THE DEVELOPMENT OF ZINC (II) SELECTIVE FLUORESCENT LIGANDS

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CONTENTS

Appendices

ABBREVIATIONS

APTE	methyl 2-(2-aminophenyl)-2-thiazoline-4-carboxylate
DNA	deoxyribonuclease
e.m.f.	electromotive force
ether	diethylether
FAB	fast atom bombardment
FLDM	fluorescence data manager
HMPA	hexamethylphosphoramide
NaPIPES	sodium piperazine-N,N'-bis-(2-ethanesulfonic acid)
n.m.r.	nuclear magnetic resonance
POEE	ethyl 2-(2-hydoxyphenyl)-oxazole-4-carboxylate
PTA	2-(2-hydoxyphenyl)-2-thiazoline-4-carboxylic acid
PTEE	ethyl 2-(2-hydoxyphenyl)-thiazole-4-carboxylate
PTME	methyl 2-(2-hydoxyphenyl)-2-thiazoline-4-carboxylate
RNA	ribonuclease
tlc	thin layer chromatography
THF	tetrahydrofuran

ABSTRACT

This thesis describes the development of Zn^{2+} selective ligands suitable for use as fluorescent probes to monitor exchangeable Zn^{2+} in biological systems. A related aim was to develop fluorescent probes for other metal ions of biological concern, such as Al^{3+} and Pb^{2+}.

A series of prospective ligands were synthesised based on the phenyl substituted thiazoline substructure of 2-(2-*o*-hydroxyphenyl-2-thiazolin-4-yl)-3-methylthiazolidine-4-carboxylic acid (pyochelin), a naturally occurring fluorescent Zn^{2+} ligand. 2-(2-Hydroxyphenyl)-2-thiazoline-4-carboylic acid and the corresponding methyl ester were synthesised along with the methyl esters of the corresponding 2-methoxyphenyl and the phenyl substituted thiazolines. The ethyl esters of the analogous thiazole carboxylic acids were also synthesised.

The chelation of these ligands to Zn^{2+} was investigated using ultraviolet spectroscopy. The selectivity of the Zn^{2+} chelating ligands was investigated using a chosen series of metal ions and ultraviolet spectroscopy. The stability of the Zn^{2+} and other complexes formed was assessed by determining values for the stability constants of formation of the complexes, using a potentiometric titration technique. Fluorescence spectroscopy was utilised to establish the fluorescent nature of the complexes.

Only the ligands which contain the 2-hydroxyphenyl substituent chelated to Zn^{2+}. These appear suitable for use as Zn^{2+} probes, since they each form stable fluorescent Zn^{2+} complexes and do not form fluorescent complexes with biologically prevalent metal ions.

2-(2-Hydroxyphenyl)-2-thiazoline-4-carboxylic acid and the corresponding methyl ester both form highly fluorescent complexes with Al^{3+} making them suitable

for use as fluorescent Al^{3+} probes.

The 2-hydroxyphenyl substituted thiazolines provide a basis for further development of Pb^{2+} fluorescent ligands, since their Pb^{2+} complexes were of high stability, but were non fluorescent.

Ethyl 2-(2-hydroxyphenyl)-oxazole-4-carboxylate and methyl 2-(2-aminophenyl)-2-thiazoline-4-carboxylate were also synthesised and their Zn^{2+} chelation and selectivity along with the fluorescence of complexes formed by these ligands were assessed. Only the Zn^{2+} complex formed by the oxazole was fluorescent. This ligand appears suitable for use as a biological Zn^{2+} probe. The 2-aminophenyl substituted thiazoline did not form fluorescent complexes with any of the metal ions tested, indicating that the 2-hydroxyphenyl substituted thiazole derivatives are more suited as biological Zn^{2+} probes.

DECLARATION

This work contains no material which has been accepted for the award of any other degree or diploma in any university or other tertiary institution and, to the best of my knowledge and belief, contains no material previously published or written by another person except where due reference has been made in the text.

I give my consent to this copy of my thesis, when deposited in the University Library, being available for loan and photocopying.

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1 The Need for a Probe for Biological Zinc (II)

1.1 The role of Zinc (II) in Human Biology

1.1.1 Clinical Aspects of Zinc (II)

Zinc (II) is an essential trace element for many forms of life.[1] Of the essential trace elements found within the human body, Zn^{2+} is nearly as abundant as Fe^{3+}.[2] Clinically, Zn^{2+} has been reported as being essential for the normal functioning of a variety of life processes.[1,3-10] Many of these reports are concerned with the various disorders of both physical and mental health, which have been related to a deficiency of Zn^{2+}.

Some of the physical disorders attributed to Zn^{2+} deficiency include growth retardation, skin problems and delayed sexual development. Prasad[5] found that Zn^{2+} deficiency was common in male patients from the Middle East, who had symptoms of dwarfism and hypogonadism. Skin disorders, such as acrodermatitis enteropathica, have also been attributed to a deficiency of Zn^{2+}.[1,5] Studies[1,5] conducted on patients suffering from such afflictions showed that the patients' conditions were improved greatly by the administration of dietary Zn^{2+} supplementation.

Reduced acuity of the senses of taste and smell, as well as vision abnormalities have also been observed in people who have a deficiency in Zn^{2+}.[1,5,10] Along with these, Zn^{2+} deficient patients have been found to suffer from mental lethargy and emotional disorders. The eating disorder anorexia nervosa tends to result in a deficiency of Zn^{2+} in the sufferer, which is then believed to escalate the symptoms of the disorder.[1] Once again, studies[1,5,10] have shown patients with such disorders improve rapidly when Zn^{2+} is supplemented into their diets.

Studies on patients with Zn^{2+} deficiency and consequent dietary Zn^{2+} supplementation have shown that Zn^{2+} is required for a variety of aspects of physical and mental health. Zinc (II) has been found to have many therapeutic uses. Apart from its use in rectifying problems caused by a deficiency of Zn^{2+} mentioned above, Zn^{2+} has proven to be beneficial in the treatment of many ailments including various dermatological conditions, male infertility, arthritis and gastrointestinal disorders.[6] Zinc (II) also aids the functioning of the immune system.[6] It has been found that Zn^{2+} has antiviral, antibacterial, antiradiation and anticancer properties.[1] Due to its antibacterial properties, Zn^{2+} has become popular for use in toothpaste and other dental hygiene products, as it inhibits plaque growth and the anti-inflammatory properties of Zn^{2+} prevents swelling in gingivitis.[6,11]

Thus, Zn^{2+} is beneficial to humans in many areas of physical and mental health. The studies on the clinical effects of Zn^{2+} quoted here and their conclusions made have mostly been based on the effects of Zn^{2+} depletion and supplementation on patients. These studies, in turn have led to suggestions as to how Zn^{2+} is utilised within the body. To obtain a greater understanding of how and why Zn^{2+} is used, a closer examination of the biochemistry of Zn^{2+} within humans is needed.

1.1.2 Biochemical Role of Zinc (II) in Man

A majority of the total Zn^{2+} within a human body is intracellular,[7] existing mainly in the cytoplasm of cells. Intracellular Zn^{2+} is unevenly distributed in a cell, producing regions of relatively high Zn^{2+} concentration.[6] Nearly all organelles of all cells throughout the human body contain Zn^{2+} at low concentration.[7] Of the intracellular Zn^{2+}, a major proportion is tightly coordinated to various proteins and enzymes in the cell.[7] There are more than 200 metalloenzymes known to require Zn^{2+} for their functions.[1] These come from a variety of enzyme classes, including oxidoreductases, transferases, hydrolases and isomerases.[2] Zn^{2+} is the only trace

element known to be utilised by various metalloenzymes from all of the six differing types of enzyme categories.[12]

Zinc (II) appears to play a role in a wide variety of biochemical functions. Uses of Zn^{2+} in these processes range from catalytic, to regulatory, to inhibitory, to structural.[2] For instance, Zn^{2+} plays a structural role in deoxyribonuclease (DNA) proteins by binding to the various nitrogen, oxygen and sulphur donor atoms of histidine, cysteine and glutamic and aspartic acid groups present in the proteins, forming what are commonly referred to as zinc fingers, zinc clusters and zinc twists.[3] Zinc (II) is also utilised in the synthesis of both DNA and ribonuclease (RNA).[13]

Zinc (II) is required in all phases of the metabolic life cycle of a cell.[13,14] It has been determined that Zn^{2+} takes part in the replication of cells and in the growth, maturation and repair of body tissue.[1] Other roles of Zn^{2+} in the biochemistry of the human body include genetic expression, transport processes, reproduction, virology and immunology.[13,15]

One of the reasons why Zn^{2+} is so widely used in biological systems is that Zn^{2+} is a strong Lewis acid, which can act to polarise a bond, making it more susceptible to reaction.[2] Hydrated Zn^{2+} is amphoteric being able to exist in both the metal hydrate and hydroxide forms.[15] Therefore, Zn^{2+} can be utilised in many base catalysed enzymatic processes.[1] Zinc (II) can readily form complexes, which are of low coordination number, binding with nitrogen, sulphur and oxygen donor atoms. Such complexes usually undergo relatively rapid exchange and have geometries which can be easily deformed.[16] Hence, Zn^{2+} may be used to catalyse various processes, by aligning substrates for reaction. Another feature of Zn^{2+} which makes it favourable for use in biological systems is its chemical inertness to redox reactions.[16] This feature of Zn^{2+} has two major benefits. Firstly, the use of Zn^{2+} in DNA protein binding to form zinc fingers and other Zn^{2+} bound tertiary protein structures provides a stable crosslink

and structure which can exist under reducing conditions, unlike the disulfide crosslinks formed by cysteine units.[1] Secondly, Zn^{2+} enzymes can be used for processes where oxidation reactions must be avoided.[17]

Zinc (II) is associated with many of life's processes, and it is believed that Zn^{2+} acts as a trigger for many of these processes to occur.[7,16-18] This implies that there exists a readily available supply of Zn^{2+} within a cell for utilisation during its life cycle. As stated previously, most of the intracellular Zn^{2+} within the body exists in a protein-bound form.[7] The excess Zn^{2+} is thought to be free or at least loosely bound, existing in small readily exchangeable 'pools' throughout the cell.[18] It is believed a rapid flux of Zn^{2+} in these 'pools', that is a rapid uptake of Zn^{2+} from, or release of Zn^{2+} into these exchangeable 'pools', is caused when a Zn^{2+} triggered metabolic process occurs within a cell. If accurate measurement of location and concentration of freely available Zn^{2+} could be made, an indication of when and where the loosely bound Zn^{2+} is required in a cell's life cycle could be obtained. Such information would lead to a greater understanding of the role Zn^{2+} plays in human biology.

1.2 Measuring Biological Zinc (II)

1.2.1 Current Methods of Measurement

Atomic absorption spectrophotometry is the most common method of analysing Zn^{2+} concentrations in biological samples used presently.[19] This method measures Zn^{2+} levels in whole tissue[7] giving a measure of the total Zn^{2+} concentration within cells, but does not differentiate between the tightly bound Zn^{2+}, which is chelated to various proteins and enzymes in the cell, and any Zn^{2+}, which is loosely coordinated to acids or water molecules and is readily available for exchange. Thus no information is obtained about the subcellular distribution of Zn^{2+}.

Other techniques which have been utilised to study intracellular Zn^{2+} status include various colorimetric methods[6] using chelating agents such as diphenylthiocarbazone. Most of these techniques involve destruction of the sample cells prior to measurement. This provides an opportunity for the distribution of Zn^{2+} within the cell to rearrange before measurement, therefore casting some doubt on the accuracy of the results obtained. A colorimetric technique, using the chromogen 4-(2-pyridylazo) resorcinol, has proven to be a simple and useful method for the measurement of Zn^{2+} in blood serum.[20] However, measurement of extracellular Zn^{2+} has shown a poor correlation between Zn^{2+} levels and the clinical diagnosis of Zn^{2+} deficiency.[6]

Studies of the use of Zn^{2+} radioisotopes to investigate the absorption of Zn^{2+} and the requirements of Zn^{2+} by biological systems have also been undertaken.[6] The method involves monitoring subjects who are given an increased dietary intake of Zn^{2+} in the form of stable radioisotopes. The high quantities of Zn^{2+} and the specialised equipment required for such measurements render this technique quite costly and decrease its accessibility. Although the method should prove useful in the monitoring of Zn^{2+} absorption, it is not practical for studying the intracellular distribution of Zn^{2+}.

The techniques used for measuring Zn^{2+} in biological samples described above are not completely satisfactory methods, each possessing unfavourable aspects. A method which can be utilised for the detection and measurement of Zn^{2+} within living cells is required to obtain a greater understanding of the status of Zn^{2+} inside cells and the requirements of a cell for Zn^{2+} throughout its metabolic life cycle. Such a method must differentiate between Zn^{2+} in different binding sites within the cell, to provide a measure of the freely exchangeable Zn^{2+}. Information provided from such measurements would assist in developing a greater understanding of the biological role of Zn^{2+} in man.

1.2.2 Zinc (II) Measurement using a Fluorescent Probe

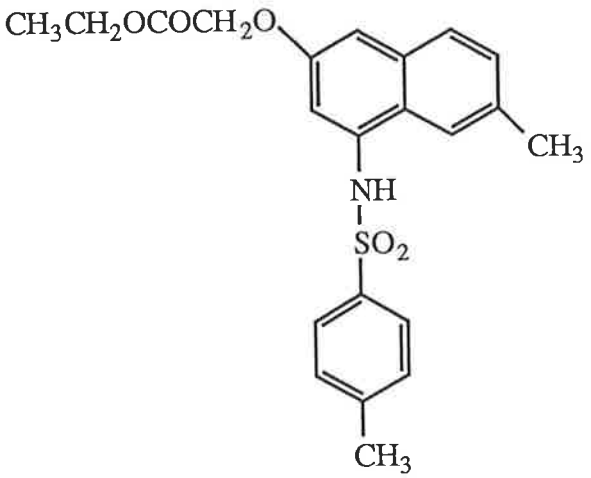
Fluorescent probes have been utilised for the detection and measurement of various metal ions in differing sample solutions.[21,22] Measurement of the quenching or enhancement of fluorescence of particular Zn^{2+} chelating agents has been utilised to determine the concentration of Zn^{2+} in various samples.[21-25] Fluorescent probes, specific for Zn^{2+}, would be useful tools for detecting and measuring the freely exchangeable Zn^{2+} within living cells.

Development of a suitable fluorescent probe for the measurement of Zn^{2+} within living cells requires certain criteria to be met. Firstly, the probe must traverse the outer membrane of a cell and once within the cell remain there to enable complexation of Zn^{2+} to occur and for measurement to be carried out. Secondly, the probe needs to bind specifically to Zn^{2+}, with a minimum interaction with other metal ions. As it is necessary to detect and measure the freely available Zn^{2+} within the cell, the probe must not compete strongly for Zn^{2+} which is already firmly bound to various metalloenzymes and proteins in the cell. Also, as fluorescence spectroscopy is to be the mode of measurement, the probe should produce a fluorescent species when bound to Zn^{2+}. It is preferable that the probe is non-fluorescent in its unbound form and produces a highly fluorescent Zn^{2+} complex, so as to avoid interference from any unbound probe remaining in the sample during measurement.

