were moderately shaken and heated for 30 minutes in a boiling water bath. The contents of the test tubes were cooled under tap water and diluted to 25 mL with distilled water in volumetric flasks. For excitation spectrum the analyte was scanned in the range of 320–470 nm and for emission spectrum it was scanned in the range of 420–600 nm using spectrofluorophotometer (figure 3.4.2).

To optimize the concentration of formaldehyde, 1.0 mL of standard sulpiride ($100\ \mu\text{g mL}^{-1}$) was taken in a set of 20 mL test tubes. To each test tube 0.5 mL of formaldehyde in the concentration range of 5.0–25% was added pursued by the addition of 1.0 mL hydrochloric acid (0.25 M) and 2.5 mL of acetyl acetone (1.4 M) and heated for 30 minutes in a boiling water bath. After cooling, the contents were transferred to 25 mL flasks and diluted to the mark with distilled water. Fluorescence intensity was measured at λ_{ex} 431 nm and λ_{em} 483 nm (figure 3.4.3).

For optimizing the volume of formaldehyde (15%) solution, only volume of formaldehyde was varied in the range of 0.25–2.5 mL and rest of the procedure was same as described above (figure 3.4.4).

To optimize the concentration of hydrochloric acid, 1.0 mL of standard sulpiride ($100\ \mu\text{g mL}^{-1}$) was taken in a set of 20 mL test tubes. To each test tube, optimized volume of formaldehyde (1.0 mL of 15%) was added. Then 1.0 mL of HCl solution in the concentration range of 0.05–0.5 M was added pursued by the addition of 2.5 mL of acetyl acetone (1.4 M) and fluorescence intensity was measured (figure 3.4.5).

For optimizing the volume of hydrochloric acid (0.25 M), only HCl volume was varied in the range of 0.5–2.5 mL and rest of the procedure was same as mentioned before and fluorescence intensity was measured (figure 3.4.6).

To optimize the concentration of acetyl acetone, only the concentrations of acetyl acetone was varied in the range of 0.6–1.8 M keeping rest of the procedure the same and fluorescence intensity was measured (figure 3.4.7).

To optimize the volume of acetyl acetone (1.4 M), only acetyl acetone volume was varied in the range of 1.0–3.5 mL keeping rest of the procedure the same. Fluorescence intensity was measured at the optimized wavelengths (figure 3.4.8).

For optimization of heating temperature, optimized amounts of all the reagents were taken in a set of test tubes and heated on water bath in the temperature range of 60–100 °C for 20 minutes and fluorescence intensity was measured (figure 3.4.9).

To optimize the heating time, only the heating time was varied from 10–40 minutes keeping rest of the conditions the same and fluorescence intensity was measured (figure 3.4.10).

For optimization of solvents used for dilution, the reagents were taken in a set of test tubes and heated in the electric thermostatic water bath for 30 minutes at boiling temperature. After cooling, the solutions were transferred to 25 mL volumetric flask and made the volume up to the mark using different solvents such as distilled water, methanol, ethanol and acetonitrile (figure 3.4.11).

The stability of the fluorescent reaction product was studied by measuring the fluorescent intensity at regular interval up to 90 minutes with 10 minutes interval. It was found that no change in fluorescence intensity was observed with time (figure 3.4.12).

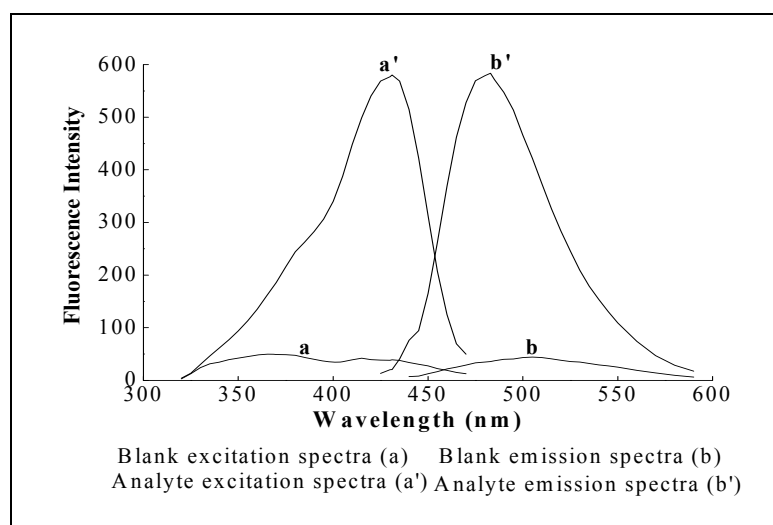


Figure 3.4.2 Excitation and emission spectra of sulpiride after condensation reaction

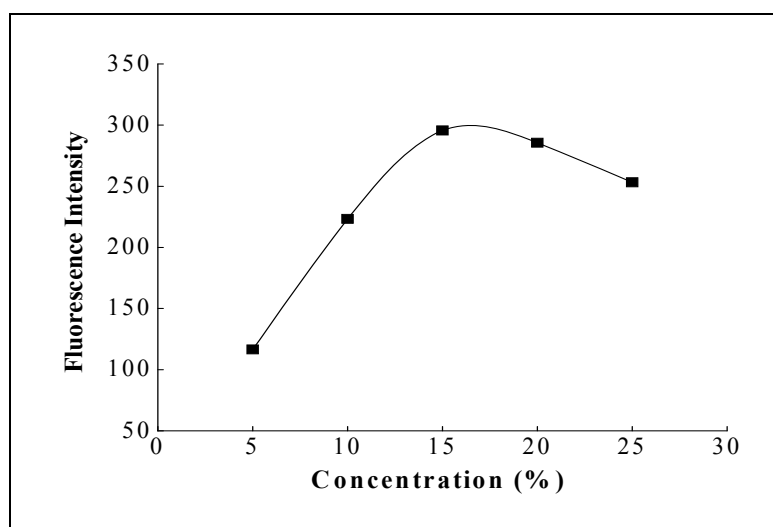


Figure 3.4.3 Effect of concentration of formaldehyde on the spectrofluorimetric determination of sulpiride

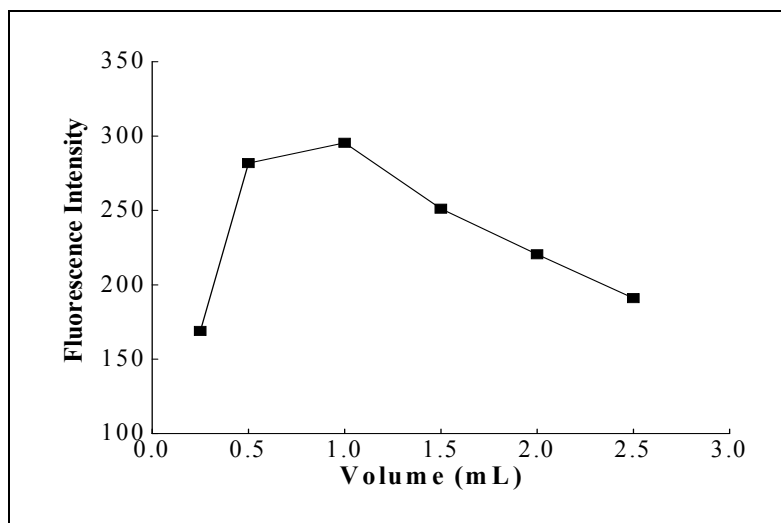


Figure 3.4.4 Effect of volume of formaldehyde on the spectrofluorimetric determination of sulpiride

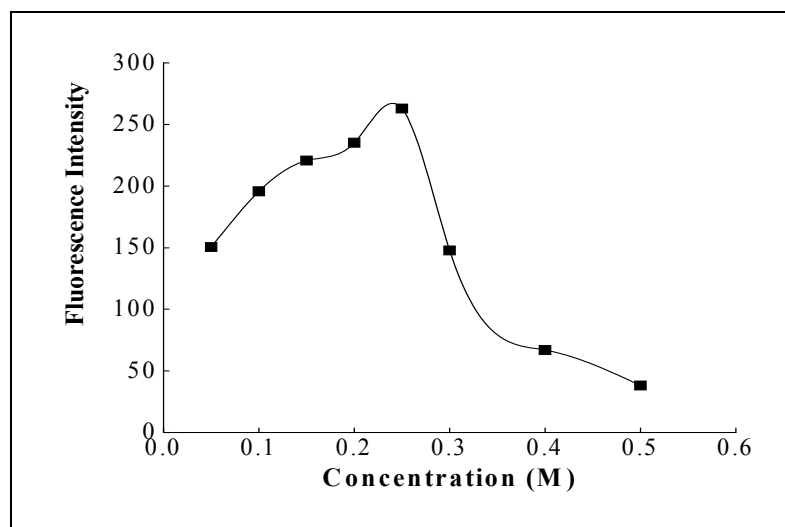


Figure 3.4.5 Effect of concentration of hydrochloric acid on the spectrofluorimetric determination of sulpiride

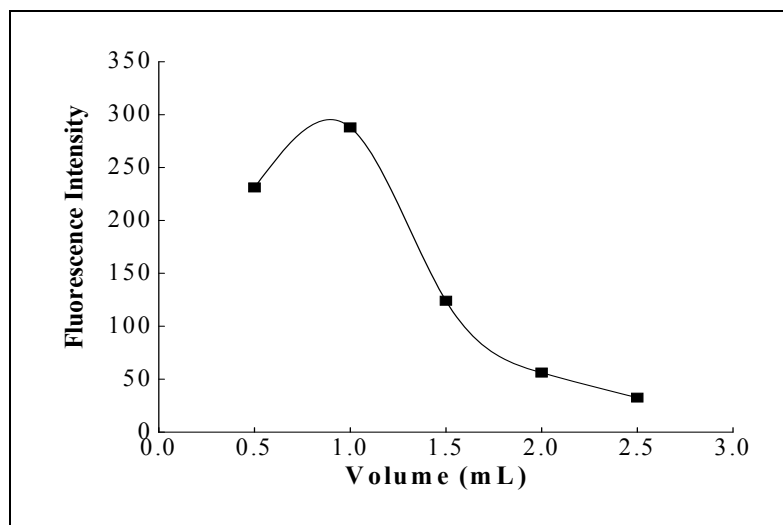


Figure 3.4.6 Effect of volume of hydrochloric acid on the spectrofluorimetric determination of sulpiride

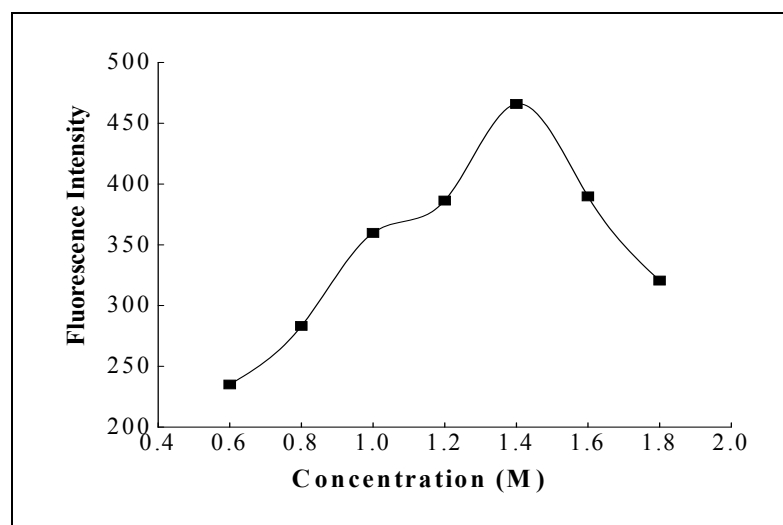


Figure 3.4.7 Effect of concentration of acetyl acetone on the spectrofluorimetric determination of sulpiride

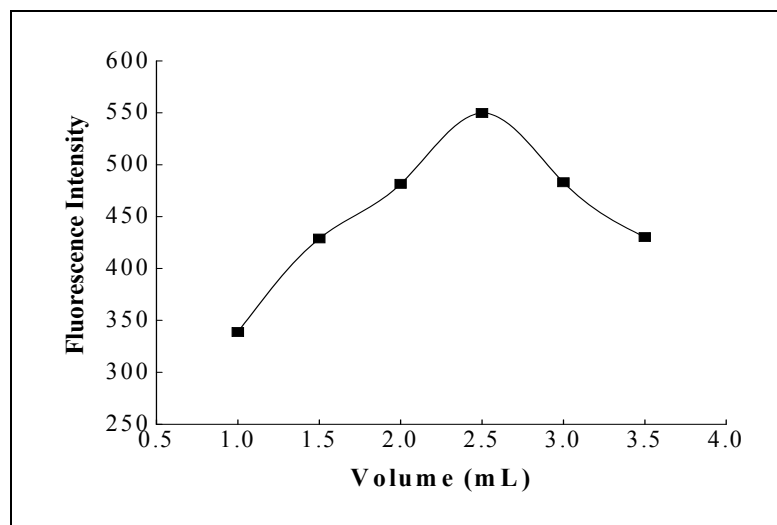


Figure 3.4.8 Effect of volume of acetyl acetone on the spectrofluorimetric determination of sulpiride

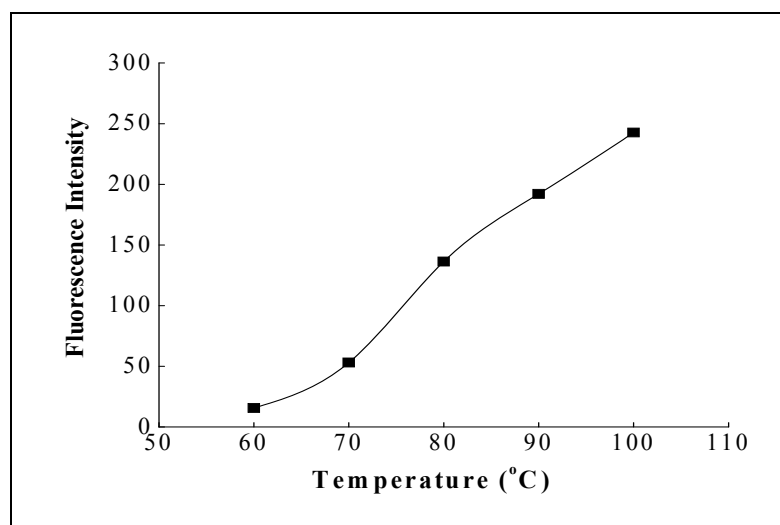


Figure 3.4.9 Effect of heating temperature on the spectrofluorimetric determination of sulpiride

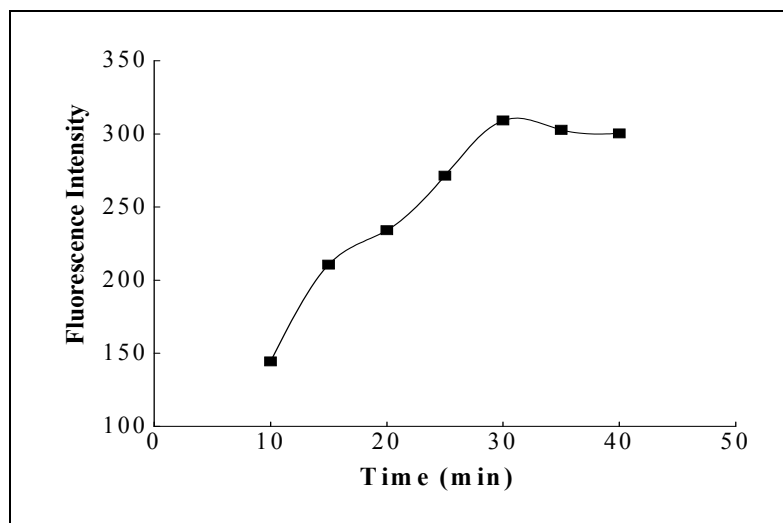


Figure 3.4.10 Effect of heating time on the spectrofluorimetric determination of sulpiride

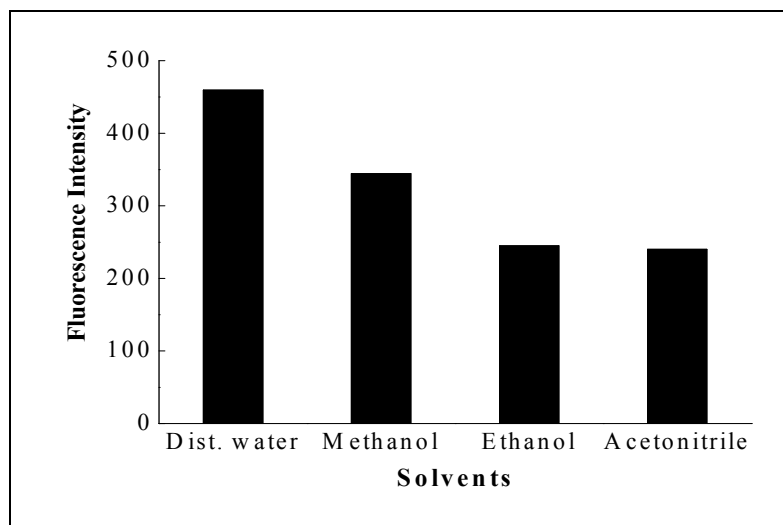


Figure 3.4.11 Effect of diluting solvent on spectrofluorimetric determination of sulpiride

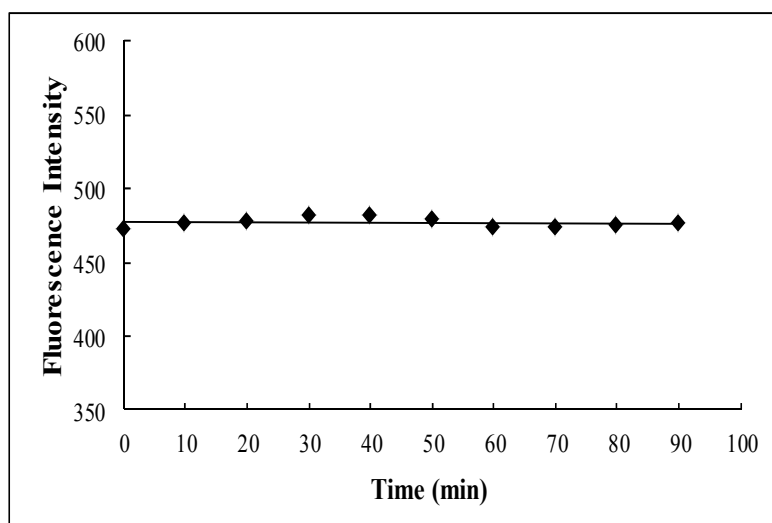


Figure 3.4.12 Stability of the fluorescent product

3.4.4 Effect of concentration of sulpiride on fluorescence intensity

Procedure

Proper volumes of the stock solution of sulpiride, to give final drug concentration of 0.1–3.5 $\mu\text{g mL}^{-1}$ were taken in a set of 20 mL test tubes. 1.0 mL of 15% formaldehyde, 1.0 mL of 0.25 M HCl and 2.5 mL of 1.4 M acetyl acetone was added to each test tube. The components of mixture were moderately shaken and heated in a boiling water bath for 30 minutes. The solutions were cooled under tap water and made the volume up to 25 mL with distilled water in volumetric flasks. The fluorescence intensity was measured at λ_{em} 483 nm using λ_{ex} 431 nm by PC RF-5301 spectrofluorophotometer against a reagent blank (table 3.4.1 and figure 3.4.13). For determination of sulpiride in pharmaceutical preparations the calibration plot of fluorescence intensity versus sulpiride concentration was constructed for lower concentration (figure 3.4.14).

Table 3.4.1 Effect of concentration on the fluorimetric behavior of sulpiride

Concentration ($\mu\text{g mL}^{-1}$)	Fluorescence Intensity
0.1	7.678
0.5	40.75
1.0	90.321
1.5	127.67
2.0	178.23
2.5	231.89
3.0	300.231
3.5	343.21

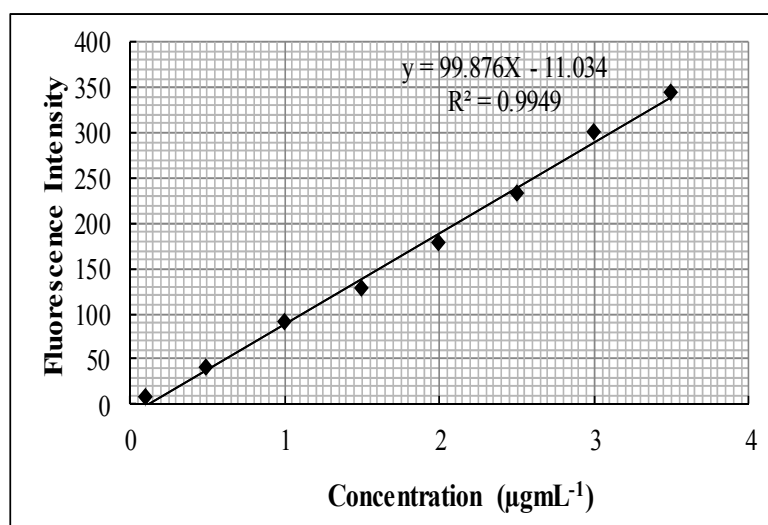


Figure 3.4.13 Effect of concentration on the fluorimetric behavior of sulpiride

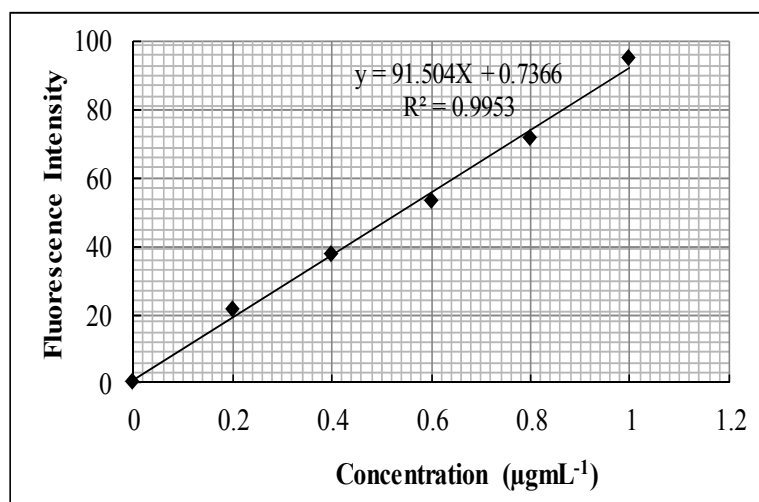


Figure 3.4.14 Calibration curve for spectrofluorimetric determination of sulpiride

Determination of LOD, LOQ and other analytical characteristics of the proposed method for determination of sulpiride

Procedure

Five replicates with lowest measurable concentration of sulpiride (0.1 µgmL⁻¹) were evaluated by the proposed method pursuing the same procedure as stated before. LOD, LOQ and other analytical characteristics were calculated (table 3.4.2).

Table 3.4.2 Analytical characteristics for the spectrofluorimetric determination of sulpiride

Analytical parameter	Value
$\lambda_{\text{ex}}(\text{nm})$	431
$\lambda_{\text{em}}(\text{nm})$	483
Linear range ($\mu\text{g mL}^{-1}$)	0.1–3.5
Limit of detection 3S ($\mu\text{g mL}^{-1}$)	1.1×10^{-2}
Limit of quantification 10S ($\mu\text{g mL}^{-1}$)	3.9×10^{-2}
Regression equation (y)	$Y = 91.504X + 0.7366$
Slope (b)	91.504
Intercept (a)	0.7366
Correlation coefficient (r^2)	0.9953
Standard deviation ($\mu\text{g mL}^{-1}$)	3.9×10^{-3}
Relative standard deviation (%)	3.81

3.4.5 Investigation of interferences on determination of sulpiride

Instrument, reagents and standard solutions used were the same as mentioned before.

Procedure

The selectivity of the proposed method for the analysis of sulpiride containing pharmaceutical preparation was evaluated by studying the interferences effect of common excipient that might be added during preparations of tablets. For this purpose a known concentration of standard sulpiride ($0.2 \mu\text{g mL}^{-1}$) was analyzed by the same procedure as stated before. Then standard sulpiride ($0.2 \mu\text{g mL}^{-1}$) was taken in a series of test tubes and excipients were added maintaining drug: excipients ratio of 1:1, 1:2 and 1:4. Then formaldehyde (1.0 mL of 15%), HCl (1.0 mL of 0.25 M) and acetyl acetone (2.5 mL of 1.4 M) was added following the same procedure as described before and fluorescence intensity was measured (table 3.4.3 and figure 3.4.15).

Table 3.4.3 Interferences effect of common excipients on determination of sulpiride (0.2 $\mu\text{g mL}^{-1}$) by the proposed method

Excipient	Amount added ($\mu\text{g mL}^{-1}$)	Drug : Excipient	%Recovery $\pm$ RSD
Glucose	0.2	1:01	95.70 $\pm$ 2.07
	0.4	1:02	95.68 $\pm$ 7.94
	0.8	1:04	101.05 $\pm$ 3.33
Sorbitol	0.2	1:01	98.68 $\pm$ 7.45
	0.4	1:02	97.38 $\pm$ 5.85
	0.8	1:04	96.62 $\pm$ 3.02
Fructose	0.2	1:01	98.59 $\pm$ 5.03
	0.4	1:02	96.23 $\pm$ 0.72
	0.8	1:04	99.86 $\pm$ 2.21
Talc	0.2	1:01	96.20 $\pm$ 1.13
	0.4	1:02	99.68 $\pm$ 5.14
	0.8	1:04	96.40 $\pm$ 2.76
Starch	0.2	1:01	98.13 $\pm$ 4.18
	0.4	1:02	97.88 $\pm$ 2.51
	0.8	1:04	98.96 $\pm$ 2.55
Lactose	0.2	1:01	99.33 $\pm$ 4.99
	0.4	1:02	99.22 $\pm$ 3.44
	0.8	1:04	95.94 $\pm$ 2.37
Mag. Stearate	0.2	1:01	98.31 $\pm$ 2.96
	0.4	1:02	100.93 $\pm$ 3.87
	0.8	1:04	96.52 $\pm$ 3.18
Sucrose	0.2	1:01	98.96 $\pm$ 4.84
	0.4	1:02	100.75 $\pm$ 2.77
	0.8	1:04	97.42 $\pm$ 1.14

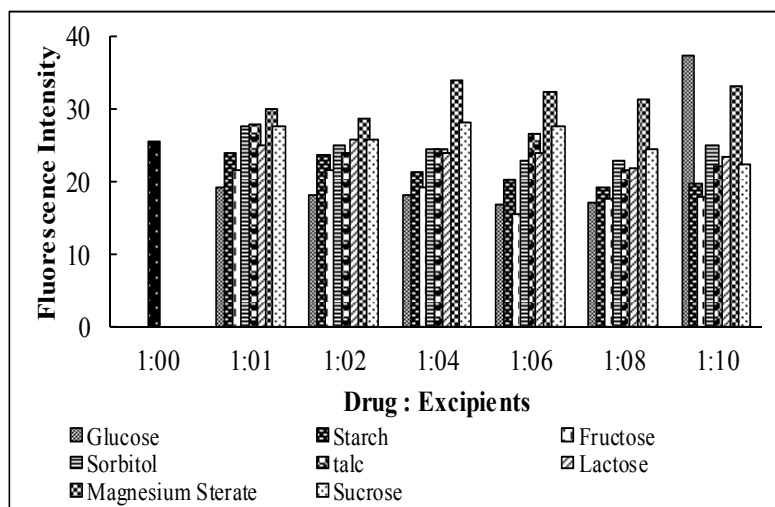


Figure 3.4.15 Effect of common excipients on determination of sulpiride by the proposed method

The effect of co-administered drugs such as Mefenamic acid, Paracetamol, Diclofenic sodium and Metronidazole on fluorescence intensity of sulpiride was also investigated. Standard sulpiride solutions and different concentration of the co-administered drugs in concentration ratio (1:1, 1:2 and 1:4) were added to a series of test tubes and fluorescence intensity was measured by the same procedure (figure 3.4.16).

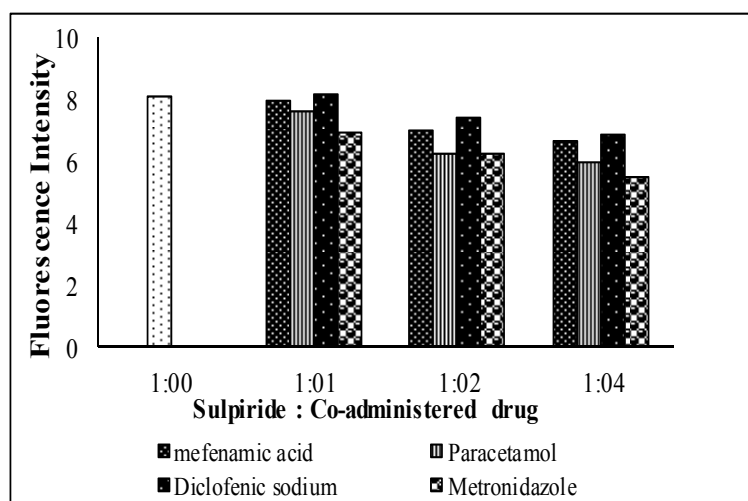


Figure 3.4.16 Effect of co-administered drugs on determination of sulpiride by the proposed method

3.4.6 Application of the investigated method for the analysis of sulpiride in different pharmaceutical formulations

A. Determination of precision and accuracy of the investigated method

Procedure

Precision and accuracy of the method was determined by analysis of four separate sample solutions of the pharmaceutical preparations (each containing 50 mg of the active ingredients of sulpiride) at three different concentrations. Calibration curve was constructed by the same procedure and known concentrations (0.2, 0.4 and 0.6 $\mu\text{g mL}^{-1}$) of these sample solution were then analyzed by the proposed method. Each set of experiment was performed in triplicates and the results are given in table 3.4.4.

Table 3.4.4 Evaluation of accuracy and precision of the proposed method for sulpiride determination

Sample	($\mu\text{g mL}^{-1}$) taken	($\mu\text{g mL}^{-1}$) found	%Recovery $\pm$ RSD
Sulpiride-50, 50 mg capsule Combitic Global (India)	0.2	0.202	101.36 $\pm$ 5.12
	0.4	0.380	95.01 $\pm$ 1.93
	0.6	0.607	101.20 $\pm$ 1.16
Sulpiride-50, 50 mg capsule Unicure (India) Pvt. Ltd	0.2	0.200	100.00 $\pm$ 2.10
	0.4	0.397	99.44 $\pm$ 2.01
	0.6	0.608	101.46 $\pm$ 0.63
Sulpiride-50, 50 mg caps Xier kangtai Pharmaceutical Co. Ltd China	0.2	0.200	100.29 $\pm$ 1.34
	0.4	0.396	99.14 $\pm$ 2.48
	0.6	0.589	98.32 $\pm$ 1.80
Sulpiride Capsules 50 mg ARBRO pharmaceuticals Ltd New Delhi (India)	0.2	0.199	99.75 $\pm$ 3.85
	0.4	0.397	99.45 $\pm$ 1.84
	0.6	0.603	100.57 $\pm$ 0.59

B. Percent recovery of sulpiride from commercial formulations (Standard addition method)

Same Instrument, reagents, standard solution and sample solutions were used.

