

**SPECTROFLUORIMETRIC QUANTIFICATION OF FURAZOLIDONE
AND NITRAZEPAM IN TABLET FORMULATIONS - DEVELOPMENT
OF SENSITIVE FLUORESCENCE TECHNIQUES FOR NON
FLUORESCENT DRUGS BY QUENCHING APPROACH**

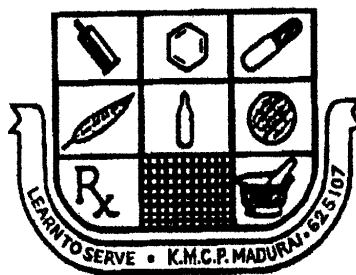
*Dissertation Submitted in partial fulfillment of the
requirement for the award of the degree of*

MASTER OF PHARMACY

IN

PHARMACEUTICAL ANALYSIS

**THE TAMILNADU Dr. M.G.R. MEDICAL UNIVERSITY
CHENNAI**



DEPARTMENT OF PHARMACEUTICAL ANALYSIS

**K.M.COLLEGE OF PHARMACY
UTHANGUDI, MADURAI - 625 107**

APRIL – 2012

CERTIFICATE

This is to certify that the dissertation entitled “**SPECTROFLUORIMETRIC QUANTIFICATION OF FURAZOLIDONE AND NITRAZEPAM IN TABLET FORMULATIONS - DEVELOPMENT OF SENSITIVE FLUORESCENCE TECHNIQUES FOR NON FLUORESCENT DRUGS BY QUENCHING APPROACH**” submitted by **Ms. M. PRINCY PRASANNA (Reg. No: 26101726)** to The Tamilnadu Dr.M.G.R.Medical University, Chennai, in partial fulfillment of the requirement for the award of **Master of Pharmacy** in Pharmaceutical analysis at K.M. College of Pharmacy, Madurai. It is a bonafide work carried out by her under my guidance and supervision during the academic year 2011-2012. This dissertation partially or fully has not been submitted for any other degree or diploma of this University or any other Universities.

GUIDE

Mr. M. S. Prakash, M.Pharm.,

Professor,

Dept. of Pharmaceutical analysis,

K.M. College of pharmacy,

Uthangudi, Madurai-625107,

Tamilnadu.

HOD

Dr. S. Meena, M.Pharm., Ph.D.,

Professor and Head,

Dept. of Pharmaceutical Analysis,

K.M.College of Pharmacy,

Uthangudi, Madurai-625107,

Tamilnadu.

PRINCIPAL

Dr. S. Jayaprakash, M.Pharm., Ph.D.,

Professor and Head,

Dept. of Pharmaceutics,

K.M.College of Pharmacy,

Uthangudi, Madurai-625107,

Tamilnadu.

CONTENTS

Chapter	Particulars	Page No.
I	INTRODUCTION	1
II	DRUG PROFILE	19
III	REVIEW OF LITERATURE	23
IV	INTRODUCTION TO PRESENT STUDY	32
V	AIM OF PRESENT STUDY	40
VI	EXPERIMENTAL WORK DONE	
	▪ Equipment & chemicals	41
	▪ Experimental work for furazolidone	43
	▪ Experimental work for nitrazeapam	77
VII	RESULTS & DICCUSSION	100
VIII	CONCLUSION	104
	REFERENCES	

ABBREVIATIONS

AA	-	Anthranilic acid
Bnk	-	Blank
Conc	-	Concentration
DM	-	Dimethyl formamide
Em	-	Emission
Ex	-	Excitation
FUZ	-	Furazolidone
I.S	-	Internal standard
mcg	-	Microgram
mg	-	Milligram
µl	-	Microlitre
ml	-	Millilitre
NED	-	Naphthyl ethylene Diamine dihydrochloride
ng	-	Nanogram
nm	-	Nanometer
NUZ	-	Nitrazepam
QS	-	Quinine sulphate
RP-HPLC	-	Reverse phase High performance liquid chromatography
Std	-	Standard
SD	-	Standard deviation
Wt	-	Weight
UPLC-MS	-	Ultra performance liquid chromatography-Mass spectroscopy

INTRODUCTION

PHARMACEUTICAL ANALYSIS

Pharmaceutical analysis is an interdisciplinary subject and derives its principles from various branches of science like chemistry, physics, microbiology, nuclear science and electronics.

Analytical determination is based on the measurement of some physical, chemical or structural properties which are related directly or indirectly to the amount of desired constituent present in the sample. Drugs or pharmaceuticals are the chemicals which are originated from either organic or inorganic. Some property of medicinal agent is used to measure them qualitatively or quantitatively.

Analytical chemistry can be defined as the science and art of determining the composition of materials, in terms of the elements or compounds contained either qualitatively or quantitatively.^{1(a)}

Analytical chemistry is concerned with the identification of a substance, the elucidation of its structure and quantitative analysis of its composition.²

Analytical chemistry is divided into two parts.

- A. Qualitative analysis
- B. Quantitative analysis.

QUALITATIVE ANALYSIS:

Chemical analysis dealing with the study of nature or quality of the compound or mixture is called qualitative analysis. It involves the identification of constituent radicals present in the inorganic mixture and presence or absence of particular element, functional group etc. in an organic compound.

QUANTITATIVE ANALYSIS:

Quantitative analysis deals with the determination of nature of the constituents of a given sample and how much of each of the constituents or of constituent specified is present. When a completely unknown sample is presented to an analyst, the first requirement is usually to ascertain what substance is present in it. This fundamental problem may sometimes

be encountered in the modified form of deciding what impurities are present in a given sample. The solution of such type of problem lies in the area of qualitative analysis.^{3(a)}

TYPES OF ANALYSIS:

MODERN CLASSIFICATION:

1. Chemical methods

- Acidimetric & Alkalimetric methods.
- Non - aqueous methods.
- Oxidation - reduction methods.
- Argentometric methods.
- Complexometric methods.
- Gravimetric methods
- Miscellaneous type of method

2. Physical Methods

- Refractometry
- Polarimetry
- Optical rotatory dispersion

3. Electro chemical Methods

- Electro Analytical methods
- Polarography

4. Spectroscopic Method

SPECTROSCOPY:

Spectroscopy is the measurement and interpretation of electromagnetic radiation absorbed, scattered or emitted by atoms, molecules or other chemical species. The absorption or emission is associated with changes in energy states of the interacting chemical species and since each species has characteristic energy states, spectroscopy can be used to identify the interacting species.

The principle types of spectroscopic techniques are

- Ultraviolet and visible spectroscopy
- Fluorescence and phosphorescence spectroscopy
- Atomic spectroscopy (emission and absorption)
- Infra red spectroscopy
- X-ray spectroscopy
- Radiochemical technique
- Nuclear magnetic resonance spectroscopy
- Electron spin resonance spectroscopy ⁴

5. Separation method

- Electrophoresis
- Chromatographic methods

CHROMATOGRAPHY:

The term chromatography (Greek kromatos-colour and Graphos-Written) means color writing.