Forbes et al.[18,26] have described one such probe which they have used to successfully detect readily exchangeable Zn^{2+} within living cells. The probe, ethyl (2-methyl-8-*p*-toluenesulphonamido-6-quinolyloxy) acetate (1), referred to as Zinquin-E, appears to have the required specificity and sensitivity for Zn^{2+} and is retained by living cells. On loading Zinquin-E into cells, cellular esterases hydrolyse the ethyl ester moiety to the corresponding acid, known as Zinquin-A. Zinquin-A then chelates the readily exchangeable Zn^{2+} within the cell and, on excitation, an intense speckled

fluorescence pattern was observed indicating the heterogeneity of available Zn^{2+} within the cells.[18] This was taken as evidence in support of the belief that there exists pools of stored freely exchangeable Zn^{2+} throughout a cell. Thus Forbes and coworkers[18,26] had successfully demonstrated the use of a fluorescent probe for the direct detection and measurement of freely exchangeable Zn^{2+} within living cells.

Zinquin-A forms 1:1 and 2:1 complexes with Zn^{2+}, whose stepwise stability constants have been determined as $2.71 \pm 0.41 \times 10^6$ dm^3 mol^{-1} and $11.7 \pm 1.9 \times 10^6$ dm^3 mol^{-1} respectively.[18] Zinquin-A is a highly specific fluorophore for Zn^{2+} in comparison with other biologically prevalent metal ions. The optimal excitation and emission wavelengths for the Zn^{2+} complexes of Zinquin-A are 364 nm and 485 nm respectively.[18]



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Although Zinquin-A appears to be useful as a fluorescent probe for readily exchangeable Zn^{2+} in living cells, there are some disadvantages with its use. Firstly, the stability constants of the Zn^{2+} complexes formed by Zinquin-A are presumably low enough to ensure little or no interaction with tightly bound structural Zn^{2+} in metalloenzymes, which have binding constants in the order of 10^{12} - 10^{13}.[26] However, it is not known whether Zinquin-A interacts with non readily exchangeable Zn^{2+}, for

instance catalytic Zn^{2+} in metalloenzymes, whose coordination sites are not completely chelated by the enzyme.[26] Secondly, no Zinquin-A fluorescence has been observed in the nucleus of various cells tested.[18] It is thought that this may be either because Zinquin-E can not traverse the nuclear membrane or because ester hydrolysis occurs inside the cell so quickly that the ester is not able to reach the nuclear membrane before being hydrolysed.[18]

It has been suggested[27] that fluctuations in the amount of freely exchangeable Zn^{2+} within a cell, at various stages throughout the cell's lifecycle, may be monitored with the use of an ultraviolet laser and confocal microscope. A confocal microscope, which has an almost planar field of focus, has the benefit of being able to focus on cells lying in one particular plane at a given time. Due to limitations in the depth of focal field of other microscopes used in fluorescence microscopy, a two dimensional field of focus cannot be achieved. The fluorescence observed using these microscopes is from a number of cells, which lie within the focal depth of the microscope. A confocal microscope provides the ability to observe fluorescence from a single cell at a time. Therefore, by using a Zn^{2+} specific probe and confocal ultraviolet laser fluorescence microscopy, changes in the concentrations of labile Zn^{2+} within a living cell could be monitored directly.

For such a process to be most easily carried out, the fluorescent probe used requires an excitation maximum equivalent to the wavelength of the laser of the confocal microscope. The standard commercially available confocal microscope is equipped with a laser of 488 nm. A specialised confocal laser microscope was used in studies with Zinquin[27] which was specially fitted with an argon ion ultraviolet laser of wavelength 364 nm. For ease of detection and measurement, it is preferred that the probe fluoresces with an emission maximum well into the visible range. This way, interference due to scattering of light from the laser itself would be minimised.

Zinquin is not a completely satisfactory Zn^{2+} probe for use in confocal microscopy, since the low excitation wavelength of its complex requires a specially fitted ultraviolet laser. Also, the emission maxima of the Zinquin Zn^{2+} complex, 485 nm, occurs at almost the same wavelength of the laser of standard confocal microscopes, 488 nm. It is preferred to have a probe which has an excitation maxima of 488 nm and an emission maxima well into the visible region, so that it can be readily used with standard confocal microscopes.

An aim of the research carried out in this thesis was to develop different fluorescent probes specific for Zn^{2+}, which could be utilised in detecting and measuring readily available Zn^{2+} within living cells. In doing this it was hoped to produce a series of Zn^{2+} specific fluorescent probes, whose Zn^{2+} complexes all had different excitation and emission maxima and a varying range of suitable stability constants. From this series, a probe could be selected which had the optimum properties for the detection and measurement of freely exchangeable Zn^{2+} within living cells.

1.3 Fluorescent Probes for Other Metal Ions

Although the main interest of this research is concerned with developing a fluorescent probe specific for Zn^{2+}, development of probes specific for other metal ions has also been considered. The use of fluorescent probes for detecting and determining concentrations of a range of metal ions in biological systems is possible, so long as each particular metal ion has a fluorescent probe which binds specifically to that metal ion. Each fluorescent probe developed needs to meet the conditions outlined previously (sec. 1.2.2). Thus, the prospects for using fluorescent probes for the detection and measurement of metal ions in biological systems are quite broad. There is strong interest in developing fast and efficient methods for detecting metal ions which are known to be, or suspected as being, harmful to humans, such as Pb^{2+} and Al^{3+}.

For decades, the toxicity of Pb^{2+} to humans has been investigated. Much has been written on the harmful effects of Pb^{2+} and knowledge of the hazards of exposure to Pb^{2+} has become common.[28-30] Currently most analysis of Pb^{2+} content in biological samples involves the use of atomic absorption spectrophotometry.[19] There is a requirement for a fast, accurate and less expensive method for determining the Pb^{2+} content in biological samples, so that populations with a high risk of Pb^{2+} poisoning, such as young children living close to industrial regions, may have their Pb^{2+} tissue concentrations more readily monitored than they are today. A fluorescent probe which is specific for Pb^{2+} and which could be used to accurately measure the concentrations of Pb^{2+} in blood samples would serve as a useful analytical tool, fulfilling the need for a faster and more efficient method of monitoring Pb^{2+} levels in human populations.

The adverse effects of Al^{3+} in biological systems has become of concern in recent years. Aluminium (III) toxicity has been linked to patients with chronic renal failure[31,32] and there is an unresolved debate as to the relationship of Al^{3+} with the pathogenesis of Alzeihmer's disease.[33-36] There is a poor understanding of the mechanisms of Al^{3+} toxicity in biological systems and much research into the role of Al^{3+} in human biology has been reported.[31,32,37] As with Pb^{2+}, current methods of measuring the content of Al^{3+} in biological samples utilise atomic absorption spectrophotometry.[31] However, there has also been recent progress in the development of metal ion specific chelators for the *in vivo* determination of Al^{3+} content.[38-40] Once again, development of a fluorescent probe specific for Al^{3+} would prove useful in the biological detection and measurement of Al^{3+} and in obtaining the information required for the further understanding of the effects of Al^{3+} in humans.

As a secondary aim of this research, investigation into the specificity of any probes developed for metal ions such as Pb^{2+} and Al^{3+} has been undertaken. It was hoped that information obtained would lead to the development of fluorescent biological probes for these metal ions.

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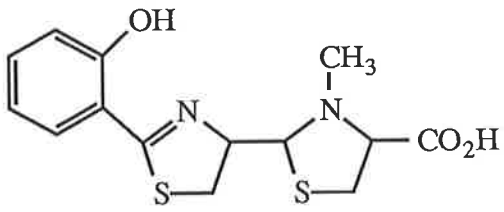
2 Design and Synthesis of Trial Ligands

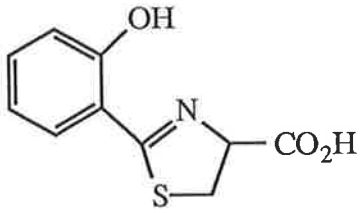
2.1 Pyochelin, a Model for Ligand Design

Pyochelin (2) is a phenolic siderophore produced by the opportunistic pathogen *Pseudomonas aeruginosa*.[1-8] Siderophores have a high affinity for Fe^{3+} and are produced and utilised by bacteria to aid in supplying Fe^{3+} to the microorganism.[1,7] Siderophores act by readily chelating to Fe^{3+}, solubilising it and, with the aid of specific cell membrane receptors, transporting the Fe^{3+} into cells,[1,7] thus, promoting the *in vivo* growth of the bacteria.[2,4,5,7]



2

Pyochelin, (2), is obtained from the ethyl acetate extracts of culture media of *Pseudomonas aeruginosa*[4] and has also been found in cultures of *Pseudomonas cepacia* and *Pseudomonas fluorescens*.[3,8] The structure of pyochelin has been determined as 2-(2-*o*-hydroxyphenyl-2-thiazolin-4-yl)-3-methylthiazolidine-4-carboxylic acid, (2).[6] The unbound form of pyochelin is fluorescent and rapidly degrades,[4] whereas pyochelin produces a non-fluorescent species when complexed to Fe^{3+}. Pyochelin promotes growth in pseudomonad pathogens but has been shown to be of low toxicity when injected into mice on its own.[7] Cuppels and coworkers[8] found that apart from readily chelating Fe^{3+}, pyochelin has a strong affinity for Zn^{2+}. Pyochelin has been shown to form a highly fluorescent stable dimeric complex with two Zn^{2+} ions. The structure of this pyochelin-Zn^{2+} complex is unclear, as is that of the pyochelin-Fe^{3+} complex, however both are thought to coordinate through the phenolic hydroxy group of at least one of the pyochelin ligands bound in the complex.[6,8]

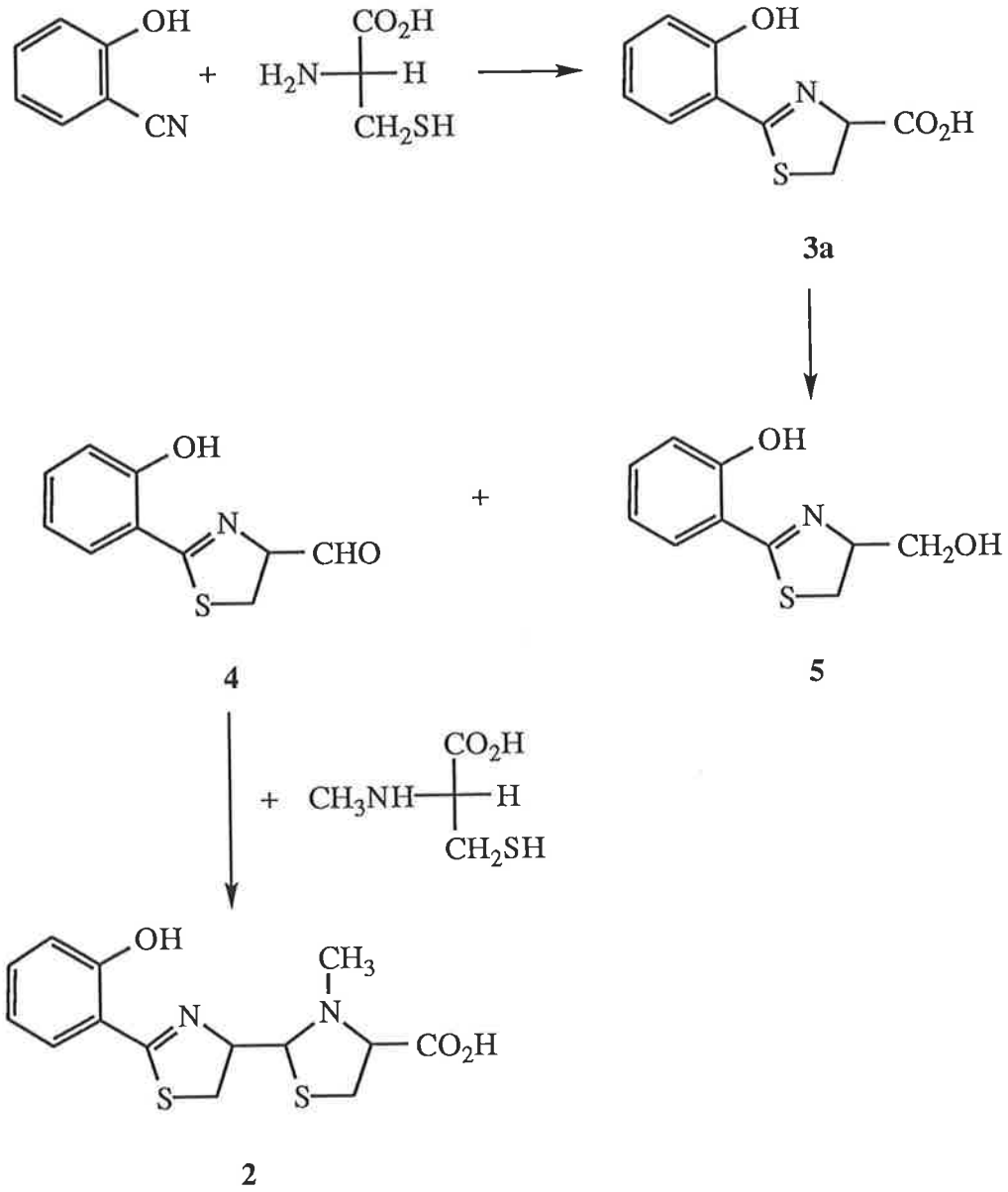


3a

The strong affinity of pyochelin for chelation with Zn^{2+}, the highly fluorescent Zn^{2+} complex it forms, the low toxicity of pyochelin and its demonstrated ability to cross cell membranes are all favourable qualities desirable for a fluorescent Zn^{2+} probe. For these reasons, pyochelin was chosen to be used as a model, on which the design of ligands for use as fluorescent Zn^{2+} probes was to be based. Clearly the chromophore of pyochelin is the conjugated phenolic substituted thiazoline ring, represented by the substructure (3a). As this substructure of pyochelin also contains the phenolic hydroxy group, which has been suggested as a coordination site for Zn^{2+}, it was decided to synthesise this compound for initial studies of fluorescence and Zn^{2+} binding properties. By making modifications to the structure of (3a), for example by removal or replacement of the hydroxy substituent on the phenyl ring with other, either coordinating or non-coordinating substituents, increasing the unsaturation in the heterocyclic ring or replacement of the heteroatoms in the ring with carbon or other heteroatoms, it was hoped to develop a series of compounds which could be assessed for their proficiency as biological Zn^{2+} probes. Results obtained from such studies were expected to give direction into the development of a fluorescent Zn^{2+} specific probe for use in biological systems.