Procedure

For determination of percent recovery of sulpiride from pharmaceutical preparations, 0.2, 0.4 and 0.6 $\mu\text{g mL}^{-1}$ standard sulpiride solution was mixed with pre-analyzed commercial formulations of sulpiride (0.2 $\mu\text{g mL}^{-1}$). The fluorescent intensity was measured at 483 nm using excitation wavelength of 431 nm. The amount of sulpiride was calculated from the calibration curve. Triplicate analysis was performed and the mean percent recovery was calculated using the following formula (table 3.4.5).

$$\% \text{ Recovery} = \frac{\mu\text{g mL}^{-1} \text{ found in the fortified sample} - \mu\text{g mL}^{-1} \text{ present in the sample}}{\mu\text{g mL}^{-1} \text{ of standard added}} \times 100$$

Table 3.4.5 Evaluation of percent recovery of sulpiride from pharmaceutical preparations by the proposed method

Samples	Sample ($\mu\text{g mL}^{-1}$)	Added ($\mu\text{g mL}^{-1}$)	Found ($\mu\text{g mL}^{-1}$)	%Recovery $\pm$ RSD
Sulpiride-50, 50 mg capsule Combitic Global (India)	0.2	0.204	0.200	98.03 $\pm$ 6.22
		0.401	0.395	98.47 $\pm$ 2.84
		0.608	0.594	97.58 $\pm$ 2.31
Sulpiride-50, 50 mg capsule Unicure (India) Pvt. Ltd	0.2	0.200	0.199	99.35 $\pm$ 4.22
		0.403	0.396	98.20 $\pm$ 4.55
		0.620	0.616	99.41 $\pm$ 3.30
Sulpiride-50, 50 mg caps Xier kangtai Pharmaceutical Co. Ltd China	0.2	0.200	0.201	100.29 $\pm$ 3.07
		0.403	0.405	100.55 $\pm$ 2.76
		0.620	0.602	97.09 $\pm$ 0.56
Sulpiride Capsules 50 mg ARBRO pharmaceuticals Ltd New Delhi	0.2	0.200	0.199	99.54 $\pm$ 2.81
		0.403	0.400	99.28 $\pm$ 4.44
		0.620	0.606	97.80 $\pm$ 2.28

C. Determination of active ingredients in the commercial formulations

Instrument, reagents, standard solution and sample solutions used were the same as mentioned before.

Procedure

Solutions of the commercial formulation of known concentration were prepared by the same way as mentioned before. Sample solution ($10 \mu\text{g mL}^{-1}$) was prepared from stock sample solution by dilution. Known volumes of this sample solution ($10 \mu\text{g mL}^{-1}$) were then analyzed by the proposed method. Triplicate analyses were performed in each case (table 3.4.6).

Table 3.4.6 Determination of active ingredients of sulpiride in the commercial formulations

Brand name	Active ingredient (mg tab^{-1})		t-test value (4.303)	F-test value (19)
	Labeled value	Found value		
Sulphiride-50, 50 mg capsule Combitic Global (India)	50	49.59 ± 1.76	0.38	0.106
Sulpiride-50, 50 mg capsule Unicare (India) Pvt. Ltd	50	50.15 ± 0.53	1.23	1.43
Sulpiride-50, 50 mg caps Xier kangtai Pharmaceutical Co. Ltd China	50	49.62 ± 0.46	1.313	0.003
Sulpiride Capsules 50 mg ARBRO pharmaceuticals Ltd New Delhi	50	49.96 ± 0.33	0.23	0.089

3.4.7 Determination of sulpiride by reference (HPLC) method

Reference HPLC method was also used for the determination of sulpiride and the results of the proposed method was compared.

Procedure

HPLC system was equilibrated with the mobile phase prior to the analysis. Then standard sulpiride solutions in the concentration range of 20–60 $\mu\text{g mL}^{-1}$ were injected one by one, and the peak height was noted. A calibration curve of peak height versus concentration was constructed (figure 3.4.17). Sample solution of four commercial formulations in three different concentrations (20, 30 and 40 $\mu\text{g mL}^{-1}$) was injected and precision of the method was determined using triplicates analysis (table 3.4.7).

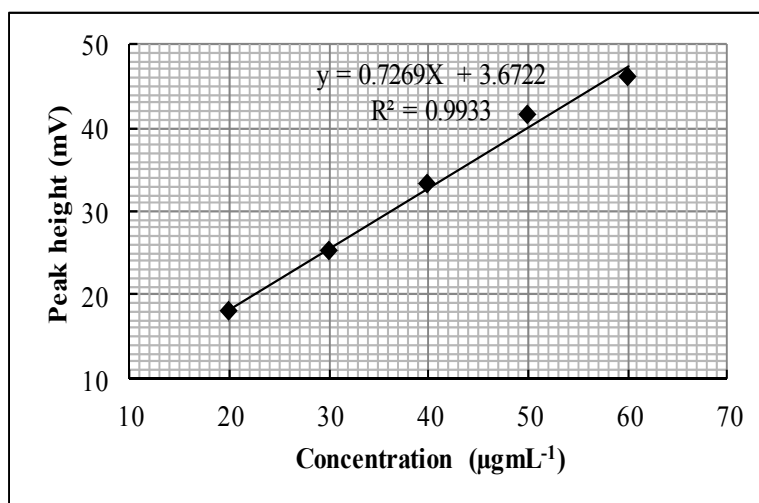


Figure 3.4.17 Calibration curve for sulpiride determination using reversed phase HPLC

Table 3.4.7 Determination of sulpiride in commercial formulations and statistical comparison with reference method

Commercial formulations		Amount determined (mg tab ⁻¹)	
		Proposed method	Reference method
Sulpiride-50, 50 mg capsule Combitic Global (India)	mean $\pm$ RSD	49.59 $\pm$ 1.76	
	F-test (19)	0.106	46.68 $\pm$ 5.53
	t-test (4.303)	0.38	
Sulpiride-50,50 mg capsule Unicure (India) Pvt. Ltd	mean $\pm$ RSD	50.15 $\pm$ 0.53	
	F-test (19)	1.43	49.11 $\pm$ 0.56
	t-test (4.303)	1.23	
Sulpiride-50, 50 mg caps Xier kangtai Pharmaceutical Co. Ltd China	mean $\pm$ RSD	49.62 $\pm$ 0.46	
	F-test (19)	0.003	46.74 $\pm$ 6.18
	t-test (4.303)	1.313	
Sulpiride Capsules 50 mg ARBRO pharmaceuticals Ltd New Delhi (India)	mean $\pm$ RSD	49.96 $\pm$ 0.33	
	F-test (19)	0.089	48.39 $\pm$ 1.17
	t-test (4.303)	0.23	

3.4.8 Results and discussion

Hantzsch reaction is known as condensation reaction that was reported in the literatures as a useful pathway for payroll and pyridine synthesis [1]. In the same way, acetyl acetone together with formaldehyde reacts with aliphatic amines by Hantzsch condensation reaction forming a fluorescent product. Sulpiride do not contain free amino group, while upon hydrolysis with alkali the cleavage of weak amide group occurs with production of primary amine. This primary amine of sulpiride was used for determination through Hantzsch condensation reaction using acetyl acetone as β -diketone and formaldehyde as an aldehyde to form a fluorescent condensation product. In the first step the acetyl acetone reacts with formaldehyde and form 3, 5-diacetylheptane-2, 6-dione. In the second step the 3, 5-diacetylheptane-2, 6-dione undergoes condensation with amino group of the sulpiride and produces a fluorescent product (figure 3.4.1). The fluorescent product produced showed maximum fluorescent intensity at λ_{em} 483 nm after excitation at λ_{ex} 431 nm against a reagent blank (figure 3.4.2).

All the parameters affecting the reaction like reagents volume and concentration, molarity of hydrochloric acid, and reaction temperature were carefully studied.

The effect of formaldehyde concentration was investigated in the range of 5.0–25%. It was found that with increase in concentration of formaldehyde to 15%, the fluorescence intensity of the product increases after which a decrease in intensity was noted (figure 3.4.3). The effect of volume of formaldehyde solution was also investigated in the range of 0.25–2.5 mL. Maximum fluorescence intensity was observed with 1.0 mL of 15% formaldehyde solution, therefore this volume was selected for further analysis (figure 3.4.4).

The effect of HCl concentration was investigated in the range of 0.05–0.5 M. It was noted that fluorescence intensity increases with HCl concentration up to 0.25 M, after which a decrease in intensity was noted (figure 3.4.5). Similarly the effect of volume of HCl solution (0.25M) was also studied in the range of 0.5–2.5 mL. Maximum fluorescent product formation occurred with 1.0 mL of 0.25 M HCl solution (figure 3.4.6). Therefore, this optimized conditions of HCl solution was used for further analysis.

The effect of concentration of acetyl acetone was studied in the range of 0.6–1.8 M. The fluorescence intensity of the fluorophore increases with increase in concentration of acetyl acetone up to 1.4 M, after which it decreased (figure 3.4.7). The effect of volume of acetyl acetone solution (1.4 M) was also investigated in the range of 1.0–3.5 mL. Maximum fluorophore formation was observed with 2.5 mL of 1.4 M acetyl acetone (figure 3.4.8). Thus, this volume of acetyl acetone solution was used throughout the work.

The effect of temperature on the condensation reaction was investigated in the range of 60–100 °C. Maximum fluorescence intensity was achieved at boiling temperature (figure 3.4.9). Heating time for the reaction was also studied in the range of 10–40 minutes (figure 3.4.10). Maximum product formation was achieved by heating the reaction mixture for 30 minutes at 100 °C. Therefore, 30 minutes heating at boiling water bath was chosen as optimum.

Different solvent (distilled water, methanol, ethanol and acetonitrile) were used for dilution after the reaction. Best fluorescence intensity was obtained by dilution with distilled water (figure 3.4.11).

The stability of the product was studied by measuring the fluorescent intensity at regular interval up to 90 minutes. It was found that no change in fluorescence intensity of the reaction product occur during this period (figure 3.4.12).

Analytical characteristics

Under the optimum experimental conditions, a linear relationship between the fluorescence intensity and sulpiride concentration was observed in the concentration range of 0.1–3.5 $\mu\text{g mL}^{-1}$ (table 3.4.1 and figure 3.4.13) with correlation coefficient of 0.9953. The LOD and LOQ were calculated for sulpiride and were found to be 1.1×10^{-2} and $3.9 \times 10^{-2} \mu\text{g mL}^{-1}$ respectively. The optical characteristic such as the linear regression equation, intercept, slope, correlation coefficient, and relative standard deviation are given in table 3.4.2.

Applications

To evaluate the selectivity of the proposed method for determination of sulpiride in pharmaceutical preparations, the interferences effect of common excipients such as glucose, sorbitol, fructose, talc, starch, lactose, magnesium stearate, and sucrose on the reproducibility of the proposed method were investigated. Solutions containing standard sulpiride and one of the excipients were taken separately at concentration level in the ratio of one, two and four time of that of sulpiride were analyzed by the proposed method (table 3.4.3 and figure 3.4.15). Percent recoveries obtained were in the range of 95.68% to 101.05%. The results showed that there were no interferences produced by these excipients substances in the determination of sulpiride by the proposed method. The effect of some common co-administered drugs like mefenamic acid, paracetamol, diclofenac sodium and metronidazole were also studied. It was found that there is no

interference of these drugs in the determination of sulpiride by the proposed method (figure 3.4.16).

The accuracy and precision of the proposed method was evaluated by determining the sulpiride in three different concentrations (0.2, 0.4, and 0.6 $\mu\text{g mL}^{-1}$) in triplicate (table 3.4.4). The percent recoveries were found in the range of 95.01% to 101.36%. The relative standard deviation value was acceptable which indicates better reproducibility of the proposed method.

The accuracy of the method in dosage form of four different brands of capsules was evaluated by performing recoveries experiments through standard addition method. Known amount of standard sulpiride solution (0.2, 0.4, and 0.6 $\mu\text{g mL}^{-1}$) was added to four different kinds of pre-analyzed dosage forms and then determined by the recommended procedure. The percent recoveries were found in the range of 97.09% to 100.55% (table 3.4.5) with low relative standard deviation.

The proposed method has been successfully applied for the determination of sulpiride in pharmaceutical preparations. The results obtained were compared statistically with respect to accuracy by student t-test at 95 % confidence level and precision by the variance ratio F-test with those of the literature HPLC method. The results obtained from both the methods were in good agreement (table 3.4.7).

Reference

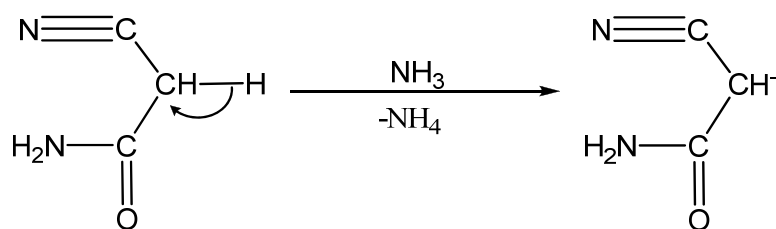
1. F.R.S.D. Bratlon, W.D. Olis. "Comprehensive Organic Chemistry, The Synthesis and Reactions of Organic Compounds", Pergamon Press London, 4, 65, 298 (1979).

3.5 SPECTROFLUORIMETRIC DETERMINATION OF SULPIRIDE IN PHARMACEUTICAL PREPARATIONS AND HUMAN PLASMA THROUGH DERIVATIZATION WITH 2-CYANOACETAMIDE

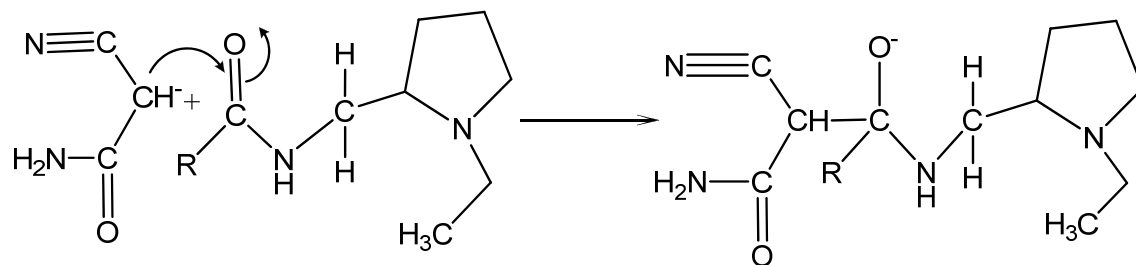
3.5.1 Proposed reaction mechanism for derivatization reaction of sulpiride with 2-cyanoacetamide

2-cyanoacetamide is a fluorogenic reagent that has been manipulated for fluorimetric determination of many pharmaceutical compounds. The developed method is based on the reaction of sulpiride with 2-cyanoacetamide in the presence of ammonia to produce a fluorescent reaction product which measure spectrofluorimetrically at 379 nm after excitation at 330 nm. The amide carbon of sulpiride on reaction with 2-cyanoacetamide in the presence of 30% ammonia produced a fluorescence product. In the first step of the proposed mechanism, ammonia acting as a base removes a proton (H^+) from the methylene group of 2-cyanoacetamide generating carbene. In the second step the carbene attacks on the amide carbon of sulpiride and an oxoprapanolate is formed. The third step comprises rearrangement producing fluorescent product and ammonia is regenerated. The proposed scheme of reaction is shown in figure 3.5.1.

Step 1



Step 2



Step 3

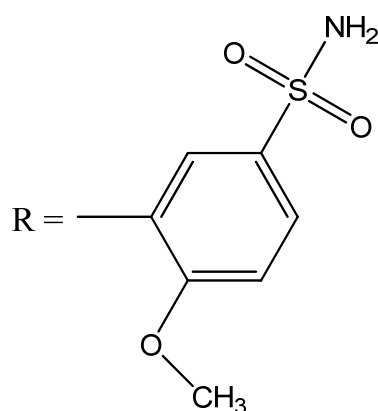
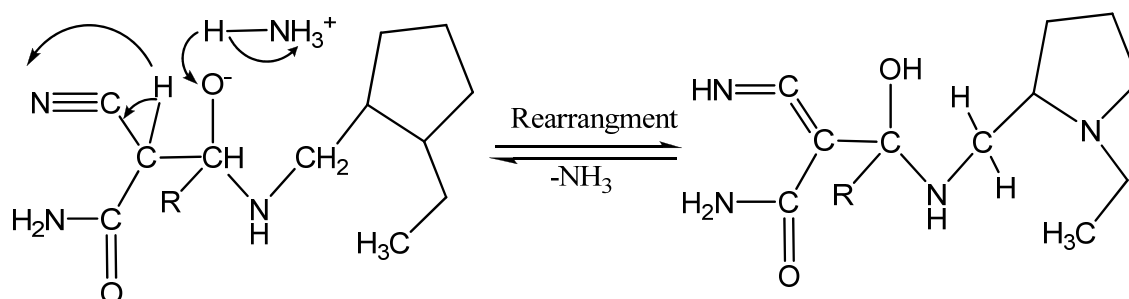


Figure 3.5.1 Proposed reaction mechanism for spectrofluorimetric determination of sulpiride

3.5.2 Preliminary investigation of the possibility of derivatization reaction of sulpiride with 2-cyanoacetamide for its spectrofluorimetric determination

Preliminary studies were carried out to study the possible derivatization reaction between sulpiride with 2-cyanoacetamide in presence of ammonia. Initially high concentration of sulpiride ($20 \mu\text{g mL}^{-1}$), 2-cyanoacetamide (1.0%) and ammonia (35%) were mixed in a 20 mL test tube and heated for 10 minutes on a boiling water bath to ensure the derivatization reaction. The reaction resulted a fluorescence product enough to be used for spectrofluorimetric determination of sulpiride.

3.5.3 Optimization studies for spectrofluorimetric determination of sulpiride using derivatization reaction

Instruments

All fluorescence intensities were measured on a RF-5301 PC Spectrofluorophotometer. Digital analytical balance and electric thermostatic water bath were used in this work.

Reagents

Analytical grade reagents were used throughout the work. Standard reference sulpiride was provided by Hang Zhou Pharma and chem. co china. Commercial formulations of sulpiride (sulpiride-50, 50 mg caps manufactured by Combitic Global D-2 industrial area Sonapat-131001 (Hr) India., Sulpiride-50, 50 mg caps manufactured by Xier kangtai Pharmaceutical Co. Ltd P.R.China., Sulpiride Capsules 50 mg manufactured by ARBRO pharmaceuticals Ltd New Delhi (India) were purchased locally. Reagents 2-cyanoacetamide (Across organics, New Jersey, USA), ammonia (BDH, Laboratory suppliers Poole, England, 355) and ethanol (Merck, Darmstadt, Germany) were used in this work. Plasma sample was granted by Khyber Teaching Hospital, Peshawar (Pakistan).

Solution preparation

2-Cyanoacetamide (4.0%) solution was prepared by dissolving 2.0 g of the reagent in distilled water and the volume was made up to 50 mL with distilled water in volumetric flask. Ammonia (30%) solution was prepared by diluting 42.85 mL of 35% ammonia in volumetric flask and the volume was made up to 50 mL with distilled water.

Standard sulpiride solution (200 $\mu\text{g mL}^{-1}$)

Standard stock solution of sulpiride (200 $\mu\text{g mL}^{-1}$) was prepared by dissolving 0.01 g of sulpiride standard in 10 mL of distilled ethanol and made the volume up to 50 mL with distilled water. Working standards solutions of 10 $\mu\text{g mL}^{-1}$ and 5.0 $\mu\text{g mL}^{-1}$ were prepared fresh on daily basis by diluting proper amount of the stock solution.

Sample solution (200 $\mu\text{g mL}^{-1}$)

The contents of five capsules of each commercial formulation were weighed to get the average weight of one capsule. An accurately weighed amount of the powder equal to 10 mg of sulpiride was dissolved in 10 mL of distilled ethanol with vigorous shaking. The solution was then filtered and made the volume up to 50 mL with distilled water. Working sample solution of 5.0 $\mu\text{g mL}^{-1}$ was prepared by dilution of the required volume of stock sample solution with distilled water.

Procedure

Ammonia solution (1.5 mL of 30%) was added to a series of separate 20 mL test tubes pursued by the addition of 2-cyanoacetamide (3.0 mL of 4.0%) and 2.5 mL of sulpiride (200 $\mu\text{g mL}^{-1}$) standard solution. These solutions were heated on a water bath at 90 °C for 25 minutes. After cooling diluted to 25 mL with distilled water in volumetric flasks. Reagent blank was prepared in the same way without the addition of sulpiride solution. Maximum excitation (λ_{ex}) and emission (λ_{em}) wavelengths for the fluorimetric behavior of the drug were investigated. For excitation spectrum the analyte was scanned in the range of 250–370 nm and for emission spectrum the analyte was scanned in the range of

350–480 nm using excitation wavelength of 330 nm using spectrofluorophotometer (figure 3.5.2).

To optimize the concentration of 2-cyanoacetamide, 1.5 mL of ammonia solution (30%) was added to a series of test tubes pursued by the addition of 1.0 mL of 2-cyanoacetamide in the concentration range of 2.0–6.0%. To each test tube 2.5 mL of 200 $\mu\text{g mL}^{-1}$ sulphiride standard solution was added. The contents of test tubes were heated on a water bath at 90 °C for 25 minutes and the rest of the procedure was the same (figure 3.5.3).

For volume optimization of 2-cyanoacetamide (4.0%), only the volume was varied in the range of 1.0–4.0 mL keeping rest of the procedure the same as described before. Fluorescence intensity was measured and the results are shown in figure 3.5.4.

To optimize the concentration of ammonia solution, only the concentration was varied in the range of 10–35% using 1.0 mL volume. The reaction was preceded in the same way as stated before and fluorescence intensity was measured (figure 3.5.5).

For volume optimization of ammonia (30%) solution, only the volume of ammonia solution was varied in the range of 0.5–2.5 mL and keeping rest of the procedure the same and fluorescence intensity was measured (figure 3.5.6).

To study the effect of different bases like sodium hydroxide, potassium hydroxide and ammonia on the reaction of sulphiride with 2-cyanoacetamide, 1.5 mL of 1.0 M of NaOH, KOH and NH_3 solutions were added separately to a series of test tubes pursued by the addition of 2-cyanoacetamide (3.0 mL of 4.0%) and 2.5 mL of sulphiride (200 $\mu\text{g mL}^{-1}$) solutions. The solutions were heated in a water bath at 90 °C for 25 minutes and after cooling diluted to 25 mL with distilled water. Fluorescence intensity was measured at 379 nm using the excitation wavelength of 330 nm (figure 3.5.7).

To optimize heating temperature for the reaction, only heating temperature was varied in the range of 60–100 °C using water bath while rest of the procedure was kept the same. Fluorescence intensity was measured at the optimized wavelengths. Similarly heating

time was optimized by varying the heating time from 10–35 minutes. Fluorescence intensity was measured (figure 3.5.8 and 3.5.9).

Stability of the reaction product was studied by measuring the fluorescent intensity for up to two hours with 10 minutes interval against a reagent blank (figure 3.5.10).

To study the effect of mixing order of the reagents, the reagents were mixed in different order and the same procedure was followed for product formation as given before and fluorescence intensity was measured (table 3.5.1).

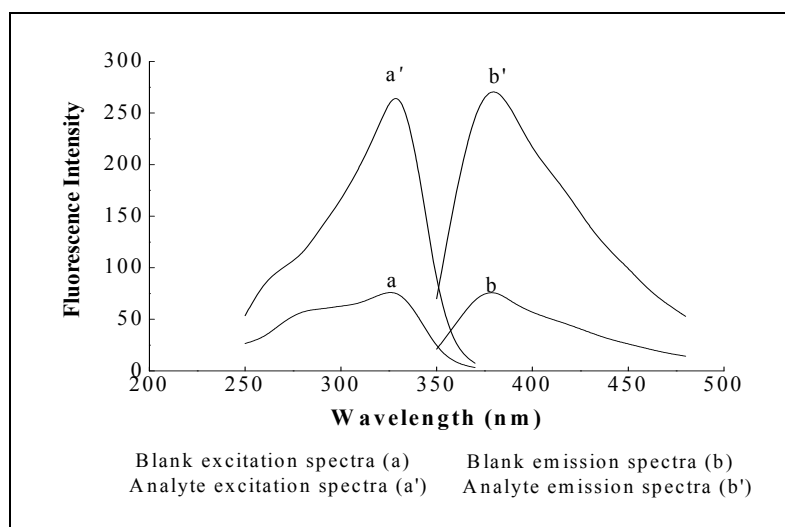


Figure 3.5.2 Excitation and emission spectra of sulpiride after derivatization reaction

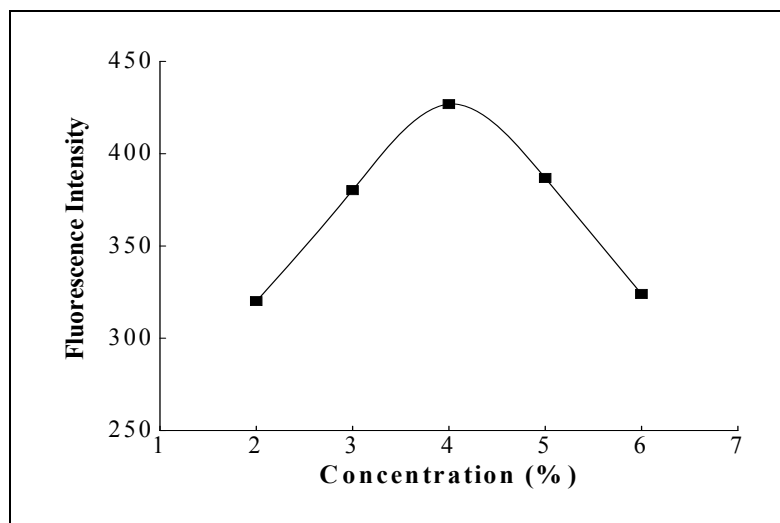


Figure 3.5.3 Effect of concentration of 2-cyanoacetamide on the derivatization reaction

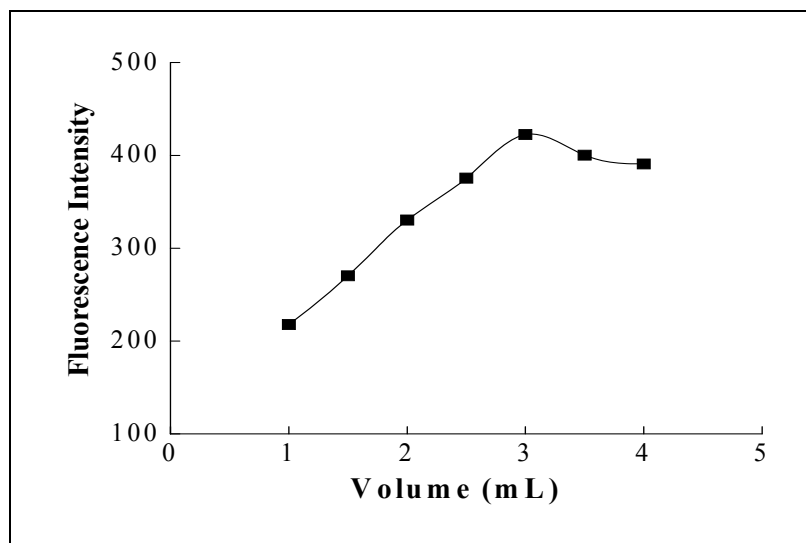


Figure 3.5.4 Effect of volume of 2-cyanoacetamide on the derivatization reaction

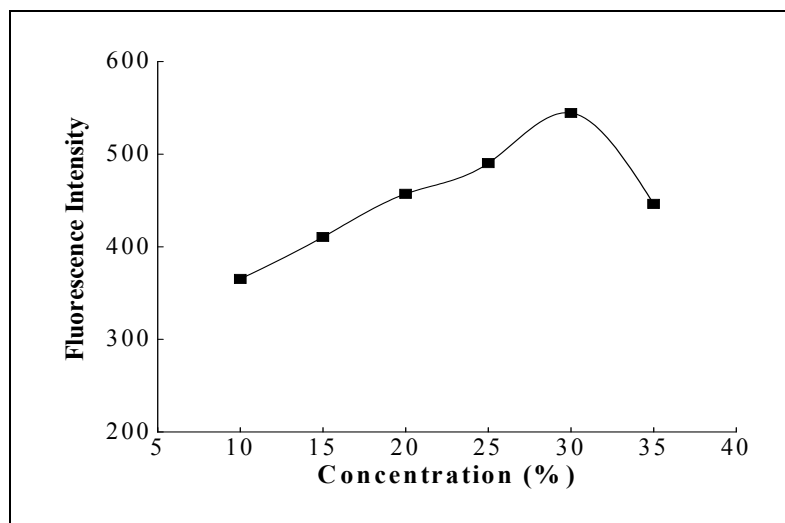


Figure 3.5.5 Effect of concentration of ammonia on the derivatization reaction

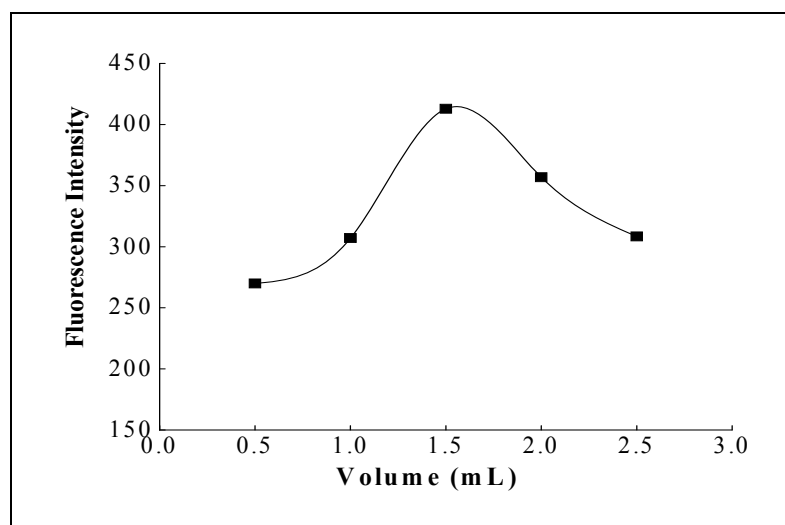


Figure 3.5.6 Effect of volume of ammonia on the spectrofluorimetric determination of sulpiride