The chromatographic principle was discovered by a Russian botanist, Michael Tswett (1906). He defined chromatography as the method in which the components of a mixture are separated on an adsorbent column in a flowing system. Recently, the IUPAC has defined the chromatography as:

Methods used primarily for the separation of the components of a sample, in which the components are distributed between two phases, one of which is stationary while the other moves. The stationary phase may be a solid or liquid supported on a solid or gel, and may be packed in a column, spread as a layer or distributed as a film. The mobile phase may be gaseous or liquid.

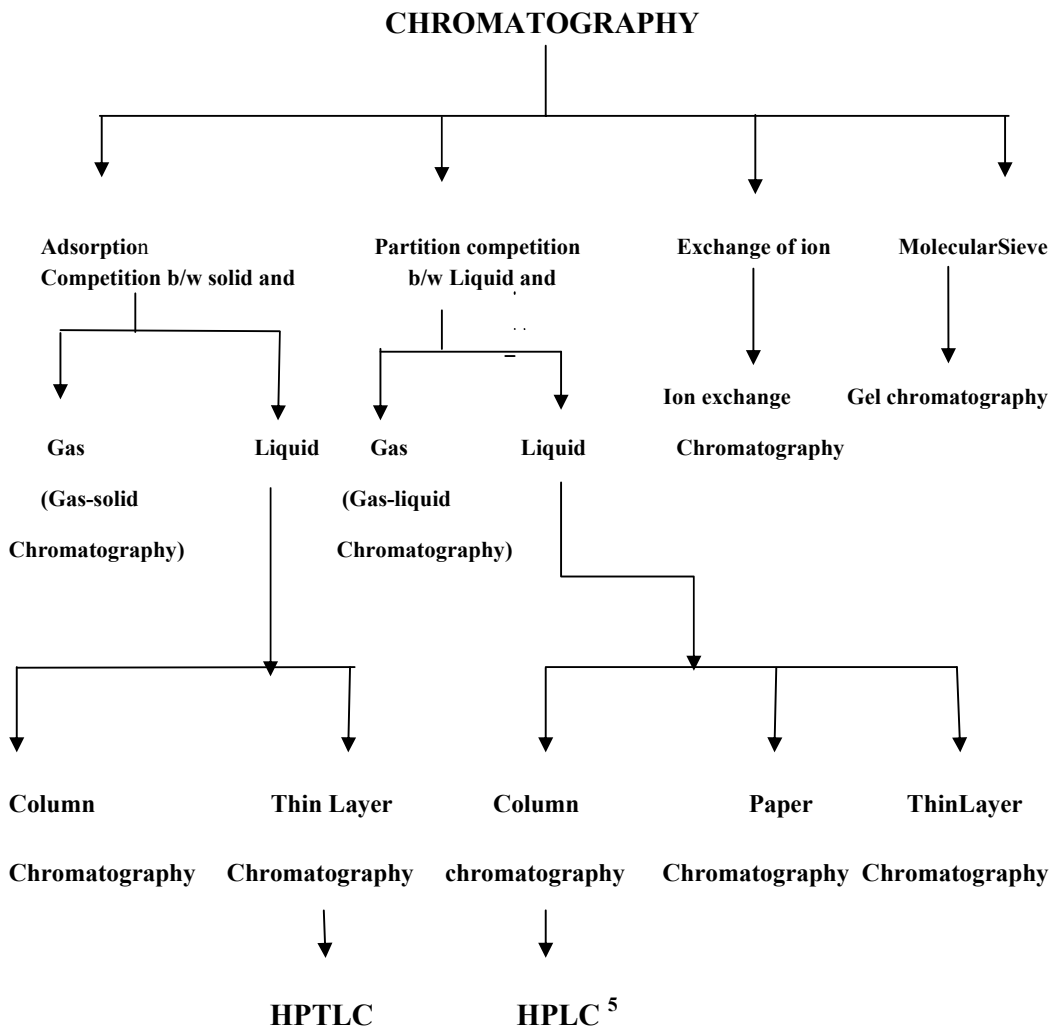
Chromatography has been used in all the fields particularly in chemistry, biology, medicine, dyes, forensic departments and clinical studies.

Advantage:

- Separations can be carried out on micro or semi micro scale. .i.e A very small quantity of mixture is required for analysis.
- Simple, rapid, and require simple apparatus.

Flow chart. 1

TYPES OF CHROMATOGRAPHY



6. Radiochemical methods

- Methods using radio isotopes

7. Miscellaneous types (Instrumental)

- Particle size analysis
- Differential thermal analysis (DTA)
- Differential scanning calorimetry (DSC)^{1 (b)}

SIGNIFICANCE OF QUANTITATIVE ANALYSIS:

The analytical chemists play a very important role in a modern industrial society. Some of the important applications of quantitative analysis are discussed here,

1. In checking the purity of sample:

The raw material used in manufacturing industries is examined to ensure that there are no unusual substances present which may appear as a harmful impurity after completion of the reaction or deleterious to the manufacturing process. The final manufactured product is subjected to quality control to ensure that its essential compounds are present within a predetermined range of composition, whilst impurities do not exceed certain specified limits.

2. In the detection of pollution:

The quantitative analysis is helpful in determine the level of pollution.

3. In farming:

In farming, the analysis of soil is done to determine the content of essential plant nutrients nitrogen, potassium, phosphorus, and of the trace elements which are necessary for healthy plant growth.

4. In medical science:

Chemical analysis has been proved to be a useful tool in diagnosing the illness and in monitoring the condition of patients.

5. Geological Application:

Geologists apply the use of analytical chemistry to determine the composition of numerous rocks and soil samples collected in the field.

6. In metallurgical process:

The composition of an alloy used in a metallurgical process can be established by analytical method.

7. In chemical kinetics:

Quantitative measurements of reactants and products involved in any reaction gives the information of rates of reactions which in turn may find to be useful in determining the mechanism of reaction.

8. In Archeology:

Archeologists identify the source of volcanic glass by measuring the concentration of minor elements in samples obtained from different places, which is used in tracing the prehistoric trade routes for tool and weapons fashioned from volcanic glass.^{3 (b)}

SPECTROFLUORIMETRY

INTRODUCTION:

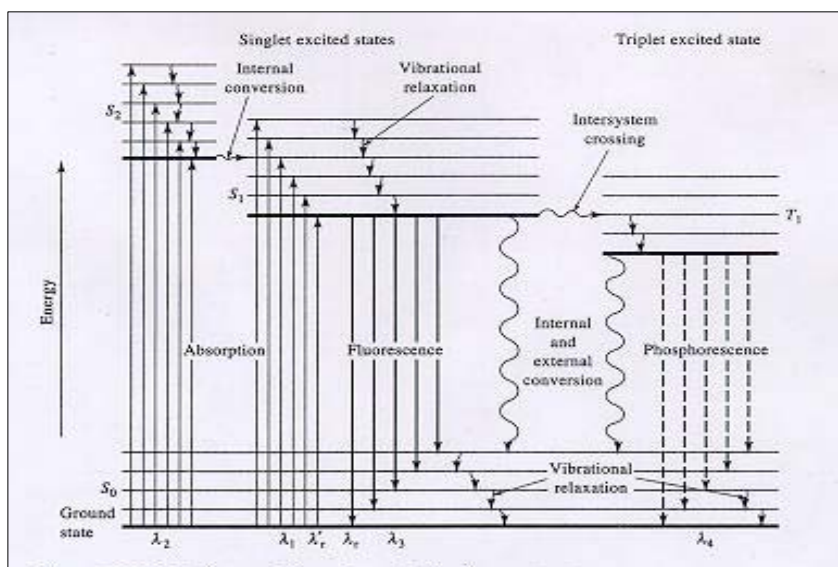
When the molecules are exposed to the electromagnetic radiation they exhibit fluorescence. Fluorescence is a process of re-emission of radiant energy absorbed in the form of visible or UV light. In this process, the light emitted is always of longer wavelength than that absorbed. In fluorescence, absorption and emission of light takes place in short time (10^{-12} to 10^{-9} seconds). The quantitative determination of fluorescence is the basis of fluorimetric analysis.^{6(a)}