2.2 Initial Ligand Synthesis

2.2.1 Selection of a Synthetic Route



Scheme 2.1

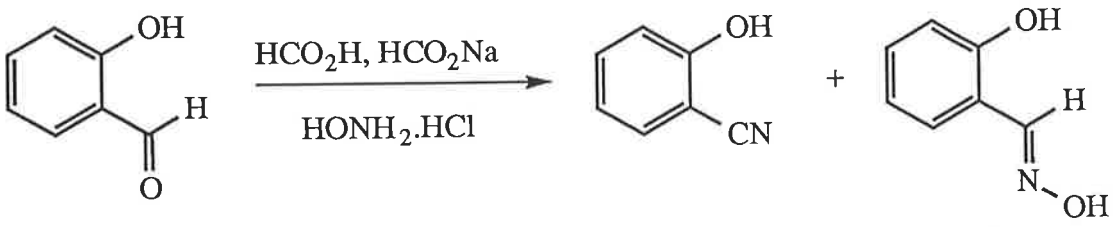
Akenbauer and coworkers[3] have described a synthesis for pyochelin which involves three main synthetic steps as shown in Scheme 2.1. Firstly, using a method reported by Mathur et al.,[9] salicylonitrile is condensed with L-cysteine hydrochloride in basic conditions, followed by reduction of the resulting thiazoline carboxylic acid, (3a),

to its corresponding aldehyde, (4). This is then treated with N-methyl-L-cysteine hydrochloride to give pyochelin, (2). This synthetic route is rather inefficient, preparing pyochelin in an overall yield of 5%. The poor overall yield is mainly due to the unstable aldehyde, (4), which is only obtained as a minor product, under mild reducing conditions. The aldehyde, (4), readily undergoes further reduction to the alcohol, (5).[3]

Initial interest was in synthesising the thiazoline, (3a), which is produced in high yield using the method described by Akenbauer et al.[3] Therefore, it was decided to follow the initial stage of the described reaction pathway to produce the desired thiazoline carboxylic acid, (3a), and various derivatives thereof.

2.2.2 Preparation of Thiazolines

In the reported synthesis of pyochelin,[3] the thiazoline carboxylic acid, (3a), was synthesised following a method established by Mathur and coworkers,[9]in which salicylonitrile was one of the starting materials. To follow this reaction procedure, salicylonitrile needed to be prepared.

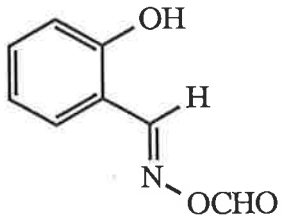


6

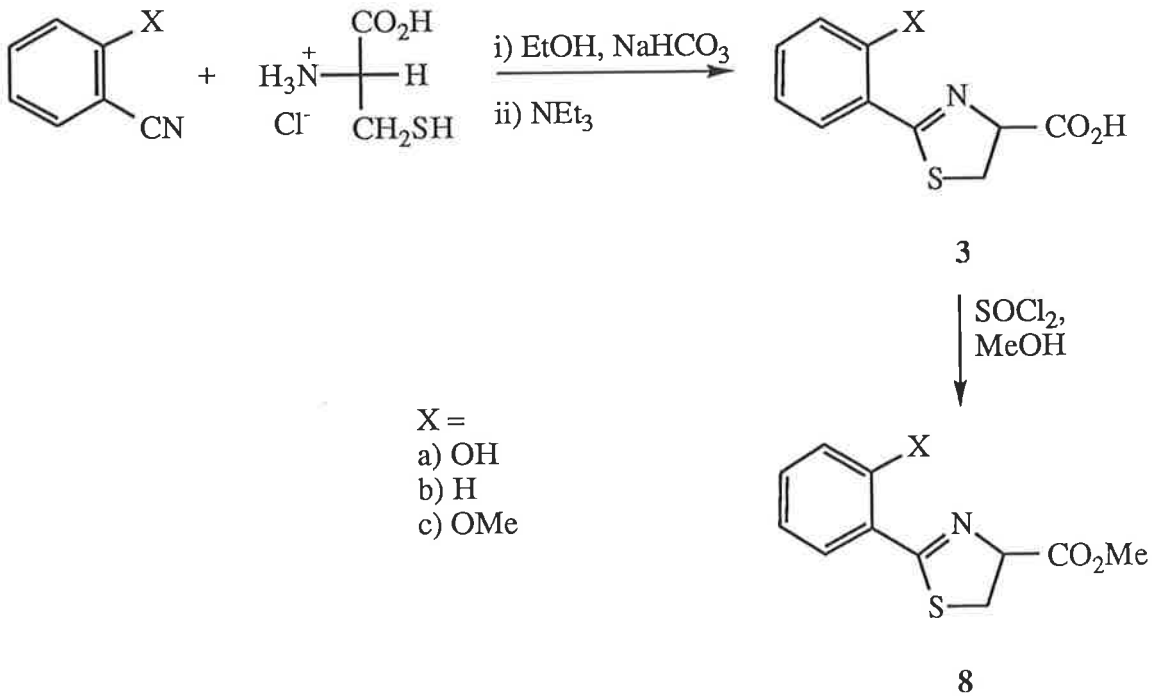
Scheme 2.2

Salicylonitrile was synthesised in high yield from the corresponding aldehyde, following a procedure described by van Es[10] (Scheme 2.2). A minor product, also obtained from the reaction, showed a molecular ion at *m/z* 137, suggesting that it was the oxime, (6), an intermediate formed in the reaction. Infrared spectral data supported this conclusion, in particular the peaks at 3372 cm^{-1} and 1620 cm^{-1} due to the O-H and

C=N stretches respectively(section 8.1.2). Van Es[10] proposed that the formation of the nitrile occurs via the oxime formate, (7). However, on following the reaction by thin layer chromatography, (tlc), only the oxime and the nitrile were detected in the reaction mixture. Thus, acid catalysed dehydration of the oxime is also a possible reaction pathway for formation of the nitrile.



7



Scheme 2.3

The salicylonitrile was then treated with L-cysteine hydrochloride using the method described by Mathur and coworkers,[9] (Scheme 2.3). The condensation is achieved through a two step process. Initially the two reagents are refluxed in absolute alcohol with one equivalent of sodium bicarbonate, allowing attack of the free amine on the nitrile carbon to occur. Then, the pH of the reaction mixture is increased to

approximately 9 by the addition of piperidine, producing an equilibrium amount of the deprotonated thiol which undergoes ring closure forming the thiazoline. As the need for piperidine appears to be only that of a base, it was replaced with triethylamine. This had no adverse effect on the reaction and the thiazoline carboxylic acid (3a) was obtained in 91% yield. The mass spectrum of the acid showed a molecular ion at m/z 223 and the ^1H n.m.r. spectrum showed three doublet of doublets at 5.23 ppm, 3.78 ppm and 3.60 ppm representing the H4 and H5 protons of the thiazoline ring respectively. The peaks at 3.78 ppm and 3.60 ppm showed characteristic geminal and vicinal coupling. Methylation of (3a) by treatment with thionyl chloride in anhydrous methanol then produced the ester, (8a), as a yellow oil which solidified on standing. Once again the product was identified by it mass spectrum with a molecular ion at m/z 237 and another peak at m/z 178 created by the characteristic loss of the methyl ester group from the molecular ion. The product was further identified by its ^1H n.m.r. spectrum which contained a broad peak at 9.24 ppm for the hydroxy proton and three doublet of doublet peaks at 5.34 ppm, 3.67 ppm and 3.58 ppm for the H4 and H5 protons of the thiazoline ring respectively.

To determine whether either of the thiazolines, (3a) and (8a), coordinated Zn^{2+}, the ultraviolet spectra of the thiazolines were recorded both in the presence and absence of Zn^{2+}. Comparison of the spectra of the thiazoline acid, (3a), collected under the specified conditions showed that, when its ultraviolet spectrum was recorded in the presence of Zn^{2+}, a bathochromic shift of the absorption maxima of the acid, (3a), occurred, as shown in Figure 2.1. This shift was taken as an indication that coordination of Zn^{2+} by the acid, (3a), was occurring in solution. A similar occurrence was observed when the absorption spectra of the thiazoline ester, (8a), measured whilst in the presence and absence of Zn^{2+}, were compared. Therefore, both of the thiazolines, (3a) and (8a), coordinated Zn^{2+}.

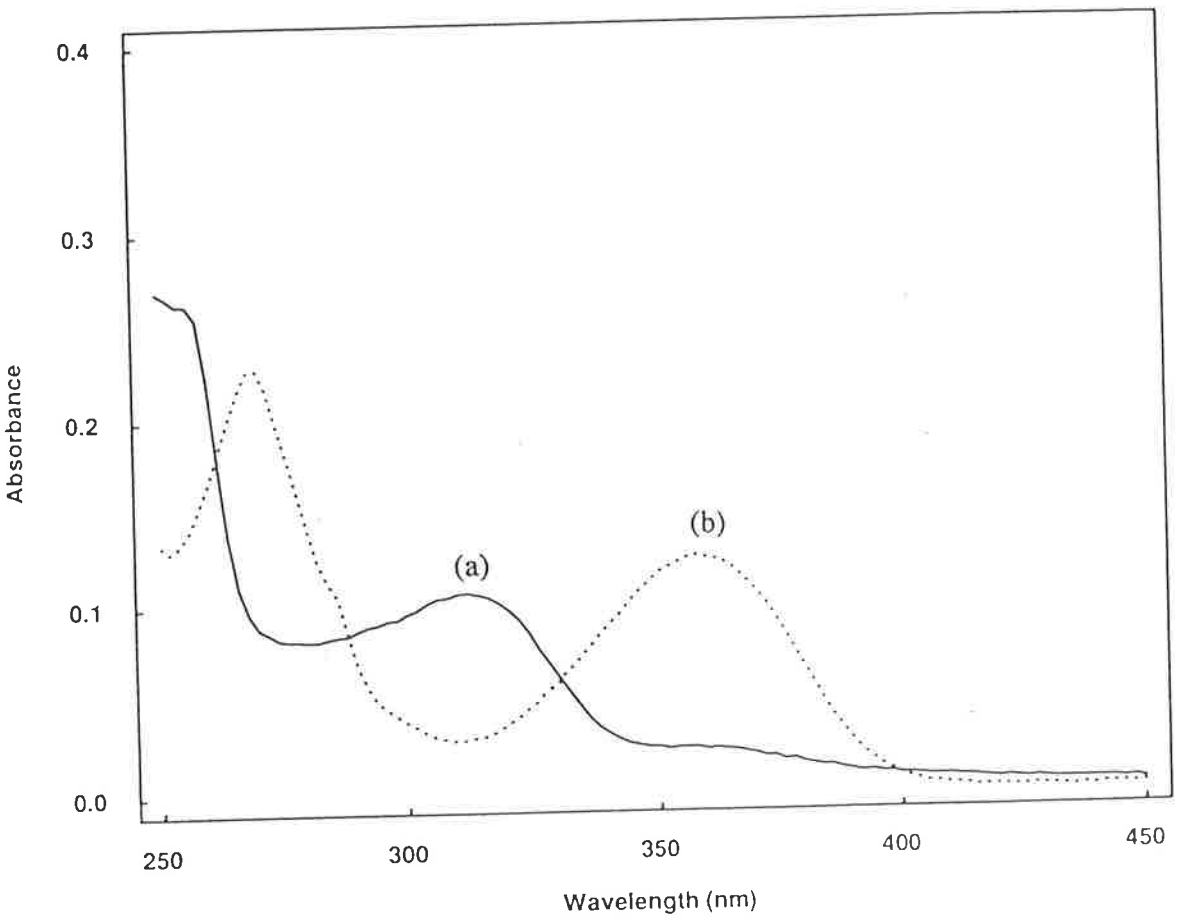


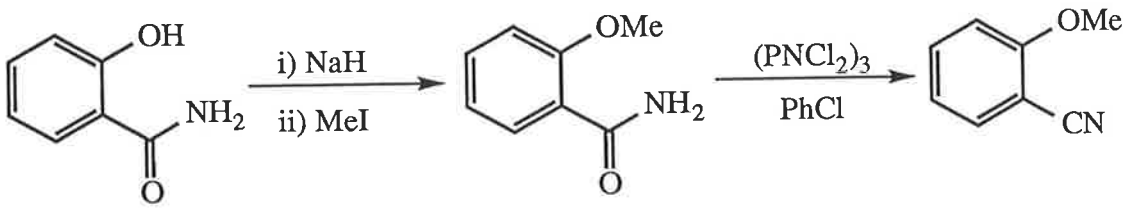
Figure 2.1 Comparison of the ultraviolet absorption spectra of the thiazoline acid, (3a), measured in the presence and absence of Zn^{2+}. The spectra are that of a) a methanolic solution of the acid, (3a), and b) the same methanolic solution of the acid, (3a), to which $Zn(ClO_4)_2$ has been added.

Initial fluorescence tests on these thiazolines were conducted by observing methanolic solutions of the thiazolines under ultraviolet light. It was found that solutions of the thiazolines themselves showed relatively little fluorescence. However, addition of Zn^{2+} to these solutions produced an intense fluorescent glow, when observed under ultraviolet light of 364 nm. Thus, the Zn^{2+} complexes formed by these thiazolines, (3a) and (8a), are highly fluorescent whereas the thiazolines themselves are not strongly fluorescent.

Although the hydroxy group on the phenyl ring of pyochelin was suspected of being involved in coordination to Zn^{2+},[8] its involvement in Zn^{2+} coordination had not been completely determined. Thus studies on the effects on the Zn^{2+} coordinating ability of the thiazolines, (3a) and (8a), by replacement of the hydroxy group with other substituents was of interest. The unsubstituted phenyl thiazoline, (3b), was synthesised from benzonitrile, using the same conditions as for (3a), shown in Scheme 2.3. On work up of the reaction mixture, the acid, (3b), could not be extracted, due to its high solubility in water. Evaporation of the reaction solvent and direct methylation of the remaining residue produced the ester, (8b), in a moderately low overall yield of 46%. Microanalysis, ^1H n.m.r., infrared and mass spectra of the product all confirmed the ester structure, (8b).

In order to determine whether the O-H bond of the phenol was necessary for complexation to occur, it was then decided to replace the hydroxy substituent of (3a) with a methoxy substituent to produce (3c). The methoxy substituent was chosen as it is the simplest ether and due to its relatively small size should not introduce great steric factors. *o*-Methoxybenzonitrile was prepared by methylation of salicylaldehyde, followed by conversion to the nitrile using the same conditions as for salicylonitrile (Scheme 2.2). Graham and Marr[11] have reported the preparation of nitriles by dehydration of their corresponding amides with phosphonitrilic chloride. They

obtained high yields of the nitriles by either directly heating the amides with phosphonitrilic chloride, or refluxing them in chlorobenzene. *o*-Methoxybenzonitrile was also prepared, again in high yield, by dehydration of its corresponding amide using this reagent in chlorobenzene (Scheme 2.4). Thus either procedure proved to be efficient for the preparation of this nitrile.

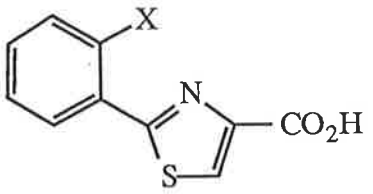


Scheme 2.4

The acid, (3c), was prepared by the method of Mathur and coworkers,[9] however, it could not be extracted from the reaction mixture. Evaporation of the reaction solvent and a mass spectrum of the crude residue confirmed the presence of the acid, (3c), showing a molecular ion at m/z 237 and a further peak at m/z 192 due to the loss of the acid group. By treatment of the reaction mixture with thionyl chloride in anhydrous methanol, the ester, (8c), was obtained in a moderate overall yield of 27%. The infrared spectrum of the product contained a carbonyl stretch at 1744 cm^{-1} and the mass spectrum contained a molecular ion peak at m/z 251, as well as a peak at m/z 192 due to the loss of the methyl ester group. The ^1H n.m.r. of the product was also consistent with the ester, (8c), showing singlets at 3.90 and 3.82 for the methyl ether and methyl ester protons, an apparent triplet at 5.17 and two doublets of doublets at 3.60 and 3.53 for the H4 and H5 protons of the thiazoline respectively.