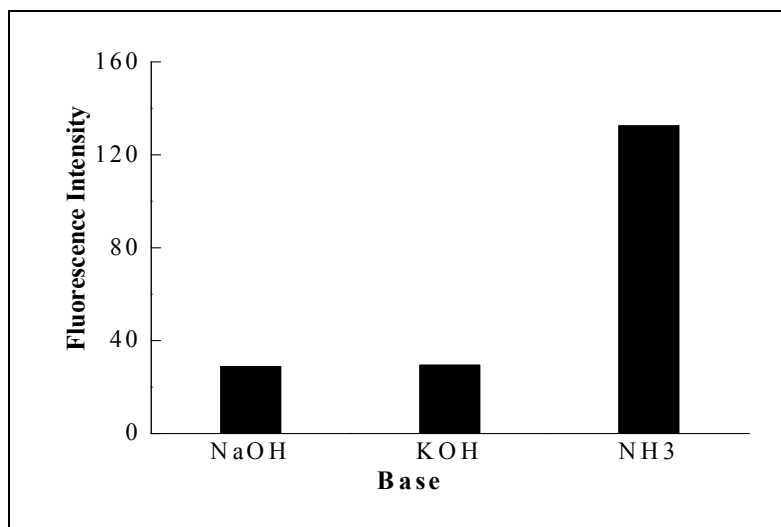


Figure 3.5.7 Effect of base on the spectrofluorimetric determination of sulpiride

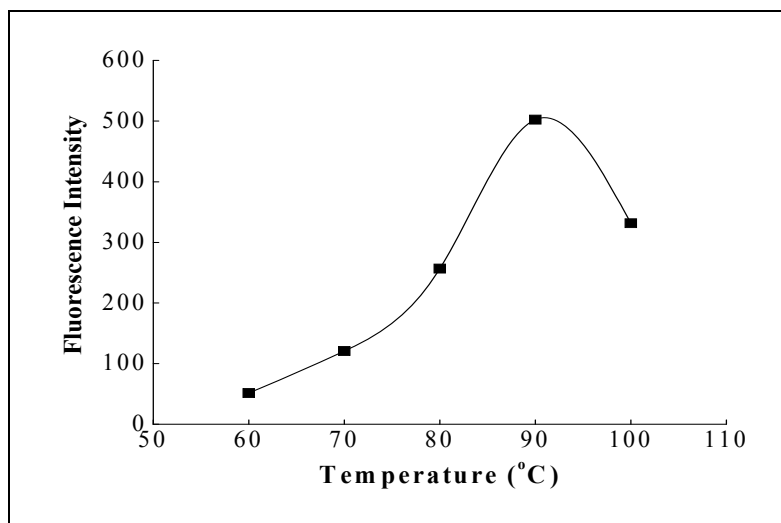


Figure 3.5.8 Effect of heating temperature on the spectrofluorimetric determination of sulpiride

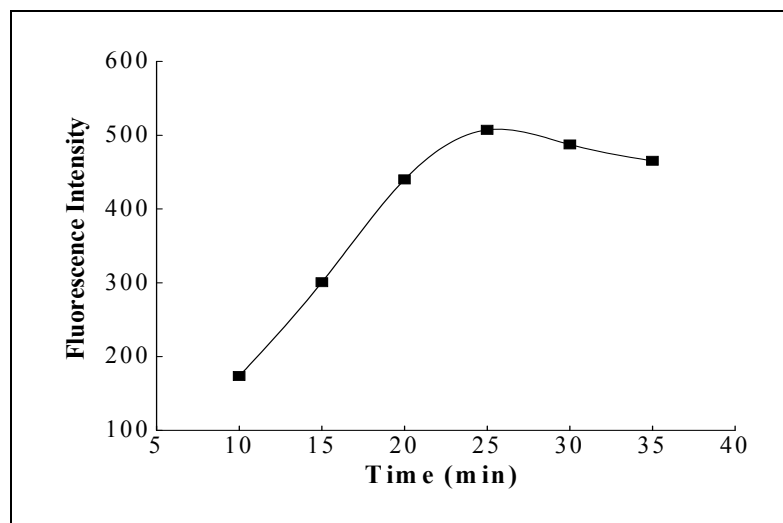


Figure 3.5.9 Effect of heating time on the spectrofluorimetric determination of sulpiride

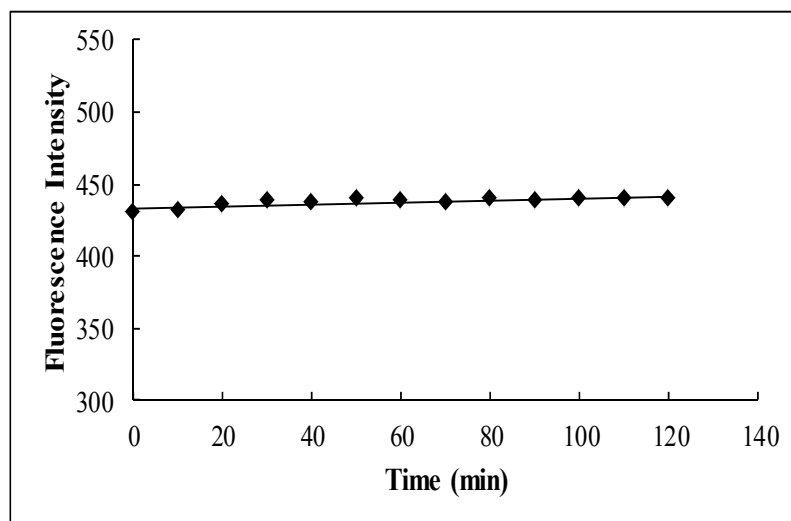


Figure 3.5.10 Effect of time on stability of fluorescent reaction product

Table 3.5.1 Effect of mixing order of the reagents on the fluorescence intensity of sulpiride

Order of reagents mixing	Fluorescence Intensity
Drug + 2-Cyanoacetamide + NH ₃	415.219
Drug + NH ₃ + 2-Cyanoacetamide	410.106
NH ₃ + 2-Cyanoacetamide + Drug	425.608

3.5.4 Effect of concentration of sulpiride on fluorescence intensity

Procedure

To a series of separate test tubes, 1.5 mL of 30% ammonia and 3.0 mL of 2-cyanoacetamide (4.0%) solution was mixed followed by the addition of sulpiride solution in the concentration range of 0.2–20 $\mu\text{g mL}^{-1}$. Reaction was carried at optimized conditions and fluorescence intensity of the product was measured at λ_{ex} 330 nm and λ_{em} 379 nm against a reagent blank. The calibration curve was attained by drawing the fluorescence intensities versus sulpiride concentration and the regression equation was derived (table 3.5.2 and figure 3.5.11). For sulpiride determination in pharmaceutical preparations and biological samples, the calibration plot for lower concentration of the drug was constructed (figure 3.5.12).

Table 3.5.2 Effect of concentration of sulpiride on the fluorescence intensity

Concentration ($\mu\text{g mL}^{-1}$)	Fluorescence Intensity
0.0	0
0.2	6.342
0.4	11.765
0.6	17.564
0.8	21.453
1.0	27.456
2.0	51.675
3.0	77.876
4.0	103.453
5.0	125.23
10	240.403
15	345.495
20	446.876

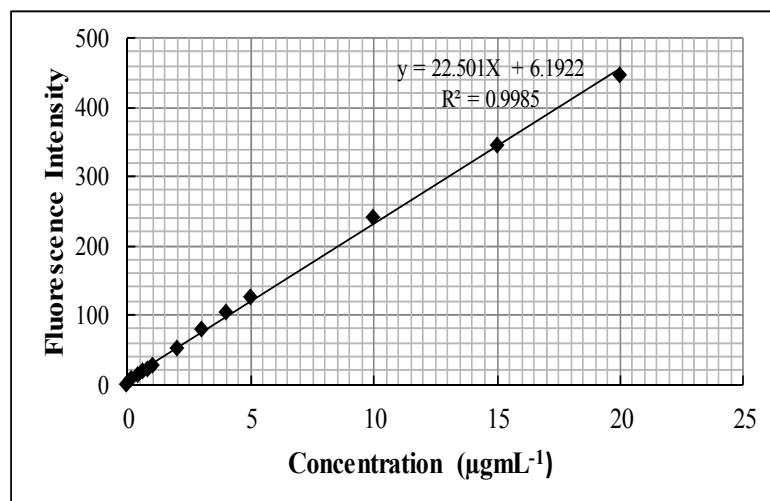


Figure 3.5.11 Effect of concentration of sulpiride on fluorescence intensity

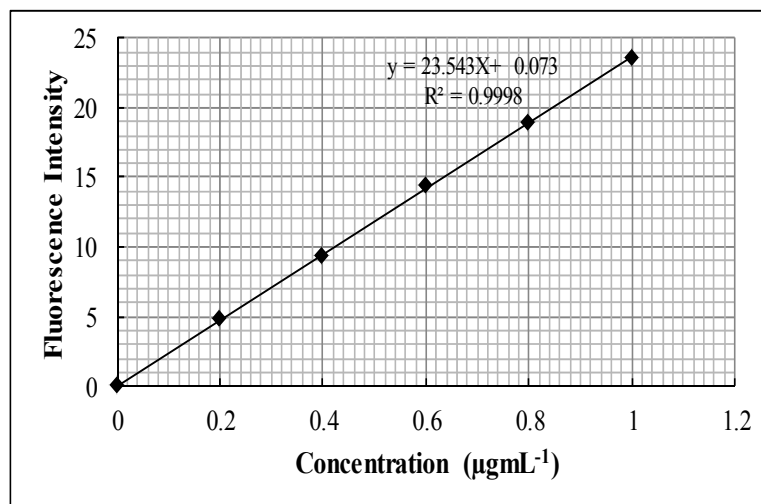


Figure 3.5.12 Effect of concentration of sulpiride on fluorescence intensity at lower concentration

Determination of LOD, LOQ and other analytical characteristics

Same Instruments, reagents and solutions were used as mentioned before.

Procedure

For determination LOD and LOQ, aliquots of 1.5 mL of ammonia (30%) solution were taken in six separate test tubes. 2-cyanoacetamide (3.0 mL of 4.0%) was added to each test tube pursued by the addition of sulpiride standard solution with minimum fluorescence intensity ($0.2 \mu\text{g mL}^{-1}$). The solutions were heated on a water bath at 90°C for 25 minutes and after cooling diluted to 25 mL with distilled water in volumetric flasks. Fluorescence intensity of the product was measured at λ_{ex} 330 nm and λ_{em} 379 nm against a reagent blank. Standard deviation, relative standard deviation (%), LOD and LOQ for the proposed method were calculated and are given in table 3.5.3.

Table 3.5.3 Analytical parameters for the spectrofluorimetric determination of sulpiride

Parameter	Value
λ_{ex} (nm)	330
λ_{em} (nm)	379
Linear range ($\mu\text{g mL}^{-1}$)	0.2–20
Limit of detection 3S/b (ng mL^{-1})	0.82
Limit of quantification 10S/b (ng mL^{-1})	2.73
Regression equation (y)	$Y = 23.543X + 0.073$
Slope (b)	23.543
Intercept (a)	0.073
Correlation coefficient (r^2)	0.9985
Standard deviation ($\mu\text{g mL}^{-1}$)	6.43×10^{-3}
Relative standard deviation (%)	2.21

3.5.5 Effect of interferences on determination of sulpiride by the proposed method

The selectivity of the proposed method for the determination of sulpiride in pharmaceutical preparation was evaluated by studying the interferences effect of common excipient that is generally added during preparations of the tablets. For this purpose a known concentration of standard sulpiride ($0.2 \mu\text{g mL}^{-1}$) was evaluated by the same procedure as described before. Then standard sulpiride ($0.2 \mu\text{g mL}^{-1}$) was taken in a series of separate test tubes and excipients were added to it keeping drug: excipients ratio of 1:2, 1:4 and 1:6. To these the optimized amounts of all reagents were added and fluorescence intensity was measured (table 3.5.4 and figure 3.5.13).

Table 3.5.4 Interferences effect of common excipients on determination of sulpiride ($0.2 \mu\text{g mL}^{-1}$) by the proposed method

Excipients	Excipients added ($\mu\text{g mL}^{-1}$)	Drug : Excipients	%Recovery $\pm$ RSD
Talc	0.4	1 : 02	98.02 $\pm$ 6.24
	0.8	1 : 04	102.66 $\pm$ 2.22
	1.2	1 : 06	101.24 $\pm$ 2.93
Sucrose	0.4	1 : 02	100.14 $\pm$ 0.93
	0.8	1 : 04	98.99 $\pm$ 2.83
	1.2	1 : 06	98.85 $\pm$ 1.04
Sorbitol	0.4	1 : 02	99.70 $\pm$ 2.02
	0.8	1 : 04	100.46 $\pm$ 1.41
	1.2	1 : 06	99.27 $\pm$ 3.70
Lactose	0.4	1 : 02	100.18 $\pm$ 1.02
	0.8	1 : 04	97.46 $\pm$ 3.68
	1.2	1 : 06	99.35 $\pm$ 0.74
Starch	0.4	1 : 02	97.35 $\pm$ 1.71
	0.8	1 : 04	99.35 $\pm$ 3.57
	1.2	1 : 06	101.36 $\pm$ 2.84
Mg. stearate	0.4	1 : 02	98.86 $\pm$ 4.00
	0.8	1 : 04	97.40 $\pm$ 2.12
	1.2	1 : 06	100.16 $\pm$ 4.26

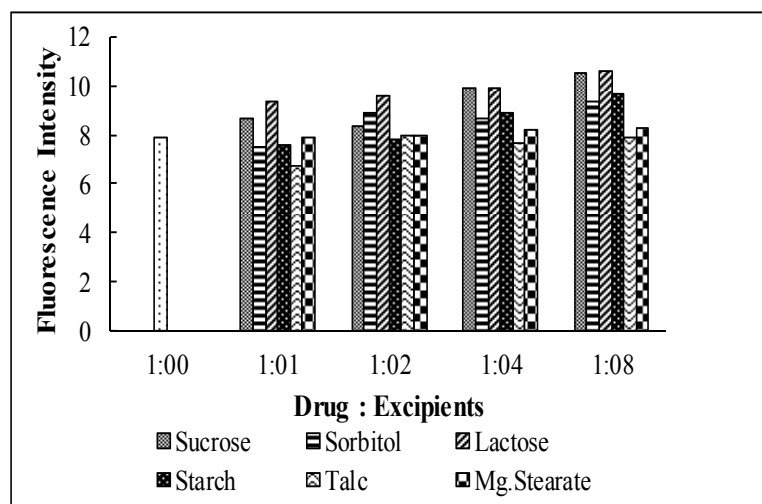


Figure 3.5.13 Effect of common excipients on determination of sulpiride

The effect of co-administered drugs such as Mefenamic acid, Ibuprofen, Acetylsalicylic acid and Metronidazole was also studied. Known concentration of standard sulpiride ($0.2 \mu\text{g mL}^{-1}$) and different concentration of the co-administered drugs in concentration ratio (1:1, 1:2 and 1:4) were added to a series of separate test tubes pursued by the addition of optimized amount of all the reagents and fluorescence intensity was measured. Each experiment was performed in triplicates (figure 3.5.14).

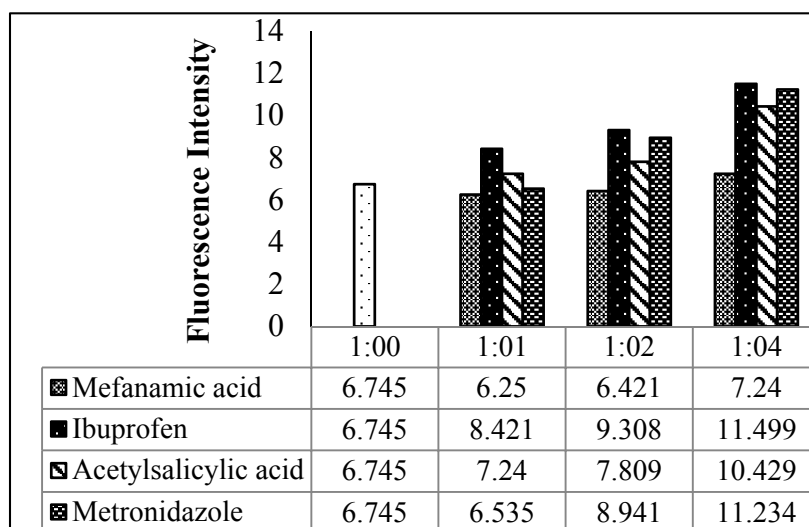


Figure 3.5.14 Effect of co-administered drugs on determination of sulpiride

3.5.6 Application of the proposed method for the analysis of sulpiride in pharmaceutical formulations

A. Determination of precision and accuracy of the proposed method

Same Instrument, reagents and standard solution were used as mentioned before.

Procedure

The precision and accuracy of the method was checked by analyzing the sulpiride in pharmaceutical preparations (each containing 50 mg of the active ingredients of sulpiride) using three different concentration of the analyte drug in triplicate with in the

calibration curve range. Calibration curve was plotted following the same procedure and known concentrations (0.2, 0.4 and 0.6 $\mu\text{g mL}^{-1}$) of sample solution were then analyzed by the proposed method (table 3.5.5).

Table 3.5.5 Evaluation of accuracy and precision of the proposed method for determination of sulpiride

Sample	($\mu\text{g mL}^{-1}$) taken	($\mu\text{g mL}^{-1}$) found	%Recovery $\pm$ RSD
Sulphiride-50, 50 mg capsule	0.2	0.202	101.00 $\pm$ 2.12
	0.4	0.401	100.25 $\pm$ 2.72
Combitic Global (India)	0.6	0.612	102.00 $\pm$ 1.63
Sulpiride Capsules 50 mg ARBRO pharmaceuticals	0.2	0.199	99.50 $\pm$ 2.57
	0.4	0.396	99.00 $\pm$ 2.05
Ltd New Delhi (India)	0.6	0.606	101.00 $\pm$ 1.55
Sulpiride-50, 50 mg caps	0.2	0.200	100.00 $\pm$ 2.63
Xier kangtai	0.4	0.392	98.10 $\pm$ 2.45
Pharmaceutical Co. Ltd China	0.6	0.586	97.67 $\pm$ 0.68

B. Percent recovery of sulpiride from commercial formulations (Standard addition method)

For determination of percent recovery of sulpiride from pharmaceutical preparations, 0.2, 0.4 and 0.6 $\mu\text{g mL}^{-1}$ standard sulpiride solution was mixed with pre-analyzed commercial sample of sulpiride (0.2 $\mu\text{g mL}^{-1}$). The fluorescent intensity was measured at 379 nm using excitation wavelength of 330 nm. The total amount of sulpiride in each sample was calculated from the calibration curve. Each experiment was performed in triplicates and percent recovery was calculated using the following relationship (table 3.5.6).

$$\% \text{ Recovery} = \frac{\mu\text{g mL}^{-1} \text{ found in the fortified sample} - \mu\text{g mL}^{-1} \text{ present in the sample}}{\mu\text{g mL}^{-1} \text{ of standard added}} \times 100$$

Table 3.5.6 Evaluation of recovery test of sulpiride in commercial formulation (capsules) by the proposed method

Sample	Sample ($\mu\text{g mL}^{-1}$)	($\mu\text{g mL}^{-1}$) added	($\mu\text{g mL}^{-1}$) found	%Recovery $\pm$ RSD
Sulpiride-50, 50 mg capsule Combitic Global (India)	0.2	0.2	0.198	99.00 $\pm$ 2.07
		0.4	0.399	99.75 $\pm$ 1.45
		0.6	0.606	101.00 $\pm$ 2.90
Sulpiride Capsules 50 mg ARBRO pharmaceuticals Ltd New Delhi (India)	0.2	0.2	0.200	100.00 $\pm$ 0.62
		0.4	0.405	101.25 $\pm$ 2.97
		0.6	0.593	99.20 $\pm$ 2.68
Sulpiride-50, 50 mg caps Xier kangtai Pharmaceutical Co. Ltd China	0.2	0.2	0.198	99.00 $\pm$ 2.85
		0.4	0.398	99.50 $\pm$ 2.36
		0.6	0.607	101.16 $\pm$ 1.08

B. Determination of active ingredients in the pharmaceutical formulations

Same instrument, reagents, standard and sample solutions were used.

Procedure

Solutions of three different commercial formulations were prepared by the same way as mentioned before. Working sample solution of $5.0 \mu\text{g mL}^{-1}$ was prepared by diluting the required volume of stock sample solution with distilled water. Proper volumes of this sample solution ($5.0 \mu\text{g mL}^{-1}$) were then analyzed by the proposed method. The results are summarized in table 3.5.7.

Table 3.5.7 Determination of active ingredients of sulpiride in the pharmaceutical preparations

Brand name	Active ingredient (mg tab ⁻¹)		t-test value (4.303)	F-test value (19)
	Labeled value	Found value		
Sulphiride-50, 50 mg capsule Combitic Global (India)	50	50.62 ± 1.86	2.37	0.003
Sulpiride Capsules 50 mg ARBRO pharmaceuticals Ltd New Delhi (India)	50	50.04 ± 2.03	0.104	0.260
Sulpiride-50, 50 mg caps Xier kangtai Pharmaceutical Co. Ltd China	50	49.08 ± 1.23	1.58	0.004

3.5.7 Validation of the proposed method in spiked human plasma

Procedure

Blood plasma was kindly granted by the Khyber Teaching Hospital, Peshawar Pakistan. In a set of 50 mL separate beakers, 1.0 mL aliquot of human plasma was spiked with 2.0 mL of 200 µg mL⁻¹ sulpiride to give the final required concentration. To each of these solution 5.0 mL diethyl ether was added. The solution was vortexed for two minutes pursued by centrifugation at 2500 rpm for 15 minutes. The resulting supernatant of organic layer was transferred into a 50 mL reaction flask with the help of pipette. The extraction was repeated with another 5.0 mL of diethyl ether. The ether extracts was evaporated to dryness. The dry residue was dissolved in 2.0 mL of distilled ethanol and the volume was made up to 50 mL with distilled water. Proper volumes of this solution to give final drug concentration in the range of 0.2–0.6 µg mL⁻¹ were taken in a series of test tubes and optimized amount of all the reagents were added. The test tubes were heated for 25 minutes at 90 °C in a water bath and after cooling diluted to 25 mL with distilled water in volumetric flasks. A reagent blank was prepared in the same way containing the same amount of plasma but without adding of drug. Fluorescence intensity was measured at

379 nm and the content of sulpiride was determined using standard calibration curve (table 3.5.8).

Table 3.5.8 Spectrofluorimetric determination of sulpiride in spiked plasma by the proposed method

Sample	($\mu\text{g mL}^{-1}$) added	($\mu\text{g mL}^{-1}$) found	% Recovery $\pm$ RSD
Plasma	0.2032	0.1944	97.50 $\pm$ 1.68
	0.3963	0.3875	97.00 $\pm$ 2.82
	0.5812	0.5724	97.80 $\pm$ 2.94

3.5.8 Determination of sulpiride by reference (HPLC) method

Instrument, reagents, standard and sample solutions used were the same as mentioned before.

Procedure

For HPLC system a calibration curve of peak height versus concentration was constructed in the same way as mentioned before (figure 3.5.15). Sample solution of three different commercial formulations in three different concentrations (20, 30 and 40 $\mu\text{g mL}^{-1}$) was injected one by one to the system and precision of the method was determined with triplicates. The amount of sulpiride in the commercial formulations was calculated and a comparison of the result of the proposed method with that of reference HPLC method is given in table 3.5.9.

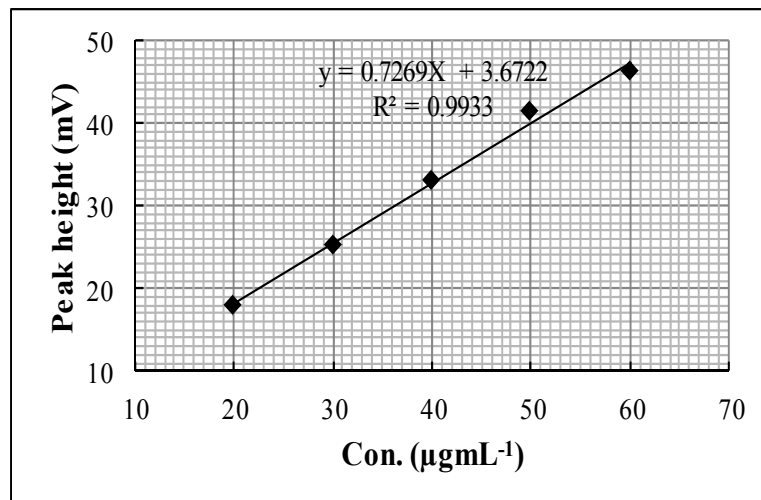


Figure 3.5.15 Calibration curve for sulpiride using reversed phase HPLC

Table 3.5.9 Comparison of determination of sulpiride contents by proposed method and reference HPLC method

Name of commercial formulation		Amount determined (mg tab ⁻¹)	
		Proposed method	Literature method
Sulpiride-50, 50 mg capsule Combitic Global (India)	Mean ± RSD	50.62 ± 1.86	
	F-test (19)	0.003	46.68 ± 2.20
	t-test (4.303)	2.37	
Sulpiride Capsules 50 mg ARBRO pharmaceuticals Ltd New Delhi (India)	Mean ± RSD	50.04 ± 2.03	
	F-test (19)	0.260	48.39 ± 1.54
	t-test (4.303)	0.104	
Sulpiride-50, 50 mg caps Xier kangtai Pharmaceutical Co. Ltd China	Mean ± RSD	49.08 ± 1.23	
	F-test (19)	0.004	46.75 ± 1.66
	t-test (4.303)	1.58	

3.5.9 Results and discussion

A simple and sensitive spectrofluorimetric method using 2-cyanoacetamide has been developed and validated for the quantification of sulpiride in bulk, pharmaceutical preparations and biological sample. The amide carbon of sulpiride on reaction with 2-

cyanoacetamide in the presence of ammonia produces a fluorescent product which was measured spectrofluorimetrically at λ_{em} 379 nm and λ_{ex} 330 nm (figure 3.5.2).

Different parameters affecting the reaction like volume and concentration of 2-cyanoacetamide, ammonia, and reaction temperature were carefully studied and optimized.

The effect of concentration of 2-cyanoacetamide was investigated in the range of 2.0 to 6.0%. It was noted that fluorescence intensity increases with increase in concentration of 2-cyanoacetamide up to 4.0% beyond which a slight decrease in fluorescence intensity was noted (figure 3.5.3). Therefore, 4.0% of 2-cyanoacetamide was selected for further analysis. Similarly the effect of volume of 4.0% 2-cyanoacetamide was also studied in the range of 1.0 to 4.0 mL and maximum fluorescence intensity was observed with 3.0 mL of 4.0% 2-cyanoacetamide solution (figure 3.5.4).

The effect of ammonia concentration on the formation of fluorescent product was also investigated in the range of 10–35%. Maximum fluorescent product was formed by using 30% of ammonia solution. Therefore this concentration of ammonia was used for further work (figure 3.5.5). The effect of volume of 30% ammonia solution was also studied in the range of 0.5–2.5 mL and 1.5 mL of this solution was found optimum (figure 3.5.6).

The effect of different bases like sodium hydroxide, potassium hydroxide and ammonia on the reaction of sulphiride with 2-cyanoacetamide was also studied. Ammonia was found to be suitable base for abstracting a proton from the methylene group and generating the carbene ion (figure 3.5.7). Therefore ammonia was used for further analysis.

The effect of temperature and heating time on the derivatization reaction was also studied. The effect of temperature was investigated in the range of 60 to 100 °C using electric thermostat water bath. Maximum fluorescent product was obtained by heating the reaction mixture at 90 °C (figure 3.5.8). Similarly the effect of heating time was investigated in the range of 10–35 minutes. Maximum product formation was observed by heating the reaction mixture for 25 minutes at 90 °C (figure 3.5.9). Therefore, 25 minutes heating time at 90 °C was selected as optimum for further analysis.

The stability of fluorescent reaction product was studied by measuring the fluorescence intensity of the product at regular interval up to two hours. It was observed that no change in fluorescence intensity with time occurred (figure 3.5.10).

Analytical characteristics

Under optimum experimental conditions, the calibration curve of fluorescence intensity versus sulpiride concentration was linear in the range of 0.2–20 $\mu\text{g mL}^{-1}$ (table 3.5.2 and figure 3.5.11) with high value of correlation coefficient ($r^2 = 0.9985$). LOD and LOQ values were calculated and found to be 0.82 ng mL^{-1} and 2.73 ng mL^{-1} respectively. The linear regression equation, intercept, slope, correlation coefficient, and RSD are given in table 3.5.3.

Applications

The selectivity of the proposed method was checked by investigating the interferences effect of common excipients like talc, sucrose, sorbitol, lactose, starch, and magnesium stearate in the analysis of the drug. Under the optimized condition, solutions of synthetic mixtures containing the analyte drug and one of the excipients in the ratio of 1:2, 1:4 and 1:6 were analyzed by the proposed method (table 3.5.4 and figure 3.5.13). No interferences were observed in the determination of sulpiride in the presences of these common excipients. Percent recoveries obtained were in the range of 97.35 % to 102.66 %. The effect of common co-administered drugs like mefenamic acid, ibuprofen, acetylsalicylic acid and metronidazole were also investigated for their interferences in the analysis of the analyte drugs. It was observed that these co-administered drugs did not interfere in the analysis of sulpiride by the proposed method (figure 3.5.14).

The accuracy and precision of the method was also evaluated by determining the sulpiride contents in three different commercial formulations at three different concentrations level (0.2, 0.4 and 0.6 $\mu\text{g mL}^{-1}$) in triplicate (table 3.5.5). The percent recoveries ranged from 97.67% to 102.00%. The RSD was acceptable which indicates good reproducibility of the proposed method.

The accuracy of the proposed method in three different brands of capsules was evaluated by performing recoveries experiments through standard addition method. A known quantity of standard sulpiride solution (0.2, 0.4 and 0.6 $\mu\text{g mL}^{-1}$) was added to three different kinds of pre-analyzed dosage forms and then determined by the proposed method. Percent recoveries were found in the range of 99.00 % to 101.25% (table 3.5.6) with low relative standard deviation. This indicates high accuracy of the developed method for the determination of sulpiride in pharmaceutical formulations. The results of the proposed method were compared statistically with respect to accuracy by student t-test at 95% confidence level and precision by the variance ratio F-test with those of the literature HPLC method [1]. There was no significant difference in precision between the proposed and literature methods (table 3.5.9).