PRINCIPLE OF FLUORESCENCE:

A Molecule in a ground state is considered to have three energy levels viz. rotational, vibrational and electronic. When an electromagnetic radiation falls on the molecule it brings changes into its energy levels and they will go to excited state. The excited molecular species are extremely short-lived and deactivation occurs due to:

- A. Internal collisions.
- b. Cleavage of chemical bonds, initiating photochemical reactions.
- c. Re-emission as light (luminescence)

Figure No. 1 Jablonski energy diagram



Molecules on excitation normally possess higher vibrational energy than they had in the ground state. This extra vibrational energy is lost by collision, after which the molecule returns to the ground state with the emission of light as fluorescence. Fluorescence is a rapid process occurring within 10^{-6} - 10^{-9} seconds of the excitation. If there is any delay in the emission of light is called as phosphorescence. The delay period may range from fraction of a second to few days. Both these processes of re-emission are generally designated as luminescence.^{7(a)}

FACTORS AFFECTING FLUORESCENCE:

$$F = 2.3I_0 abc \phi$$

For a fixed set of instrumental (I_0 and b) and sample (a and ϕ) parameter, the fluorescence is proportional to concentration.

$$F = kc \quad \text{where } K = 2.3 I_0 ab\phi$$

There are number of factors which or directly or indirectly affect the fluorescence and decrease its intensity and sensitivity which is termed as “quenching”.

1. Concentration:

Fluorescence is best in dilute solution. In concentrated solution the intensity of fluorescence is reduced and is not quantitative. This is called as concentration quenching.

2. Quantum yield of fluorescence (ϕ):

$$\Phi = \text{no of photons emitted/no of photons absorbed}$$

Highly fluorescent substances have ϕ values near 1, which shows that most of the absorbed energy is re-emitted as fluorescence. Non fluorescent substance have $\phi = 0$.

3. Intensity of incident light:

An increase in the intensity of light incident on the sample produces a proportional increase in the fluorescent intensity. The intensity of incident light and sensitivity of fluorescence measurement are increased by increasing the width of the excitation slit.

4. Oxygen:

The presence of oxygen may interfere in two ways: By direct oxidation of the fluorescent substance to non-fluorescent products, or by quenching of fluorescence.

5. pH:

Alteration of pH brings changes in the ionized and non-ionized form of fluorogenic material.

6. Temperature and viscosity:

An increase in temperature or decrease in viscosity is likely to decrease fluorescence by deactivation of the excited molecules by collision. Low temperature and appropriate dilute solution are necessary for quantitative analysis.

7. Quenchers:

❖ Collisional quenchers:

Collisional quenchers reduce fluorescence by dissipating absorbed energy as heat due to collisions with the quenching species.

E.g.: Quinine

Highly fluorescent in 0.05M sulphuric acid.

Non-fluorescent in 0.1M hydrochloric acid.

❖ Static quenchers:

Static quenchers form a chemical complex with the fluorescent substance and alter its fluorescence characteristics.

E.g.: Caffeine reduces the fluorescence of riboflavin by static quenching.

INSTRUMENTATION:

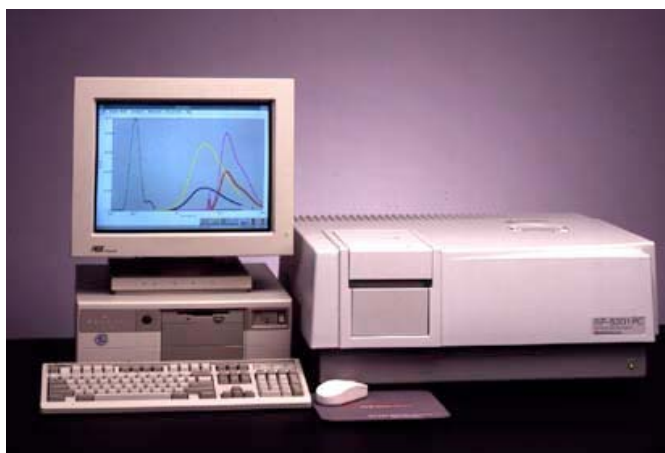
Two primary kinds of instruments are used to measure the fluorescence

❖ Filter fluorimeters

❖ Spectrofluorometer

Instruments that measure the intensity of fluorescence are called fluorimeters. Those that measure the fluorescence intensity at variable wavelength of excitation and emission, and are able to produce fluorescence spectra are called as Spectro fluorimeters.^{8 (a)}

Figure No. 2 Spectrofluorometer



1. Source of light:

- i. Mercury vapour lamp
- ii. Xenon-arc lamp
Providing light output from 190-1200nm.
Versatile and powerful.

2. Filter and monochromator:

In Spectrofluorometer, excitation and emission monochromators are used.

Excitation Monochromator:

Provides a suitable radiation for excitation of the molecules

Emission Monochromator:

Isolate only the radiation emitted by the fluorescent molecule.

3. Cuvettes/cells:

Four sides polished cuvettes are used. Made up of borosilicate or quartz glass.

4. Detector:

Photo multiplier tube (PMT) used.

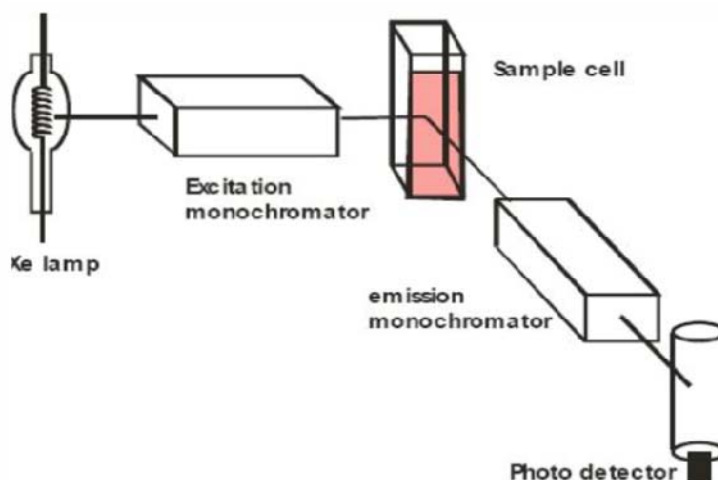
Advantage:

- ✓ Standard device
- ✓ Large signal
- ✓ Fast rise time possible

Disadvantage:

- ✓ High voltage required
- ✓ Sensitive to magnetic field

Figure no: 3 Working Principle



The light source produces light photons over a broad energy spectrum, typically ranging from 200 to 900nm. When photons fall on the excitation monochromator, which selectively transmits light in a narrow range. The transmitted light passes through adjustable slit that control magnitude and resolution by further limiting the range of transmitted light. The filtered light passes in to the sample cell causing fluorescent emission by fluorophores

with in the sample. Emitted light enters the emission monochromator, which is positioned at 90° angle from the excitation light path to eliminate background signal and minimise noise due to stray light. Again the emitted light is transmitted through adjustable slits, finally entering the PMT. The signal is amplified and create a voltage that is proportional to the measured emitted intensity.^{9(a)}

Advantages of fluorimetry:

- * Hundred to Thousands times more sensitive than absorbance techniques
- * Possible to analyse sample in the nanogram to picogram level ^{8(b)}

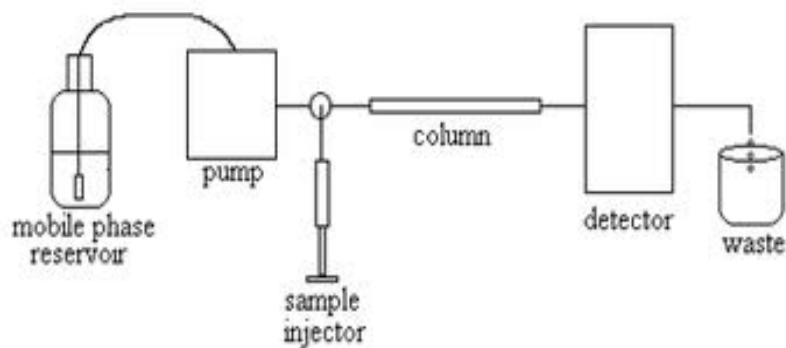
HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

The name High Performance Liquid Chromatography (HPLC) is employed to distinguish these newer procedure from the basic methods which are still used for preparative purposes.

SCOPE OF HPLC:

HPLC is the most widely used of all analytical separation techniques. The reasons for the popularity of the method is its sensitivity, its ready adoptability to accurate quantitative determinations, It is suitable for separating non volatile species or thermally fragile compounds. This method is applicable to the substance which include protein, aminoacids, carbohydrates, antibiotics, steroids, pesticides, and variety of inorganic substances.

Figure no: 4 Instrumentation



Various components of HPLC are,

- * Mobile phase reservoir, including pump
- * Sample injection system
- * Chromatographic column
- * Detector

MOBILE PHASE RESERVOIRS:

A Modern HPLC apparatus is equipped with one or more glass or stainless steel reservoirs, each of contain 200 to 1000ml of solvent. The solvents used should be free from dissolved gases. Because these bubbles causes band broadening and also interfere with the performance of the detector.

Degassers may consist of a vaccum pumping system, a distillation system, devices for heating and stirring the solvents. Often the system also contains filtering unit to remove dust and particulate matter from the solvent. In analytical HPLC the mobile phase is pumped through the column at 1-5ml/min. ^{7 (b)}

MODES OF SEPARATION:

Isocratic:

A Single solvent in constant composition is used.

Gradient:

Two or three solvent systems that differ significantly in solvents and also ratio of the solvents are varied. Frequently the separation was greatly enhanced by gradient elution and also reduces the retention time of the eluates.¹⁰

PUMPING SYSTEM:

Pumps are required to deliver a constant flow of mobile phase at pressure ranging from 1 to 550 bar. Pumps are capable of withstanding the pressure level up to 800psi.

TYPES OF PUMPS:

- ★ Reciprocating pump
- ★ Syringe or displacement pump
- ★ Pneumatic or constant pressure pump^{7(c)}

SAMPLE INJECTORS:

One of the limiting factors, the precision of liquid chromatographic measurements lies in the reproducibility with which samples can be introduced in to the column packing. Because of the overloading of the sample causes band broadening.

TYPES OF SAMPLE INJECTORS:

- ◆ Syringe injectors

This is the earliest and simplest technique. Hence a sample is injected through a self-sealing elastomeric septum and the syringes are designed to with stand pressure up to 1500psi.

Disadvantage:

The reproducibility is poor

- ◆ Stop flow injection

This too is a syringe injection but the solvent flow is stopped momentarily. Sample is injected directly on to the head of the column packing.¹¹

- ◆ Solvent flowing

This is now- a- days used in all automatic systems where the sample more than 10 μ l.

Modern injectors are based on injection valves which allow the sample at atmospheric pressure to be transferred to the high-pressure mobile phase immediately before the column inlet. When, the injector in the LOAD position, the sample is injected from a syringe through the needle port in to the loop. The valve lever is then turned through 60⁰ to the INJECTION position and the sample is swept in to the flowing mobile phase. If an excess of sample is flushed through the loop in the LOAD position.^{8 (c)}

SEPARATION	VOL. OF. INJECTION
Analytical column	10-20 μ l
Semi-preparative column	0.1-1ml

COLUMN:

HPLC columns are made up of high quality stainless steel, polished internally to a mirror finish.

Analytical columns Length: 25to100cm with Internal Diameter 2to 6mm.

Preparative columns Length: 25to100cm with Internal Diameter 6mm or more.^{11 (b)}

PACKING MATERIAL:

Adsorption HPLC:

Unmodified silica most widely used in adsorption HPLC. The functional group responsible for the adsorption is the silanol group. This interacts with the sample solutes by hydrogen bonding.

Partition HPLC:

In partition HPLC, silica (support) was coated with a polar liquid stationary phase e.g. Ethane 1, 2-diol.

Now a day's such packing materials have been replaced by chemically bonded packing materials.

Normal-Phase Partition HPLC:

In this type of chromatography, Mobile phase is less polar than the stationary phase. Stationary phase is a bonded siloxane with a polar functional group. Polarity order (cyano< diol< amino).

Here, Substance is eluted in the order of their increasing polarities. I.e. the least polar substance is eluted first.

Reverse Phase-HPLC:

This type of HPLC is opposite to that of normal phase-HPLC. The stationary phase is less polar than the mobile phase, and consequently the solutes are eluted in the order of their decreasing polarities.

The stationary phase is silica, chemically bonded through a siloxane linkage to a low polar functional group.

Untreated silanol groups may be capped by treatment with trimethyl chlorosilane to eliminate adsorption effects. Water and less polar organic solvent modifier e.g.: Methanol or acetonitrile used as a mobile phase in RP-HPLC. ^{12(a)}

DETECTORS:

The detection of the separated components from the column is based on the bulk property of the eluate or the solute property of the individual components.

REQUIREMENTS OF DETECTORS:

- * Sensitive to at least 10^{-8} gm^{-1} .