2.2.3 Preparation of Thiazoles

It was considered likely that since the conjugation was increased on going from the thiazolines, (3), to their corresponding fully aromatic thiazoles, (9), the thiazoles would have greater fluorescence properties.



X =
a) OH
b) H
c) OMe

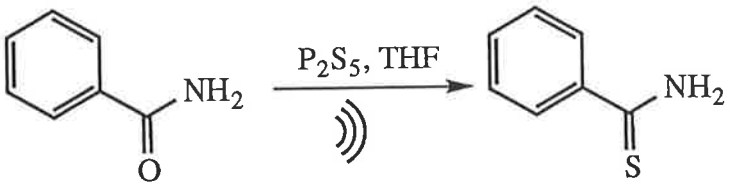
9

The thiazole, (9a), is commonly known as aeruginoic acid. It is a naturally occurring compound and, like pyochelin, is isolated from the culture medium of *Pseudomonas aeruginosa*, along with its methyl ester.[12] Yamada and coworkers[12] have prepared the ethyl ester of aeruginoic acid by treatment of *o*-hydroxythiobenzamide with ethyl bromopyruvate as shown in Scheme 2.7.

To prepare the thiazoles, (13), the corresponding thioamides, (10), needed to be prepared. There have been numerous reports in the literature on the preparation of thioamides, using various reagents. For example, thioacetamide has been used to convert nitriles into thioamides through an exchange reaction.[13] Ethoxycarbonyl isothiocyanate produces aromatic thioamides through a Friedel-Crafts thioacylation.[14] Another method used for the preparation of thioamides is the Wilgerodt-Kindler reaction, which converts aldehydes and ketones to thioamides using elemental sulfur and an amine.[15]

Direct thiation of amides is normally carried out by refluxing the amides with phosphorus pentasulfide for extended periods. Raucher and Klein[16] have found that ultrasonic irradiation of the reaction mixture accelerates the conversion of an amide to its thioamide, reducing the need for long reaction times and excessive heating. Using this procedure,[16] thiobenzamide, (10b), was obtained as a yellow powder in 83% yield from benzamide (Scheme 2.5). Unfortunately this method could not be employed for the preparation of *o*-hydroxythiobenzamide, (10a), as the hydroxy oxygen

competitively binds with the phosphorus of phosphorus pentasulfide, usually forming a mixture of products with O-P bonds.



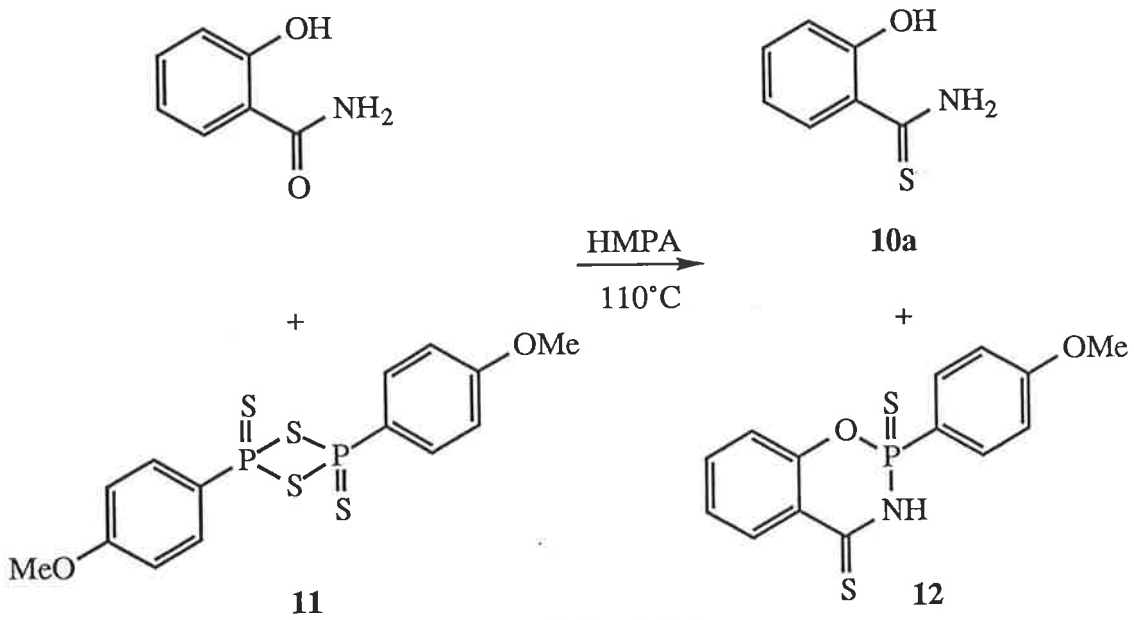
10b

Scheme 2.5

Scheibye et al.[17,18] have synthesised the thioamide, (10a), and various other substituted aromatic thioamides using the thioating reagent 2,4-bis(*p*-methoxyphenyl)-1,3-dithiadiphosphetane-2,4-disulfide, (11), commonly called Lawesson's reagent. Lawesson's reagent is very useful for converting various carbonyl containing compounds into the corresponding thiocarbonyl system.[19] It is readily prepared in high yields by refluxing phosphorus pentasulfide in anisole.[20] Lawesson's reagent is relatively insoluble in most organic solvents and it is common for reactions which utilise Lawesson's reagent to be carried out at temperatures elevated to around 100°C, in high boiling solvents, such as hexamethylphosphoramide (HMPA).[17,18] By heating with Lawesson's reagent in HMPA, a wide variety of hydroxy and aminobenzamides have been successfully thioated.[17,18] It is believed that the HMPA binds to the hydroxy or amino group of the benzamide and acts as a protecting group, preventing any interaction between these groups and the phosphorus of Lawesson's reagent.

The thiobenzamide, (10a), was obtained in moderate yield from the reaction of salicylamide with Lawesson's reagent[20] (Scheme 2.6). The sulfide, (12), was also obtained from the reaction. Presumably, it is formed by the further reaction of the thioamide, (10a), with Lawesson's reagent. The sulfide, (12), which showed a molecular ion at *m/z* 231, is similar in structure to the sulfides obtained as byproducts from thiation reactions carried out by Scheibye et al.[17] Along with these sulfides,

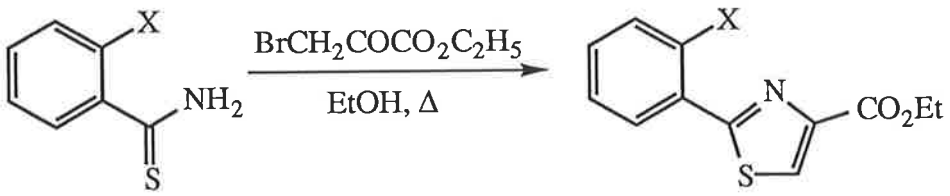
Scheibye and coworkers[17] also obtained another byproduct in their thiation reactions, which is similar in structure to the sulfides, but with an oxygen bonded to the phosphorus, instead of a sulfur atom. No corresponding oxide was extracted from the reaction mixture of (10a). ^{1}H n.m.r. and ^{13}C n.m.r. spectra obtained for the sulfide, (12), agreed well with those of the sulfides reported by Scheibye and coworkers.[17]



Scheme 2.6

In an attempt to improve the yield of the thioamide, (10a), its formation was followed by tlc. This indicated that the reaction may not have gone to completion in the time stated by the literature procedure,[17] since there was starting material remaining in the reaction mixture. Increasing the reaction time, produced increased yields of both products, (10a) and (12). However, after twelve hours the reaction appeared to cease with a maximum obtainable yield for (10a) of around 40%. Further increases in the reaction time did not produce any increase in the yields of either of the products of the reaction, even though there was still some salicylamide remaining in the reaction mixture. The termination of the reaction could be due to the instability of Lawesson's reagent at high temperatures, causing it to decompose during prolonged heating.

To test the reactivity of the prepared Lawesson's reagent, *o*-methoxythiobenzamide, (10c), was synthesised from *o*-methoxybenzamide, using the same procedure as that for (10a) (Scheme 2.6). *o*-Methoxybenzamide was prepared by methylation of salicylamide using sodium hydride and methyl iodide. The thioamide, (10c), was obtained in 77% yield after a reaction time of three hours, showing that the problems faced in preparing (10a) could not be attributed to the prepared Lawesson's reagent being of poor quality.



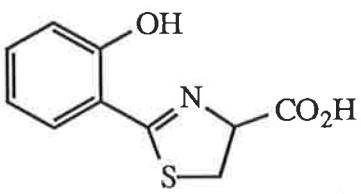
10 X= a) OH b) H c) OMe **13**

Scheme 2.7

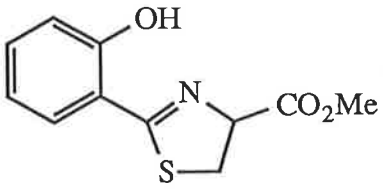
All the thioamides, (10), were reacted with ethyl bromopyruvate using Yamada and coworkers' procedure,[12] (Scheme 2.7), to form the thiazoles, (13), in high yields of up to 87%. The ^{1}H n.m.r. spectra of all three thiazoles, (13), each contained a singlet peak between 8.1 - 8.2 ppm for the H5 proton of the thiazole ring as well as a quartet near 4.4 - 4.5 ppm and a triplet in the region 1.40 - 1.43 ppm for the protons of the ethyl ester. All other peaks in the ^{1}H n.m.r. spectrum were consistent with the proposed structures of the respective thiazole products. The infrared spectra of the products confirmed the presence of the ester group with each showing a carbonyl stretch at 1728 - 1730 cm^{-1}. The mass spectrum of each thiazole, (13), contained the relevant molecular ion.

2.3 Initial Tests on Trial Ligand Series

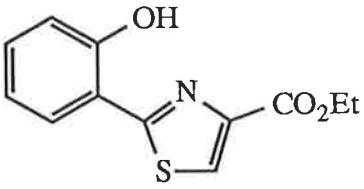
A series of seven thiazoles and thiazolines, (3a), (8a-c) and (13a-c), had been produced, whose Zn^{2+} coordination and fluorescence properties needed to be assessed, in order to determine their possible usefulness as biological fluorescent probes for Zn^{2+}. It had already been ascertained (sec 2.2.2) that the thiazoline acid, (3a), and its corresponding methyl ester, (8a), both bound Zn^{2+} and produced fluorescent Zn^{2+} complexes.



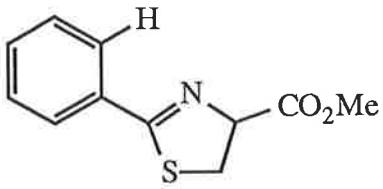
3a



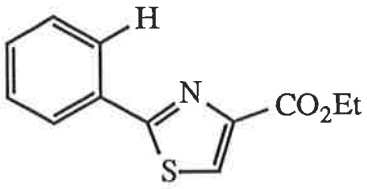
8a



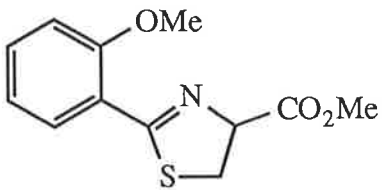
13a



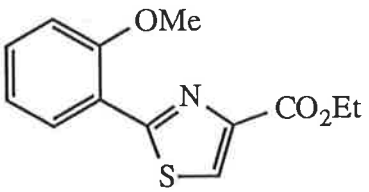
8b



13b



8c



13c

Using the methods used previously to determine the Zn^{2+} coordination and fluorescence properties of (3a) and (8a) (sec 2.2.2) on the remaining series of thiazoles and thiazolines synthesised, revealed that only those compounds which had an *o*-hydroxy substituent on the phenyl ring, for both the thiazoles and thiazolines, coordinated Zn^{2+}. Similarly, only these compounds, (3a), (8a) and (13a), produced a

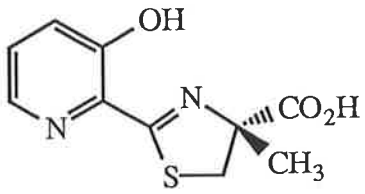
strongly fluorescent species when complexed to Zn^{2+}. All of the thiazolines and thiazoles were not strongly fluorescent in their unbound form. The methoxy substituted and the unsubstituted phenyl thiazolines and thiazoles, (8b), (8c), (13b) and (13c), showed no tendency to coordinate Zn^{2+}. Thus, it is apparent that the ortho hydroxy substituent on the phenyl ring is necessary for Zn^{2+} coordination by these thiazole and thiazoline compounds.

The lack of fluorescence of the ligands in their unbound form and the highly fluorescent species formed by those which coordinated Zn^{2+}, suggests Zn^{2+}, on coordination, 'locks' the thiazoline and thiazole compounds into a rigid but still conjugated structure. The uncomplexed thiazolines and thiazoles are non-fluorescent mainly because the greater degrees of freedom available for these structures allows excess excited energy to dissipate through various vibrational and rotational modes.

The necessity of the phenolic group for coordination of Zn^{2+} by these compounds indicates that Zn^{2+} may replace the hydroxy hydrogen on coordination. If it was the case that Zn^{2+} merely coordinates to the phenolic oxygen of these compounds, (3a), (8a) and (13a), without replacement of the hydroxy hydrogen, then observation of Zn^{2+} coordination by the methoxy substituted compounds, (8c) and (13c), would be expected, since coordination by Zn^{2+} to the ether oxygen of the methoxy substituent should be similar to that of an oxygen of a hydroxy substituent. However, the possibility of the lack of coordination to Zn^{2+} by the methoxy substituted phenyl thiazole and thiazoline being due to steric factors caused by the methyl substituent on the oxygen cannot be ruled out.

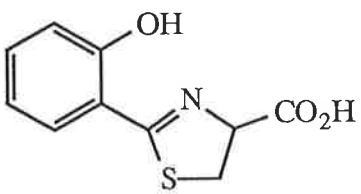
Which other coordination sites, of those available in the thiazoline and thiazole compounds, are involved in Zn^{2+} chelation is not clear. A compound of similar structure to the acid, (3a), is 2-(3-hydroxypyrid-2-yl)-4-methyl-Δ^2-thiazoline-4(S)-carboxylic acid, (14), more commonly referred to as desferriferrithiocin.[21]

Desferriferrithiocin is another naturally occurring siderophore which is isolated from *Streptomyces antibioticus*. It has been reported[21] that desferriferrithiocin coordinates to Fe^{3+} through its phenolic oxygen, the nitrogen of the thiazoline ring and its carboxylate moiety, forming a terdentate species. It is expected that the coordination of the thiazolines, (3a) and (8a), and the thiazole, (13a), to Zn^{2+} would be quite similar to that of desferriferrithiocin, (14), to Fe^{3+}, since Zn^{2+} and Fe^{3+} tend to form similar complexes, both being from the first transition series of metal ions and near each other in the periodic table.