Good sensitivity, high precision and accuracy achieved by the proposed method recommended the quantification of sulpiride in spiked plasma samples. A proper volume of plasma was spiked with standard drug solution. The percent recovery was found to be in the range of 97.00 to 97.80%. The results are given in table 3.5.8. The method can be successfully used for the determination of sulpiride in biological samples in clinical analysis.

Reference

1. H. Tokunaga, K. Kudo, N. Jitsufuchi, Y. Ohtsuka, T. Imamura, "Sensitive determination of sulpiride in human plasma by high performance liquid chromatography" *Journal of Chromatography B*, 691, 203-207 (1997).

3.6 MICELLAR-ENHANCED SPECTROFLUORIMETRIC METHOD FOR DETERMINATION OF SULPIRIDE IN PHARMACEUTICAL FORMULATIONS AND BIOLOGICAL SAMPLES

3.6.1 Method development strategy for the spectrofluorimetric determination of sulpiride

Surfactants are frequently employed to solubilize hydrophobic compounds and it also increases the fluorescence intensity of weakly fluorescent compounds as micellar solutions provide a microenvironment with lower polarity than water. Sulpiride is fluorescence active and addition of a micelle can enhance the fluorescence intensity of the fluorophore..

3.6.2 Preliminary investigation of the possibility of enhancement in the fluorescence intensity of sulpiride by sodium dodecyl sulphate (SDS)

Preliminary studies were carried out to investigate the possible enhancement of fluorescence intensity of sulpiride using different micelles such as anionic (SDS), non-ionic (Triton X-100) and methyl cellulose and different buffer system in the pH range of 2.2–8.0. Initially 2.0 mL of sulpiride standard solution ($20 \mu\text{g mL}^{-1}$) and 2.0 mL of each micelle with and without the addition of buffer solution were taken in volumetric flask and made the volume up to 25 mL with distilled water. Fluorescence intensity of each solution was measured (figure 3.6.1).

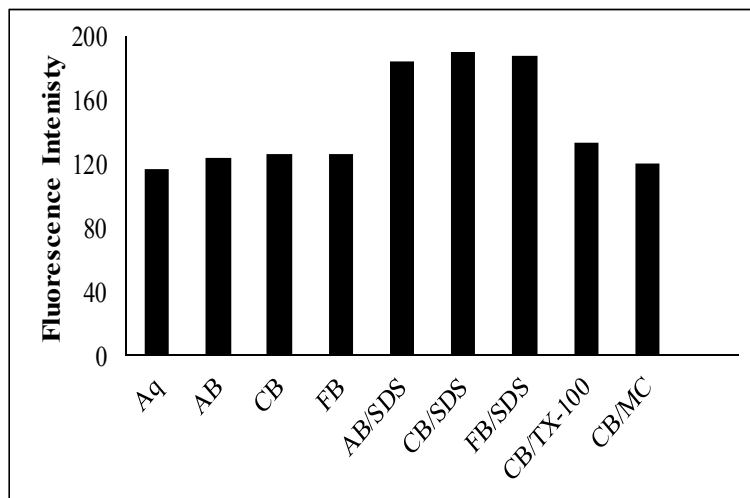


Figure 3.6.1 Fluorescence intensity of the analyte as a function of medium. Aq, aqueous medium; AB, acetate buffer (pH 4.0); CB, citrate buffer, FB, formate buffer (pH 4.0); SDS, sodium dodecyl sulphate (1.0%); MC, methyl cellulose (1.0%); T-X100 = Triton-X100 (1.0%)

3.6.3 Optimization studies for spectrofluorimetric determination of sulpiride using micelle

After the preliminary investigation, it was observed that fluorescence intensity of sulpiride could be increased by the addition of micelle. For quantitative determination of sulpiride various experimental parameters of the method was optimized.

Instruments

All fluorescence intensities were measured on a RF-5301 PC Spectrofluorophotometer. Digital analytical balance and electric thermostatic water bath were used in this work. A WISSENSCHAFTLICH-TECHNISCHE WERKSTATTEN 8120 WEILHEM pH meter was used for pH measurements. A clinical centrifuge (Model 800, China with maximum speed 4000 rpm) was used for centrifugation of plasma and urine samples.

Reagents

Analytical grade purity reagents were used. Sodium dodecyl sulphate (BDH Poole, England), Triton X-100, and methyl cellulose (Scharlau, Barcelona, Spain) were used.

Disodium phosphate and citric acid (Merck KGaA, 64271 Darmstadt, Germany). Standard reference sulpiride was provided by Hang Zhou Pharma and chem. co china. Commercial formulations of sulpiride (sulpiride-50, 50 mg caps manufactured by Combitic Global D-2 industrial area Sonapat-131001 (Hr) India, Sulpiride-50, 50 mg caps manufactured by Xier kangtai Pharmaceutical Co. Ltd North zone, P.R.China. Sulpiride Capsules 50 mg manufactured by ARBRO pharmaceuticals Ltd (India), Sulpiride-50, 50 mg capsule manufactured by Unicure (India) Pvt. Ltd) were purchased from local market. Blank urine sample was obtained from healthy male volunteer, and the plasma samples were supplied by the Khyber Teaching Hospital Peshawar (Pakistan).

Solutions preparation

Sodium dodecyl sulphate (SDS) solution (0.2 M)

Sodium dodecyl sulphate (0.2 M) solution was prepared by dissolving 2.88 grams of the reagent in distilled water and diluted to 50 mL with distilled water in volumetric flask. Citrate buffer solution (pH=4.0) was prepared by mixing 0.2 M disodium phosphate with 0.1 M citric acid solution and the pH was adjusted to 4.0 with pH meter.

Standard sulpiride solution (100 $\mu\text{g mL}^{-1}$)

Standard stock sulpiride solution (100 $\mu\text{g mL}^{-1}$) was prepared on daily basis by dissolving 0.01g of sulpiride standard in 1.0 mL of 0.2 M HCl and the volume was made up to 100 mL with distilled water. Working standard solutions (10 $\mu\text{g mL}^{-1}$ and 5.0 $\mu\text{g mL}^{-1}$) were prepared daily by diluting appropriate amount of the stock solution with distilled water.

Sample solution

Contents of the five capsules of sulpiride were weighed separately, and average weight of the powder in one capsule was calculated. An accurate weighed portion of the sample claimed to contain 10 mg of sulpiride was dissolved in 1.0 mL of 0.2 M HCl, filtered and made the volume up to 100 mL with distilled water. 5.0 $\mu\text{g mL}^{-1}$ sample solutions were prepared from stock sample solution by dilution with distilled water.

Procedure

2.0 mL of 20 $\mu\text{g mL}^{-1}$ sulpiride standard solutions and 1.0 mL of 0.1 M SDS solution was mixed in 25 mL volumetric flask and the volume was made up to the mark with distilled water. Fluorescence intensity of the drug with and without the presence of micelle was recorded by scanning the analyte solution for excitation spectra in the range of 250–330 nm and for emission spectra in the range of 300–450 nm using spectrophotofluorimeter (figure 3.6.2).

For optimizing the concentration of SDS solution, 2.0 mL of sulpiride standard solution (20 $\mu\text{g mL}^{-1}$) and 1.0 mL of citrate buffer solution (pH= 4.0) was taken in a series of 25 mL volumetric flasks. To each flask 1.0 mL of SDS solution in the concentration range of 0.01–0.4 M was added and the volume was made up to the mark with distilled water and fluorescence intensity was measured (figure 3.6.3).

For optimization the volume of SDS (0.20 M), only volume of SDS was varied in the range of 0.5–3.0 mL keeping rest of the procedure the same and fluorescence intensity was measured (figure 3.6.4).

For finding the most suitable pH of citrate buffer solution, 2.0 mL of sulpiride solution (20 $\mu\text{g mL}^{-1}$) and 1.0 mL of citrate buffer solution in the pH range 2.2–8.0 were taken in 25 mL volumetric flasks. Then 1.0 mL of SDS (0.2 M) solution was added and the volume was made up to the mark with distilled water and fluorescence intensity was measured (figure 3.6.5).

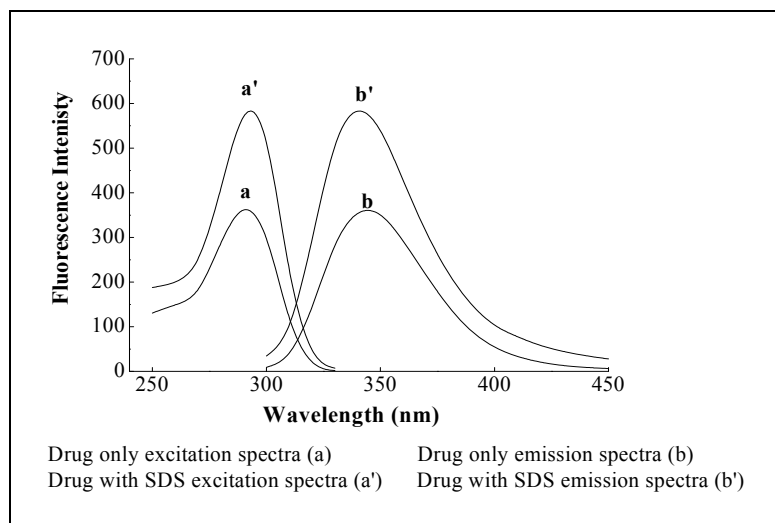


Figure 3.6.2 Excitation and emission spectra of drug with and without SDS

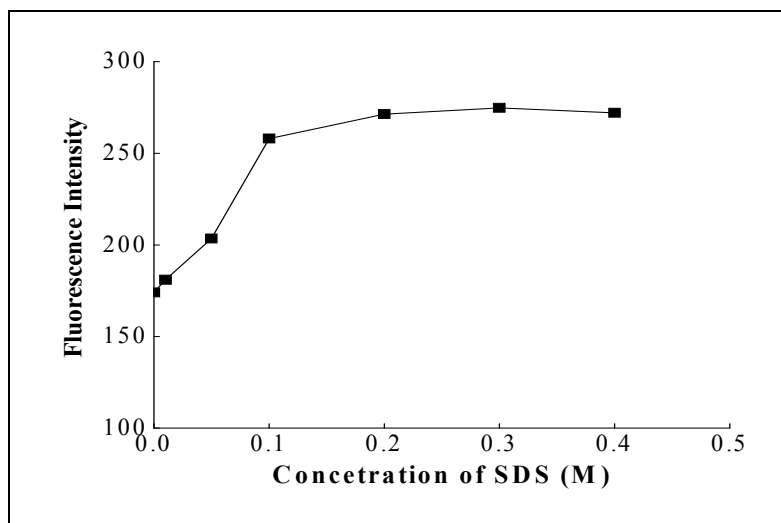


Figure 3.6.3 Effect of concentration of SDS on the spectrofluorimetric determination of sulphiride

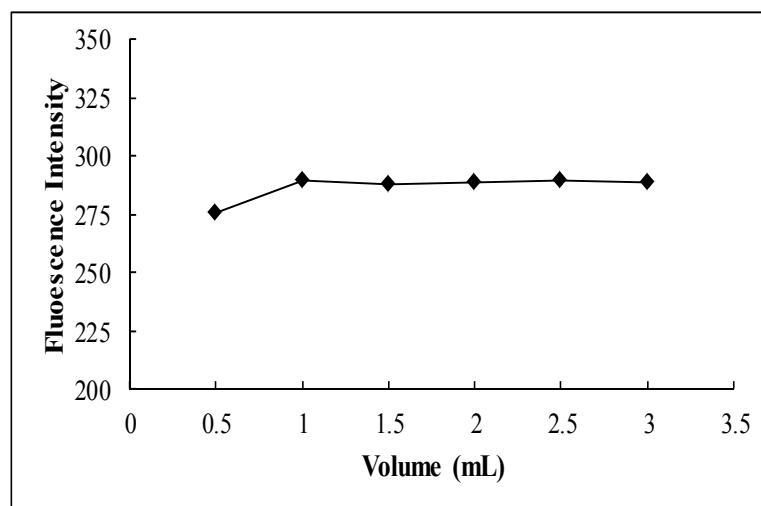


Figure 3.6.4 Effect of volume of SDS (0.2 M) on the spectrofluorimetric determination of sulpiride

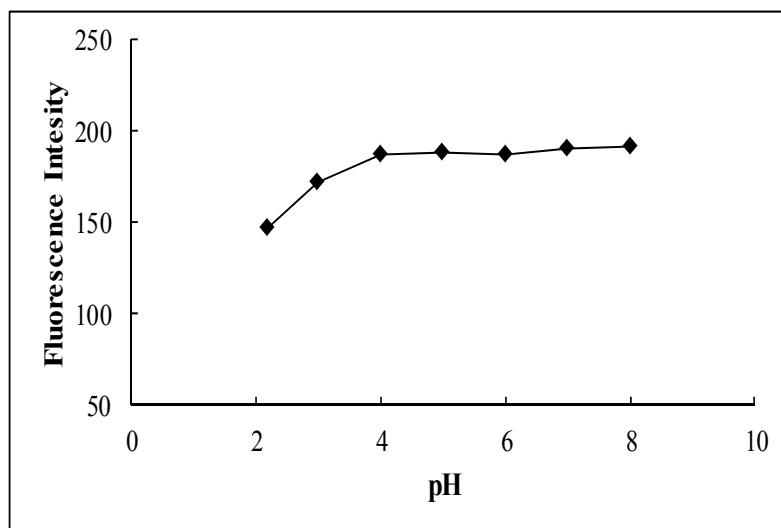


Figure 3.6.5 Effect of pH of on the spectrofluorimetric determination of sulpiride

3.6.4 Effect of sulpiride concentration on fluorescence intensity

Instruments, reagents and solution used were the same as mentioned before.

Procedure

In a series of 25 mL volumetric flasks, proper volumes of the sulpiride solution were taken to give a final concentration in the range of 0.02–7.0 $\mu\text{g mL}^{-1}$. Then 1.0 mL of citrate buffer solution of pH 4.0 and 1.0 mL of 0.2 M SDS were added to each flask. The contents of the flasks were diluted to 25 mL with distilled water and fluorescence intensity was measured at optimum excitation and emission wavelengths using PC RF-5301 spectrofluorophotometer against a reagent blank (table 3.6.1 and figure 3.6.6). To determine sulpiride in pharmaceutical preparations and biological samples, the calibration plot for lower concentration of the drug was constructed (figure 3.6.7).

Table 3.6.1 Effect of concentration of sulpiride on the fluorescence intensity

Concentration ($\mu\text{g mL}^{-1}$)	Fluorescence intensity	Concentration ($\mu\text{g mL}^{-1}$)	Fluorescence intensity
0.02	3.072	2.4	367.412
0.04	6.978	2.6	398.791
0.06	9.326	2.8	426.528
0.08	12.954	3.0	459.305
0.10	15.900	3.2	486.555
0.2	31.736	3.4	499.657
0.4	62.400	3.6	541.428
0.6	98.369	3.8	583.935
0.8	126.279	4.0	594.373
1.0	158.394	4.4	662.340
1.2	186.892	4.8	716.528
1.4	222.131	5.2	778.953
1.6	252.347	5.6	824.981
1.8	283.109	6.0	883.208
2.0	315.291	6.5	938.998
2.2	344.350	7.0	999.985

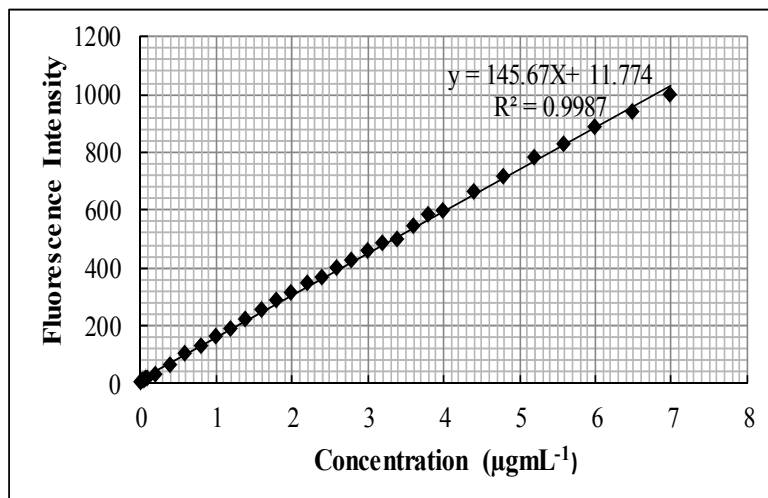


Figure 3.6.6 Effect of concentration of sulpiride on the fluorescence intensity

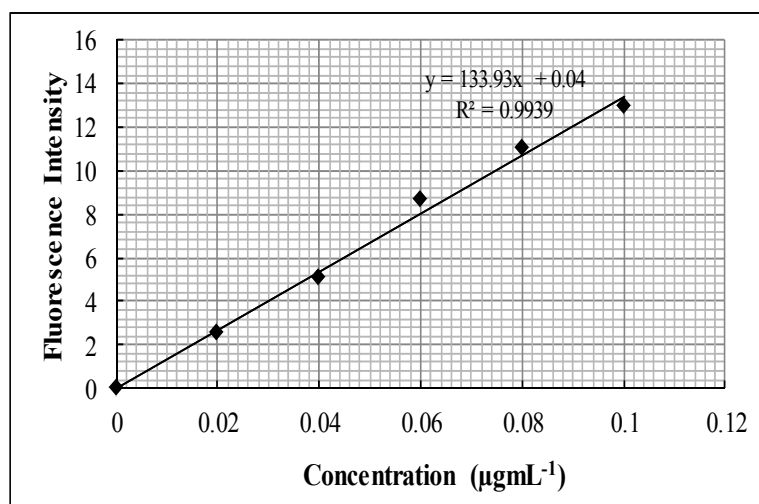


Figure 3.6.7 Calibration curve for spectrofluorimetric determination of sulpiride at lower concentration

Determination of LOD, LOQ and other analytical characteristics for the proposed spectrofluorimetric method for determination of sulpiride

Six replicates with lowest quantifiable concentration of sulpiride ($0.02 \mu\text{g mL}^{-1}$) which can be measured with acceptable precision and accuracy were investigated by the

proposed method pursuing the same procedure as mentioned before. LOD, LOQ and other analytical characteristics were calculated and are given in table 3.6.2.

Table 3.6.2 Analytical characteristics for the spectrofluorimetric determination of sulpiride

Parameter	Value
λ_{ex} (nm)	292
λ_{em} (nm)	343
Linear range ($\mu\text{g mL}^{-1}$)	0.02–7.0
Limit of detection 3S/b ($\mu\text{g mL}^{-1}$)	1.79×10^{-5}
Limit of quantification 10S/b ($\mu\text{g mL}^{-1}$)	5.9×10^{-5}
Regression equation (y)	$Y=133.93X+0.04$
Slope (b)	133.93
Intercept (a)	0.04
Correlation coefficient (r^2)	0.993
Standard deviation ($\mu\text{g mL}^{-1}$)	8.9×10^{-4}
Relative standard deviation %	4.7

3.6.5 Investigation of interferences effect on determination of sulpiride by the proposed method

Same instrument, reagent and standard solution were used as mentioned before.

Procedure

The selectivity of the proposed method for the analysis of sulpiride containing pharmaceutical preparation was evaluated by studying the interferences effect of common excipients that are generally added during preparations of tablets. Known concentration of standard sulpiride ($0.02 \mu\text{g mL}^{-1}$) was analyzed by the same procedure as stated before. Then standard sulpiride ($0.02 \mu\text{g mL}^{-1}$) and different concentrations of excipients were mixed in a series of 25 mL volumetric flasks maintaining drug: excipients ratio of 1:50, 1:100 and 1:500. Then optimized amount of SDS solution (1.0 mL of 0.2 M) was added and the volume was made up to the mark with distilled water. The fluorescence intensity

was measured at the optimized wavelengths. The analysis were performed in triplicates and mean fluorescence intensity was calculated (table 3.6.3 and figure 3.6.8).

Table 3.6.3 Effect of common excipients on determination of sulpiride ($0.02 \mu\text{g mL}^{-1}$) using the proposed method

Excipient	Excipients added ($\mu\text{g mL}^{-1}$)	Drug: Excipient	%Recovery $\pm$ RSD
Talc	1.00	1:50	103.33 ± 2.79
	2.00	1:100	101.67 ± 2.83
	10.0	1:500	105.83 ± 3.03
Magnesium stearate	1.00	1:50	98.33 ± 2.92
	2.00	1:100	95.00 ± 5.26
	10.0	1:500	101.67 ± 2.83
Sorbitol	1.00	1:50	106.66 ± 2.70
	2.00	1:100	103.33 ± 2.88
	10.0	1:500	102.16 ± 2.69
Starch	1.00	1:50	93.33 ± 3.08
	2.00	1:100	98.33 ± 2.92
	10.0	1:500	96.67 ± 2.97
Lactose	1.00	1:50	98.33 ± 5.86
	2.00	1:100	101.67 ± 2.83
	10.0	1:500	103.33 ± 2.78
Glucose	1.00	1:50	105.26 ± 4.98
	2.00	1:100	105.26 ± 0.00
	10.0	1:500	99.99 ± 9.11
Fructose	1.00	1:50	96.48 ± 3.15
	2.00	1:100	105.96 ± 3.85
	10.0	1:500	98.24 ± 3.09
sodium stearate	1.00	1:50	101.67 ± 5.67
	2.00	1:100	105.00 ± 4.76
	10.0	1:500	100.00 ± 5.0
Sucrose	1.00	1:50	93.01 ± 3.47
	2.00	1:100	98.60 ± 3.19
	10.0	1:500	101.04 ± 3.39

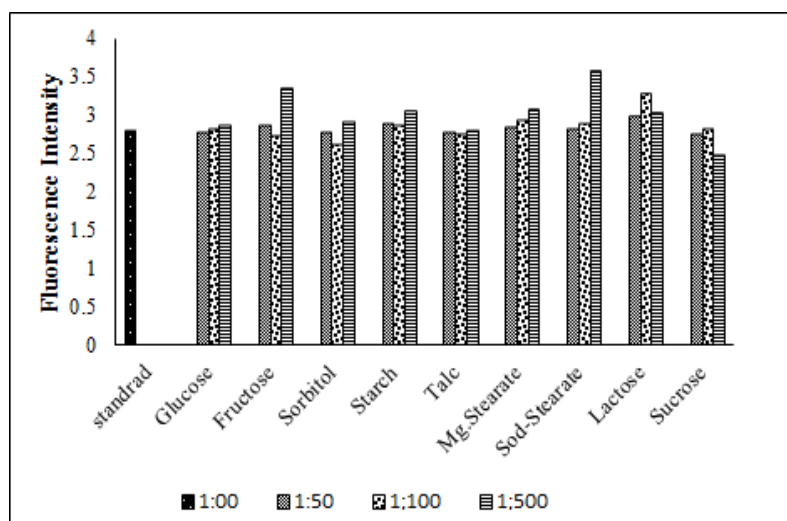


Figure 3.6.8 Effect of common excipients on determination of sulpiride by the proposed method

The effect of some co-existing substances normally found in human urine and blood samples was also investigated. For this purpose a known concentration ($0.02 \mu\text{g mL}^{-1}$) of standard sulpiride solution was analyzed. Then the same concentration of standard sulpiride solution and different concentration of the possible cations in different concentration ratio maintaining drug: cations ratio of (1:10, 1:50 and 1:100) were added to a series of 25 mL volumetric flasks pursued by the addition of SDS (1.0 mL of 0.2 M) and made the volume up to the mark with distilled water and fluorescence intensity was measured (table 3.6.4 and figure 3.6.9).

Table 3.6.4 Interferences effect of co-existing substances on the determination of sulphiride

Co-existing substances	Amount added ($\mu\text{g mL}^{-1}$)	Drug: coexisting substance	%Recovery $\pm$ RSD
Na^+	0.2	1:10	102.50 ± 5.53
	1.0	1:50	101.00 ± 5.01
	2.0	1:100	100.98 ± 4.56
K^+	0.2	1:10	102.50 ± 2.50
	1.0	1:50	94.00 ± 5.05
	2.0	1:100	93.50 ± 3.47
Ca^{2+}	0.2	1:10	96.07 ± 4.70
	1.0	1:50	94.14 ± 4.08
	2.0	1:100	97.31 ± 3.38
Mg^{2+}	0.2	1:10	100.52 ± 5.08
	1.0	1:50	106.84 ± 2.90
	2.0	1:100	102.00 ± 2.45
Urea	0.2	1:10	104.61 ± 2.99
	1.0	1:50	103.58 ± 2.72
	2.0	1:100	103.58 ± 2.5
Uric acid	0.2	1:10	90.47 ± 2.38
	1.0	1:50	95.23 ± 1.62
	2.0	1:100	95.23 ± 2.32

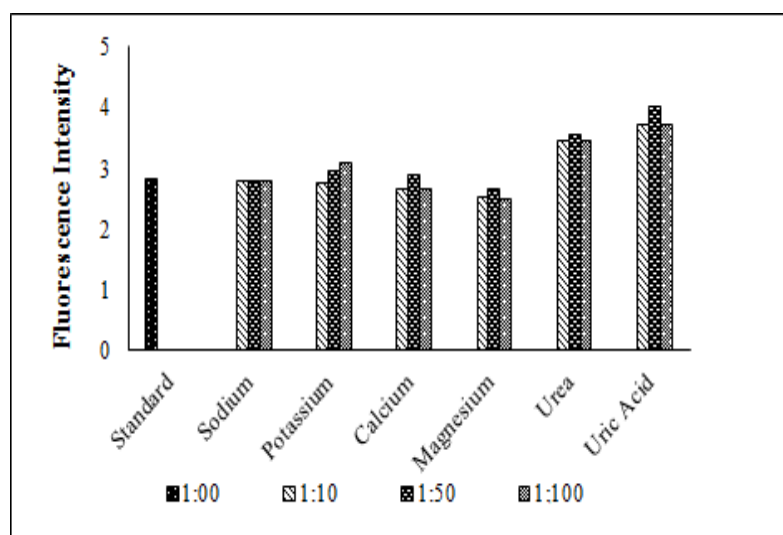


Figure 3.6.9 Effect of co-existing substances on determination of sulphiride by the proposed spectrofluorimetric method

3.6.6 Application of the investigated method for the analysis of sulpiride in pharmaceutical preparations

A. Precision and accuracy of the investigated method

Instrument, reagents and standard solution used were the same as mentioned before.

Procedure

Precision and accuracy of the method was determined by analysis of three separate sample solutions of the pharmaceutical preparations (each containing 50 mg of the active ingredients of sulpiride) at three different concentration levels. Calibration curve was plotted by the same procedure as given before and known concentrations (0.02, 0.04 and 0.06 $\mu\text{g mL}^{-1}$) of these sample solutions were then analyzed by the proposed method in triplicate (table 3.6.5).

Table 3.6.5 Evaluation of the accuracy and precision of the proposed method for sulpiride determination

Pharmaceutical formulations	($\mu\text{g mL}^{-1}$) taken	($\mu\text{g mL}^{-1}$) found	%Recovery $\pm$ RSD
1	0.02	0.0195	97.50 $\pm$ 2.56
	0.04	0.0392	98.00 $\pm$ 1.02
	0.06	0.0560	93.34 $\pm$ 1.78
2	0.02	0.0196	98.00 $\pm$ 3.57
	0.04	0.0382	95.50 $\pm$ 3.66
	0.06	0.0577	96.16 $\pm$ 1.03
3	0.02	0.0199	99.50 $\pm$ 1.50
	0.04	0.0391	97.75 $\pm$ 3.06
	0.06	0.0592	98.67 $\pm$ 3.37
4	0.02	0.0196	98.00 $\pm$ 2.04
	0.04	0.0398	99.50 $\pm$ 2.51
	0.06	0.0585	97.50 $\pm$ 1.70

B. Percent recovery of sulpiride from commercial formulations (Standard addition method)

Same instrument, reagents, standard and sample solutions were used.

Procedure

Sulpiride standard solution 0.02, 0.04 and 0.06 $\mu\text{g mL}^{-1}$ was mixed with pre-analyzed commercial sample of sulpiride (0.02 $\mu\text{g mL}^{-1}$). The fluorescent intensity of was measured at 343 nm using excitation wavelength of 292 nm. The total amount of sulpiride in each fortified sample was calculated from calibration curve. The experiment was performed in triplicates and the mean percent recovery was calculated using the following formula. The results are given in table 3.6.6.

$$\% \text{ Recovery} = \frac{\mu\text{g mL}^{-1} \text{ found in the fortified sample} - \mu\text{g mL}^{-1} \text{ present in the sample}}{\mu\text{g mL}^{-1} \text{ of standard added}} \times 100$$

Table 3.6.6 Evaluation of recovery test of sulpiride in commercial formulation (capsules) by the proposed method

Pharmaceutical formulations	Sample ($\mu\text{g mL}^{-1}$)	($\mu\text{g mL}^{-1}$) added	($\mu\text{g mL}^{-1}$) found	%Recovery $\pm$ RSD
1	0.02	0.02	0.0201	100.50 $\pm$ 2.06
		0.04	0.0369	92.30 $\pm$ 3.61
		0.06	0.0608	101.30 $\pm$ 1.26
2	0.02	0.02	0.0201	100.50 $\pm$ 1.24
		0.04	0.0384	97.30 $\pm$ 2.25
		0.06	0.0599	99.80 $\pm$ 2.52
3	0.02	0.02	0.0193	96.50 $\pm$ 1.54
		0.04	0.0386	96.50 $\pm$ 1.54
		0.06	0.0588	98.00 $\pm$ 1.02
4	0.02	0.02	0.0210	100.50 $\pm$ 2.51
		0.04	0.0396	99.00 $\pm$ 1.01
		0.06	0.0599	99.80 $\pm$ 0.88

C. Determination of active ingredients in the commercial formulations

Instrument, reagents, standard solution and sample solutions used were the same as mentioned before.

Procedure

Solutions of four different commercial formulations ($100 \mu\text{g mL}^{-1}$) were prepared by the same way as mentioned before. Working sample solution of $5.0 \mu\text{g mL}^{-1}$ was prepared from stock sample solution by dilution of the fixed volume with distilled water. Proper volumes of this sample solution ($5.0 \mu\text{g mL}^{-1}$) were then analyzed by the proposed method Triplicate analyses were performed in each case (table 3.6.7).