- * Should give linear response over a wide concentration range
- * Should have low dead volume
- * Good stability
- * Non-destructive, cheap, reliable and easy to use

Table no: 1 TYPICAL DETECTOR CHARACTERISTICS IN HPLC

TYPE	RESPONSE	Conc.(gcm ⁻³)	LINEAR RANGE
UV/VIS absorption	Selective	10 ⁻⁸	10 ⁴ -10 ⁵
Conductometric	Selective	10 ⁻⁷	10 ³ -10 ⁴
Refractive index	Universal	10 ⁻⁶	10 ³ -10 ⁴
Fluorimetry	Selective	10 ⁻¹¹	10 ² -10 ³
Amperometric	Selective	10 ⁻¹⁰	10 ⁴ -10 ⁵
IR absorption	Selective	10 ⁻⁶	10 ³ -10 ⁴

APPLICATIONS OF HPLC:

1. In Inorganic chemistry:

It is used to separate the anions like F⁻, Cl⁻, Br⁻, NO₂⁻, NO₃⁻, HPO₄⁻, SO₄⁻, CH₃CHOO⁻, HCOO⁻ and cations like Mg²⁺, Ca²⁺, Sr²⁺, Ba²⁺.

2. In organic chemistry:

- * To determine the composition of nucleic acid, and in purification of nucleic acids
- * Separation of lipids and fatty acids.
- * To resolve mixture of corticosteroids has been resolved.

3. Most of the pharmaceutical drugs have been separated by HPLC method.

- * Scopolamine, Ergotamine, Hyoscyamine using Mercosorp column
- * Phenothiazines, benzodiazepine derivatives, Barbiturates, Rauwolfia alkaloids using C₁₈-RP-HPLC.

4 .In Forensic chemistry:

A number of addictive drugs such as Cannabis products, LSD, Heroin, Opiates and poisons have been analysed by HPLC.

5. Identification of Psychotropic drugs in body fluids:

Phenothiazine, Benzodiazepine, and Butyrophenones in body fluids such as plasma, CSF, Saliva.

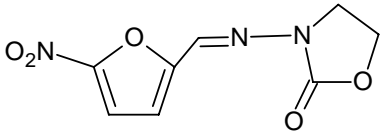
6. Separation of fat soluble vitamins has been carried out on silica columns.

7. A mixture of sugar containing glucose, fructose, sucrose, maltose, and lactose has been separated.^{13 (a)}

8. The principle area of pharmaceutical analysis by HPLC to quality testing of drugs and medicines, stability testing, therapeutic monitoring, drug metabolism studies and pharmacokinetic studies.

DRUG PROFILE

FURAZOLIDONE^{14(a)}

CAS NO: 67-45-8	MW :225.16
	MF :C₈H₇N₃O₅
	MP :255-259°C
	CATEGORY: Anti infective

Chemical Formula:

3 - { [(5-nitrofuranyl) methylene] amino }-1, 3-oxazolidin-2-one

Physical and Chemical Properties:

Physical state - yellowish crystalline powder
Odour - Odourless or almost odourless
Solubility in water - 60 mg/l

Solubility:

Almost insoluble in water and ethanol, slightly soluble in chloroform, soluble in Dimethyl formamide.^{15(a)}

Pharmacokinetics:

Ash colored metabolites is found in the urine, it is clearly absorbed to some extent. Because furazolidone is used to treat enteric infections, absorption becomes important only when discussing adverse reactions and drug interaction issues. Furazolidone is reported to be

distributed into the CSF. Absorbed furazolidone is rapidly metabolized in the liver and the majority of absorbed drug is eliminated in the urine. ¹⁶

Half life:

10 Minutes

Mechanism of Action:

Furazolidone has a broad antibacterial spectrum which is active against susceptible organisms including E. coli, staphylococci, giardia sp. and against salmonella, shigella, proteus, and V. cholerae. Its bactericidal action is by interfering with bacterial enzyme systems.

Contra indication:

Hypersensitivity, infants <1 month, Alcohol. Special Precautions Pregnancy, lactation, G6PD deficiency.

Adverse Drug Reaction:

Hypersensitivity, hypo glycaemia, orthostatic hypotension, dizziness, drowsiness, headache, malaise, nausea, vomiting, rash, fever, arthralgia, brown urine, haemolytic anaemia.

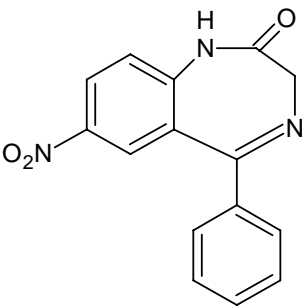
Dosage:

100-mg tablet four times daily.

Formulation:

- Tablet
- Capsule
- Oral suspension
- Aerosol powder

NITRAZEPAM^{14(b)}

CAS NO: 146-22-5	MW: 281.27
	MF : C₁₅H₁₁N₃O₃
	MP : 227 °C
	CATEGORY : Anti-anxiety agents Hypnotics & Sedatives

Chemical Formula:

1, 3-Dihydro-7-nitro-5-phenyl-2H-1,4-benzodiazepin-2-one

Physical and Chemical Properties:

Physical nature	- Light yellow crystalline powder
Odor	- odorless
Taste	- Tasteless

Solubility:

Soluble: In Acetone, Chloroform

Insoluble: In Water, Ethanol, Ether^{15(b)}

Pharmacokinetics:

Nitrazepam is largely bound to plasma proteins Benzodiazepines such as nitrazepam are lipid soluble and have a high cerebral uptake. The time for nitrazepam to reach peak

plasma concentrations is about 2 hours. Nitrazepam breaks down into a nitro benzophenone and quinolone compound. Nitrazepam has a much longer half life in the cerebrospinal fluid. Concomitant food intake has no influence on the rate of absorption of nitrazepam or on its bioavailability. Therefore nitrazepam can be taken with or without food.¹⁶

Half Life:

10 Minutes.

Mechanism of action:

Benzodiazepines act selectively on GABA - A receptor which mediate fast inhibitory synaptic transmission throughout CNS, Benzodiazepines enhance the response to GABA by facilitating the opening of GABA activated chloride channel. They bind specifically to a regulatory site of the receptor.^{17 (a)}

Contra indication:

Myasthenia gravis, narrow-angle glaucoma, severe respiratory insufficiency, sleep apnoea syndrome, severe hepatic impairment and porphyria

Adverse drug reactions:

Hypotension, palpitation; agitation, aggressiveness, amnesia, ataxia, confusion, delusions, disorientation, dizziness, fatigue, hallucination, hangover, headache, irritability, nightmares, psychoses, rage, restlessness, sedation; rash; changes in libido; constipation, diarrhoea, excessive salivation, heartburn, nausea, vomiting; granulocytopenia, leukopenia; falling, muscle weakness; blurred or double vision; tinnitus (associated with withdrawal); aspiration, increased bronchial secretion, dyspnoea¹⁸

Dosage:

5mg at bed time

Formulations:

- Tablet
- Capsule

LITERATURE REVIEW

FURAZOLIDONE

Furazolidone is official in Indian pharmacopeia^{15 (b)}, British pharmacopeia^{23 (a)}, and in United States^{24 (a)} Pharmacopeia. Official assay methods reported that bulk drug and its formulations were quantified spectrophotometrically by measuring the absorbance at 367nm. The literature also reports some spectrometric and chromatographic methods that are given below:

- ◆ **Chen Li – Xin et. al.**, synthesized an aminobenzthiazole as a new fluorescent carrier. It was utilized for preparing an optical sensor, for determining furazolidone by fluorescence quenching. This sensor showed satisfactory results in reversibility, repeatability, and selectivity. The range and detection limit of the sensor are 1.0×10^{-6} - 1.0×10^{-3} M and 6.0×10^{-7} M. This sensor could be used for the direct determination of furazolidone in pharmaceutical preparations and urine samples.²⁵
- ◆ **LI Wei-bo, ZHANG Guo-wen et. al.**, investigated the interaction between furazolidone and bovine serum albumin (BSA) in physiological buffer solution (pH 7.4) by fluorescence spectroscopy and UV –Vis absorption spectra. The results showed that there was a strong fluorescence quenching of BSA by furazolidone. The probable quenching mechanism of fluorescence of BSA by furazolidone was a static quenching by forming the BSA –furazolidone complex.²⁶
- ◆ **C.V.N. Prasad et. al.**, developed a second- derivative spectrophotometric procedure for the simultaneous determination of tinidazole, furazolidone, diloxanide furoate in a commercial preparation. Tablet was extracted in distilled water and then determination of analyte concentration was carried out by zero-crossing (ZC) and ratio-compensation (RC) techniques. Linearity was obeyed in the concentration range of 2.5-10 mcg ml⁻¹. The results were accurate and free from interference.²⁷
- ◆ **V. Bharadwaj, et. al.**, established a derivative spectrophotometric procedure for the simultaneous determination of tinidazole and furazolidone in combined formulations. Tablet was extracted in dimethyl formamide and ratio of the analyte concentration in the mixture was determined by the compensation technique. Zero crossing point was utilized to compare the technique.²⁸

- ◆ **XIE Qing-juanl, ZHOU Lin 2, TANG Qian, et. al.,** determined furazolidone tablets by HPLC. Using Hypersil ultimate column and mobile phase Methanol-Water at a flow rate of 1.0 ml /min. The detection wavelength was 364nm. Linearity was obeyed in the range of 20-300mcg. The average recovery was 100.9%. The method is simple, reproducible, and suitable for the quality control of furazolidone tablets.²⁹
- ◆ **Miao Shu et. al.,** determined the content of furazolidone and doxycycline in compound Weikening capsules by HPLC. Analytical column packed with YWG, C18 was used and the mobile phase consisted of 0.025% sodium laurylsulfonate methanol, 0.025%mol/L sodium dihydrogen phosphate and triethylamine (38:62:04) at pH 3. The detection wavelength was at 259nm. The average recovery of furazolidone was 100.2%. This method was simple, accurate and highly sensitive.³⁰
- ◆ **Zhang Y et. al.,** developed a RP-HPLC method with gradient elution to separate and determine furazolidone, carbenoxolone sodium and berberine hydrochloride in Wei Kang Tablets. This method utilized Nova-Pak C18 column and 3, 5-dinitro benzoic acid as the internal standard. Mobile phase was acetonitrile-phosphate buffer solution (pH 7.0). Linearity was obeyed in the concentration range of 141.2mg/L-1,270.8mg/L. The recovery and RSD s were 99.5% and 1.38 % respectively for furazolidone.³¹
- ◆ **Nickos A. Botsoglou et. al.,** developed a high- performance liquid chromatographic method to determine the furazolidone in eggs. Egg homogenate was acidified and extracted with dichloromethane. After solvent evaporation extract was subjected to clarification. HPLC determination was performed on a reversed phase C₈ column. The accuracy was found to be 92.9%.³²
- ◆ **S.M Hassan , F. A Ibrahim et. al.,** developed stability-indicating HPLC assay for the determination of furazolidone with some other pharmaceutically important nitro compounds. Method was developed on a Lichrosorb RP 18 column with methanol – water- buffer pH 3 (40:55:5) and detection at 365, 375, 367,368, 340nm. Recovery from bulk drugs were 99.94-100%.³³
- ◆ **T Galeano Diaz, A Guiberteau Cabanillas et. al.,** established a HPLC method with coulometric detection to separate the three nitro furan derivatives, nitrofurantoin, furazolidone, and furaltadone in milk. Nova-pak C18 column had been used.

Acetonitrile-0.1M aqueous solution sodium per chlorate (28:72) with 0.5% glacial acetic acid was used as the mobile phase. Nitrogen stream was used to remove the oxygen from the samples. The method was used with good results for the determination of three compounds in milk.³⁴

- ◆ **Smallidge RL et. al.**, developed a HPLC method for the determination of furazolidone in feed and feed premixes. Furazolidone was separated from finished feeds and the residue was dissolved in dimethyl formamide-5%tetra ethyl ammonium bromide. Sample was injected on a C₁₈ reverse phase column with acetonitrile-2% acetic acid as a mobile phase and detected at 365 nm. The developed method was compared with the AOAC colorimetric assay. Recovery was 97.5% and linearity was excellent up to 0.06% of furazolidone.³⁵
- ◆ **A Valadez- Salazar et. al.**, measured concentrations of furazolidone in several biological fluids by HPLC method. This was a rapid and sensitive method for the quantification of furazolidone in biological fluids. The minimum detectable concentration was 0.05mg/l, with a precision of 6% from peak area and an average recovery of 98%.³⁶
- ◆ **L. Lopez Martinez et. al.**, described a new and rapid method to determine furazolidone, nitrofurantoin and furaltadone in formulations, feed and milk by RP-HPLC method. A high speed C₁₈ 3*3 column was used with a mobile phase of HAC/NaAC was found to be the optimum to carry out the analytical separation. A photometric detection at 360nm was selected.³⁷
- ◆ **J. Robert et. al.**, determined furazolidone in animal feeds by LC with U.V and thermo spray and mass spectrometric detection. The LC-UV Procedure was used to quantify furazolidone at medicated levels (200mg/kg) and at contaminated levels of 5 and 20mg/kg. Overall mean recoveries from fortified samples were, 93.4, 98.2and 98.0% at concentrations of 5, 20and 200mg/kg, respectively. The mass spectrometric procedure was used to analyse sample extracts at concentrations <5mg/kg. Therefore this assay method was capable of determining the furazolidone in animal feeds at both high medicated concentrations and at low concentration.³⁸

- ◆ **N. M. Tendolkar et. al.**, developed a HPTLC & HPLC methods for the determination of tinidazole and furazolidone in suspension. The following chromatographic conditions were used:

HPTLC:

St. Phase	- Glass plate coated with silica gel 60 _{F254}
M. Phase	- Chloroform: Methanol: Ammonia (9:1:0.1%v/v)
R _f Value	- 0.63

HPLC:

Column	- Bondapak C ₁₈
M. Phase	- Water: Acetonitrile: Triethyl amine (80: 20: 0.1%v/v)
R _t Time	- 7.82 min ³⁹