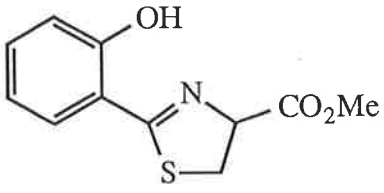


14

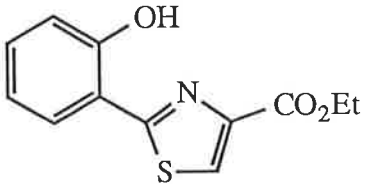
Of the series of compounds synthesised, only those three which contained a phenolic group, (3a), (8a) and (13a), of the seven compounds prepared, coordinated Zn^{2+}. Therefore these compounds were the only candidates of the series likely to be useful as fluorescent probes for biological Zn^{2+}. Further investigations into the selectivity and specificity of these compounds for Zn^{2+} and also the relative stability of the complexes formed with Zn^{2+} needed to be undertaken. From this point on the thiazoline acid, (3a), will be referred to as PTA, its methyl ester, (8a), as PTME and the thiazole ethyl ester, (13a), as PTEE.



3a
PTA



8a
PTME



13a
PTEE

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3 Investigation of the Specificity of the Thiazole Derivatives for Chelation with Zinc (II)

3.1 Introduction

3.1.1 Selection of a Test Series of Metal Ions

For a ligand to be a suitable probe for the detection and measurement of Zn^{2+}, it is preferable that the ligand is specific for Zn^{2+}. That is, the ligand should only coordinate Zn^{2+} and no other metal ions. This is the optimum situation and a more general expectation is that a ligand will selectively complex Zn^{2+}. Ideally the stability constant of the Zn^{2+} complex should be at least 10^3 times greater than those of competitor metal ions. Therefore, whether a ligand will be selective for Zn^{2+} in a particular system depends on the other competitor metal ions present, the relative stability of the complexes formed and the relative concentrations of Zn^{2+} and the other metal ions in the system.

To investigate the selectivity of the thiazole derivatives, PTA, PTME and PTEE, a series of possible competitor metal ions needed to be chosen and the coordination of these ions by the ligands tested. As the main interest in developing a fluorescent probe for Zn^{2+} was for the detection and measurement of readily available Zn^{2+} in human cells, whether the ligands coordinate other metal ions found in these cells needed to be examined. Therefore, the biologically prevalent metal ions, Na^+, Mg^{2+}, K^+ and Ca^{2+}, were selected as members of the series of metal ions to be tested with the thiazole derivatives.

The transition metal ions, Co^{2+}, Ni^{2+} and Cu^{2+}, being from the same period as Zn^{2+} and closest to Zn^{2+} on the periodic table, tend to have similar binding properties

to Zn^{2+}. Since these three ions are trace elements found in biological systems, they were also included in the series of metal ions selected.

As discussed previously (section 1.3), there is growing interest in developing a fast efficient method for measuring levels of metal ions known to be toxic to humans, such as the heavy metal ion Pb^{2+}. The current methods of detecting and measuring Pb^{2+} in biological systems[1] have proven to be expensive and time consuming. The use of a fluorescent probe which is selective for Pb^{2+} would provide a fast efficient and accurate measurement of Pb^{2+} levels in blood cells and other biological samples. For this reason, there was interest in investigating the coordination properties of the thiazole derivatives with Pb^{2+} and other heavy metal ions. Thus, the heavy metal ions Pb^{2+} and Cd^{2+} were selected as members of the test series. Similarly, as recent research has linked high concentrations of Al^{3+} in biological systems to such ailments as chronic renal failure and the pathogenesis of Alzheimer's disease,[2-4] there is growing interest in developing a fluorescent probe for the detection and measurement of Al^{3+} levels in biological samples. Aluminium (III) was the final ion included in the series of metal ions with which the thiazole derivatives, PTA, PTME and PTEE, coordination properties were to be studied.

The series of eleven metal ions chosen to investigate the selectivity of the thiazole derivatives consisted of Na^{+}, Mg^{2+}, Al^{3+}, K^{+}, Ca^{2+}, Co^{2+}, Ni^{2+}, Cu^{2+}, Zn^{2+}, Cd^{2+} and Pb^{2+}. Ultraviolet absorption spectroscopy was the method chosen to investigate whether complex formation of these ions occurred with the thiazole derivatives, PTA, PTME and PTEE.

3.1.2 Ultraviolet Absorption Spectroscopy

A compound's ultraviolet absorption spectrum is characteristic of the compound itself, or the class of compounds to which the compound belongs,[5] as it represents the

differences in energy between the electronic ground state of the compound and its various excited states. The ultraviolet absorption of an organic compound in solution, at a particular wavelength, has been related to the concentration of the compound in solution by Beer's Law (eq. 3.1),[5] where ε is the molar absorptivity of the compound at that wavelength and l is the sample length through which the light passed. Hence the concentration of a species in solution can be determined using Beer's Law and ultraviolet absorption spectroscopy. It is because of this and the sensitivity of ultraviolet absorption spectroscopy, that ultraviolet absorption spectroscopy has proven to be useful in many applications such as the determination of acid - base dissociation constants and other equilibrium constants.[5,6]

$$A = \varepsilon c l \tag{3.1}$$

The wavelengths at which absorption maxima occur and the relative intensities of absorption peaks in a spectrum of a compound are characteristic for a compound in a particular solution.[5] Factors which have an effect on the electrons of a compound, especially the π and non-bonding electrons, such as solvent polarity and addition of substituents onto the compound, produce a marked effect on the ultraviolet absorption spectrum of the compound, as they also effect the separation of electronic energy levels of the compound. Those which reduce the number or delocalisation of π or non-bonding electrons tend to increase the energy differences between the ground and excited electronic states of the compound. Hence the compound will absorb light quanta of higher energy and the absorption maxima of the compound will shift to shorter wavelengths. Such a displacement is referred to as a hypsochromic shift. On the other hand, if the number or delocalisation of π and non-bonding electrons is increased, the energy differences between a compound's ground and excited electronic states are reduced. In this case the compound will absorb light quanta of lower energy and the absorption maxima of the compound will be shifted to longer wavelengths. This is known as a bathochromic shift. Thus, the absorption spectrum of a compound is

very dependent on the substituents on the compound, which effect the conjugation of the π and non-bonding electrons, and the solvent of the solution.

3.1.3 Using Ultraviolet Spectroscopy to Detect Complex Formation

Coordination of a ligand to a metal ion may place the ligand in a particular conformation which is of a much more rigid geometry than the unbound ligand itself. This is especially the case when the denticity of the ligand is greater than one. The conformational restriction of the ligand and the sharing of excess electron density of the ligand with a metal ion through coordination affect the energy differences between the ground and excited electronic states of the ligand. Provided the ligand absorbs ultraviolet light, a shift of the absorption maxima of the ligand should be observed in its ultraviolet absorption spectrum if it coordinates a metal ion. Such a shift occurs so long as the separation of the electronic energy levels in the chromophore part of the ligand are affected by coordination. Therefore, it is possible to determine whether a complex is formed between a ligand and a metal ion by comparing ultraviolet absorption spectra of the ligand which were recorded in the absence and presence of the metal ion.

The thiazole derivatives, PTA, PTME and PTEE, all absorb light in the ultraviolet region between 200 - 400 nm, due to the extensive conjugation within their ring systems. It was expected that determination of complex formation between these compounds and the series of metal ions selected (section 3.1.1) could be achieved using ultraviolet absorption spectroscopy. The absorption spectra of each of the derivatives were measured in the presence of each metal ion of the series chosen. A change in the absorption spectrum of the derivatives, measured in this way, as compared with the absorption spectrum of the respective derivative alone, would indicate that complex formation had occurred.

3.2 Results and Discussion

The ultimate aim of this research is to develop a fluorescent probe which would be suitable for detecting and monitoring readily exchangeable intracellular Zn^{2+} within human cells. Because of this, it was preferable to record the ultraviolet absorption spectra of the thiazole derivatives, PTA, PTME and PTEE, in solutions which maintained the conditions of biological cells as closely as possible. To do this, it was decided to use sample solutions which had an ionic strength and pH similar to that within a biological cell and were made up of a predominantly water based solvent. The values of ionic strength and pH for biological systems are known to be approximately 0.1 and 7.0, respectively.

The ionic strength of a solution, I, is defined as the sum of half the molarity of each ionic species in solution, M, multiplied by the square of their formal charge, z, (eq. 3.2). When an electrolyte is present in a solution at relatively high concentrations and all other ionic species in the solution are dilute, the value of the ionic strength of the solution is predominantly based on the contribution of the highly concentrated species, with little effect being made by all other dilute species present. Therefore it can be said that the ionic strength of the solution is controlled by this electrolyte. For the purpose of maintaining a constant ionic strength, it is common to use an inert electrolyte in sample solutions, in far greater concentrations than other ionic species in the solution. In this way, the ionic strength of the solution is controlled by the inert electrolyte and any changes to the other ionic species in solution, for example by reaction or complex formation, have minimal effect on the ionic strength of the solution.

$$I = \Sigma(Mz^2)/2 \qquad (3.2)$$

The inert electrolyte requires a cation that does not coordinate with any of the derivatives, PTA, PTME and PTEE. Preliminary qualitative measurements of the

ultraviolet absorption spectra of the thiazole derivatives, PTA, PTME and PTEE, in methanolic solutions were carried out as in sections 2.2.2 and 2.3, with the perchlorate salts of the biologically prevalent metal ions, Na^+, Mg^{2+}, K^+ and Ca^{2+}. The perchlorate salts were used because the perchlorate ion, ClO_4^-, does not form strong associations to metal ions in solution.[7,8] Therefore, the metal ions of concern would be freely available to interact with the thiazole derivative and not complexed to perchlorate ions present. From these investigations, it was found that on addition of Na^+ ions in the form of sodium perchlorate to methanolic solutions of PTA, PTME and PTEE, no change in the absorption spectra of these derivatives was observed. This indicated that no significant coordination of Na^+ occurs. From this result, Na^+ was chosen as the cation to control the ionic strength of the sample solutions to be used for further ultraviolet absorption spectra measurements.

Although using an aqueous solvent for the sample solutions was favoured, the three derivatives PTA, PTME and PTEE did not dissolve easily in water. A solvent system made up of 75% methanol and 25% water was found to be that which contained the highest percentage of water in which the thiazole derivatives, PTA, PTME and PTEE, readily dissolved. A relatively high percentage of water was required not only to maintain conditions which were as similar as possible to those within a biological cell, but also to ensure the dissolution of the salts of the metal ions which were to be added to solutions of the thiazole derivatives. This solvent system was used to prepare all sample solutions used in the ultraviolet absorption spectroscopy measurements.

Solutions used for ultraviolet absorption spectroscopy measurements were prepared as described in section 8.2.2. All sample solutions had an ionic strength of 0.100 mol dm^{-3} controlled by sodium perchlorate. Preliminary absorption spectra measurements of the solutions of PTME found that a concentration of 2.00×10^{-5} mol dm^{-3} PTME was convenient for use in the spectral measurements carried out. This

ligand concentration was used for all sample solutions of all three thiazole derivatives, PTA, PTME and PTEE.

To ensure substantial complex formation between the test metal ions and the thiazole derivatives, PTA, PTME and PTEE, the test metal ions needed to be present in the sample solutions at a concentration far in excess of that of the thiazole derivatives themselves. Ultraviolet absorption spectra were recorded of trial solutions which contained 2.00×10^{-5} mol dm^{-3} PTME and varying concentrations of $Zn(ClO_4)_2$, with ratios of PTME to Zn^{2+} ranging from 1:10 through to 1:500. The solution which contained a PTME to Zn^{2+} ratio of 1:200 was that which contained the lowest concentration of Zn^{2+} and whose absorption spectrum only showed peaks attributable to the Zn^{2+} complex of PTME, therefore indicating that PTME was completely complexed in solution. This ligand to metal ion ratio was used for all sample solutions. Hence, the concentration of the test metal ions added to the sample solutions was 4.00×10^{-3} mol dm^{-3}.

To buffer the pH of the sample solutions to a value of approximately 7.0, 1.00×10^{-3} mol dm^{-3} sodium piperazine-N,N'-bis-(ethanesulfonic acid), NaPIPES, was added. Although the concentration of test metal ions added to these sample solutions was in excess of the buffer, those which contained the metal ions Mg^{2+}, K^{+}, Ca^{2+}, Co^{2+}, Ni^{2+}, Zn^{2+} or Cd^{2+} did not have an adverse effect on the buffer and the pH of their solutions lay within the range 6.9 to 7.1. Solutions containing the metal ions Al^{3+}, Cu^{2+} or Pb^{2+}, which form highly acidic hydrated species, adversely affected the buffer and the pH of their solutions were found to be 3.72 ± 0.01, 5.33 ± 0.02 and 5.88 ± 0.03 respectively. Increasing the concentration of the buffer in an attempt to increase the pH of these solutions, created precipitation problems with the test metal ions. Therefore, the spectral measurements were carried out on these solutions at the above pH values.

Under the conditions used (section 8.2.2), precipitation of an hydroxide species in sample solutions which contained the metal ions Al^{3+}, Cu^{2+}, Zn^{2+} and Pb^{2+}, occurred, if the sample solutions were allowed to stand for more than a day or so. To avoid this situation, all solutions were prepared and all spectral measurements of the solutions were recorded within eight hours of each other. This practice eliminated any precipitation problem with all solutions containing metal ions except for those containing Pb^{2+} ions. For those solutions which contained Pb^{2+} ions, problems with precipitation of an hydroxide species could not be avoided and the solutions needed to be filtered through a millipore filter before spectral measurements could be performed. Thus, the total concentration of Pb^{2+} within these solutions was not accurately known. Since only a small extent of precipitation of hydroxide species in these Pb^{2+} solutions occurred, the concentration of Pb^{2+} ions present in solution was still far in excess of the ligand concentration. Hence conditions within these solutions were still favourable to ensure that any likely coordination between the ligand and Pb^{2+} would occur. Thus, it was still possible to observe any coordination of Pb^{2+} by the ligands and draw the relevant deductions from the qualitative spectra obtained.

The ultraviolet absorption spectra of the thiazole derivatives PTA, PTME and PTEE were recorded by the method described in section 8.2.2. In addition, the absorption spectra of each of the thiazole derivatives were recorded, whilst individually in the presence of each individual metal ion, previously selected as members of the cation series. A comparison of the spectra of PTA measured in the absence and presence of the metal ions was then made to determine which metal ions coordinated to PTA. Similar comparisons of the spectra of PTME and PTEE were also made for the same purpose. A shift in the absorption maxima wavelengths of the thiazole derivatives of greater than 5 nm was taken as indicating that a significant coordination had occurred between the thiazole derivative and the metal ion. Figures 3.1 to 3.3 show the absorption spectra of the thiazole derivatives, PTA, PTME and PTEE, and that of the three compounds when Zn^{2+} had been added to the solution. The shifts in absorption

maxima of the three compounds caused by coordination of Zn^{2+} are typical of those observed if the compounds coordinated any of the metal ions tested. All other absorption spectra of the metal complexes formed by PTA, PTME and PTEE are presented in Appendix A.

In Table 3.1, the ultraviolet absorption maxima of the thiazole derivatives, PTA, PTME and PTEE, between 450 - 250 nm, are listed. Also listed are the absorption maxima of the metal complexes formed by the thiazole derivatives. The absorption maxima values of the thiazole derivatives measured in the presence of non-coordinating metal ions were identical to those of the thiazole derivatives themselves and have not been included in the table.

As can be seen from Table 3.1, the wavelength values, at which absorption maxima of PTA occurred, were shifted to longer wavelengths when any one of the metal ions Al^{3+}, Ca^{2+}, Co^{2+}, Ni^{2+}, Cu^{2+}, Zn^{2+}, Cd^{2+} or Pb^{2+} were added to a solution of PTA, signifying coordination of these metal ions. No shift in the ultraviolet absorption maxima of PTA was observed when either of the metal ions Mg^{2+} or K^{+} were added to a solution of PTA, indicating PTA does not coordinate these metal ions.