Table 3.6.7 Determination of active ingredients of sulpiride in selected commercial formulations

Pharmaceutical preparations	Active ingredient (mg tab^{-1})		t-test value (4.303)	F-test value (19)
	Labeled value	Found value		
1	50	47.50 ± 1.28	2.86	0.041
2	50	48.12 ± 0.564	3.51	1.30
3	50	49.25 ± 0.437	2.64	0.005
4	50	49.12 ± 0.520	2.76	0.288

3.6.7 Validation of the proposed method in spiked biological samples

Procedure

Urine sample was taken from a healthy volunteer and 3.0 mL sample was spiked with 2.5 mL of sulpiride standard solution ($100 \mu\text{g mL}^{-1}$). The solution was then deproteinized by adding 9.0 mL of acetonitrile and centrifuged for 5 minutes at 3000 rpm. The supernatant was transferred to 50 mL volumetric flask and made the volume up to the mark with distilled water. Proper volume of this solution was taken in volumetric flask (25 mL) and 1.0 mL of 0.2 M SDS solution was added. The volume was made up to the mark with distilled water to give final drug concentration in the range of $0.02\text{--}0.06 \mu\text{g mL}^{-1}$. Fluorescence intensity of the resultant solution was measured at the optimized wavelengths. The results are given in table 3.6.8.

Similarly 3.0 mL of plasma sample was spiked with 2.5 mL of sulpiride standard solution ($100 \mu\text{g mL}^{-1}$) to give final drug concentration in the range of $0.02\text{--}0.06 \mu\text{g mL}^{-1}$ and the rest of the procedure was kept the same as for urine sample (table 3.6.9).

Table 3.6.8 Spectrofluorimetric determination of sulpiride in spiked urine by the proposed method

Sample	Spiked urine		
	($\mu\text{g mL}^{-1}$) taken	($\mu\text{g mL}^{-1}$) found	%Recovery $\pm$ RSD
Urine	0.02	0.0201	100.50 ± 6.96
	0.04	0.0406	101.67 ± 4.92
	0.06	0.0574	95.67 ± 2.61

Table 3.6.9 Spectrofluorimetric determination of sulpiride in spiked plasma by the proposed method

Sample	Spiked plasma		
	($\mu\text{g mL}^{-1}$) taken	($\mu\text{g mL}^{-1}$) found	%Recovery $\pm$ RSD
Plasma	0.02	0.0198	99.00 ± 3.03
	0.04	0.0399	99.75 ± 5.01
	0.06	0.0590	98.33 ± 1.35

3.6.8 Determination of sulpiride by reference method (HPLC)

Instrument, reagents, standard and sample solutions used were the same as mentioned in before.

Procedure

HPLC system was equilibrated with mobile phase prior to the analysis. Then standard sulpiride solution in the concentration range of 20–60 $\mu\text{g mL}^{-1}$ were injected one by one and the peak height was noted for each concentration. A calibration curve of peak height was constructed and is shown in figure 3.6.10. Sample solution of three commercial formulations in three different concentrations (20, 30 and 40 $\mu\text{g mL}^{-1}$) was injected to the system one by one and precision of the method was determined using triplicates readings. The results are given in table 3.6.10.

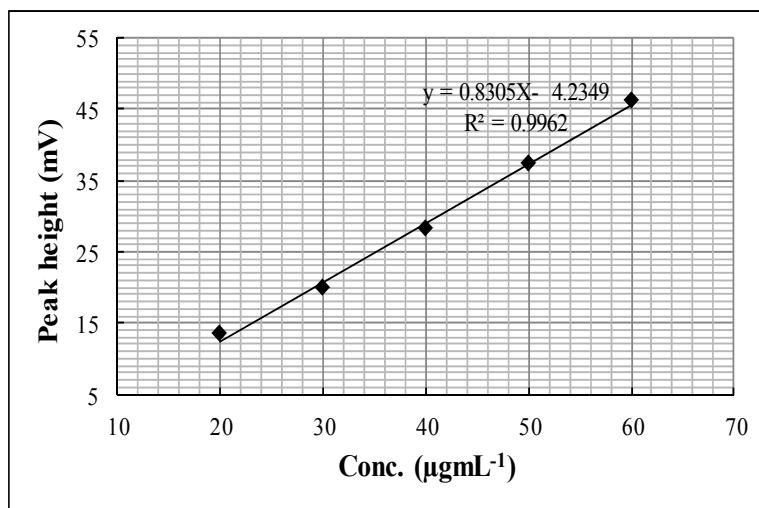


Figure 3.6.10 Calibration curve for sulpiride determination using reversed phase HPLC

Table 3.6.10 Determination of sulpiride in commercial formulations and validation by reference method

Commercial Formulation		Amount determined (mg ^{tab} l ⁻¹)	
		Proposed method	Literature method
Sulpiride-50, 50 mg capsule Combitic Global (India)	Mean ± RSD	47.50 ± 1.281	
	F-test (19)	0.041	46.68 ± 5.53
	t-test (4.303)	2.86	
Sulpiride-50,50 mg capsule Unicure (India) Pvt. Ltd	Mean ± RSD	48.12 ± 0.647	
	F-test (19)	1.30	49.11 ± 0.57
	t-test (4.303)	3.51	
Sulpiride-50, 50 mg caps Xier kangtai Pharmaceutical Co. Ltd China	Mean ± RSD	49.25 ± 0.438	
	F-test (19)	0.005	46.74 ± 6.18
	t-test (4.303)	2.64	
Sulpiride Capsules 50 mg ARBRO pharmaceuticals Ltd New Delhi (India)	Mean ± RSD	49.12 ± 0.520	
	F-test (19)	0.288	48.39 ± 0.97
	t-test (4.303)	2.76	

3.6.9 Results and discussion

A simple and accurate micelle enhanced spectrofluorimetric method has been developed for the determination of sulpiride in bulk, pharmaceutical formulations and biological samples. Sulpiride is native weak fluorescent and its fluorescence intensity can be greatly enhanced with SDS (figure 3.6.2).

For investigating the most favorable conditions for the enhanced fluorescence intensity of sulpiride, all parameters affecting the reaction such as types and concentration of micellar media, pH of the medium and effect of various buffer solutions were optimized.

The fluorimetric properties of sulpiride were investigated in the presence of different micelle such as SDS (1.0%), triton X-100 (1.0%) and methyl cellulose (1.0%) and in different buffer solutions such as acetate, citrate and formate. It was found that fluorescence intensity of sulpiride is greatly enhanced in the presence of SDS (figure 3.6.1) as compared to aqueous solution and other micellar media.

The effect of concentration of SDS solution was investigated in the range of 0.01 to 0.4 M and it was observed that the fluorescence intensity of sulpiride increases with increase in concentration of SDS up to 0.2 M, after which it remain constant. This enhancement may be due to protection of lower excited state of fluorescence compound from quenching process in SDS micellar that can readily occurs in aqueous solutions. Therefore 0.2 M SDS was preferred as optimum concentration for further work (figure 3.6.3). The effect of volume of 0.2 M SDS solution was also investigated in the range of 0.5 to 3.0 mL and 1.0 mL was found as the optimum volume (figure 3.6.4).

The effect of pH was investigated in the range of 2.2–6.0. The fluorescence intensity increase with increase in pH up to 4.0 after which it remains constant (figure 3.5.4). Therefore pH 4.0 was selected for further measurements in micellar media. The effect of different buffers solutions at the optimum pH value (4.0), including acetate, citrate, and formate was also studied in the micellar media. It was found that the citrate buffer offers the highest sensitivity. Therefore a pH 4.0 citrate buffer solution was selected for further study.

Analytical Characteristics

Under the optimal experimental conditions the calibration plot of fluorescence intensity versus the concentration of sulpiride was linear in the range of 0.02–7.0 $\mu\text{g mL}^{-1}$ with a correlation coefficient of 0.998 (figure 3.6.6). The LOD and LOQ were calculated and found to be $1.79 \times 10^{-5} \mu\text{g mL}^{-1}$ and $5.9 \times 10^{-5} \mu\text{g mL}^{-1}$ respectively. The linear regression equation, intercept, slope, correlation coefficient, and relative standard deviation are given in table 3.6.2.

Applications

To evaluate the selectivity of the proposed method for analysis of pharmaceutical formulations containing sulpiride, the interferences effect of various pharmaceutical additives such glucose; talc, fructose, magnesium stearate, lactose, sorbitol, starch, sodium stearate and sucrose on the efficiency of the presented method were investigated. Under the optimized condition, solutions containing sulpiride and one of the excipients

taken separately at the concentration ratio of fifty, hundred and five hundred of that of sulphiride were analyzed by the proposed method (table 3.6.3 and figure 3.6.8). The results showed that there was no significant interferences effect of these excipients on the determination of sulphiride by the proposed method.

The effect of some coexisting substances normally found in human urine and blood samples such as Na^+ , K^+ , Ca^{2+} , Mg^{2+} , urea and uric acid on fluorescence intensity of sulphiride was also investigated. The results showed that there was no effect of these substances on the determination of sulphiride (table 3.6.4 and figure 3.6.9).

The accuracy and precision of the proposed method was evaluated by determining sulphiride in four different commercial formulations at three different concentrations (0.02, 0.04 and 0.06 $\mu\text{g mL}^{-1}$) in triplicate. The results are summarized in table 3.6.5. The percent recoveries were found to be ranged from 93.34% to 99.50%. The RSD was acceptable which indicates better reproducibility of the proposed method.

The validity of the proposed method in dosage form of four different brands of capsules was evaluated by performing recoveries experiments through standard addition method. Known amount of standard sulphiride solution (0.02, 0.04 and 0.06 $\mu\text{g mL}^{-1}$) was added to four different kinds of pre-analyzed dosage forms and then determined by the recommended procedure. The percent recoveries were in the range of 96.76% to 102.74% (table 3.6.6) with low relative standard deviation. This indicates high accuracy of the developed method for the determination of sulphiride in pharmaceutical formulations.

The proposed method has been applied for the determination of sulphiride in pharmaceutical formulations and the results of the proposed method were compared statistically with those of the literature HPLC methods [1, 2]. There was no difference in precision between the proposed and literature methods (table 3.6.10).

Good sensitivity, high precision and accuracy attained by the proposed method recommended the applicability to the quantification of sulphiride in urine and plasma samples. A proper volume of urine and plasma was spiked with standard drug solution. In case of urine the percent recovery was found to be in the range of 95.67% to 101.672% while in case of plasma, it was found 98.33% to 99.75% (table 3.6.8 and 3.6.9). The

method can be used for determination of sulpiride concentration in biological samples in clinical analysis.

References

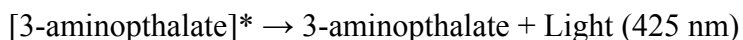
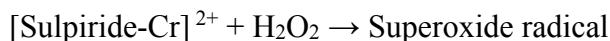
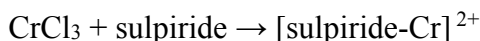
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3.7 DETERMINATION OF SULPIRIDE IN PHARMACEUTICAL PREPARATIONS AND BIOLOGICAL FLUIDS USING Cr (III) ENHANCED CHEMILUMINESCENCE METHOD

3.7.1 Method development strategy for the determination of sulpiride by CL method

It has been reported that some transition metal chelates are capable of catalyzing the formation of hydroxyl radical in the presence of H₂O₂ through a modified Fenton reaction [1-3]. The oxidation reactions of luminol by H₂O₂ produce CL light at 425 nm under alkaline conditions. The initial CL signal of the Cr (III) catalyzed luminol-H₂O₂ reaction was measured in the presence and absence of sulpiride keeping Cr (III) ion concentration constant. As shown in figure 3.7.1, Cr (III)-sulpiride complex produced a CL signal that was enhanced by over 4-four times that of Cr (III) present as a free ion in the luminol-H₂O₂ system. These findings indicate that the enhanced CL signal is due to a chelating ligand complexation reaction.

Cr (III) ion on reaction with sulpiride form a chelate that might serves as a center for production of superoxide radical from the decomposition of H₂O₂ by its strong catalytic activity because homogeneous transition metal complexes contribute to the rapid decomposition of H₂O₂ [4]. Based on the above description, the following mechanism is suggested.



3.7.2 Preliminary investigation of the possibility of Cr (III) enhanced CL method

Preliminary studies were conducted to investigate the possible enhancement of the CL intensities of luminol-H₂O₂ system by Cr (III). Initially high concentration of sulpiride (4.0 µgmL⁻¹), Cr (III) (1.0×10^{-3} molL⁻¹), sodium hydroxide (0.1 M), luminol solution (1.0×10^{-3} molL⁻¹), and H₂O₂ (2.0×10^{-3} molL⁻¹) were injected in the reaction cell and CL intensity was measured. Further studies were focused on optimization of various parameters leading to the possible enhancement of CL intensity of the drug and are given below.

3.7.3 Optimization studies for the determination of sulpiride using Cr (III) enhanced CL method

Instruments

A Hitachi F-4500 spectrofluorometer was used to detect and record the CL intensity produced by the luminol-H₂O₂-Cr (III) system during each process. For measurement of the CL, the light source of the spectrofluorometer was switched off. The slit width of the emission monochromators was 5 nm. A UV-1800 Shimadzu UV-Vis spectrophotometer was used for absorption spectra.

Reagents

Analytical grade purity reagents were used. Luminol (5-Amino-2, 3-dihydro-1, 4-phthalazinedione) (Sigma-Aldrich) H₂O₂, CrCl₃·6H₂O and sodium hydroxide (Junsei Chemical Co. Ltd. Japan) were used in this study. Standard reference sulpiride was provided by Hang Zhou Pharma and Chem. Co., China. Commercial formulations of sulpiride (sulpiride-50, 50 mg caps manufactured by Combitic Global D-2 industrial area Sonepat-131001 (Hr) India, and Sulpiride 50 mg capsules manufactured by ARBRO Pharmaceuticals Ltd; India, were purchased locally.

Solution preparation

Luminol stock solution (4.0×10^{-3} M) was prepared by dissolving 0.035 g of luminol in 4.5 mL of 0.1 M NaOH and diluted to 50 mL with deionized (DI) water and was stored at 4 °C. Hydrogen peroxide stock solution (4.0 M) was prepared by diluting 10.2 mL of 30% H₂O₂ to 25 mL with DI water. CrCl₃·6H₂O stock solution (10×10^{-3} M) was prepared by dissolving 0.02665 g of CrCl₃·6H₂O into 100 mL DI water. NaOH (1.0 M) solution was prepared by dissolving 2.0 g in 50 mL DI water. Working solutions were prepared daily fresh from the stock solution by proper dilution with DI water.

Preparation of Standard Solution

Standard stock solution of sulphuride ($100 \mu\text{g mL}^{-1}$) was prepared by dissolving 0.005 g of sulphuride standard in 5.0 mL of distilled ethanol with vigorous shaking, after which it was diluted to 50 mL with DI water. Working sample solutions of $20 \mu\text{g mL}^{-1}$ and $10 \mu\text{g mL}^{-1}$ were prepared daily by diluting an appropriate amount of the stock solution with DI water.

Preparation of sample solution ($100 \mu\text{g mL}^{-1}$)

Contents of five capsules of each sample containing 50 mg of active ingredients were weighed to get the average weight of one capsule. Amount of the powdered drug equal to 0.005 g was then dissolved in 5.0 mL ethanol, filtered and made the volume up to 50 mL with DI water. Working sample solutions of $20 \mu\text{g mL}^{-1}$ and $10 \mu\text{g mL}^{-1}$ were prepared daily by diluting an appropriate amount of the sample solution with DI water.

Procedure

Analytical procedures of the CL involve the addition of the following quantities of solutions to the reaction cell. An appropriate volume of stock solution of sulphuride was added to give final concentrations in the range of $0.068\text{--}4.0 \mu\text{g mL}^{-1}$, 0.5 mL of CrCl₃ (4.0×10^{-5} molL⁻¹) solution, 0.5 mL NaOH (6.0×10^{-3} molL⁻¹) solution, and 0.5 mL luminol (2.4×10^{-3} molL⁻¹) solution. The solutions added to the reaction cell were let to mix for 10 s prior to the injection of 0.5 mL (3.0×10^{-2} molL⁻¹) H₂O₂ solution. The reaction

chamber was closed immediately after placing the cell inside the chamber. The CL spectrum was recorded and the CL intensity ΔI was calculated by $\Delta I = I_s - I_o$, where I_s and I_o are the CL signal in the presence and absence of sulpiride, respectively. CL emission spectra of the luminol- H_2O_2 system catalyzed by Cr (III) in the absence and presence of sulpiride were scanned in the range of 350–600 nm and are presented in figure 3.7.1.

For optimizing the concentration of NaOH, aliquots of 0.5 mL ($4.0 \mu\text{g mL}^{-1}$) of sulpiride was injected in the reaction cell. Then 0.5 mL of $1.0 \times 10^{-3} \text{ mol L}^{-1}$ Cr (III), 0.5 mL of NaOH in the concentration range of $3.0\text{--}10 \times 10^{-3} \text{ mol L}^{-1}$, 0.5 mL of $1.0 \times 10^{-3} \text{ mol L}^{-1}$ luminol were injected. The solutions added to the reaction cell were allowed to mix for 10 s prior to the injection of 0.5 mL ($1.0 \times 10^{-2} \text{ mol L}^{-1}$) H_2O_2 solution and the CL intensity was measured at 425 nm (figure 3.7.2).

For optimization of concentration of H_2O_2 , only the concentration of H_2O_2 was varied in the range of $1.0\text{--}8.0 \times 10^{-2} \text{ mol L}^{-1}$, keeping rest of the procedure the same. CL intensity was measured and the results are shown in figure 3.7.3.

Luminol concentration was optimized in the range of $1.2\text{--}4.0 \times 10^{-3} \text{ mol L}^{-1}$ (figure 3.7.4).

For optimization of concentration of Cr^{3+} , only the concentration of Cr^{3+} was varied in the range of $1.0\text{--}10 \times 10^{-5} \text{ mol L}^{-1}$ (figure 3.7.5).

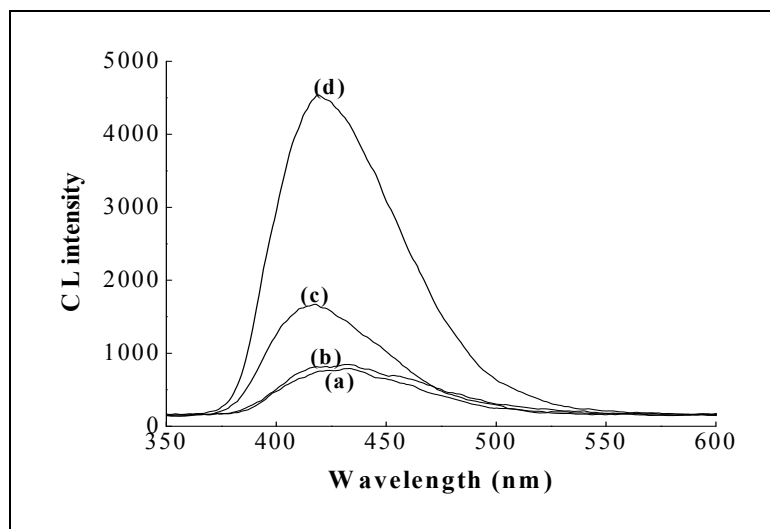


Figure 3.7.1 CL emission spectra (a) luminol-H₂O₂; (b) luminol-H₂O₂-sulpiride; (c) luminol-H₂O₂-Cr (III); (d) luminol-H₂O₂-Cr (III)-sulpiride.

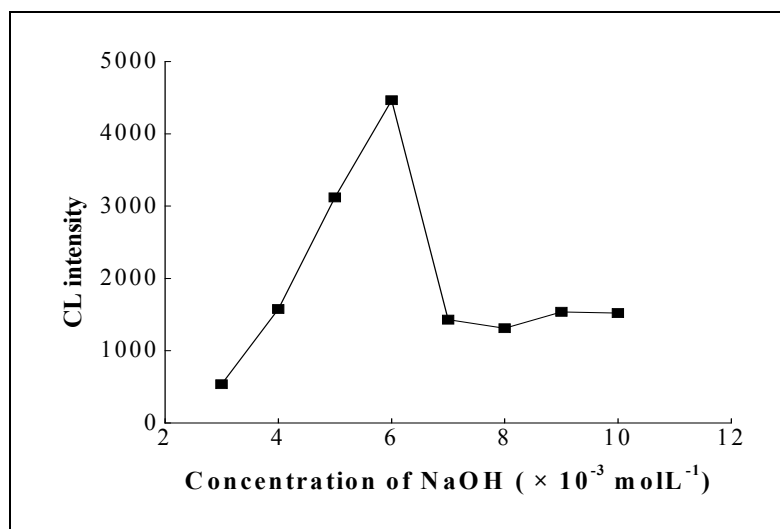


Figure 3.7.2 Effect of concentration of NaOH on the CL determination of sulpiride

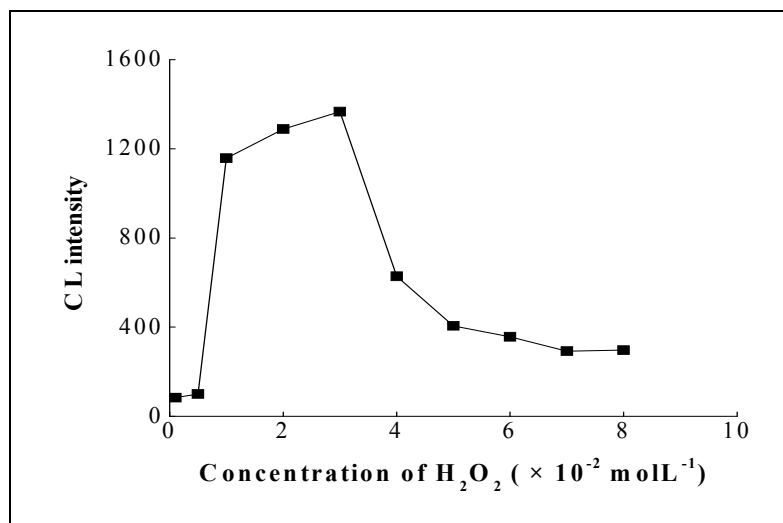


Figure 3.7.3 Effect of concentration of H_2O_2 on the CL intensity of sulpiride

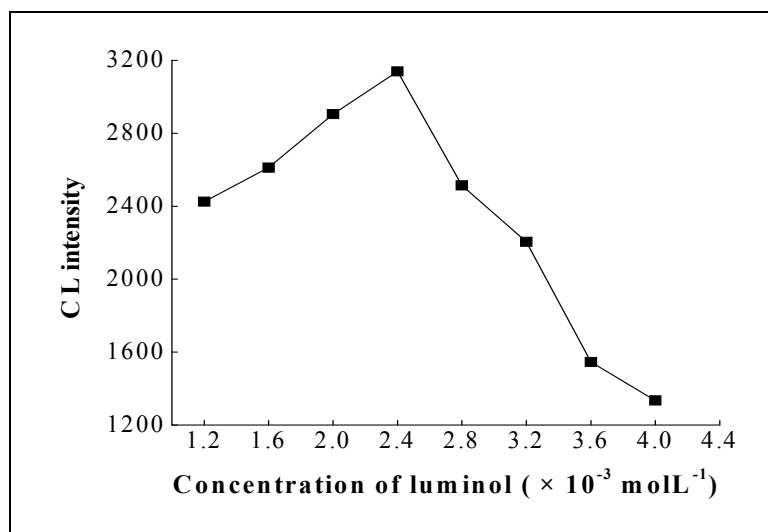


Figure 3.7.4 Effect of concentration of luminol on the CL intensity of sulpiride

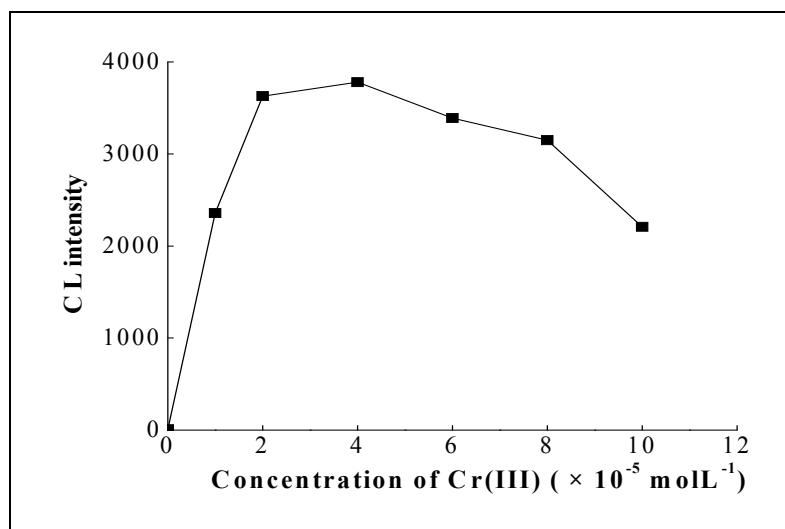


Figure 3.7.5 Effect of concentration of Cr (III) on the CL intensity of sulpiride

3.7.4 Effect of concentration of sulpiride on CL intensity

Instrument, reagents and solutions were the same as mentioned before.

Procedure

Analytical procedures of the CL consisted addition of the following quantities of the solutions to the reaction cell. An appropriate volume of stock solution of sulpiride ($0.068\text{--}4.0 \mu\text{g mL}^{-1}$), 0.5 mL of CrCl_3 ($4.0 \times 10^{-5} \text{ mol L}^{-1}$) solution, 0.5 mL NaOH ($6.0 \times 10^{-3} \text{ mol L}^{-1}$) solution, and 0.5 mL luminol ($2.4 \times 10^{-3} \text{ mol L}^{-1}$) solution. The solutions added to the reaction cell were mix for 10 s in the cell compartment prior to the injection of 0.5 mL ($3.0 \times 10^{-2} \text{ mol L}^{-1}$) H_2O_2 solution. The computer program was started immediately to record the CL signal. The CL intensity ΔI was calculated by $\Delta I = I_s - I_0$, where I_s and I_0 are the CL signal in the presence and absence of sulpiride, respectively (table 3.7.1 and figure 3.7.6).

Table 3.7.1 Effect of concentration of sulpiride on CL intensity

Concentration ($\mu\text{g mL}^{-1}$)	CL Intensity
0.068	463
1.068	1010
2.068	1632
3.068	2345
4.068	3023

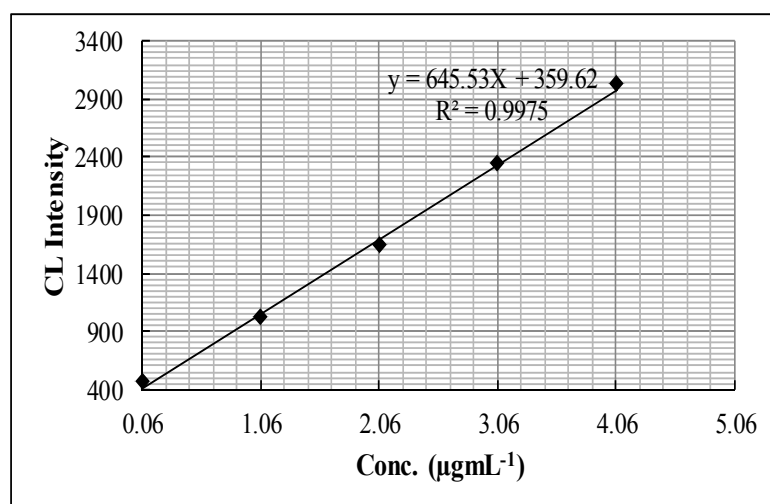


Figure 3.7.6 Effect of concentration of sulpiride on CL intensity

Determining LOD, LOQ and other analytical characteristics of the proposed CL method for determination of sulpiride

Seven replicate solution of lowest measurable concentration of sulpiride ($0.068 \mu\text{g mL}^{-1}$) were prepared and evaluated by the proposed method pursuing the same procedure as stated before. LOD, LOQ and other analytical characteristics were calculated (table 3.7.2).

Table 3.7.2 Analytical characteristics for the CL determination of sulpiride

Parameter	Value
λ_{em} (nm)	425
Linear range ($\mu\text{g mL}^{-1}$)	0.068–4.0
Limit of detection 3S/b ($\mu\text{g mL}^{-1}$)	8.50×10^{-6}
Limit of quantification 10S/b ($\mu\text{g mL}^{-1}$)	2.83×10^{-5}
Regression equation (y)	$Y = 654.04X + 350.9$
Slope (b)	654.04
Intercept (a)	350.9
Correlation coefficient (r^2)	0.996
Standard deviation ($\mu\text{g mL}^{-1}$)	1.85×10^{-3}
Relative standard deviation (%)	2.70

3.7.5 Investigation of interferences on determination of sulpiride by the proposed method

Procedure

The selectivity of the proposed method for the analysis of sulpiride containing pharmaceutical preparation was evaluated by studying the interferences effect of metal ions and common excipient. For this purpose a known concentration of standard sulpiride ($0.068 \mu\text{g mL}^{-1}$) one directly and the other having different concentrations of metal ions and excipients were analyzed by the same procedure. The analysis were performed in triplicates and mean CL intensity was calculated. The results are given in table 3.7.3.

Table 3.7.3 Percent recoveries of sulpiride ($0.068 \mu\text{g mL}^{-1}$) in the presence of interfering substances

Interfering substances	Amount added ($\mu\text{g mL}^{-1}$)	Drug : Interfering substances	% Recovery $\pm$ RSD
Co^{2+}	0.136	1:02	99.02 ± 3.24
	0.272	1:04	100.70 ± 4.24
	0.408	1:06	98.40 ± 3.33
Ni^{2+}	0.136	1:02	96.80 ± 2.22
	0.272	1:04	97.40 ± 2.41
	0.408	1:06	97.30 ± 2.70
Cu^{2+}	0.136	1:02	99.80 ± 3.54
	0.272	1:04	98.40 ± 2.76
	0.408	1:06	97.20 ± 2.89
K^{+}	0.272	1:04	100.20 ± 1.02
	0.408	1:06	97.60 ± 2.68
	0.680	1:10	99.80 ± 1.74
Fe^{3+}	0.272	1:04	97.80 ± 3.71
	0.408	1:06	99.40 ± 2.57
	0.680	1:10	101.40 ± 3.84
Glucose	3.40	1:50	98.80 ± 2.00
	6.80	1:100	97.40 ± 3.12
	34.0	1:500	99.40 ± 2.25
Starch	3.40	1:50	98.40 ± 2.24
	6.80	1:100	99.50 ± 3.11
	34.0	1:500	98.80 ± 3.43
Lactose	3.40	1:50	97.80 ± 3.26
	6.80	1:100	98.50 ± 2.17
	34.0	1:500	99.00 ± 3.35
Fructose	3.40	1:50	99.70 ± 2.87
	6.80	1:100	97.40 ± 3.19
	34.0	1:500	99.90 ± 4.41

3.7.6 Application of the investigated method for the analysis of sulpiride in various pharmaceutical brands

A. Determination of precision and accuracy of the investigated method

Instrument, reagents and standard solution used were the same as mentioned before.