- ◆ **SHEN Mei-fang et. al.**, established an ELISA method for the estimation of furazolidone metabolites in aquatic products. Samples were derivated with 2- nitro benzaldehyde and extracted with ethyl acetate. Then they were purified with n-hexane and determined by ELISA. The average recoveries were 99.2-100%. The relative standard deviation for intra – assay were 1.76-12.57% and co efficient of variation for inter- assay were 5.43- 8.58%. Three positive samples were analysed by ELISA and confirmed by LC-MS. No false positive results were found.⁴⁰
- ◆ **D. A. Volotskaya et. al.**, developed a method for assaying nitro furan drugs (furazolidone, furadonine, furagin) using an amperometric biosensor based on a printed platinum electrode and immobilized monoamine oxidase was developed for analysis of medicinal formulations and urine. Nitrofurans derivatives were found to have inhibitory action, weaker than those of classical tricyclic antidepressants.⁴¹

NITRAZEPAM

Nitrazepam is official in Indian pharmacopeia 15^(c) and in British pharmacopeia^{23 (b)}. Official method reported that bulk drug of nitrazepam were assayed by potentiometrically. Formulations were assayed by spectrophotometrically in which absorbance of methanolic HCl solution was measured at 280nm. The literature also reports some of spectrometry and chromatographic methods are given below

- ◆ **WANG Zhi- hong, HUANG Yong-gui et. al.**, developed a new method for the determination of nitrazepam by photochemical fluorescence analysis (PCFA). In weak alkaline medium (pH=9.5), photochemical reaction of nitrazepam can occur under the irradiation of U.V light and produce a fluorescence product. The fluorescence intensity was linearly increased with the concentration of nitrazepam over the range of 0.25-4µg/ml. The relative standard deviation and detection limit were 2.0% and 45ng/ml. ⁴²
- ◆ **Salwa Risk El-Shabouri**, reported a sensitive spectrophotometric method for the determination of nitrazepam either in pure form or in tablets. Reduction of nitrazepam with zinc dust followed by reaction with pentacyanoaminoferrate (II) gave a violet product having an absorbance maximum at 560nm. Beer's law was obeyed over the concentration range 1-20µg/ml. The recovery and precision were similar to that of the B.P method. ⁴³
- ◆ **M. I. Walash et.al.**, reported a colorimetric method for the determination of six nitro derivatives, including nitrazepam and furazolidone. Orange to purple colour was developed when these nitro compounds reacted with tetra butyl ammonium hydroxide in dimethyl formamide. The mean percentage recoveries ranged from 98.9- 100.4. The proposed method was also applied to the determination of furazolidone and nifuroxime in mixture. ⁴⁴
- ◆ **F. Randez-Gil et. al.**, developed a direct derivative spectrophotometric method for the determination of nitrazepam and clonazepam in biological fluids. A fifth order derivative spectra was used to determine nitrazepam in urine at 288nm while fourth order derivative spectra was used for its determination in plasma at 402nm. A Sixth order derivative spectra was utilized to determine clonazepam in urine sample at 384nm while fourth order derivative spectra was used for its determination in plasma in the same wavelength. ⁴⁵
- ◆ **Karen Mollen Jensen**, developed a gas chromatographic method with electron capture detection for the analysis of nitrazepam in urine. Nitrazepam was isolated from serum and converted in to 2-amino-5-nitrobenzophenone by acid hydrolysis. N-DesMethyl diazepam was used as internal standard. The limit of detection was 5ng/ml

of nitrazepam in serum. Recovery experiments showed that the method was quantitative.⁴⁶

- ◆ **Mavis S. Greaves**, described accurate methods for the qualitative and quantitative determination of nitrazepam, diazepam, and medazepam in whole blood by flame-ionization gas-liquid chromatography. Medazepam and diazepam were analyzed intact and nitrazepam was chromatographed as its trimethylsilyl (TMS) derivative by flame-ionization gas-chromatography on “1% OV-17”. A Supplementary column of “2% OV-1” was used to separate nitrazepam TMS from diazepam when both were present in same extract.⁴⁷
- ◆ **Lauri Kangas**, developed a gas-liquid chromatographic method for the determination of nitrazepam and its metabolites in urine with the use of electron capture and nitrogen-selective detector. Nitrazepam and the metabolites were extracted with benzene dichloromethane from a 1ml sample. Methylnitrazepam and methylbromazepam served as internal standards. The detection limits were about 0.2ng/ml for nitrazepam and 50ng/ml for the metabolites. Recoveries were better than 90%.⁴⁸
- ◆ **Ho, Paul C, Triggs et. al.**, reported a rapid and simple HPLC for the analysis of nitrazepam in plasma with temazepam as an internal standard. Alternatively, this assay method can also be used to determine temazepam in plasma concentrations with nitrazepam as the internal standard. Chloroform was used as an organic solvent for the extraction process. The percentage recoveries of nitrazepam and temazepam were 79% and 85%.⁴⁹
- ◆ **Mehdi Bakavoli, Massoud Kaykhaiir**, described a reliable assay for diazepam, nitrazepam and flunitrazepam in tablets by HPLC and TLC- densitometry. Tablet powder was sonicated in methanol, mixed with appropriate internal standard, filtered and either injected directly into the liquid chromatograph, or spotted on a silica gel thin-layer plate.⁵⁰

HPLC:

Column : C₁₈ reverse phase column

Mobile phase : 1:1(v/v) mixture of methanol (40⁰C) and
0.01M phosphate buffer

Detector : UV 254nm

TLC:

Mobile phase : Chloroform-acetone (9:1)

The TLC densitometry, yielded slightly higher values than the HPLC method, was preferred due to its simplicity, ease and low cost.

- ◆ **G. P. Beharell et. al.**, developed a sensitive GC method for the analysis of nitrazepam in plasma, using clonazepam as an internal standard. After extraction, the benzodiazepines were hydrolysed, and chromatographed as the benzophenones. Drug recovery from plasma was quantitative (>95%) and the sensitivity limit of detection was about 0.1ng/ml in plasma.⁵¹
- ◆ **B. Moore, G.Nickless et.al.**, reported HPLC method for the analysis of nitrazepam and its metabolites. Nitrazepam was converted into its methyl derivative and chromatographed on silica phases and eluted with a solvent mixture of ethyl acetate-hexane (3:7) at flow rate of 1ml/ min.⁵²
- ◆ **Tadashi kozu**, reported HPLC method for the determination of nitrazepam and its metabolites in human urine. Nitrazepam and its metabolite were extracted from the urine sample. The following chromatographic conditions were used,

Column : Steel column packed with Nucleosil -5 C₁₈

Mobile phase : Methanol: water-(35:65)

Flow rate : 1.3ml/min

Detector : UV at 254nm⁵³

- ◆ **Matthias kleinschnitz et. al.**, established a sensitive and selective HPLC method to determine diazepam, nitrazepam, flunitrazepam, and medazepam in human serum and in urine by HPLC- electro spray tandem mass spectroscopy (HPLC-ESI-MS). Diazepam was used as an internal standard. Recoveries were between 90.4% and 109.7%. The routine quantification limit was set at 2ng/ml for serum.⁵⁴
- ◆ **Einosuke Tanaka et. al.**, developed RP-HPLC method for the simultaneous analysis of 12 frequently used benzodiazepines (BZPs).