The corresponding methyl ester, PTME, coordinated the metal ions Al^{3+}, Co^{2+}, Ni^{2+}, Cu^{2+}, Zn^{2+}, $Cd^{2+}$and Pb^{2+}. This was shown by a shift in the ultraviolet absorption maxima of the ester, PTME, observed when the absorption spectra of solutions of the ester, to which these metal ions had been added, were recorded. The wavelengths at which absorption maxima of PTME occurred, when complexed to these metal ions, are listed in Table 3.1. No change in the absorption spectrum of the ester, PTME, was observed when the metal ions, Mg^{2+}, K^{+} or Ca^{2+} were added to sample solutions of the ester, indicating PTME does not coordinate these metal ions.

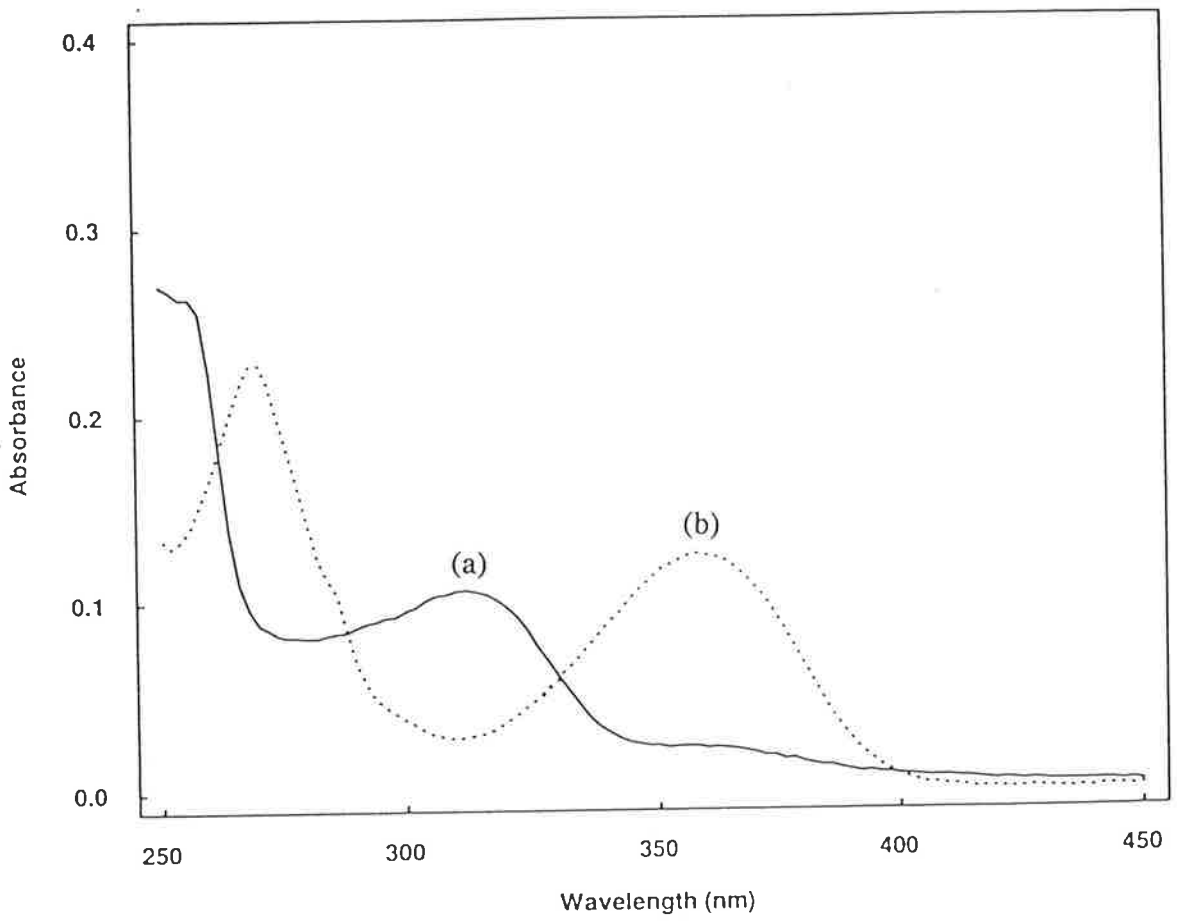

Figure 3.1 Comparison of the ultraviolet absorption spectra of PTA measured in the absence and presence of Zn^{2+}. The solutions contained (a) 2.00 x 10^{-5} mol dm^{-3} PTA, 1.00 x 10^{-3} mol dm^{-3} NaPIPES and 0.100 mol dm^{-3} $NaClO_4$ and (b) 2.00 x 10^{-5} mol dm^{-3} PTA, 4.00 x 10^{-3} mol dm^{-3} $Zn(ClO_4)_2$, 1.00 x 10^{-3} mol dm^{-3} NaPIPES and 0.100 mol dm^{-3} $NaClO_4$ in 75% CH_3OH and 25% H_2O. Spectra were recorded at 298.2 K.

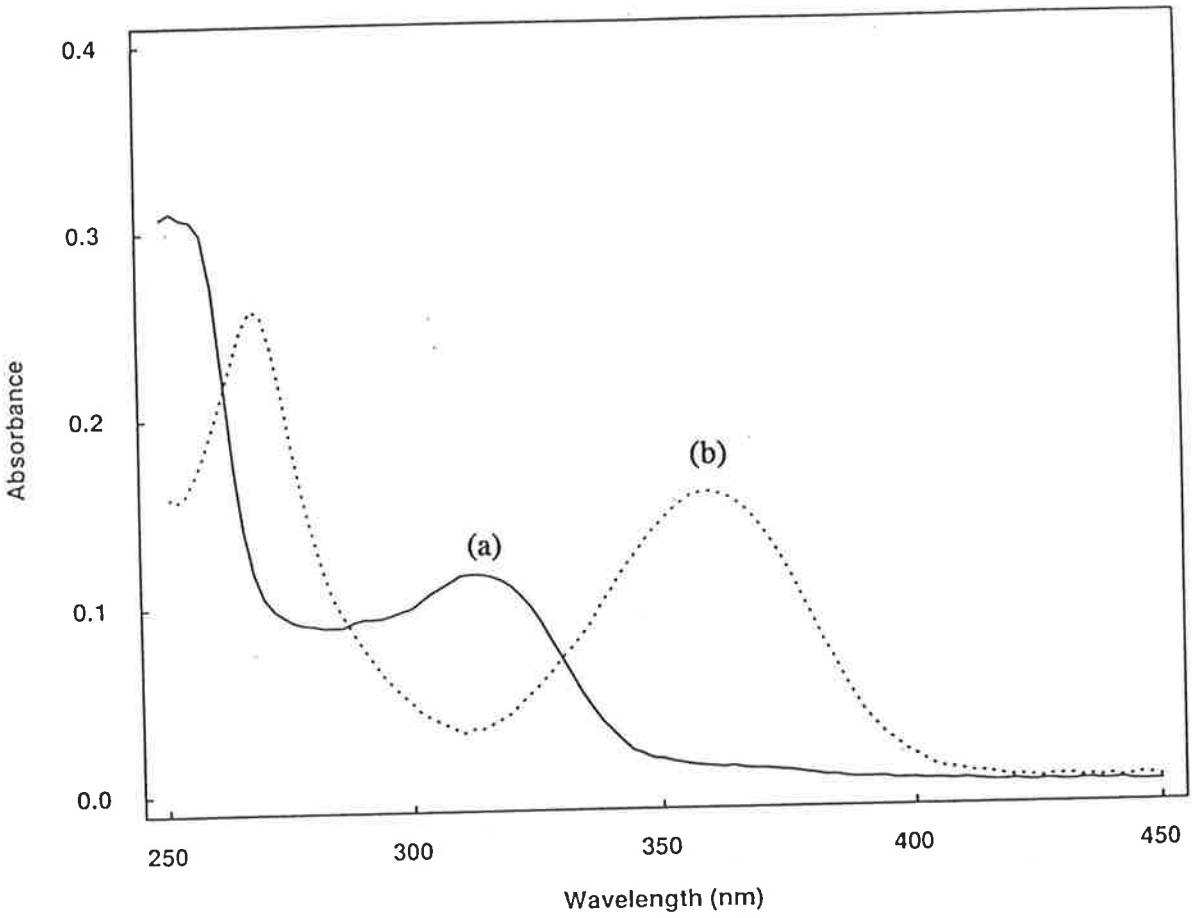

Figure 3.2 Comparison of the ultraviolet absorption spectra of PTME measured in the absence and presence of Zn^{2+}. The solutions contained (a) 2.00 x 10^{-5} mol dm^{-3} PTME, 1.00 x 10^{-3} mol dm^{-3} NaPIPES and 0.100 mol dm^{-3} $NaClO_4$ and (b) 2.00 x 10^{-5} mol dm^{-3} PTME, 4.00 x 10^{-3} mol dm^{-3} $Zn(ClO_4)_2$, 1.00 x 10^{-3} mol dm^{-3} NaPIPES and 0.100 mol dm^{-3} $NaClO_4$ in 75% CH_3OH and 25% H_2O. Spectra were recorded at 298.2 K.

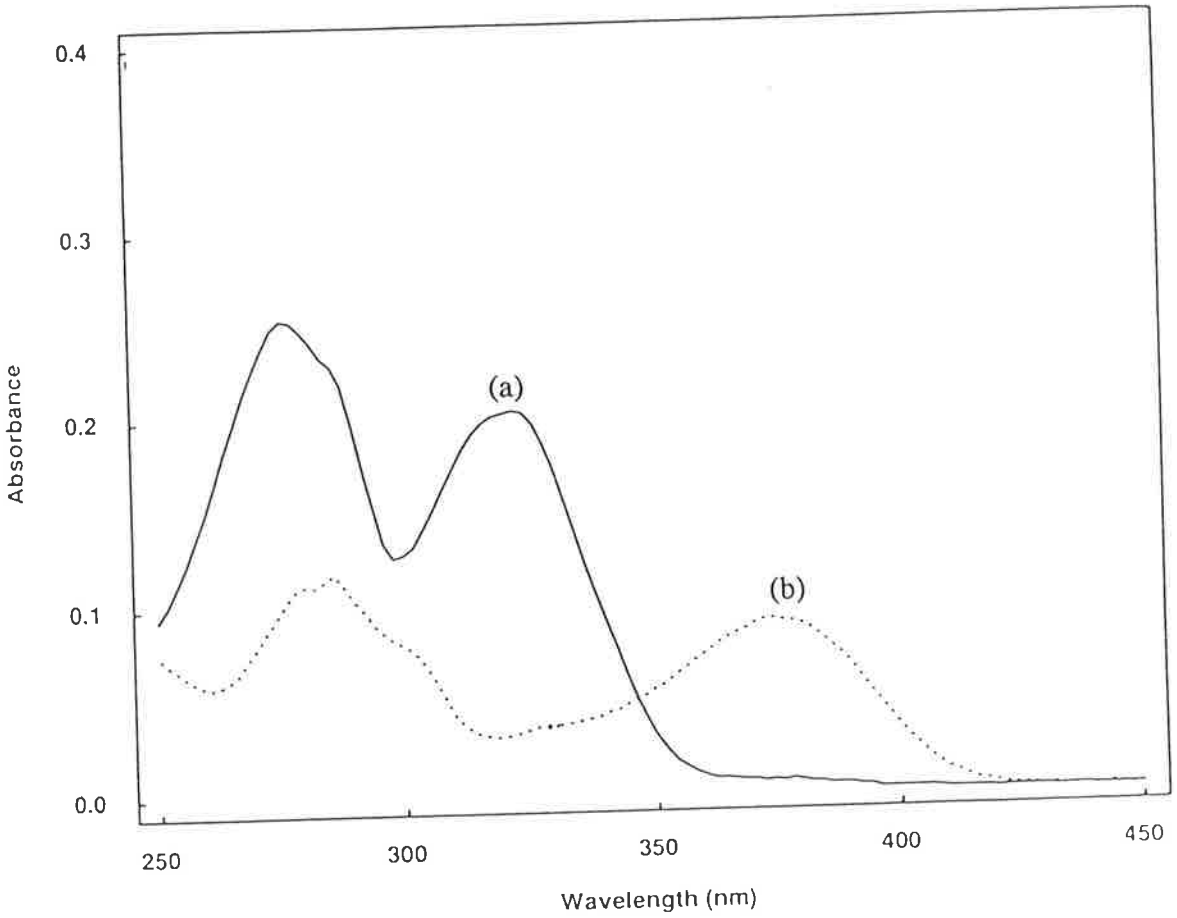

Figure 3.3 Comparison of the ultraviolet absorption spectra of PTEE measured in the absence and presence of Zn^{2+}. The solutions contained (a) 2.00 x 10^{-5} mol dm^{-3} PTEE, 1.00 x 10^{-3} mol dm^{-3} NaPIPES and 0.100 mol dm^{-3} $NaClO_4$ and (b) 2.00 x 10^{-5} mol dm^{-3} PTEE, 4.00 x 10^{-3} mol dm^{-3} $Zn(ClO_4)_2$, 1.00 x 10^{-3} mol dm^{-3} NaPIPES and 0.100 mol dm^{-3} $NaClO_4$ in 75% CH_3OH and 25% H_2O. Spectra were recorded at 298.2 K.

Table 3.1 Ultraviolet absorption maxima of the thiazolines, PTA and PTME, and the thiazole, PTEE, and their metal complexes.[a]

Ligand[b]	Metal Ion[c]	Absorption Maxima between 450 - 250 nm[d] (nm)
PTA		310; 250
	Al^{3+}	350; 270
	Ca^{2+}	358; 256
	Co^{2+}	356
	Ni^{2+}	362; 268
	Cu^{2+}	360; 265
	Zn^{2+}	356; 268
	Cd^{2+}	357; 266
	Pb^{2+}	362; 272
PTME		313; 252
	Al^{3+}	356; 260
	Co^{2+}	356; 262
	Ni^{2+}	360; 268
	Cu^{2+}	362; 268
	Zn^{2+}	358; 270
	Cd^{2+}	356; 264
	Pb^{2+}	365, 266
PTEE		322; 276
	Co^{2+}	374; 286
	Ni^{2+}	379; 288
	Cu^{2+}	376; 286
	Zn^{2+}	372; 284
	Cd^{2+}	379; 325; 280
	Pb^{2+}	374; 284

a All solutions contained 1.00×10^{-3} mol dm^{-3} NaPIPES, 0.100 mol dm^{-3} $NaClO_4$ in 75% methanol and 25% water. Spectra were recorded at 298.2 K.

b The concentration of the ligand in solution was 2.00×10^{-5} mol dm^{-3}.

c The concentration of metal ions in solution was 4.00×10^{-3} mol dm^{-3}.

d Errors in the absorption maxima values are $\pm$ 1 nm.