Procedure

The precision and accuracy of the proposed method was evaluated by determining sulpiride in pharmaceuticals preparations using two different brands (each containing 50 mg of the active ingredients of sulpiride) in three different concentrations within the calibration curve range in triplicate. Calibration curve was constructed using the same procedure as mentioned before and known concentrations (0.068, 1.068 and 2.068 $\mu\text{g mL}^{-1}$) of these sample solution were then analyzed by the proposed method. The results are given in table 3.7.4.

Table 3.7.4 Evaluation of accuracy and precision of the proposed method for the determination of sulpiride

Pharmaceutical preparations	($\mu\text{g mL}^{-1}$) taken	($\mu\text{g mL}^{-1}$) found	%Recovery $\pm$ RSD
Sulphiride-50, 50 mg capsule, Combitic Global (India)	0.068	0.069	102.10 $\pm$ 3.43
	1.068	1.065	99.70 $\pm$ 0.73
	2.068	2.027	98.02 $\pm$ 0.83
Sulpiride Capsules 50 mg ARBRO Pharmaceuticals Ltd. New Delhi (India)	0.068	0.068	100.00 $\pm$ 5.45
	1.068	1.049	98.30 $\pm$ 1.77
	2.068	2.049	99.08 $\pm$ 0.63

B. Percent recovery of sulpiride from commercial formulations (Standard addition method)

Instrument, reagents, standard solution and sample solutions used were the same as mentioned before.

Procedure

For determination of percent recovery of sulpiride from pharmaceutical preparations, 0.068, 1.068 and 2.068 $\mu\text{g mL}^{-1}$ standard sulpiride solution was mixed with pre-analyzed commercial sample of sulpiride (0.068 $\mu\text{g mL}^{-1}$). The CL intensity of the resulting solution was measured at 425 nm. The total amount of sulpiride in each fortified sample was calculated from calibration curve. Each experiment was performed in triplicates and the mean percent recovery was calculated using the following formula. The results are given in table 3.7.5.

$$\% \text{ Recovery} = \frac{\mu\text{g mL}^{-1} \text{ found in the fortified sample} - \mu\text{g mL}^{-1} \text{ present in the sample}}{\mu\text{g mL}^{-1} \text{ of standard added}} \times 100$$

Table 3.7.5 Evaluation of percent recovery of sulpiride from commercial formulations by the proposed method

Pharmaceutical preparations	Sample ($\mu\text{g mL}^{-1}$)	($\mu\text{g mL}^{-1}$) added	($\mu\text{g mL}^{-1}$) found	%Recovery $\pm$ RSD
Sulphiride-50, 50 mg capsule, Combitic Global	0.068	0.068	0.067	99.40 $\pm$ 2.59
		1.068	1.070	100.20 $\pm$ 1.43
		2.068	2.068	100.00 $\pm$ 0.32
Sulpiride Capsules 50 mg ARBRO Pharmaceuticals Ltd. New Delhi	0.068	0.068	0.066	98.10 $\pm$ 4.67
		1.068	1.067	99.90 $\pm$ 1.05
		2.068	2.064	99.80 $\pm$ 0.47

C. Determination of active ingredients in the commercial formulations

Instrument, reagents, standard solution and sample solutions used were the same as mentioned before.

Procedure

Solutions of the commercial formulation ($100 \mu\text{g mL}^{-1}$) were prepared by the same way as mentioned before. Known volume of this solution was then analyzed as and the nominal content of the capsule was calculated from the calibration plot. Triplicate analyses were performed in each case. The results are given in table 3.7.6.

Table 3.7.6 Determination of active ingredients of sulpiride in the commercial formulations

Brand name	Active ingredient (mg/capsule)	
	Label value	Found value $\pm$ %RSD
Sulphiride-50, 50 mg capsule, Combitic Global (India)	50	49.90 ± 0.40
Sulpiride Capsules 50 mg ARBRO Pharmaceuticals Ltd. New Delhi (India)	50	49.50 ± 1.01

3.7.7 Validation of the proposed method in spiked human plasma

Procedure

A 1.0 mL plasma sample was placed in a centrifuge tube and deproteinized with 5.0 mL of 20% trichloroacetic acid. The mixture was then centrifuged for 15 min at 3500 rpm. The supernatant was dissolved in DI water and $100 \mu\text{g mL}^{-1}$ of sulpiride solution was added into the protein free plasma sample and diluted to 50 mL with DI water. To make the concentration of sulpiride within the linear range, an appropriate volume of this solution was diluted with DI water and analyzed as described above. The results are given in table 3.7.7 and table 3.7.8.

Table 3.7.7 Determination of sulpiride in spiked plasma by the proposed method

Sample	($\mu\text{g mL}^{-1}$) taken	($\mu\text{g mL}^{-1}$) found	% Recovery $\pm$ RSD
Plasma	0.068	0.067	98.50 ± 5.41
	1.068	1.045	97.80 ± 0.73
	2.068	2.047	99.00 ± 0.74

Table 3.7.8 Recovery of sulpiride in spiked plasma samples by the standard addition method

Sample	($\mu\text{g mL}^{-1}$) added	($\mu\text{g mL}^{-1}$) found	% Recovery $\pm$ RSD
Plasma	0.068	0.0689	101.40 ± 2.38
	1.068	1.0455	97.90 ± 2.24
	2.068	2.0479	99.03 ± 1.29

3.7.8 Results and discussion

A fast and sensitive CL method for sulpiride determination in pharmaceutical formulations and biological samples has been developed based on the sensitizing effect of sulpiride on the CL of the luminol- H_2O_2 -Cr(III) system. The CL intensities of the luminol- H_2O_2 system catalyzed by Cr (III) in the absence and presence of sulpiride were recorded and are presented in figure 3.7.1. As shown in figure 3.7.1 a, the luminol- H_2O_2 system produced very weak CL intensity and the intensity of the system did not increase in response to the addition of sulpiride (figure 3.7.1b). While the CL intensity was only slightly enhanced in response to the addition of Cr (III) to the luminol- H_2O_2 system, it was greatly enhanced by the addition of sulpiride (figure 3.7.1 c and d). In addition, the degree of CL intensity enhancement was proportional to the concentration of sulpiride in a sample solution. Based on these findings, sulpiride was sensitively determined by the CL method.

To establish the most favorable conditions for luminol- H_2O_2 -Cr (III)-sulpiride system, all the parameters affecting the reaction like concentration of NaOH, H_2O_2 , luminol and Cr (III) were carefully studied and optimized.

The effect of sodium hydroxide concentration was investigated in the range of $3.0\text{--}10 \times 10^{-3} \text{ molL}^{-1}$. The CL intensity increases as the concentration increased up to $6.0 \times 10^{-3} \text{ molL}^{-1}$ beyond which the CL intensity starts decreasing (figure 3.7.2). Therefore, the optimum concentration of NaOH was taken to be $6.0 \times 10^{-3} \text{ molL}^{-1}$ for further experiments.

H₂O₂ acts as a strong oxidizing agent in the luminol CL reaction. The effects of H₂O₂ concentration were investigated in the range of $1.0\text{--}8.0 \times 10^{-2} \text{ molL}^{-1}$. The CL intensity increases as the concentration of H₂O₂ increased to $3.0 \times 10^{-2} \text{ molL}^{-1}$, beyond which the intensity decreased (figure 3.7.3).

The effects of the luminol concentration were investigated in the range of $1.2\text{--}4.0 \times 10^{-3} \text{ molL}^{-1}$. The CL intensity of the system increases as the concentration of luminol increased to $2.4 \times 10^{-3} \text{ molL}^{-1}$, after which started to decrease (figure 3.7.4). Too much high concentration of luminol decreases the CL intensity because luminol forms a bidentate chelate that could bind with chromium ion and decrease the concentration of 3-aminophthalate ions. Thus, $2.4 \times 10^{-3} \text{ molL}^{-1}$ was selected as the optimum concentration for further study.

Transition metal ions act as catalysts in the luminol-H₂O₂ system. The effects of five different transition metal ions i.e. Cu (II), Co (II), Ni (II), Fe (II), and Cr (III), were investigated in this study. Cr (III) ion exhibited the strongest CL intensity. The effects of Cr (III) concentration were examined in the range of $1.0\text{--}10 \times 10^{-5} \text{ molL}^{-1}$. The CL intensity increases as the Cr (III) concentration increased up to $4.0 \times 10^{-5} \text{ molL}^{-1}$, after which the intensity decreased (figure 3.7.5). Accordingly, $4.0 \times 10^{-5} \text{ molL}^{-1}$ Cr (III) was selected for further experiments

Analytical Characteristics

Under the optimum experimental conditions, a linear relationship between the concentration of sulphide and CL intensity was observed in the range of $0.068\text{--}4.0 \mu\text{g mL}^{-1}$, with a good correlation coefficient of 0.996 (figure 3.7.6). LOD as defined by IUPAC is given by $C_{\text{LOD}} = 3 S_b/m$, where S_b is the standard deviation of the blank signals

and 'm' is the slope of the calibration graph. In this study, the LOD was found to be $8.50 \times 10^{-6} \mu\text{g mL}^{-1}$. Similarly, LOQ which is defined by IUPAC as $C_{\text{LOQ}} = 10 S_b/m$ was found to be $2.83 \times 10^{-5} \mu\text{g mL}^{-1}$. The linear regression equation, slope, intercept, correlation coefficient and relative standard deviation of the response factor are given in table 3.7.2.

Application

In an actual sample, a chemical species might interfere with the luminol CL reaction. Specifically, the interfering species might enhance or suppress the CL signal. To evaluate the proposed method, the interference effect of several metal ions and common excipients was investigated. To accomplish this, interference studies were carried out by analyzing a set of sample solutions of $0.068 \mu\text{g mL}^{-1}$ sulpiride that were amended with increasing amounts of various substances. The results showed that 6-fold increases in Co^{2+} , Ni^{2+} and Cu^{2+} , 10-fold increases in K^{1+} , and Fe^{3+} and 500-fold increases in glucose, starch, lactose and fructose concentration resulted in no interferences in the determination of sulpiride. The average recoveries obtained ranged from 96.80–101.40% (table 3.7.3).

The precision of the present method was examined by identifying the sulpiride in pharmaceutical preparations and spiked plasma samples using three different concentrations in triplicate within the calibration curve range. The results are summarized in table 3.7.4 for pharmaceutical preparations and table 3.7.7 for spiked plasma samples. The percent recoveries obtained ranged from 98.02–102.10% for pharmaceutical preparations and 97.80–99.0% for spiked plasma samples with good relative standard deviations indicating good reproducibility of the present method.

The validity of the present method was evaluated by standard addition method using two different brands of capsules (Sulpiride-50, 50 mg capsules manufactured by Combitic Global, India, and Sulpiride 50 mg Capsules manufactured by ARBRO Pharmaceuticals Ltd New Delhi, India, as well as spiked plasma samples. The percent recoveries ranged from 98.10–100.20% for pharmaceutical preparations and 97.90–101.40% for spiked plasma samples (table 3.7.5 and 3.7.8). These good recoveries indicate that the proposed method had a high accuracy for determination of sulpiride in pharmaceutical preparations and spiked plasma samples.

The present method was successfully applied to the determination of sulpiride active ingredients in two different types of pharmaceutical preparations. The results of the proposed method were in close agreement with the labeled quantities (table 3.7.6). Due to the high specificity of the present method and absence of interferences with common excipients and transition metal ions, the method was applied for the quantification of sulpiride in biological samples. The results are given in table 3.7.7 and 3.7.8.

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3.8 QUANTITATIVE DETERMINATION OF FLUOXETINE IN PHARMACEUTICALS AND PLASMA SAMPLES USING BROMATOMETRIC METHOD

3.8.1 Method development strategy for the spectrophotometric determination of fluoxetine

The present method deals with the spectrophotometric determination of fluoxetine using bromate-bromide mixture as the oxidimetric reagent. The proposed method is based on the determination of unreacted bromine (*insitu* generated) after bromination reaction of fluoxetine in acidic media with an excess amount of bromate-bromide mixture via electrophilic substitution reaction. The excess bromine was determined by reacting it with a known amount of methyl orange dye. The method is based on bleaching action of bromine on methyl orange due to the oxidative destruction of methyl orange and absorbance was measured at 505 nm. The proposed scheme of reaction is shown in figure 3.8.1.

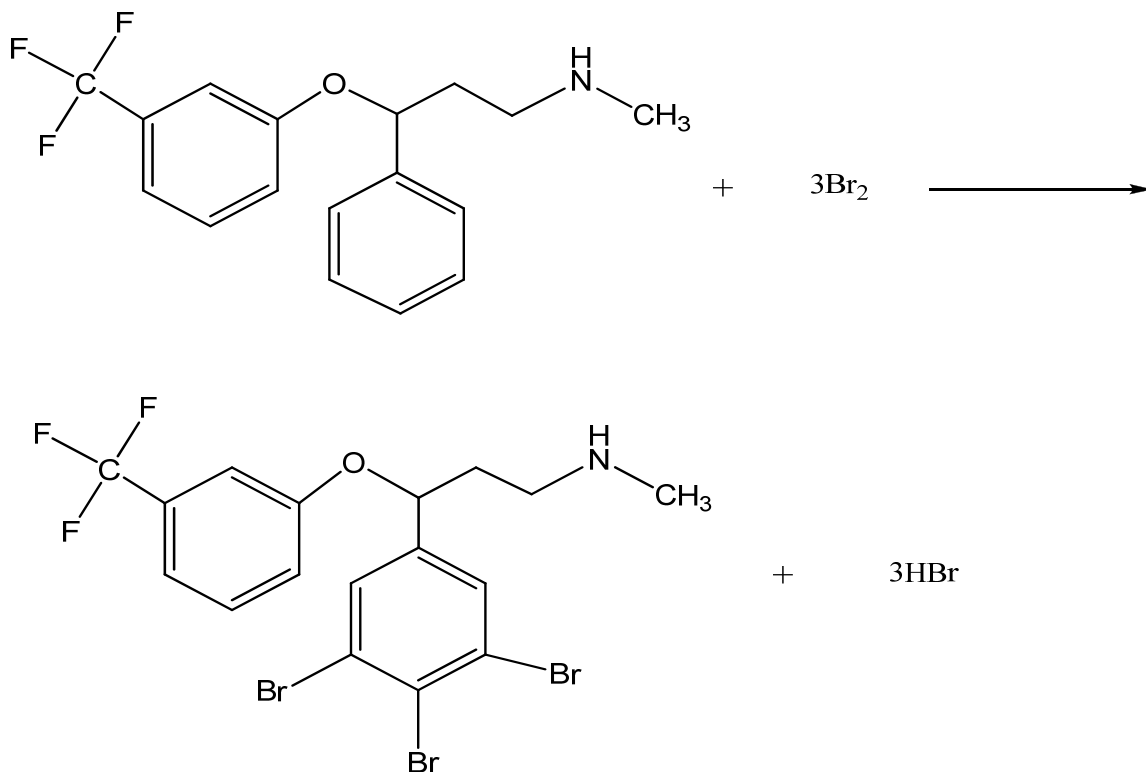


Figure 3.8.1 Proposed reaction scheme for reaction of fluoxetine with bromine

3.8.2 Preliminary investigation of the possibility of bromination reaction of fluoxetine with bromate-bromide mixture for its spectrophotometric determination

Preliminary studies were conducted to investigate the possible reaction of fluoxetine with bromine in the presence of hydrochloric acid. Initially high concentration of fluoxetine solution (100 µg mL⁻¹), KBrO₃ (2.0 mM), KBr (2.0 mM) and HCl (5.0 M) were mixed in a 25 mL volumetric flask. The contents of the flasks were mixed thoroughly and let to stand for 10 minutes with occasional shaking. This was followed by the addition of methyl orange solution (500 µg mL⁻¹) and absorbance of the resultant solution was measured. Further studies were focused on optimization of various parameters leading to the complete bromination of fluoxetine and are presented below.

3.8.3 Optimization studies for spectrophotometric determination of fluoxetine using bromate-bromide mixture

After the preliminary reaction, it was evident that fluoxetine could undergo bromination reaction with bromate-bromide mixture and by adding methyl orange produced a red colored product. The colored product could be determined by spectrophotometric method. For quantitative determination of fluoxetine, it was important to optimize various experimental parameters of the method.

Instrument

UV/Vis spectrophotometer Sp-3000 plus Optima (Tokyo, Japan) with 1-cm matched quartz cells was used for absorbance measurements.

Materials and reagents

Reagents used were of analytical grade or of high grade purity. Potassium bromate, potassium bromide, hydrochloric acid, methanol (Merck KGaA 64271 Darmstadt, Germany) and methyl orange (BDH Laboratory Supplies Poole, England) were used in this work. Standard reference fluoxetine was provided by Libra Pharmaceutical Industry pvt. Ltd., Peshawar, Pakistan. Commercial formulations of fluoxetine (Dopex capsules 20 mg, manufactured by Merck (Pvt) Ltd, Quetta, Pakistan), Flux capsules 20 mg (manufactured by Hilton Pharma (pvt) Ltd, Karachi, Pakistan), Depricap capsules 20 mg (manufactured by Pharma Nabi Qasim industries (Pvt) Ltd, Karachi Pakistan were purchased locally.

Solutions preparation

The bromate-bromide mixture (3.0 mM KBrO_3 –30 mM KBr) was prepared by dissolving 0.025 g of KBrO_3 and 0.1785 g of KBr in distilled water and diluting to 50 mL. The solution was diluted to obtain 0.119 mM solutions with respect to KBrO_3 for further use. Methyl orange solution ($300 \mu\text{g mL}^{-1}$) was prepared by dissolving 0.03 g of methyl orange dye and diluted to 100 mL with distilled water. HCl solution (5.0 M) was prepared by diluting 20.8 mL of 36% hydrochloric acid to 50 mL with distilled water.

Standard fluoxetine solution (1000 $\mu\text{g mL}^{-1}$)

1000 $\mu\text{g mL}^{-1}$ stock standard solution of fluoxetine was prepared by dissolving 0.05 g of authentic standard in 0.5 mL methanol and made the volume up to 50 mL with distilled water. Working standard solutions were freshly prepared by diluting proper volume of the stock solution with distilled water.

Sample preparation

Contents of five capsules and tablets containing 20 mg of active ingredient were weighed and average mass of the powder in one capsule was calculated. An accurately weighed amount of the powder equivalent to 0.01 g of fluoxetine was dissolved in 0.5 mL methanol and distilled water was added to it. The solution was filtered to separate any suspended particles and finally the volume of filtrate was adjusted to 100 mL with distilled water. Sample solution (10 $\mu\text{g mL}^{-1}$) was prepared from stock sample solution by dilution of the required volume with distilled water.

Procedure

1.0 mL of Bromate-bromide mixture (0.089 mM with respect to KBrO_3) was taken in a set of 25 mL flasks. Then 1.0 mL of 4.0 M HCl solution and 5.0 $\mu\text{g mL}^{-1}$ fluoxetine standard solution was added to it. The contents of the flasks were mixed thoroughly and allowed to stand for 15 minutes with occasional shaking. After equilibration, 1.0 mL of methyl orange solutions (300 $\mu\text{g mL}^{-1}$) was added to each flask and diluted to the mark with distilled water. For optimization of wavelength, the absorbance of the red colored product was measured from 400 to 600 nm against a reagent blank prepared by the same procedure but without addition of the drug (figure 3.8.2).

For optimization of bromate concentration in the presence of excess of bromide, 1.0 mL of the KBrO_3 -KBr mixture in the concentration range of 0.029–0.179 mM was taken in a series of 25 mL volumetric flasks. Then 1.0 mL of HCl (4.0 M) and 4.0 mL of fluoxetine (100 $\mu\text{g mL}^{-1}$) solution was added to it. The flasks were allowed to stand for 15 minutes and then 1.0 mL of (100 $\mu\text{g mL}^{-1}$) methyl orange solution was added. The contents of the flask were mixed well and made the volume up to the mark with distilled water. The

absorbance of the solution was measured at 505 nm by adjusting zero with blank (figure 3.8.3).

For volume of KBrO_3 (0.089 mM), only volume of the reagent was varied in the range of 0.5–2.5 mL while rest of the procedure was kept the same and absorbance was measured at 505 nm (figure 3.8.4).

For optimization of concentration of HCl, only the HCl concentration was varied in the range of 1.0–6.0 M keeping rest of the procedure the same. Absorbance of the solution was measured and the results are shown of in figure 3.8.5.

For optimization of volume of HCl (4.0 M), only volume of the reagent was varied in the range of 0.5–2.5 mL and rest of the procedure was kept the same. Absorbance was measured at 505 nm and the results are shown in figure 3.8.6.

For optimization of concentration of methyl orange, only the concentration of methyl orange solution was varied in the range of 200–450 $\mu\text{g mL}^{-1}$ keeping rest of the procedure the same. Absorbance was measured and the results are shown in figure 3.8.7.

For optimization of volume of methyl orange (300 $\mu\text{g mL}^{-1}$), only volume of the reagent was varied in the range of 0.5–3.0 mL and rest of the procedure was kept the same. Absorbance was measured at 505 nm and the results are shown in figure 3.8.8.

Stability of the colored product was also investigated by measuring the absorbance up to two hours with 10 minutes interval (figure 3.8.9).

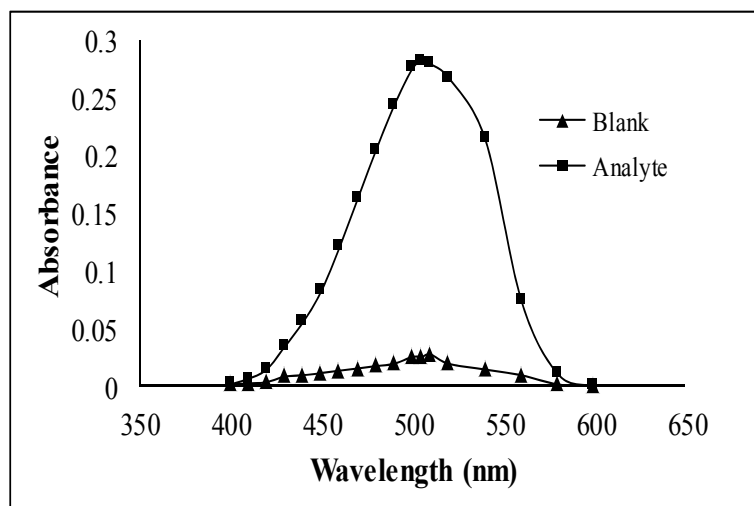


Figure 3.8.2 Wavelength optimization for the spectrophotometric determination of fluoxetine

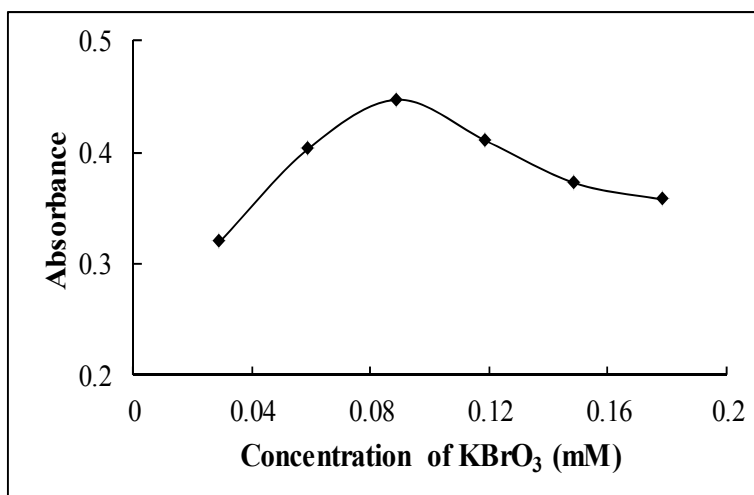


Figure 3.8.3 Effect of KBrO₃ concentration on the spectrophotometric determination of fluoxetine

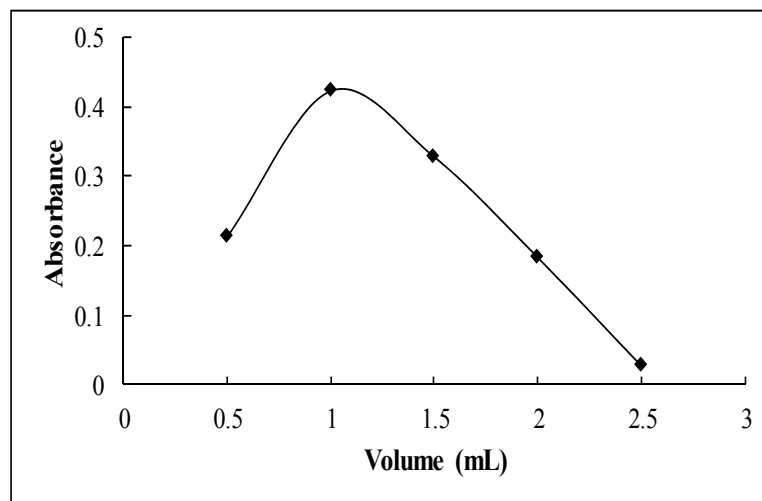


Figure 3.8.4 Effect of volume of KBrO₃ on the spectrophotometric determination of fluoxetine

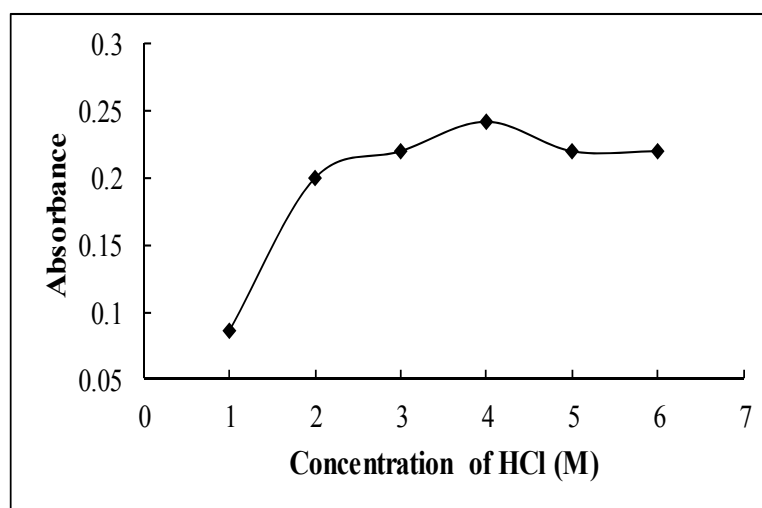


Figure 3.8.5 Effect of HCl concentration on the spectrophotometric determination of fluoxetine

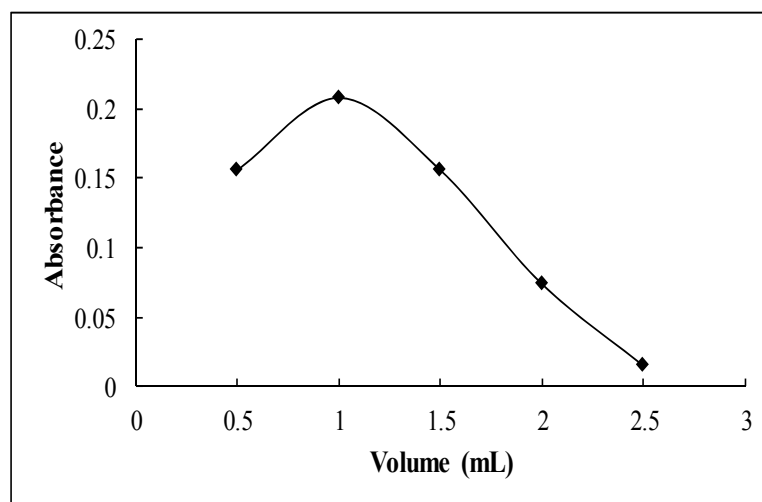


Figure 3.8.6 Effect of volume of HCl on the spectrophotometric determination of fluoxetine

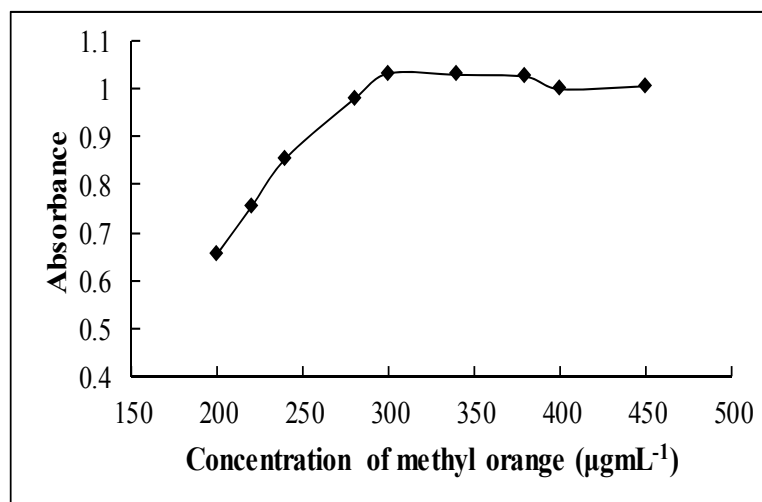


Figure 3.8.7 Effect of concentration of methyl orange on the spectrophotometric determination of fluoxetine

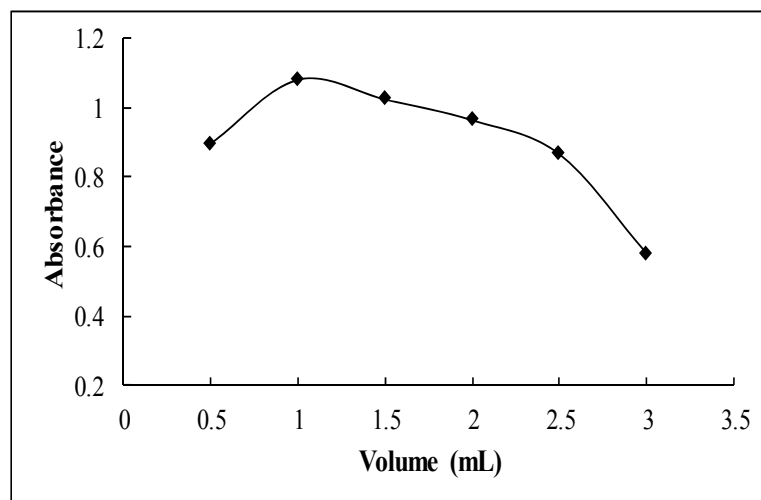


Figure 3.8.8 Effect of volume of methyl orange on the spectrophotometric determination of fluoxetine

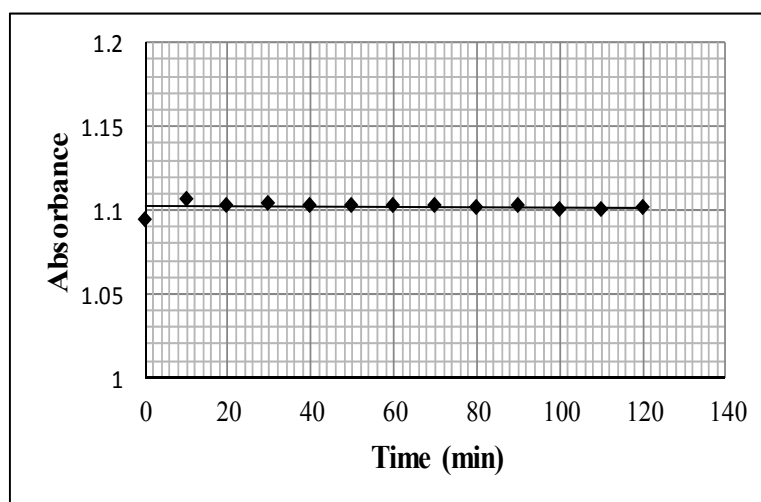


Figure 3.8.9 Effect of time on stability of colored product

3.8.4 Verification of Beer's law and determination of LOD and LOQ

Instrument, reagent and solutions used were the same as mentioned before.