Column : Hypersil ODS-C₁₈

Mobile phase : Methanol-phosphate buffer (45:55)

Flow rate : 0.65ml/min

Detector : UV at 254 nm⁵⁵

- ◆ **C. Pistos et. al.**, developed HPLC method to determine selected benzodiazepines in plasma. Linearity was obeyed in the concentrations range of 0.5- 18µg/ml. The following chromatographic conditions were used.

Column : Hisep column

Mobile phase : ACN- 0.18 M ammonium acetate pH 2.5 (15:85 v/v)

Flow rate : 2ml/min⁵⁶

- ◆ **Kazutoshi Suzuki et. al.**, reported HPLC method to determine nitrazepam in plasma and its application to pharmacokinetic studies in rat.

Column : LC – 6A chromatographic system packed with
Zorbax ODS

Mobile phase : Acetonitrile: Phosphate buffer (2:3 v/v) pH: 3

Detector : UV at 221 nm.⁵⁷

- ◆ **E. N. Sauv , M. Langodegard et. al.**, developed a solid supported liquid-liquid extraction and UPLC-MS/MS (ultra performance liquid chromatography tandem mass spectrometry) method for the determination of benzodiazepines in ante-mortem and post-mortem in whole blood. A 500µl aliquot of whole blood was saturated with borate buffer pH 11, extracted and evaporated at 40°C. Reconstitution was made with acetonitrile: 5mM ammonium acetate buffer pH 5(25:75). Sample was injected in to the UPLC-MS/MS system having the following chromatographic conditions.⁵⁸

Column : UPLC BEH C18 Vanguard pre-column

Flow rate : 0.6ml/min

Mobile phase : Acetonitrile: 5mM ammonium acetate buffer (pH -5)

- ◆ **Boukhabza A, Lugnier A. A. J et. al.**, developed a simultaneous HPLC method for the determination of hypnotic benzodiazepines Nitrazepam, Estazolam, Flunitrazepam and triazolam in plasma. Biological samples and calibrated standards were extracted at basic pH in to diethyl ether- methylene chloride (2:1 v/v). The reconstituted extract

was separated on a Novapak C18 column with acidic buffer (6mM) - acetonitrile-methanol (64:23:13 v/v). The detection was performed at 242nm.⁵⁹

INTRODUCTION TO PRESENT STUDY

Fluorescence:

Fluorescence is a photoluminescence process in which atoms or molecules are excited by absorption of electromagnetic radiation. The excited species then relax to the ground state, giving up their excess energy as photons.

One of the most attractive features of molecular fluorescence is its inherent sensitivity, which is often one to three orders of magnitude better than absorption spectroscopy. For selected species under controlled conditions, single molecules have been detected by Fluorescence spectroscopy. Another advantage is the large linear concentration range of fluorescence methods, which is significantly greater than those encountered in absorption spectroscopy.^{19(a)}

Introduction:

An electronically excited molecule, that does not undergo any chemical reaction, has to get rid of its excess energy in order to return to the ground state. The ways of energy release are divided into two categories; Radiative and non-radiative transitions according as energy is released as light. The energy of the emitted radiation is usually lower than the excitation energy since in solution the energy resulting in vibrational excitation is rapidly absorbed by the solvent. When the molecule arrives at the lowest vibrational level and the solvent is not capable of accepting such a big quantum of energy as would be necessary in order to return to the ground state, the energy is emitted as photons.

Applications:

- ❖ Molecular fluorescence is used in various applications; many clothes and other gadgets are colored with fluorescing dyes in order to achieve desired effects in the U.V-light of a discotheque.

- ❖ The government and the police using fluorescing molecules in documents and money for U.V-light authenticity checks.
- ❖ In the chemical laboratory fluorescence is a valuable effect for the qualitative as well as quantitative analysis of certain substances. In addition fluorescence is used to visualize the dots on a “TLC” plate or as a detector in chromatography.

Fluorescence Spectroscopy:

Fluorescence spectroscopy has assumed a major role in analysis, particularly in the determination of trace contaminants in our environment, industries and bodies. Because for applicable compounds fluorescence gives high sensitivity and high specificity.

High sensitivity results from the differences in wavelengths between the exciting and fluorescence radiation. High specificity results from dependence on two spectra, the excitation and emission spectra.

The apparatus for fluorescence spectroscopy consists of a light source, (usually U.V light) of which a certain excitation wavelength is selected with a monochromator. The resulting monochromatic light is passed through the sample cuvette. The emitted light is measured orthogonal to the incoming beam in order to minimize the measurement of excitation radiation which was not absorbed or scattered from the sample. With the use of another monochromator one wavelength of the emitted light is selected and detected with a photomultiplier tube. The measured signal is transferred to a read out. ^{19(b)}

Development of Fluorimetric Method:

Fluorescence in a molecule is brought about by absorption of photons. The molecule is promoted to a higher electronic state called singlet excited state. The excited molecule returns to the ground state by emitting longer wavelength radiation. The phenomenon can occur in certain molecules in dilute solutions upon excitation by suitable wavelength.

In the second type, the emission spectrum of an excited species is formed in course of a chemical reaction. The excited species is the product of a reaction between the analyte and a suitable reagent. ^{6(b)}

The equations for the process of energy transfer were derived for conditions where the distance between donor and acceptor is large compared to molecular dimensions and does not change within the life time of the excited state of the donor.

When the life time of the donor is long relative to the diffusion processes the equations must be modified.²¹

On the above basis, a number of organic fluorescent compounds were tried as the “Donor Molecule” suitable for quenching experiments. Out of which quinine sulphate, anthranillic acid, and Naphthyl ethylene diamine dihydrochloride have been used in this experiment.

AIM OF PRESENT STUDY

Furazolidone and Nitrazepam were selected for the indirect fluorimetric determination.

Furazolidone is official in IP, BP, and USP. Determination of furazolidone in official method is based on the measurement of absorbance in aqueous DMF at 367nm and use of specific extinction coefficient value.

Literature review reveals that, different technique such as second-derivative spectrophotometry, quenchofluorimetric methods, ELISA and chromatographic methods like HPLC and GC have been utilised for the determination of furazolidone in formulations and in biological matrix.

Nitrazepam is official in IP and in BP. Pharmacopoeial methods use UV spectrophotometric method, absorbance being measured at 280nm in methanolic HCl medium.

The literature survey reveals that, different analytical techniques for the determination of nitrazepam in pure form, formulated drugs and the drug in biological samples by PCFA, UV, colorimetric method, chromatographic methods such as HPLC, GC and hyphenated techniques like LC-MS, UPLC- MS/MS are available.

CONCLUSION

Furazolidone and nitrazepam were selected for the quenchofluorimetric determination. It is an indirect spectrofluorimetric method.

- ♣ The method has been found to be sensitive one, as the technique is being a type of spectrofluorimetric method

These developed methods could be used in establishing the quality control of **furazolidone tablet and nitrazepam tablet**.