Although the thiazoline carboxylic acid, PTA, coordinates Ca^{2+} at pH 7, the corresponding methyl ester, PTME, does not. It is common for Ca^{2+} ions to bind to carboxylate anions,[9] which is a likely explanation why PTA, with its carboxylic acid group predominantly in a carboxylate anionic form at pH 7, binds to Ca^{2+} ions, and the corresponding ester, PTME, does not. Thus the ester, PTME, appears to suit the selectivity requirements for a Zn^{2+} probe, at pH 7, better than the corresponding acid, PTA.

Of all three thiazole derivatives prepared, PTA, PTME and PTEE, the thiazole ester, PTEE, coordinates the fewest of the metal ions from the series chosen to test the binding properties of these derivatives. PTEE coordinates the metal ions Co^{2+}, Ni^{2+}, Cu^{2+}, Zn^{2+}, Cd^{2+} and Pb^{2+}. No indication of coordination of Mg^{2+}, Al^{3+}, K^{+} or Ca^{2+} by the ester, PTEE, was observed.

It was shown earlier (section 2.3) that the ortho hydroxy group on the phenyl ring of each of the thiazole derivatives, PTA, PTME and PTEE, was necessary for coordination of Zn^{2+} by these compounds. It is expected that coordination of the thiazole derivatives to metal ions, such as Zn^{2+}, involves the phenolic oxygen and possibly either the ring nitrogen or sulfur. In the case of PTA, one of the carboxylic oxygens may also be involved. Molecular modelling studies[10] have confirmed that such coordination is possible.

Computer aided molecular modelling studies[10] showed the 'bite' distance between the oxygen on the phenyl ring and the heterocyclic nitrogen is greater for the thiazole ester, PTEE, than the thiazoline acid, PTA, being 266 pm and 255 pm respectively (Figure 3.4). This increased distance between the probable coordination sites in the thiazole, PTEE, is most likely responsible for it not chelating to relatively small metal ions, such as Al^{3+}, and having a preference for larger cations. Hence this

explains the greater selectivity observed of this compound for coordination of the metal ions tested.

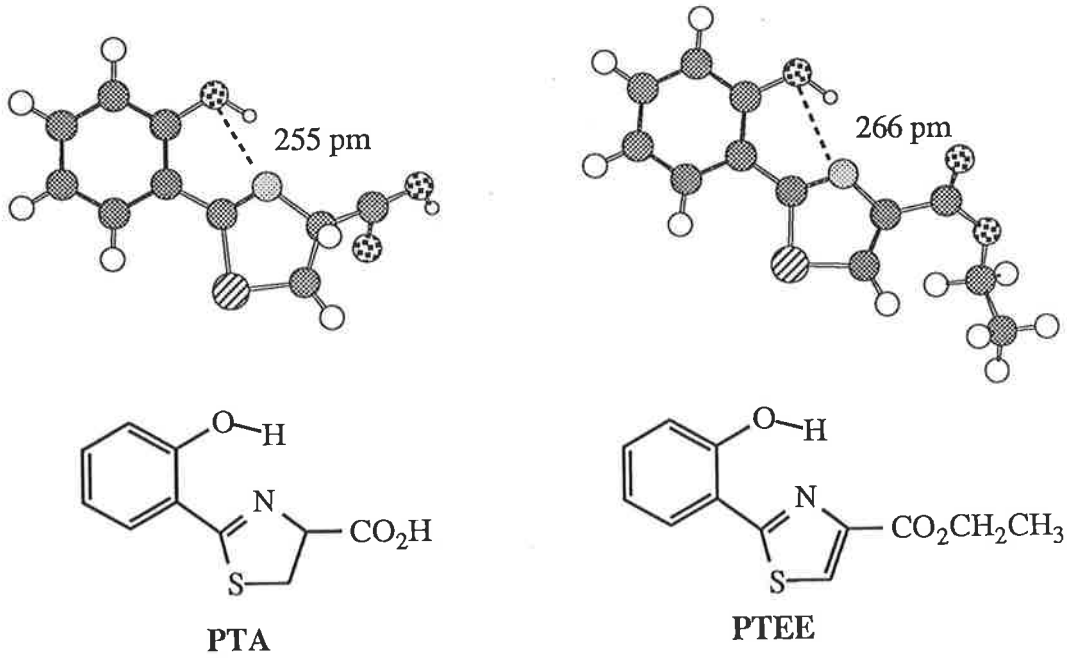

Figure 3.4 Molecular model[10] representation of the thiazoline acid, PTA, and the thiazole ester, PTEE, indicating the spatial distance between the phenyl oxygen and the ring nitrogen when in a planar arrangement.

None of the three thiazole derivatives, PTA, PTME and PTEE, coordinated the biologically prevalent metal ions Na^{+}, Mg^{2+} and K^{+}. This result shows promise for these compounds to be useful as probes in biological systems as no significant interference should occur from the above mentioned metal ions when using these compounds as probes. However, the acid, PTA, does coordinate to Ca^{2+}. All three thiazole derivatives, PTA, PTME and PTEE, coordinated the transition metal ions Co^{2+}, Ni^{2+} and Cu^{2+} as well as Zn^{2+}. They also coordinated the heavy metal ions Cd^{2+} and Pb^{2+}. Only the two thiazolines, PTA and PTME, coordinated the relatively small Al^{3+} ion. Therefore, it is apparent that the thiazole derivatives are not specific for Zn^{2+}. However, they may still prove useful as probes for biological Zn^{2+}, since the

other metal ions, Al^{3+}, Co^{2+}, Ni^{2+}, Cu^{2+}, Cd^{2+} and Pb^{2+}, are not normally found in significant concentrations where Zn^{2+} is found in biological systems.[9]

The selectivity of PTA, PTME and PTEE needed to be investigated. To do this, determination of the stability constants of the metal complexes formed by these ligands was required. Also, since measurement of readily exchangeable Zn^{2+} within living cells is to be done using fluorescence spectroscopy, studies into the fluorescence of PTA, PTME and PTEE were required. Information obtained from these studies would aid in assessing the suitability of these compounds as fluorescent Zn^{2+} probes.

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4 Acid Dissociation and Stability Constant Determination

4.1 Introduction

4.1.1 The Required Affinity of a Probe for Zinc (II)

For a ligand to be a suitable probe for measuring readily exchangeable Zn^{2+} within a cell, certain restrictions are placed on the ability of the ligand to form complexes with Zn^{2+}. As discussed previously in section 1.1.2, the ligand must readily form complexes of suitable stability with Zn^{2+}, so that all the freely available Zn^{2+} within a cell is rapidly coordinated. The stability of these Zn^{2+} complexes should be such that little, if any, competitive displacement reactions, by other biologically prevalent cations, such as Ca^{2+} and Mg^{2+}, occur. However, the Zn^{2+} complexes' stability should not be so high that the ligand competes for Zn^{2+} which is tightly complexed to chelating sites within the cell.

Hence, the stability of the Zn^{2+} complexes formed by the ligands, PTA, PTME and PTEE, needed to be examined. Comparison of their stabilities with that of complexes formed, by the ligands, with other biologically relevant ions was also required. Such information is necessary to assess the applicability of PTA, PTME and PTEE as probes for measuring readily exchangeable Zn^{2+} in biological systems. Also, the potential of these compounds as probes for the measurement of other metal ions, such as the heavy metal ions, could be assessed by the determination of the affinity of these compounds for such ions.

Stability constants for the formation of metal complexes are a quantitative measure of the stability of a complex.[1]

4.1.2 Definition of Acid Dissociation and Stability Constants

A thermodynamic equilibrium constant ${}^{T}K_{eq}$ is defined as

$$aA + bB \rightleftharpoons cC + dD \qquad {}^{T}K_{eq} = \frac{\{C\}^c \{D\}^d}{\{A\}^a \{B\}^b} \qquad (4.1)$$

where {X} denotes the activity of species X at equilibrium.

A stoichiometric equilibrium constant K_{eq} is defined as

$$aA + bB \rightleftharpoons cC + dD \qquad K_{eq} = \frac{[C]^c [D]^d}{[A]^a [B]^b} \qquad (4.2)$$

where [X] denotes the concentration, usually molar, of species X at equilibrium.

The stoichiometric equilibrium constant is related to the thermodynamic equilibrium constant by

$$K_{eq} = {}^{T}K_{eq} \cdot \frac{\upsilon_A{}^a \cdot \upsilon_B{}^b}{\upsilon_C{}^c \cdot \upsilon_D{}^d} \qquad (4.3)$$

where υ_X is the activity coefficient of species X.

When the ionic strength of a solution is maintained by an inert electrolyte, the activity coefficient of an ionic solute, present in more dilute concentrations, is held constant.[2] Slight changes to the concentration of this solute have little effect on its activity coefficient. Thus, concentrations tend to parallel activities of dilute ionic solutes in solutions where the ionic strength is controlled by an inert electrolyte.[1] This assumes

that the inert electrolyte is present in far greater concentration than that of any of the reacting ionic species of concern.

For reasons of practicality, it has become the general practice to maintain a constant ionic strength of a solution by using an inert electrolyte when measuring equilibrium constants of coordination compounds.[1] The inert electrolyte must not interfere with any of the ionic species under investigation, nor with the physical or chemical properties being measured. It must have a significantly high solubility in the solvent used and readily dissociate into ionic form.[3] All equilibrium constants then quoted are those of concentration, K_{eq}, and not the thermodynamic ${}^{T}K_{eq}$. This practice has been adopted throughout this study.

A ligand which has an acidic proton, LH, has an acid dissociation constant, K_a, given by

$$LH \rightleftharpoons L^- + H^+ \qquad K_a = \frac{[L^-]\ [H^+]}{[LH]} \qquad (4.4)$$

and is a quantitative measure of how readily the ligand will ionise, releasing a proton. It is more common to speak of the acidity of a compound in terms of its pK_a, where

$$pK_a = -\log K_a \qquad (4.5)$$

The more acidic a compound, the lower its pK_a.

When a fully deprotonated ligand, L^-, forms a complex with a metal ion, M^{n+}, a quantitative measure of the affinity of the ligand for the metal ion is given by the equilibrium constant for complex formation. This constant is referred to as a stability constant and is defined by

$$M^{n+} + L^- \rightleftharpoons ML^{(n-1)+} \qquad \beta_1 = \frac{[ML^{(n-1)+}]}{[M^{n+}]\ [L^-]} \tag{4.6}$$

$$M^{n+} + 2L^- \rightleftharpoons ML_2^{(n-2)+} \qquad \beta_2 = \frac{[ML_2^{(n-2)+}]}{[M^{n+}]\ [L^-]^2} \tag{4.7}$$

$$M^{n+} + nL^- \rightleftharpoons ML_n \qquad \beta_n = \frac{[ML_n]}{[M^{n+}]\ [L^-]^n} \tag{4.8}$$

The stability constant, β_n, is the overall stability constant for the formation of the complex ML_n. The stepwise stability constant, K, for successive addition of a ligand to a metal ion complex, is given by

$$M^{n+} + L^- \rightleftharpoons ML^{(n-1)+} \qquad K_1 = \frac{[ML^{(n-1)+}]}{[M^{n+}]\ [L^-]} \tag{4.9}$$

$$ML^{(n-1)+} + L^- \rightleftharpoons ML_2^{(n-2)+} \qquad K_2 = \frac{[ML_2^{(n-2)+}]}{[ML^{(n-1)+}]\ [L^-]} \tag{4.10}$$

$$ML_{n-1}^{+} + L^- \rightleftharpoons ML_n \qquad K_n = \frac{[ML_n]}{[ML_{n-1}^{+}]\ [L^-]} \tag{4.11}$$

Notice that

$$\beta_1 = K_1 \tag{4.12}$$

and

$$\beta_n = K_1 \,.\, K_2 \,.\, .\, .\, K_n \tag{4.13}$$

The constants defined in equations 4.4 - 4.11 are those that are used throughout this study for the determination of acid dissociation and stability constants.

4.1.3 Potentiometry

Stability constants can be determined by measurement of any parameter which alters due to complex formation, provided the composition of the equilibrium state can be calculated from such measurement. Many methods have been employed for the determination of stability constants of various complexes.[1-3] Potentiometry has become particularly widely used, as it is a convenient, widely applicable technique for the accurate study of ionic equilibria.

In potentiometry the e.m.f. of a series of solutions of an equilibrium process containing the ionic species of interest is measured using a relevant sensing electrode. Each solution contains a different total concentration of one species, which affects complex formation, and measurement is made at equilibrium. Depending on the choice of electrode, the equilibrium concentration of one species present in solution can be determined. From data collected, the equilibrium concentrations of all species in solution and the equilibrium formation constants of species formed in solution can be calculated.

If equilibrium is reached quickly, it is often convenient to use a titration technique in which a large variation in e.m.f. is induced through the variation of the concentrations of the reactant species in solution over a wide range.

Specific ion electrodes and metal electrodes have been used to monitor the equilibrium concentration of a metal ion in solution. However, as many complex formation equilibria involve protonic acid - base equilibria, it is usually convenient to monitor the hydrogen ion concentration of the solution throughout a potentiometric

titration.[1,3] The glass electrode provides a highly accurate and reproducible measurement of pH.

Such a potentiometric titration process involves the addition of a strong base to a solution containing strong acid and ligand in the presence, and absence, of varying total metal ion concentrations. All total concentrations of the base and acid solutions are known. From the pH data collected, the equilibrium concentrations of all components of the solution and the equilibrium stability constants can be calculated.

The process of calculating stability constants is known as stability constant refinement. This is where a set of fixed and adjustable parameters are placed through an algorithm, usually with the aid of a computer, in order to obtain the best possible least squares fit between the calculated and observed data.[1] Many computer programs have been written for this purpose.[1,4]

4.1.4 Determination of Stability Constants

As the thiazolines, PTA and PTME, and the thiazole, PTEE, contained acidic protons, complexation with metal ions was expected to alter the position of acid - base equilibria in solution. The potentiometric titration technique was chosen to monitor any pH changes resulting from complex formation of these ligands with metal ions. From this, values for the stability constants of the respective complexes could be determined. Of main interest was the affinity of these compounds for Zn^{2+}. However, as seen previously (section 3.2), the thiazole derivatives, PTA, PTME and PTEE, coordinate other metal ions, which are found in biological systems. Determination of the stability constants of these complexes would provide information required to assess the selectivity of the ligands for Zn^{2+}.

4.2 Results and Discussion

A solvent system comprising of 75% methanol and 25% water was chosen to prepare the solutions required for the potentiometric titrations. A relatively high percentage of water was required for two main reasons. Firstly, the glass electrode requires the presence of a sufficient percentage of water, in a solution, in order for it to function properly. Ensuring a solvent system has a relatively high content of water, minimises the risk of the electrode's glass bulb dehydrating and producing poor electrode response.[5] Secondly, due to the nature of this research, it was desirable to maintain conditions which were as similar as possible to the environment within a living cell.

Initial tests found that a methanol based solvent system containing 25% water by volume, was that which contained the highest percentage of water, yet readily dissolved the thiazole derivatives, PTA PTME and PTEE, and also minimised precipitation of zinc hydroxide or zinc ligand complexes at high pH. This solvent system was then used for all analytical measurements of all prospective ligands throughout this research and allowed for a direct comparison of results.