Procedure

Bromate-bromide mixture of 1.0 mL (0.089 mM with respect to KBrO_3) was taken in a set of 25 mL flasks. Then 1.0 mL of 4.0 M HCl solution and fluoxetine standard solution in the range of $0.4\text{--}12\ \mu\text{g mL}^{-1}$ was added to each flask. The contents of the flasks were mixed thoroughly and allowed to stand for 20 minutes. After equilibration, 1.0 mL of methyl orange solutions ($300\ \mu\text{g mL}^{-1}$) was added to and diluted to the mark with distilled water. A blank solution was prepared by the same method without addition of the drug. The absorbance of analyte was measured against a blank at 505 nm (table 3.8.1 and figure 3.8.10). For determination of fluoxetine in pharmaceutical preparations and biological fluids, the calibration plot was constructed for lower concentration of the drug (figure 3.8.11).

Table 3.8.1 Effect of concentration on the absorption behavior of fluoxetine

Concentration($\mu\text{g mL}^{-1}$)	Absorbance
0.0	0.000
0.4	0.044
0.8	0.061
1.2	0.100
1.6	0.150
2.0	0.193
2.4	0.235
2.8	0.294
3.2	0.321
3.6	0.370
4.0	0.403
5.0	0.488
6.0	0.607
7.0	0.677
8.0	0.782
9.0	0.820
10	0.915
11	0.985
12	1.102

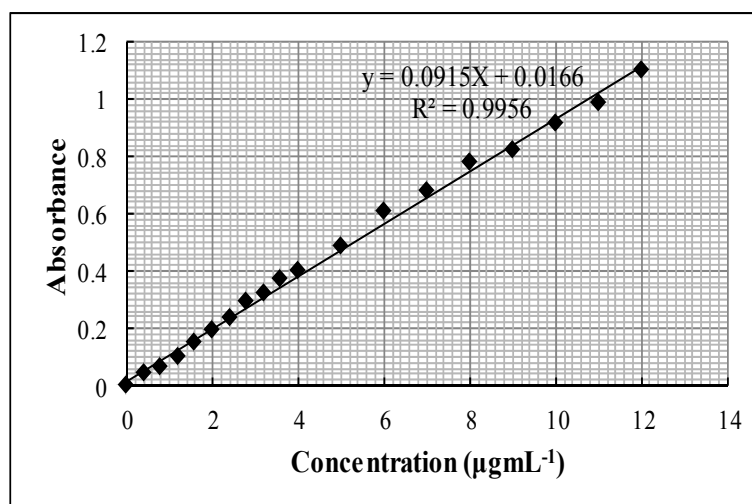


Figure 3.8.10 Effect of concentration on the absorption behavior of fluoxetine

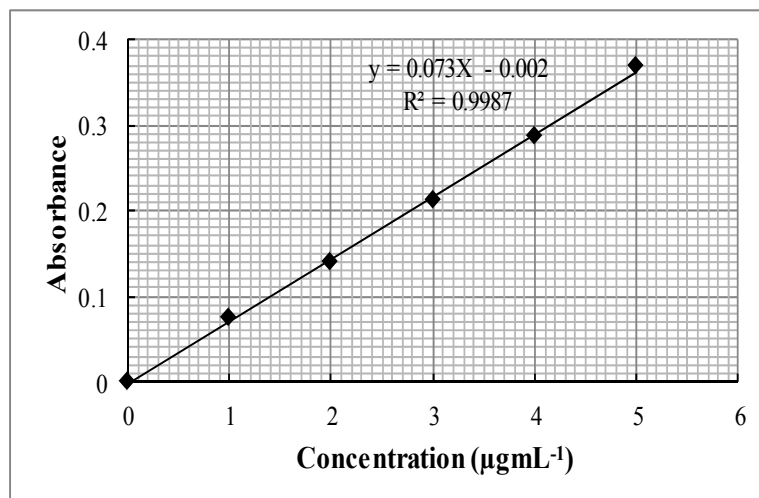


Figure 3.8.11 Calibration curve for spectrophotometric determination of fluoxetine at lower concentration

Standard deviation and relative standard deviation (RSD %) were calculated by analyzing six replicate of analytes having lowest quantifiable concentration. LOD and LOQ were calculated using the following equations.

$$\text{LOD} \quad (\text{For concentration}) = 3 \times S$$

$$\text{LOQ} \quad (\text{For concentration}) = 10 \times S$$

Where S = Standard deviation of the method

All the optical parameters and statistical values calculated for the proposed method are given in table 3.8.2.

Table 3.8.2 Analytical parameters for the spectrophotometric determination of fluoxetine

Parameter	Value
$\lambda_{\max}$ (nm)	505
Beer's law limit ($\mu\text{g mL}^{-1}$)	0.4–12
Molar absorptivity ($\text{L mol}^{-1}\text{cm}^{-1}$)	3.80×10^4
Limit of detection ($\mu\text{g mL}^{-1}$)	0.32
Limit of quantification ($\mu\text{g mL}^{-1}$)	1.0
Regression equation (y)	$Y=0.073X-0.002$
Slope (b)	0.073
Intercept (a)	0.002
Correlation coefficient (r^2)	0.9956
Relative standard deviation (%)	2.04

3.8.5 Effect of common excipients found in commercial formulations on determination of fluoxetine using the proposed method

Instrument, reagent and standard solution were the same as mentioned before.

Procedure

To assess the selectivity of the proposed method, the interferences effects of common excipients like starch, glucose, sucrose, talc, sorbitol, lactose, and magnesium stearate were tested for the determination of fluoxetine in commercial formulations. To a fixed amount of fluoxetine solution, one of the excipients in ratio of 1:2, 1:4 and 1:8 was added. Then the optimized amounts of all the reagents were added and color was developed by the same procedure as mentioned before. Absorbance was measured at 505 nm against a reagent blank. The experiments were performed in triplicate and mean absorbance was calculated (table 3.8.3).

Table 3.8.3 The effect of common excipients on determination of fluoxetine using the proposed method

Excipients	Excipients added ($\mu\text{g mL}^{-1}$)	Drug : Excipients	%Recovery $\pm$ RSD
Sorbitol	2.0	1:2	101.75 $\pm$ 1.76
	4.0	1:4	102.63 $\pm$ 3.05
	8.0	1:8	101.75 $\pm$ 2.00
Glucose	2.0	1:2	100.89 $\pm$ 3.17
	4.0	1:4	98.21 $\pm$ 2.65
	8.0	1:8	101.78 $\pm$ 0.52
Lactose	2.0	1:2	101.73 $\pm$ 2.55
	4.0	1:4	100.00 $\pm$ 3.02
	8.0	1:8	101.73 $\pm$ 1.53
Magnesium Stearate	2.0	1:2	100.00 $\pm$ 3.27
	4.0	1:4	103.77 $\pm$ 2.15
	8.0	1:8	100.94 $\pm$ 0.96
Starch	2.0	1:2	100.97 $\pm$ 0.69
	4.0	1:4	101.94 $\pm$ 2.09
	8.0	1:8	102.91 $\pm$ 1.93
Talc	2.0	1:2	103.84 $\pm$ 2.84
	4.0	1:4	102.88 $\pm$ 0.59
	8.0	1:8	97.11 $\pm$ 1.31
Sucrose	2.0	1:2	101.96 $\pm$ 2.21
	4.0	1:4	99.01 $\pm$ 2.42
	8.0	1:8	100.00 $\pm$ 1.95

3.8.6 Application of the investigated method for the determination of fluoxetine in various pharmaceutical brands

A. Determination of precision and accuracy of the investigated method

Instrument, reagents and standard solution used were the same as mentioned before.

Procedure

The precision and accuracy of the method was checked by determining fluoxetine in pure form and pharmaceutical preparations using four different brands of capsules Dopex, Depricape, Faxetine and Flux (each containing 20 mg of the active ingredient of fluoxetine) at three different concentrations in triplicate with in the Beer's law range. Calibration curve was constructed using the same procedure as mentioned before and known concentrations (1.0, 1.5 and 2.0 $\mu\text{g mL}^{-1}$) of standard solution and (1.0, 2.0 and 3.0 $\mu\text{g mL}^{-1}$) of sample solution were then analyzed by the proposed method. The results are given in table 3.8.4 for the standard and in table 3.8.5 for pharmaceutical preparations.

Table 3.8.4 Evaluation of accuracy and precision of the proposed method using solutions of pure fluoxetine

Amount taken ($\mu\text{g mL}^{-1}$)	Amount found ($\mu\text{g mL}^{-1}$)	%Recovery $\pm$ RSD	Confidence limit
1.0	0.996	99.6 $\pm$ 4.4	99.60 $\pm$ 0.10
1.5	1.50	100.0 $\pm$ 2.0	100.00 $\pm$ 0.07
2.0	1.99	99.5 $\pm$ 4.0	99.50 $\pm$ 0.19
X'=		99.70 %	
$\pm$ SD =		0.264	
t-test =		1.96 (4.303)	

Table 3.8.5 Evaluation of accuracy and precision of the proposed method for fluoxetine determination in pharmaceutical preparations

Pharmaceutical preparations	Amount taken ($\mu\text{g mL}^{-1}$)	Amount found ($\mu\text{g mL}^{-1}$)	%Recovery $\pm$ RSD
Dopex capsules 20 mg	1.0	1.00	100.00 $\pm$ 3.0
	2.0	2.00	100.00 $\pm$ 2.0
	3.0	2.91	97.00 $\pm$ 2.5
Depricape capsules 20 mg	1.0	1.05	101.00 $\pm$ 1.0
	2.0	2.01	100.50 $\pm$ 2.3
	3.0	2.96	98.70 $\pm$ 3.1
Faxetine tablets 20 mg	1.0	0.96	96.70 $\pm$ 2.3
	2.0	1.97	98.50 $\pm$ 2.3
	3.0	3.01	100.40 $\pm$ 2.9
Flux capsules 20mg	1.0	0.99	99.00 $\pm$ 2.5
	2.0	1.98	99.00 $\pm$ 3.0
	3.0	2.89	96.30 $\pm$ 3.7

B. Percent recovery of fluoxetine from commercial formulations (Standard addition method)

Instrument, reagents, standard solution and sample solutions used were the same as mentioned before.

Procedure

For determination of percent recovery of fluoxetine from pharmaceutical preparations, 1.0, 1.5 and 2.0 $\mu\text{g mL}^{-1}$ standard fluoxetine solution was mixed with pre-analyzed commercial sample of fluoxetine (1.0 $\mu\text{g mL}^{-1}$). Color was developed following the same procedure as mentioned before and absorbance was noted. The total amount of fluoxetine in each fortified sample was calculated from calibration curve. Each experiment was performed in triplicates and the mean percent recovery was calculated using the following formula. The results are given in table 3.8.6.

$$\% \text{ Recovery} = \frac{\mu\text{gmL}^{-1} \text{ found in the fortified sample} - \mu\text{gmL}^{-1} \text{ present in the sample}}{\mu\text{gmL}^{-1} \text{ of standard added}} \times 100$$

Table 3.8.6 Fluoxetine percent recovery in commercial formulations (capsules) using the standard addition method

Pharmaceutical preparation	Sample (μgmL^{-1})	Amount added (μgmL^{-1})	Amount found (μgmL^{-1})	RE %	% Recovery $\pm$ RSD
Dopex capsules 20 mg	1.0	1.0	0.97	3.00	97.00 $\pm$ 2.05
		1.5	1.49	0.67	99.30 $\pm$ 1.57
		2.0	2.02	-1.00	101.0 $\pm$ 1.16
Depricape capsules 20 mg	1.0	1.0	0.98	2.00	98.00 $\pm$ 1.02
		1.5	1.46	2.67	97.30 $\pm$ 2.60
		2.0	2.04	-2.00	102.0 $\pm$ 0.49
Faxetine tablets 20 mg	1.0	1.0	0.97	3.00	97.00 $\pm$ 2.37
		1.5	1.48	1.34	98.70 $\pm$ 2.83
		2.0	1.98	0.67	99.30 $\pm$ 2.12
Flux Capsules 20 mg	1.0	1.0	1.03	-3.00	103.0 $\pm$ 2.91
		2.0	1.98	1.00	99.00 $\pm$ 1.76
		3.0	2.99	0.34	99.70 $\pm$ 1.20

C. Determination of active ingredients in the commercial formulations

Instrument, reagent, standard solution and sample solution were the same as mentioned before.

Procedure

Solutions of the commercial formulation ($100 \mu\text{gmL}^{-1}$) were prepared by the same way as mentioned before. Sample solution ($10 \mu\text{gmL}^{-1}$) was prepared from stock sample solution by dilution of the required volume with distilled water. Known volumes of this sample solution were then analyzed by the proposed method in triplicate. The results are given in table 3.8.7.

Table 3.8.7 Determination of active ingredients in the commercial formulations

Brand name	Active ingredient (mg/cap or tab)		t-test value (4.303)
	Labeled value	Found value	
Dopex	20	19.80 ± 1.50	1.65
Depricape	20	20.01 ± 1.80	2.00
Faxetine	20	19.85 ± 0.98	1.88
Flux	20	19.90 ± 1.20	1.26

3.8.7 Validation of the proposed method in spiked human plasma

To check the applicability of the proposed method in different matrices, recovery test was performed on spiked human plasma sample.

Procedure

In a set of 15 mL centrifugation tubes; 3.0 mL aliquots of plasma was taken and 2.5 mL of 100 µg/mL⁻¹ of fluoxetine solution was added to it. It was then deproteinized by mixing with 9.0 mL of acetonitrile and centrifuged for five minutes at a rate of 3000 rpm. The supernatant was transferred to 50 mL volumetric flask and diluted up to the mark with distilled water. To make the concentration of fluoxetine within the linear range, an appropriate volume of this solution was diluted with distilled water. The same procedure was followed for colour development as mentioned before and the content of fluoxetine was calculated using standard calibration plot. Each result is the average of three replicates (table 3.8.8).

Table 3.8.8 Recovery test of fluoxetine from Plasma (standard addition method)

Amount added (µg/mL ⁻¹)	Amount found (µg/mL ⁻¹)	% RE	%Recovery ± RSD
1.0	0.97	3.0	97.0 ± 2.03
2.0	1.98	2.0	99.0 ± 2.76
3.0	2.97	3.0	99.0 ± 3.12

3.8.8 Results and discussion

The present method deals with the spectrophotometric determination of fluoxetine using bromate-bromide mixture as the oxidimetric reagent. The proposed spectrophotometric method is based on the determination of unreacted bromine (insitu generated) after bromination reaction of fluoxetine in acidic media with a measured excess amount of bromate-bromide mixture via electrophilic substitution reaction. The excess bromine was determined by reacting it with a known amount of methyl orange dye. The method is based on bleaching action of bromine on methyl orange due to the oxidative destruction of methyl orange (Figure 3.8.1).

Effect of Reagents concentration

The concentrations and volume of different reagents affecting the bromination reactions were carefully studied and optimized. The effect of concentration of bromate solution in the presence of excess of bromide was investigated in the range of 0.029 mM to 0.179 mM. It was observed that absorbance increases with increase in concentration up to 0.089 mM of bromate (figure 3.8.3). The effect of volume of bromate solution (0.089 mM) was also investigated. Maximum absorbance was obtained when 1.0 mL of 0.089 mM bromate solution was used (figure 3.8.4).

For acidity of solution, different mineral acids like hydrochloric acid, nitric acid, perchloric acid and sulphuric acids were tried. Among the acids, HCl was found to be the suitable medium for bromination of fluoxetine by insitu generated bromine due to accurate and precise results. The effect of hydrochloric acid concentration was studied in the range of 1.0–6.0 M. The absorbance increases with increase in concentration of hydrochloric acid up to 4.0 M after which a slight decrease in absorbance was observed (figure 3.8.5). The effect of volume of HCl was also studied and it was found that 1.0 mL of 4.0 M HCl give maximum absorbance (figure 3.8.6).

The effect of methyl orange concentration was also investigated in the range of 200–450 $\mu\text{g mL}^{-1}$ and absorbance increased with increase in concentration of methyl orange up to 300 $\mu\text{g mL}^{-1}$ after which it remain constant (figure 3.8.7). The effect of volume of methyl

orange was also investigated and it was observed that 1.0 mL of 300 $\mu\text{g mL}^{-1}$ methyl orange produced maximum absorbance (figure 3.8.8).

Stability of the reaction product

The stability of the reaction product was also studied by measuring the absorbance at regular interval up to 120 min (figure 3.8.9). It was observed that no change in absorbance of the colored product was produced. Thus the colored product is stable and will not affect the results of analysis even if the absorbance is measured after two hours of the dilution.

Analytical characteristics

Under the optimum experimental conditions of the proposed method, a linear correlation was found between absorbance and fluoxetine concentration. Beer's law was obeyed in the concentration range of 0.4– 12 $\mu\text{g mL}^{-1}$ with a good correlation of 0.9956 (figure 3.8.10). The LOD and LOQ values were calculated and found to be 0.32 $\mu\text{g mL}^{-1}$ and 1.0 $\mu\text{g mL}^{-1}$ respectively. The linear regression equations, correlation coefficients, intercepts, slope and molar absorptivity are given in table 3.8.2.

Applications

To evaluate the selectivity of the proposed method, the interferences effects of common excipients like starch, glucose, sucrose, talc, sorbitol, lactose and magnesium stearate were examined. To a fixed amount of fluoxetine solution one of the excipients in ratio of 1:2, 1:4 and 1:8 were added and analyzed by the proposed method. No interferences were observed in the analysis of fluoxetine in the presence of these excipients. The percentage recoveries obtained were in the range of 97.11 to 103.84% (table 3.8.3).

The precision of the method was checked by evaluating fluoxetine in pure form and pharmaceutical preparations at three different concentrations level in triplicate with in the Beer's law range. The results are summarized in table 3.8.4 for pure form and table 3.8.5 for dosage form. The percentage recoveries obtained were in the range of 99.50 to

100.0% for pure form and 96.30 to 101.00% for dosage form. The RSD value was found to be satisfactory ($RSD \leq 3.0$) specifying better reproducibility of the proposed method.

Accuracy of the method was evaluated by standard addition method using four different brands of capsules (Dopex, Depricape, Faxetine and Flux each containing 20 mg of fluoxetine) and spiked plasma sample. Percent recoveries were found in the range of 97.00 to 103.00% for pharmaceutical formulations (table 3.8.6) and from 97.0 to 99.0% for spiked plasma samples with a $\%RE \leq 3.0$ (table 3.8.8). Good percent recovery with a small $\%RE$ indicates high accuracy of the proposed method for the quantification of fluoxetine in pharmaceutical preparations and spiked plasma samples.

The proposed method has been successfully applied for the determination of fluoxetine in four different brands of capsules and tablets. The result showed good recoveries and close agreement with the label claims without any interference. The results were also compared statistically using student's t-test for accuracy. The calculated value of 't' was found to be less than the tabulated value for three degrees of freedom (table 3.8.7).

Good sensitivity, high precision and accuracy obtained by the proposed method indicate the applicability for the quantification of fluoxetine in plasma samples. A fixed volume of plasma was spiked with standard drug solution. Percent recovery was found to be in the range of 97.00 to 99.00% (table 3.8.8).

CONCLUSION

The aim of the current research work was to develop fast, simple, sensitive, selective and inexpensive spectrofluorimetric, chemiluminescence and spectrophotometric methods for quantification of selected antipsychotic drugs that are widely available in the market and manufactured by the indigenous units. Five different spectrofluorimetric methods were developed for determination of citalopram and sulpiride, two chemiluminescence methods for citalopram and sulpiride and one spectrophotometric method was developed for the determination of fluoxetine. The developed methods were applied for the quantification of the selected drugs in commercial formulations and biological fluids.

The first spectrofluorimetric method is for quantification of citalopram in commercial formulation and biological fluids and is based on the oxidation of citalopram by Ce (IV) in acidic media to produce fluorescent Ce (III). The fluorescence intensity of Ce (III) was measured at 353 nm after excitation at 252 nm. The proposed method is simple, fast and sensitive. The method was validated for determination of citalopram in bulk, pharmaceutical preparations and biological fluids (plasma and urine) without interferences. As compared to HPLC and GC methods, the proposed method is also useful from an economic point of view as the reagents used are inexpensive and easily available. The proposed method can be recommended for the routine analysis of citalopram in quality control laboratory.

The second method for citalopram is based on the enhancement of the weak fluorescence signal of the Tb (III)-citalopram system by silver nanoparticles. Fluorescence intensities were measured at 555 nm after excitation at 281 nm. The enhancement of the fluorescence signal of Tb³⁺-CIT complex by Ag NPs enable the assay of citalopram with high sensitivity and selectivity. The proposed method was found to have a wider linear range and lower limits of detection and quantification than other reported methods. The method was successfully used for quantification of citalopram in commercial tablet formulations with good recovery and excellent reproducibility. The interferences studies indicate that excipients commonly found in pharmaceutical preparations did not interfere with the analysis.

The third method for citalopram determination based on silver nanoparticles enhanced chemiluminescence. The enhancement of the weak CL signal arising from the reaction between alkaline luminol and H_2O_2 after the addition of citalopram in the presence of silver nanoparticles is utilized. The proposed method is rapid, sensitive and was developed for the determination of citalopram concentrations based on the enhancement of the CL of the luminol- H_2O_2 -AgNPs system by citalopram. The present method was found to have a wider linear range and lower limits of detection and quantification than other reported methods of determining citalopram concentrations. The proposed method was successfully applied to the determination of citalopram in pharmaceutical preparations and in human plasma.

The fourth proposed method is for determination of sulpiride in commercial formulations using Hantzsch condensation reaction. The method is based on the condensation of the primary amino group of alkaline hydrolytic product of sulpiride with acetyl acetone and formaldehyde in acidic medium to form a fluorescent product. The excitation and emission wavelength are 431 nm and 483 nm respectively. Reaction conditions affecting the condensation reaction were optimized and a linear range of $0.1\text{--}3.5\ \mu\text{g mL}^{-1}$ with good correlation coefficient between fluorescent intensity and concentration of sulpiride was found at optimum parameters. The method is simple, precise, accurate, linear, and selective. It also offers advantages of reagent availability, stability and minimum time consumption with high sensitivity. Thus it can be extended for routine analysis of sulpiride in pharmaceutical industries and research laboratories. Compared with the LC/MS technique and HPLC procedure, the developed spectrofluorimetric method is simple, low cost, and easy to operate.

The fifth spectrofluorimetric method is also for determination of sulpiride in pharmaceutical preparations and human plasma. The method is based on the derivatization reaction of 2-cyanoacetamide with sulpiride in the presence of ammonia. The proposed method is simple, sensitive, precise and accurate and has been successfully applied for the determination of sulpiride in pure form and pharmaceutical preparations. The proposed method has also been applied for the analysis of sulpiride in spiked human

plasma. As compared to LC/MS techniques and HPLC method, the developed spectrofluorimetric method is simple, low cost, does not require any pretreatment and tedious extraction procedures and has wide linear range. Therefore the proposed method can be used as an alternative to HPLC method for the routine analysis of sulpiride in industrial and research institutional laboratories.

The sixth proposed method is micellar-enhanced spectrofluorimetric quantification of sulpiride in pure, commercial formulations, human plasma and urine in the presence of sodium dodecyl sulphate surfactant micelle. Experimental parameters like buffer, surfactants type and volume were optimized. A linear relationship was found between sulpiride concentration and fluorescence intensity in the range of 0.02– 7.0 $\mu\text{g mL}^{-1}$ containing 0.2 M SDS with λ_{ex} 292 nm and λ_{em} 343 nm with a correlation coefficient of 0.9987. The proposed method is simple, rapid, sensitive and was applied for the determination of sulpiride in pure form, in pharmaceutical formulations and spiked human plasma and urine samples. As compared to HPLC method, the proposed method is simple, fast and does not involve complicated sample preparation step, and is less time consuming. The method is good alternative to the determination of sulpiride through HPLC methods with good sensitivity.

The seventh proposed method is for the determination of sulpiride in pharmaceutical preparations and biological fluids using a Cr (III) enhanced chemiluminescence method. The method is based on increased CL intensity of a luminol- H_2O_2 system in response to the addition of Cr (III) under alkaline conditions. Various parameters affecting the CL intensity were systematically investigated and optimized for determination of the sulpiride in a sample. The present method produced a wide linear range with low limits of detection and quantification when compared to previously reported CL methods applied for the determination of sulpiride. The developed method was successfully applied for the analysis of active ingredients of sulpiride in pharmaceutical preparations and spiked human plasma. The method can be used for the analysis of sulpiride in general research and industrial fields.

Conclusion

The last proposed method is spectrophotometric determination of fluoxetine in pharmaceuticals and plasma samples using bromatometric method. The method is based on the addition of measured excess amount of bromate-bromide mixture to fluoxetine in hydrochloric acid medium. The residual bromine was determined by reacting with a fixed amount of methyl orange and absorbance was measured at 505 nm. Linear relationship between absorbance and fluoxetine concentration was found and Beer's law was obeyed in the concentration range of 0.4–12 $\mu\text{g mL}^{-1}$. The proposed method is sensitive, accurate and has been validated for the analysis of fluoxetine in pure form and in its capsules and tablets according to the guidelines of ICH. The main advantage of the bromatometric method is the use of nontoxic bromate-bromide instead of hazardous liquid bromine reagent which is easily available and environment friendly. The proposed method does not involve tedious sample preparation, heating or extraction like other non-spectrophotometric methods.

LIST OF PUBLICATIONS

1. **Muhammad Naeem Khan**, Muhammad Rasul Jan, Jasmin Shah and Sang Hak Lee, "A Validated Silver Nanoparticle Enhanced Chemiluminescence Method for the Determination of Citalopram in Pharmaceutical Preparations and Human Plasma," Accepted in *Luminescence* (The Journal of Biological and Chemical Luminescence).
2. Jasmin Shah, Muhammad Rasul Jan, **Muhammad Naeem Khan** and Sultan Shah, "Validated Spectrofluorimetric Method for Determination of Sulpiride in Commercial Formulations using Hantzsch Condensation Reaction," Accepted in *Pakistan Journal of Pharmaceutical Sciences*
3. **Muhammad Naeem Khan**, Jasmin Shah, Muhammad Rasul Jan, Sang Hak Lee, "A Validated Spectrofluorimetric Method for the Determination of Citalopram in Bulk and Pharmaceutical Preparations Based on the Measurement of the Silver Nanoparticles-Enhanced Fluorescence of Citalopram/Terbium Complexes," *Journal of Fluorescence*, (2013) 23: 161–169.
4. Jasmin Shah, M. Rasul Jan, **M. Naeem Khan** and Inayatullah, "Spectrofluorimetric Method for Quantification of Citalopram in Pharmaceutical Preparations and Biological Fluids through Oxidation with Ce (IV)," *JOURNAL OF APPLIED SPECTROSCOPY*, 79(6), 922–928 (2013).
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A Validated Spectrofluorimetric Method for the Determination of Citalopram in Bulk and Pharmaceutical Preparations Based on the Measurement of the Silver Nanoparticles-Enhanced Fluorescence of Citalopram/Terbium Complexes

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Abstract A simple, sensitive, and accurate spectrofluorimetric method was developed for the determination of citalopram in bulk and pharmaceutical preparations. The method is based on the enhancement of the weak fluorescence signal (FL) of the Tb (III)-citalopram system in the presence of silver nanoparticles. Fluorescence intensities were measured at 555 nm after excitation at 281 nm. Prepared silver nanoparticles (AgNPs) were characterized by UV-Visible spectra and transmission electron microscopy (TEM). Various factors affecting the formation of citalopram-Tb (III)-AgNPs complexes were studied and optimized. The fluorescence intensity versus concentration plot was linear over the range 0.02–14 $\mu\text{g mL}^{-1}$, with an excellent correlation coefficient of 0.9978. The limit of detection (LOD) and limit of quantification (LOQ) were found to be $7.15 \times 10^{-6} \mu\text{g mL}^{-1}$ and $2.38 \times 10^{-5} \mu\text{g mL}^{-1}$ respectively. The proposed method was found

to have good reproducibility with a relative standard deviation of 3.66 % ($n=6$). The interference effects of common excipients found in pharmaceutical preparations were studied. The developed method was validated statistically by performing recoveries studies and successfully applied for the assay of citalopram in bulk powder and pharmaceutical preparations. Percent recoveries were found to range from 98.98 % to 100.97 % for bulk powder and from 96.57 % to 101.77 % for pharmaceutical preparations.

Keywords Fluorescence · Citalopram · Colloidal silver nanoparticles · Terbium

Introduction

Depression is a major issue in today's society because of the inherent personal, social, and economic issues, and the treatment of depression usually involves the administration of medications. Tricyclic antidepressants (TCAs) and selective serotonin reuptake inhibitors (SSRIs) are commonly used in psychiatry for the treatment of major depression. The SSRIs are a recently introduced group of antidepressants and differ from the TCAs in terms of chemical structure and mode of action. The SSRIs are similar to the TCAs in terms of clinical ability but have more favorable pharmacological profiles. Furthermore, they are considered safe and well-tolerated [1, 2].

Citalopram, (CIT) 1-(3-dimethylaminopropyl)-1-(4-fluorophenyl)-1, 3-dihydroisobenzofuran-5-carbonitrile [3], is a second generation antidepressant and one of the recently introduced SSRIs. It is used for managing depression, social

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Quantitative Determination of Fluoxetine in Pharmaceuticals and Plasma Samples Using Bromatometric Method

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ABSTRACT

Asensitive and simple spectrophotometric method has been developed for quantitative determination of fluoxetine using bromatometric method. The method is based on the addition of measured excess amount of bromate-bromide mixture to fluoxetine in hydrochloric acid medium. The residual bromine was determined by reacting with a fixed amount of methyl orange and absorbance was measured at 505 nm. The amount of bromine reacted corresponds to the amount of fluoxetine. Linear relationship between absorbance and fluoxetine concentration was found and Beer's law was obeyed in the concentration range of 0.4 - 12 $\mu\text{g}\cdot\text{mL}^{-1}$. The molar absorptivity was found to be $3.8 \times 10^4 \text{ L}\cdot\text{mol}^{-1}\cdot\text{cm}^{-1}$. The limit of detection and limit of quantification was calculated and found to be 0.32 $\mu\text{g}\cdot\text{mL}^{-1}$ and 1.0 $\mu\text{g}\cdot\text{mL}^{-1}$ respectively. The common excipients were investigated for their interferences effect in the assay. The validity of the developed method was checked through recoveries studies and successfully applied to the determination of fluoxetine in bulk powder, pharmaceutical formulations and spiked human plasma samples. The percent recoveries were found to be in the range of 97.0% - 101.0% for pharmaceutical formulations and from 97.0% - 99.0% for spiked human plasma.

Keywords: Fluoxetine; Bromatometric Method; Methyl Orange; Spectrophotometry

1. Introduction

Fluoxetine hydrochloride, ($\pm$)-N-methyl-3-Phenyl-3-[(α , α , trifluoro-*p*-tolyl)] propylamine hydrochloride (Figure 1), is an antidepressant drug used for the handling of unipolar mental depression. Fluoxetine (FLX) is the most widely prescribed selective serotonin reuptake inhibitor antidepressant drug [1]. FXT has been shown to have comparable efficacy to tricyclic antidepressants but with fewer cardiovascular and anticholinergic side effects [2,3]. It is also effective in treatment of the obsessive compulsive disorders [4]. Fluoxetine is extensively metabolized by N-demethylation in liver into its active metabolite norfluoxetine [5]. It is well absorbed after oral administration, and it takes 6 - 8 hours to reach the plasma peak.

Since FXT is a compound of great pharmacological and analytical importance there has been an interest to develop accurate analytical method for the quantification of FXT in biological and pharmaceutical samples. Several instrumental methods developed for the determination of fluoxetine in pharmaceutical formulation include titrimetric [6], nuclear magnetic resonance spectrometric

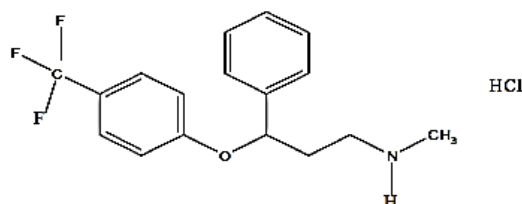


Figure 1. Chemical structure of fluoxetine.

[7], potentiometric [8], and thin-layer chromatographic [9]. Similarly several other methods for fluoxetine determination in biological fluids have been reported in the literature. These methods include liquid chromatography with different detection system [10-14], gas chromatography [15-17], and capillary electrophoresis [18,19]. Unfortunately, these methods are time consuming and require complicated analytical procedures, particularly due to the laborious pretreatment of plasma sample and also not chemically precise.

Comparatively limited number of spectrophotometric methods has been described in the literature for determination of fluoxetine in pharmaceutical formulations and biological samples. These methods require lengthy pro-

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SPECTROFLUORIMETRIC METHOD FOR QUANTIFICATION OF CITALOPRAM IN PHARMACEUTICAL PREPARATIONS AND BIOLOGICAL FLUIDS THROUGH OXIDATION WITH Ce(IV)

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A sensitive and simple spectrofluorimetric method for the quantification of citalopram in a pure form, in pharmaceutical preparations, and in human blood plasma and urine has been described. The proposed method was based on the oxidation of citalopram by Ce(IV) in acidic media to produce fluorescent Ce(III), and on the subsequent measurement of its fluorescence intensity at 353 nm after excitation at 252 nm. All variables affecting the oxidation of citalopram such as Ce(IV) concentration, the type and concentration of acid, temperature and heating time were studied and optimized. Under the optimized experimental conditions the linear range of concentration versus fluorescence intensity was found to lie between 0.02 and 1.4 g/ml. Limits of detection and quantification were determined to be $6.9 \cdot 10^{-3}$ g/ml and $2.3 \cdot 10^{-2}$ g/ml respectively. Effects of excipients commonly used in the quantification of citalopram have been studied and no interferences were found. The proposed method was successfully applied to determine citalopram in pure form, in pharmaceutical preparations, and in biological fluids. Good recoveries in the ranges of 95.31–101.67%, 88.50–96.67%, and 90.0–96.67% were obtained for the pharmaceutical preparations (tablets), blood plasma, and human urine respectively.

Keywords: citalopram, spectrofluorimetry, cerium(IV), urine, blood plasma.

Introduction. Depression is a major issue in today's society because of the inherent personal, social, and economic repercussions it causes. The treatment of depression usually involves the administration of medications. Selective serotonin reuptake inhibitors (SSRIs) such as citalopram, paroxetine, fluvoxamine, fluoxetine, and sertraline have gained huge popularity in this treatment and are the most frequently prescribed drugs worldwide.

One of them, citalopram (1-[3-(dimethylamino)propyl]-1-(4-fluorophenyl)-1,3-dihydroisobenzofuran-5-carbonitrile), is a chemically bicyclic phthalate. It has been used to treat depression, panic disorder, social anxiety disorder, and obsessive-compulsive disorder [1–3]. It offers therapeutic efficacy similar to that of tricyclic antidepressants [4] yet a more favorable tolerability profile. Citalopram is sold as a racemic mixture, consisting of 50% *R*-(–)-citalopram and 50% *S*-(+)-citalopram. The *S*-(+)-enantiomer has the desired antidepressant effect [5], so it is now available in the market under the generic name of escitalopram. It has been shown that the *R*-enantiomer present in citalopram counteracts the activity of escitalopram. Citalopram and escitalopram have different pharmacological and clinical effects [6]. Recommended daily doses for citalopram range from 20 to 40 mg; the corresponding drug concentration was detected between 30 and 130 ng/ml [7]. Maximum plasma levels are reached after 4 h and the plasma half-life is 37 h. Citalopram is metabolized by the cytochrome P enzyme in the liver [8].

In the literature, several methods can be found for the determination of citalopram in pharmaceutical and biological fluids. Most of these are based on the use of HPLC with UV [9–12], fluorimetric [13–16], and mass spectrometric [17–19] detectors. GC [20], GC-MS [21–24], and electrophoretic methods for the determination of citalopram in a pharmaceutical formulation [25–27] and biological fluids [28–31] were also used.

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Development and Validation of Micellar-Enhanced Spectrofluorimetric Method for Determination of Sulpiride in Pharmaceutical Formulations and Biological Samples

Sensitive and simple spectrofluorometric method has been developed and validated for the quantification of sulpiride in pure, commercial formulations, human plasma and urine in the presence of sodium dodecyl sulphate (SDS) surfactant micelle. The experimental parameters like buffer, surfactant type and volume were studied. A linear relationship was found between sulpiride concentration and fluorescence intensity in the range of $0.02 \mu\text{g mL}^{-1}$ – $7.0 \mu\text{g mL}^{-1}$ containing 0.2 M sodium dodecyl sulphate with λ_{ex} 292 nm and λ_{em} 343 nm with a correlation coefficient of 0.9987. The limit of detection (LOD) and limit of quantification (LOQ) was found to be $1.9 \times 10^{-2} \mu\text{g mL}^{-1}$ and $5.9 \times 10^{-2} \mu\text{g mL}^{-1}$ respectively. The proposed method was successfully applied for the determination of sulpiride in pure and dosage form; the percentage recoveries agreed well with the label value. The effect of common excipients used as additive in pharmaceutical formulations was studied and no interferences was found. Similarly the effect of some common cations and compounds present in urine and plasma were also investigated and the method was found free of interferences. The method was further extended to the in-vitro determination of sulpiride in spiked human plasma and urine samples with percentage recoveries from 98.33%–99.75% and 95.67%–101.67% respectively. The results of the proposed method have been compared with the literature HPLC method and no significant difference was found between them.

Key words: Sulpiride, sodium dodecyl sulphate, micelle-enhanced spectrofluorimetry, pharmaceutical formulations, biological fluids

Entwicklung und Validierung einer mizellar verstärkten spektralfluorimetrischen Methode zur Bestimmung von Sulpirid in pharmazeutischen Formulierungen und in biologischen Proben. Es wurde eine einfache und empfindliche spektrofotometrische Methode zur quantitativen Bestimmung von Sulpirid in reiner Form, in kommerziellen Formulierungen, im menschlichen Blutplasma und Urin in Gegenwart von Natriumdodecylsulfonat (SDS)-Mizellen entwickelt und validiert. Experimentelle Parameter wie Puffer, Tensidtyp und Volumen wurden untersucht. Es wurde eine lineare Beziehung zwischen der Sulpiridkonzentration im Bereich von $0.02 \mu\text{g mL}^{-1}$ – $7.0 \mu\text{g mL}^{-1}$ und der Fluoreszenzintensität mit den Wellenlängen λ_{ex} 292 nm und λ_{em} 343 nm gefunden. Die SDS-Konzentration betrug dabei $0,2 \text{ M}$ und der Korrelationskoeffizient lag bei 0,9987. Die entwickelte Methode konnte erfolgreich zur Bestimmung von Sulpirid in reiner Form und in Dosierungen eingesetzt werden. Die prozentualen Wiederfindungen stimmten gut mit den Angaben überein. Bei der Unter-

suchung der Wirkung der üblichen Arzneihilfsmittel, die in pharmazeutischen Formulierungen als Additive eingesetzt werden, wurden keine Störungen festgestellt. In ähnlicher Weise wurde der Einfluss einiger üblicher Kationen und Verbindungen untersucht, die in Urin und Blutplasma vorliegen. Auch sie störten die Methode nicht. Weiterhin wurde die Methode auf die In-Vitro-Bestimmung von Sulpirid in Additionsproben aus Blutplasma und Urin ausgedehnt. Die Wiederfindung lag zwischen 98,33%–99,75% bzw. 95,67%–101,67%. Die Ergebnisse der entwickelten Methode wurden mit denen der in der Literatur beschriebenen HPLC-Methode verglichen. Es wurde zwischen ihnen kein signifikanter Unterschied gefunden.

Stichwörter: Sulpirid, Natriumdodecylsulfonat (SDS), mizellar verstärkte Spektralfluorimetrie, pharmazeutische Formulierungen, biologische Flüssigkeiten

1 Introduction

Sulpiride, 5-(aminosulfonyl)-N-[(1-ethyl-2-pyrrolidinyl)ethyl]-2-methoxy-benzamide is a selective dopamine D2 competitor with antipsychotic and antidepressant actions, and has found prevalent medical applications for the handling of severe and prolonged schizophrenia as well as other similar mental disorders, such as depression and hallucination with a low frequency of extrapyramidal side effects [1–3]. The recommended oral dose of sulpiride in the treatment of schizophrenia is 200–300 mg three times a day with a gradual increase to a maximum of 1200 mg daily [4].

Taking into account its relatively widespread practicability and possible health effects, it is thus necessary to quantify sulpiride in biological fluids. Several analytical methods have been published for the determination of sulpiride. These methods include gas chromatography (GC) with mass spectrometric [5, 6] or oscillographic detection [7], high performance liquid chromatography (HPLC) with ultraviolet [8–10], fluorescence [11], or mass spectrometric detection [12], and capillary electrophoresis [13, 14].

Two methods are available in the literature for fluorimetric determination of sulpiride [15, 16]. The first method is based on the incorporation of excitation-emission matrix (EEM) fluorescence and second order calibration methodologies based on the alternating fitting residue (AFR) and self-weighted alternating trilinear decomposition (SWATLD) algorithms. The second method is based on the simultaneous determination of sulpiride and its alkaline degradation product by second derivative synchronous fluorescence spectroscopy. A spectrophotometric method is also available for sul-

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Determination of sulpiride in pharmaceutical preparations and biological fluids using a Cr (III) enhanced chemiluminescence method

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ABSTRACT: A highly sensitive and simple method for identifying sulpiride in pharmaceutical formulations and biological fluids is presented. The method is based on increased chemiluminescence (CL) intensity of a luminol-H₂O₂ system in response to the addition of Cr (III) under alkaline conditions. The CL intensity of the luminol-H₂O₂-Cr (III) system was greatly enhanced by the addition of sulpiride and the CL intensity was proportional to the concentration of sulpiride in a sample solution. Various parameters affecting the CL intensity were systematically investigated and optimized for determination of the sulpiride in a sample. Under the optimum conditions, the CL intensity was proportional to the concentration of sulpiride in the range of 0.068–4.0 µg/mL, with a good correlation coefficient of 0.997. The limit of detection (LOD) and limit of quantification (LOQ) were found to be 8.50×10^{-6} µg/mL and 2.83×10^{-5} µg/mL, respectively. The method presented here produced good reproducibility with a relative standard deviation (RSD) of 2.70% ($n = 7$). The effects of common excipients and metal ions were studied for their interference effect. The method was validated statistically through recovery studies and successfully applied for the determination of sulpiride in pure form, pharmaceutical preparations and spiked human plasma samples. The percentage recoveries were found to range from 99.10 to 100.05% for pure form, 98.12 to 100.18% for pharmaceutical preparations and 97.9 to 101.4% for spiked human plasma. Copyright © 2012 John Wiley & Sons, Ltd.

Keywords: chemiluminescence; sulpiride; Cr (III); luminol; spiked human plasma

Introduction

Sulpiride, 5-(aminosulfonyl)-N-[(1-ethyl-2-pyrrolidinyl)methyl]-2-methoxybenzamide, is a selective dopamine D₂ antagonist with antipsychotic and antidepressant activity. Sulpiride has an affinity for the D₂ and D₄ brain dopamine receptor with a low frequency of extrapyramidal side effects (1). Sulpiride has been widely applied in the treatment of depression, schizophrenia and psychopathology of senescence, gastric or duodenal ulcers, as well as irritable colon due to psychosomatic stress and various vertigo syndromes (2). The recommended oral dose of sulpiride in the treatment of schizophrenia is 200 to 400 mg twice daily with a gradual increase based on the clinical response to a maximum of 1200 mg daily. Sulpiride can be slowly and poorly absorbed from the gastrointestinal tract, with peak serum levels occurring in 2–6 h (3). Additionally, sulpiride does not appear to be metabolized completely, as indicated by 70–90% of intravenous doses and 15–25% of oral doses being excreted unchanged in the urine (4,5). Moreover, a high percentage of oral doses of sulpiride have been recovered in feces. The usual half-life of sulpiride is 6 to 10 h (6). In cases of overdoses or long-term therapy, sulpiride can induce serious adverse reactions, such as cardiac muscle damage or death (7).

A number of analytical techniques for determination of sulpiride in pharmaceutical formulations and biological samples have been reported. These include gas chromatography (GC) (8,9), spectrofluorimetric techniques (10,11), high-performance liquid chromatographic (HPLC) methods with UV detection (12–14), HPLC with fluorescence detection (15,16), capillary

electrophoresis (17,18) and adsorptive stripping voltammetry (19). Two chemiluminescence (CL) methods using flow injection systems were recently reported for the analysis of sulpiride in pharmaceutical formulations and biological samples using two different systems, sulpiride-Ce (IV)-Na₂SO₃ (20) and sulpiride-KMnO₄-Tween 80 (21). Considering its relatively extensive applications and possible health effects, the development of a simple, fast and economic method for quantification of sulpiride in commercial formulations and biological samples is highly desirable. Application of CL is gaining interest in analytical chemistry because it has a number of advantages including a low limit of detection in the nanogram or sub-nanogram per

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Spectrofluorimetric method for the determination of sulpiride in pharmaceutical preparations and human plasma through derivatization with 2-cyanoacetamide

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ABSTRACT: A sensitive and accurate spectrofluorimetric method has been developed for the determination of sulpiride in pharmaceutical preparations and human plasma. The developed method is based on the derivatization reaction of 2-cyanoacetamide with sulpiride in 30% ammoniacal solution. The fluorescent derivatized reaction product exhibited maximum fluorescence intensity at 379 nm after excitation at 330 nm. The optimum conditions for derivatization reactions were studied and the fluorescence intensity versus concentration plot was found to be linear over the concentration range 0.2–20.0 µg/mL with a correlation coefficient of 0.9985. The limit of detection (LOD) and limit of quantification (LOQ) were found to be 0.82 and 2.73 ng/mL, respectively. The proposed method was validated according to ICH guidelines. The effects of common excipients and co-administered drugs were also studied. The accuracy of the method was checked using the standard addition method and percent recoveries were found to be in the range of 99.00–101.25% for pharmaceutical preparations and 97.00–97.80% for spiked human plasma. The method was successfully applied to commercial formulations and the results obtained for the proposed method were compared with a high-performance liquid chromatography reference method and statistically evaluated using the Student's *t*-test for accuracy and the variance ratio *F*-test for precision. A reaction pathway was also proposed. Copyright © 2012 John Wiley & Sons, Ltd.

Keywords: sulpiride; 2-cyanoacetamide; derivatization; spiked human plasma

Introduction

Depression is one of the most common psychiatric syndromes; at any given moment, around 5–6% of the population is depressed and an expected 10% of people may become depressed during their lifetime (1). Antidepressant medications are thought to be most effective in patients with clearly vegetative characteristics, comprising psychomotor retardation, sleep disturbance, poor appetite, weight loss and loss of libido.

5-(Aminosulfonyl)-*N*-[(1-ethyl-2-pyrrolidinyl) ethyl]-2-methoxybenzamide (sulpiride) is the most commonly recommended antipsychotic drug. It is a selective dopamine D2 antagonist with antipsychotic and antidepressant activities and has widespread medical applications for the treatment of severe and prolonged schizophrenia, as well as other related syndromes such as depression and hallucination, with a low frequency of extrapyramidal side effects (2–4). The recommended oral dose of sulpiride in the treatment of schizophrenia is 200–400 mg two times a day with a gradual increase in dosage to a maximum of 1200 mg daily (5).

Sulpiride is slowly and poorly absorbed from the gastrointestinal tract, with the peak serum level occurring in 2–6 h (6). The usual half-life is 6–8 h. Sulpiride does not appear to be metabolized completely and 70–90% of an intravenous dose and 15–25% of an oral dose is excreted unchanged in the urine (7,8).

A number of analytical methods have been published in the literature for the analysis of sulpiride in pharmaceutical formulations and biological fluids. These methods include

gas chromatography (GC) (9,10), spectrophotometry (11,12), high-performance liquid chromatography (HPLC) with UV detection (13–15), HPLC with fluorescence detection (16,17), HPLC-mass spectrometry (MS) (18), GC-MS (19), capillary electrophoresis (20,21) and adsorptive stripping voltammetry (22). An osilopolarographic method has also been reported for the determination of sulpiride in the presence of acetic acid:sodium acetate buffer at pH 5 (23). All these methods are either not appropriately sensitive or tedious and need expensive, sophisticated and dedicated instrumentation.

In the literature, a few methods are available for the spectrofluorimetric determination of sulpiride (24,25) within a linear range of 0.05–1.0 µg/mL, 2–10 µg/mL and 0.05–1.0 µg/mL. These methods have a very narrow analytical range and also suffer from interferences by other compounds.

2-Cyanoacetamide is a fluorogenic reagent and has been used in post column derivatization in HPLC for the determination of catecholamines (26) and carbohydrates (27). It has also been used for fluorimetric determination of pharmaceutical compounds like oxamniquine (28), ascorbic acid (29), prenalterol HCl (30), cephalixin (31), aminoglycosides (32), 3,4-dihydroxyphenylalanine (33) and cefixime (34).

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Spectrofluorimetric Protocol for Ceftriaxone in Commercial Formulation and Human Plasma After Condensation with Formaldehyde and Ethyl Acetoacetate

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Abstract A new spectrofluorimetric method has been developed and validated for the quantification of ceftriaxone in bulk powder, pharmaceutical formulations and spiked human plasma. The developed method is reproducible, accurate, sensitive and cost effective. In this method, ceftriaxone was converted into a fluorescent compound by reacting with 0.8 M ethyl acetoacetate and 25% formaldehyde in a buffered medium (pH=4.2) at 90 °C. The excitation and emission wavelengths of the fluorescent reaction product are 316 nm and 388 nm respectively. Optimization of the experimental conditions affecting the condensation reaction were carefully carried out and the optimum experimental conditions were incorporated in the procedure. The developed method has a broad linear range ($0.2\text{--}20\text{ }\mu\text{g mL}^{-1}$) with a correlation coefficient of 0.9992. The limit of detection (LOD) and limit of quantification (LOQ) was found to be $1.94\times 10^{-2}\text{ }\mu\text{g mL}^{-1}$ and $6.47\times 10^{-2}\text{ }\mu\text{g mL}^{-1}$ respectively. The common excipients and co-administered drugs were investigated for their interferences effect in the assay. The developed method was validated statistically through recovery studies and successfully applied to ceftriaxone determination in bulk powder, pharmaceutical formulations and spiked human plasma samples. The percent recoveries were found to be in the range of 99.04–100.26% for bulk powder, 98.88–99.92% for pharmaceutical formulations and

94.22–98.48% for spiked human plasma. The results were verified by comparing with reference literature HPLC method and were found in good agreement.

Keywords Ethyl acetoacetate · Formaldehyde · Spectrofluorimetric · Ceftriaxone · Spiked human plasma

Introduction

The first and second generation cephalosporins are unsuccessful in the treatment of meningitis because they are unable to penetrate the central nervous system. However the third generation cephalosporin not only crosses the blood brain barrier but reaches the therapeutic concentration that are sufficient for the treatment of meningitis caused by aerobic gram-negative bacteria [1].

Ceftriaxone, (6R, 7R)-7-[(2E)-2-(2-amino-1, 3-thiazol-4-yl)-2-methoxyimino-acetyl] amino-3-[(2-methyl-5, 6-dioxo-1H-1, 2, 4-triazin-3-yl) sulfanylmethyl]-8-oxo-5-thia-1-azabicyclo [4.2.0] oct-2-ene-2-carboxylic acid is third generation semisynthetic cephalosporin antibiotic. It is effective against a wide variety of gram positive and gram negative bacteria including Enterobacteriaceae, H. influenza, N. meningitides, N. gonorrhoeae, and Streptococcus pyogenes. It has promising activity against strains of Pseudomonas aeruginosa. [2–4]. It inhibits the growth of microbes by hindering the mucopeptide synthesis in bacterial cell wall [5–7]. 90% decrease in growth was shown by ceftriaxone concentration as low as $0.03\text{ }\mu\text{g mL}^{-1}$ for Escherichia coli, $0.06\text{ }\mu\text{g mL}^{-1}$ for Salmonella spp. and $0.2\text{ }\mu\text{g mL}^{-1}$ for Pasteurella multocida in their isolates from infected Israeli-Friesian male calves [8].

After administering one gram dose of ceftriaxone by intramuscular injection and intravenous infusion, mean peak

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Quantification of sparfloxacin in pharmaceutical dosages and biological samples

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Abstract: A simple and fast method for spectrophotometric determination of sparfloxacin using *p*-dimethylaminobenzaldehyde (DMAB) has been developed. A yellow coloured product formed from reaction between sparfloxacin and DMAB as a result of condensation reaction at room temperature. The maximum absorbance was found at 392 nm with molar absorptivity of $4.9 \times 10^3 \text{ L mol}^{-1} \text{ cm}^{-1}$. All parameters for the reaction, as concentration of DMBA reagent, molarity of sulphuric acid, and reaction temperature were studied. Under the conditions studied, a linear relationship between absorbance of the condensation product and concentration of sparfloxacin in the range of 2.0-80.0 $\mu\text{g mL}^{-1}$ was found with good correlation coefficient (0.9997). The limits of detection (LOD) and quantification (LOQ) for the proposed method were found to be 0.22 and 0.75 $\mu\text{g mL}^{-1}$ respectively. The repeatability and accuracy (model) of the method was studied at three different concentrations of sparfloxacin and found with value of relative standard deviation less than 2.0%. The method was found selective for determination of sparfloxacin in the presence of commonly used excipients in dosage forms. The developed method was validated statistically and applied successfully to the analysis of the drug in pure form, pharmaceutical preparations, and spiked blood plasma and urine samples with good accuracy (real) and precision. The percentage recovery was found from 99.0-100.0% with relative standard deviation less than 1%. The results of the proposed method were compared statistically with the results of literature HPLC method.

Keywords: Sparfloxacin, *p*-dimethylaminobenzaldehyde, spectrophotometry, formulations.

INTRODUCTION

Fluoroquinolones are one of the most important antibacterial agents used in human medicines, which are active through inhibition of their DNA gyrase against both Gram positive and Gram negative bacteria.

Sparfloxacin has a broad spectrum of activity and is more potent in vitro than the non-fluorinated quinolone and more favourable pharmacokinetics allowing its use in systemic infections. It has been used in the treatment of urinary tract infection, lung infection and cutaneous allergy (Martindale 2009).

Analytical methods so far reported for determination of sparfloxacin in pharmaceutical preparation are DC polarography (Jain *et al.*, 2003), HPLC (Marona *et al.*, 1999, Kowalczyk *et al.*, 2003, Cao *et al.*, 2001) and capillary zone electrophoresis (Faria *et al.*, 2006, Sun *et al.*, 1999, Fierrens *et al.*, 2000). Spectrophotometric methods have also been reported based on the oxidation of sparfloxacin (Jan *et al.*, 2010), indirect determination of sparfloxacin with N-bromosuccinimide (Askal *et al.*, 2007), ion-pair complex with bromothymol blue (Marona and Schapoval, 2001), coloured product with ammonium reineckate as a precipitate and redissolution in acetone (Al-Ghamam 2008), Pd (II) and eosin ternary complex (El-Didamony 2007) and UV-spectrophotometry (Kaur *et al.*, 2008). Some spectrofluorimetric methods have also

been reported based on intrinsic fluorescence property (El-Didamony 2011) or complexation reaction with aluminum chloride (Rizk *et al.*, 2000), lutetium (III)-sparfloxacin (Wang 2004) and terbium (III)-sparfloxacin complex (Fangtian *et al.*, 1999).

The present work was carried out with the aim to develop a selective, precise, accurate, and sensitive method for spectrophotometric determination of sparfloxacin in pure, different pharmaceutical preparations and biological samples. The validated method should also be according to the guidelines of ICH. The method is based on the spectrophotometric analysis of the studied fluoroquinolones through its condensation reaction with *p*-dimethylaminobenzaldehyde (DMAB) in acidic medium at room temperature.

MATERIALS AND METHODS

Instruments

UV/Vis Spectrophotometer (Model SP-3000 plus, Optima, Japan) with matched 1 cm quartz cells for all spectrophotometric measurements and thermostatically controlled water bath (Yu Jia, China) were used. Centrifugation of plasma and urine samples was carried out on a clinical centrifuge (Model 800, Jiangsu, China).

Reagents and solutions

All reagents used were of high grade purity. *p*-

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Full Length Research Paper

Micellar-enhanced spectrofluorometric quantification of moxifloxacin in pharmaceutical formulations, human urine and plasma samples

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Simple, rapid, specific and highly sensitive spectrofluorometric method has been developed for the quantification of moxifloxacin in pure, commercial formulations, human urine and plasma in the presence of sodium dodecyl sulphate surfactant micelle. The different experimental parameters that effect fluorescence intensity of the drug were studied and optimized. A linear relationship was found between fluorescence intensity and moxifloxacin concentration in the range of 1.25 to 320 ngml⁻¹ containing 0.03 M sodium dodecyl sulphate with λ_{ex} 294 nm and λ_{em} 494 nm with a good correlation coefficient between fluorescence intensity and concentration of the drug. The detection and quantification limits were found to be 0.48 and 1.6 ngml⁻¹, respectively. The proposed method was applied for quantification of moxifloxacin in tablets, infusion and eye drops with mean percentage recovery of 99.89%. Common excipients used as additive in pharmaceutical formulations do not interfere with the method. The method was successfully applied for quantification of moxifloxacin in spiked human urine and plasma. The effect of some common cations and compounds present in urine and plasma were also investigated and the method was found free of interferences. The effect of co administered analgesic and other drugs were also studied and no interferences were found.

Key words: Moxifloxacin, micellar-enhanced, spectrofluorometry, pharmaceutical formulations, urine sample.

INTRODUCTION

Moxifloxacin, 1-cyclopropyl-7-(2,8-diazabicyclo [4,3,0] nonane)-6-fluoro-8-methoxy-1,4-dihydro-4-oxo-3-quinoline carboxylic acid, is a new enantiomerically pure 8-methoxy quinolone, which was first synthesized by Peterson and colleagues (Dalhoff et al., 1996; Peterson et al., 1996) and that has potent and enhanced antimicrobial activity against Gram positive and maintained activity against Gram negative and anaerobes. It is a fourth generation fluoroquinolones which is one of the most important and successful classes of manmade antibacterial used in the treatment of lungs infections, urinary tract infections, respiratory tract infections, cutaneous allergy, pneumonia, abdominal and eye infections caused by bacteria. *In vitro*, moxifloxacin is highly

effective against a wide spectrum of respiratory tract pathogens, showing enhanced activity against gram positive microorganism compared to those of other antibiotics. It has excellent efficacy against *pneumococcal* strain resistant to beta-lactum and macrolides antibiotics and good activity against common, less common and typical respiratory tract pathogens (Rouse et al., 1996; Stass et al., 1998).

Moxifloxacin has an excellent pharmacokinetics, bioavailability and penetration into respiratory tissues and other body fluids. The drug is rapidly absorbed, with peak plasma concentration reached within 1 to 4 h after treatment, and the long half life (11.4 to 15.8 h) makes it suitable for once daily administration (Bienenbach et al., 1998). Moxifloxacin is receiving great attention due to its broad spectrum activity, efficacy and potency.

Wide spread use of moxifloxacin requires development of a number of analytical methods for its determination in various matrices. However, not many methods have been

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