Representative experimental titration profiles resulting from potentiometric titrations carried out are shown in Figure 4.1. The curve (b), resulting from the titration of the uncomplexed ligand PTME, deviates significantly from the titration curve of a calibration (a). This deviation arises because of ligand deprotonation. Similarly, for a titration of an acidic solution containing the ligand, PTME, in the presence of metal ions, such as Zn^{2+}, formation of a complex in greater than 5% proportions results in a significant deviation of its titration profile, (c), from that of the titration profile of the ligand alone, (b). Data collected at such points are used to calculate the pK_a values of the ligand's acidic protons and stability constants of the metal complexes formed. If less than 5% of a species is formed throughout a titration, its effect on the pH of a solution is usually minimal and it's formation constant cannot be determined accurately.

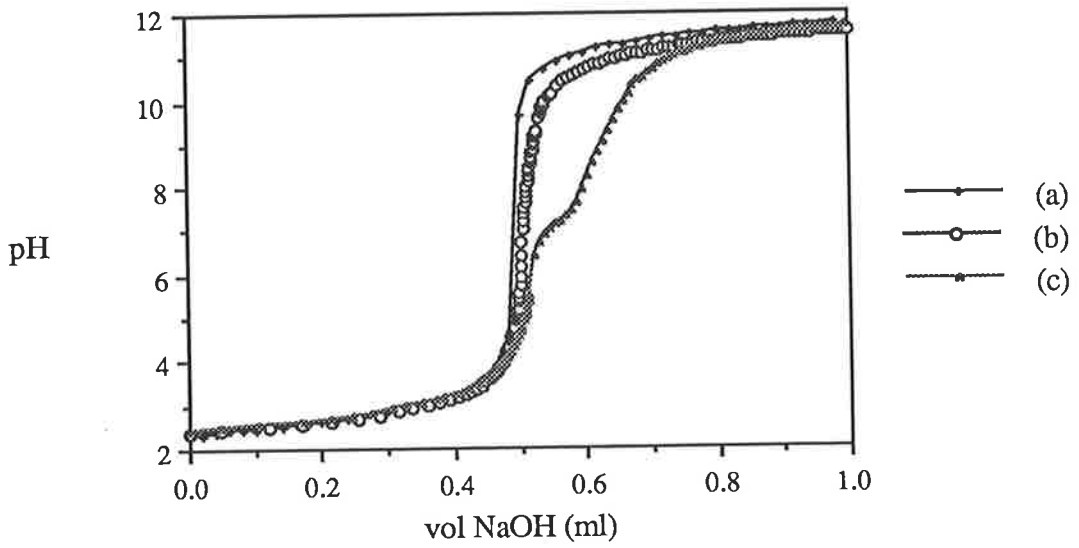


Figure 4.1 Experimental titration profiles of

a) 5.00 x 10^{-3} mol dm^{-3} $HClO_4$

b) 1.00 x 10^{-3} mol dm^{-3} PTME and 5.00 x 10^{-3} mol dm^{-3} $HClO_4$

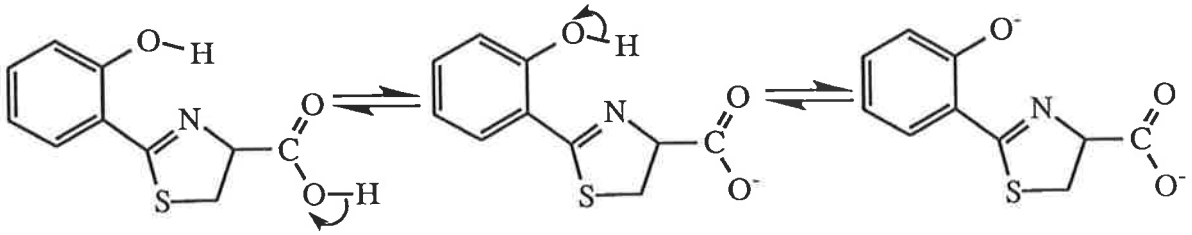
c) 9.00 x 10^{-4} mol dm^{-3} $Zn(ClO_4)_2$, 1.00 x 10^{-3} mol dm^{-3} PTME and 5.00 x 10^{-3} mol dm^{-3} $HClO_4$

in 75% CH_3OH, 25% H_2O and 0.100 mol dm^{-3} $NaClO_4$ at 298.2 K against 0.100 mol dm^{-3} NaOH.

The curves, running through the experimental points collected, represent the best fit of the data over the pH range of the titration.

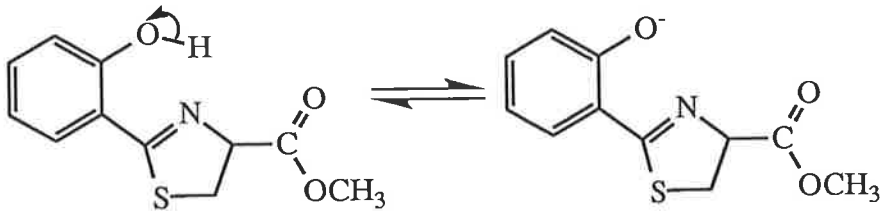
The pK_a values for the acidic protons of PTA, PTME and PTEE, represented in schemes 4.1 - 4.3 respectively, were derived from the best fit of the experimental data (section 8.2.3) to equations 4.4 and 4.5. PTA was found to have pK_a values of 4.62 ± 0.01 and 11.98 ± 0.01, for its carboxylic and phenolic protons respectively (Scheme 4.1). The corresponding methyl ester, PTME, had a determined pK_a of 10.99 ± 0.01 for its phenolic proton (Scheme 4.2). The decreased acidity of the phenolic proton of PTA as compared with that of the corresponding methyl ester, PTME, is probably due to the

unfavourable electron density increase caused by the formation of a double negatively charged species.



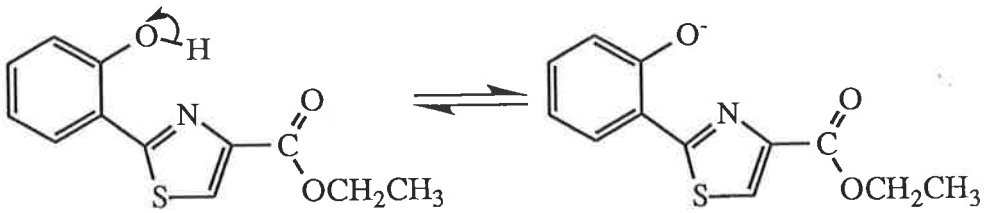
PTA

Scheme 4.1



PTME

Scheme 4.2



PTEE

Scheme 4.3

The pK_a values of the phenolic protons of the thiazolines are higher than that for phenol itself (9.82 ± 0.04 at 298K in an aqueous solution of 0.1 mol dm^{-3} ionic strength[6]). Apart from the different solvent system used in this study, a major cause of the reduced acidity of the phenolic protons of the thiazolines, PTA and PTME, is the existence of a hydrogen bonding interaction between the nitrogen of the thiazoline ring and the phenolic hydrogen as shown in Figure 4.2. Molecular modelling[7] (section 8.2.4)

has shown that when the phenyl and thiazoline rings of PTA lie in the same plane, the distance between the nitrogen of the thiazoline ring and the phenolic hydrogen is 176 pm. This distance is within the limits of hydrogen bonding distance which, for the modelling package used,[7] has an upper limit of 225 pm. Therefore it is possible for these two atoms to be involved in a hydrogen bond. For proton dissociation to occur, this favourable 1,6-interaction would need to be broken as well as the O-H bond. Hence the phenolic protons of the thiazolines, PTA and PTME, are less acidic than that of phenol itself.

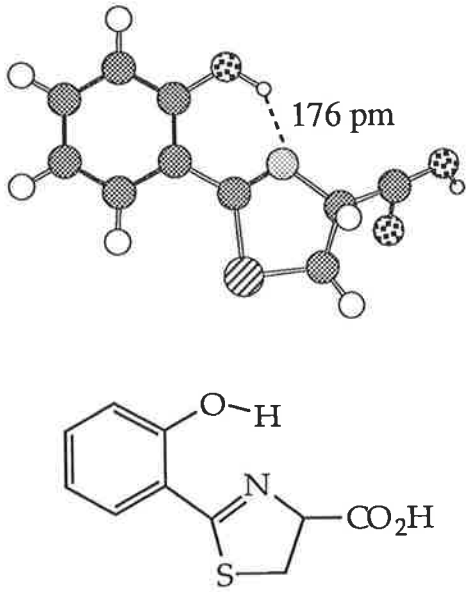
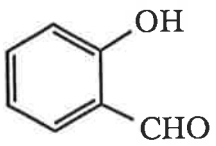


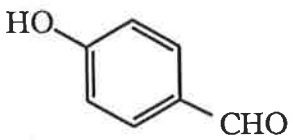
Figure 4.2 Molecular model[7] representation of the thiazoline acid PTA.

Support for this reasoning can be seen by comparison of the pK_a values of salicylaldehyde and 4-hydroxybenzaldehyde. Salicylaldehyde, with a pK_a for its phenolic proton of 8.13 ± 0.01 in an aqueous solution of ionic strength 0.1 mol dm^{-3} and at 298 K, is over five times less acidic than 4-hydroxybenzaldehyde, which has a pK_a of 7.41.[6] Both phenols have the same electron withdrawing effects favourable for acid dissociation. However, salicylaldehyde, with its aldehyde substituent ortho to the hydroxy group, can have a hydrogen bonding interaction occur between the oxygen of the aldehyde moiety and the phenolic hydrogen. Such an interaction cannot occur in

4-hydroxybenzaldehyde. Hence, the reduced acidity of salicylaldehyde can be attributed to the possible hydrogen bonding interaction between the aldehyde oxygen and the phenolic hydrogen, reducing the likelihood of proton dissociation.



Salicylaldehyde



4-Hydroxybenzaldehyde

The pK_a of the phenolic proton of the thiazole, PTEE, was determined to be 9.43 ± 0.01. This increased acidity as compared with the thiazolines, PTA and PTME, is most likely due to the increased conjugation in the thiazole, which creates more stabilisation of a negative charge, through greater delocalisation of electron density and makes proton dissociation more favourable. As well as this, the protonated nitrogen of the fully aromatic thiazole ring is less acidic than the protonated nitrogen of the thiazoline ring. The pK_a values of these protonated nitrogens have been quoted as 2.44 and 3.64, for thiazole and thiazoline respectively.[8] Due to the increased ring strain and shorter bonding distance of the extra π bond in the thiazole, the distance between the thiazole nitrogen and the phenolic hydrogen is slightly greater than that of the thiazolines. Computer aided molecular modelling[7] (section 8.2.4) showed this distance to be 186 pm, when the two rings lie in the same plane (see Figure 4.3). Thus, the reduced acidity of the thiazole protonated nitrogen and the increased distance between the thiazole nitrogen and the phenolic hydrogen, reduces the strength of the hydrogen bond between these two atoms. Therefore the acidity of the phenolic hydrogen on the thiazole, PTEE, is greater than that of the corresponding thiazoline.

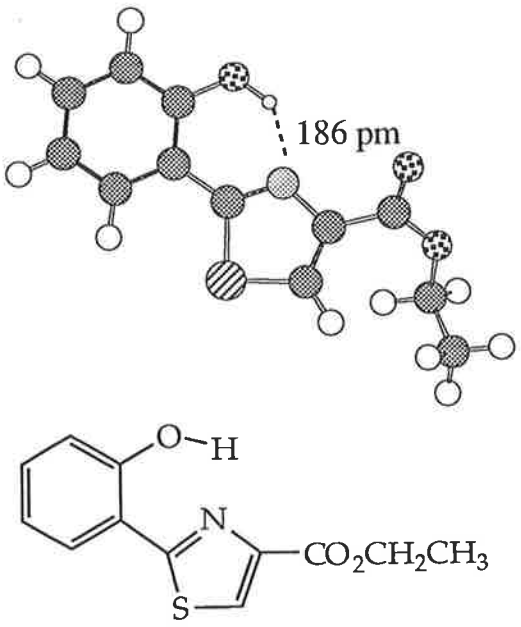


Figure 4.3 Molecular model[7] representation of PTEE.

As stated above, the pK_a of the protonated nitrogen of thiazole has been quoted as 2.44 and thiazoline as 3.64.[8] It is expected that the presence of the carboxyl substituent on the thiazole derivatives, PTA, PTME and PTEE, should increase the acidity of the protonated nitrogens on their thiazole rings. In the titrations carried out on the thiazole derivative, no dissociation of the protonated ring nitrogen was detected. This suggests that the acidity of the protonated ring nitrogen of these derivatives have been increased by the substituents on the heterocyclic rings. Due to the limitations of the glass electrode,[1,3] the low pK_a values characterising these dissociations could not be accurately measured. This did not inconvenience further calculations of stability constants of complexes formed by these compounds with various metals, as all calculations for stability constants were made using data collected at pH values higher than the expected pK_a values of the protonated nitrogens. Hence the thiazoline and thiazole nitrogens of PTA, PTME and PTEE were assumed to be fully deprotonated.

The thiazoline carboxylic acid, PTA, was chosen as the ligand for which the stability constants of complexes it formed with a series of metal ions were determined.

The acid, PTA, was selected in preference to the esters, PTME and PTEE, because within a cell, it would be more likely for a carboxyl group on a compound to be in the acid form rather than an ester form, due to the action of esterases present which hydrolyse ester functional groups to the corresponding acid. For the other two thiazole derivatives, PTME and PTEE, stability constants for their complexes formed only with Zn^{2+} and Pb^{2+} were determined, as these complexes were of the most interest.

In Table 4.1, the log values of the stepwise stability constants for metal complexes formed with the thiazole derivatives, PTA, PTME and PTEE, are listed. These values were derived from the best fit of the experimental data to equations 4.9 and 4.10. Stability constant values were determined for both the mono and bis complexes of the deprotonated thiazole derivatives with the metals listed. No other complexes were detected in the titration solutions.

Under the conditions used, precipitation of some metal hydroxide species occurred at high pH values, especially when the titration solutions either contained relatively high total concentrations of metal ions or where the affinity of the ligand for the metal ion was relatively low. The pH at which such precipitation occurred was dependent on the metal ion, its concentration and the readiness of complex formation between the metal ion and ligand in the solution. Data collected at and above the pH at which precipitation had occurred were ignored when calculating stability constants, since accurate total concentrations of the metal ion in solution above this pH could not be determined.

For the metal ions Al^{3+} and Cu^{2+}, precipitation of an hydroxide species occurred in titration solutions whose pH were as low as 4 and 3.5 respectively. Below these pH values, complexes of these metal ions with the thiazoline carboxylic acid, PTA, had not formed to a significant extent. Therefore, accurate determination of stability constants of such complexes was not possible. An upper limit was estimated for the stability constants of the mono complexes formed by PTA with Al^{3+} and Cu^{2+}. These limiting

Table 4.1 Stepwise stability constants for the complexes formed by the thiazolines PTA and PTME and the thiazole PTEE with various metal ions.[a]

Ligand[b]	Metal Ion	log K_1[c]	log K_2[c]
PTA	Al^{3+}	< 9.2[d]	
	Ca^{2+}	6.50 ± 0.01	5.19 ± 0.01
	Cu^{2+}	< 12.3[d]	
	Zn^{2+}	7.63 ± 0.01	6.36 ± 0.01
	Cd^{2+}	11.93 ± 0.02	8.50 ± 0.04
	Pb^{2+}	13.04 ± 0.01	10.32 ± 0.01
PTME	Zn^{2+}	7.09 ± 0.01	7.42 ± 0.01
	Pb^{2+}	8.43 ± 0.01	7.52 ± 0.02
PTEE	Zn^{2+}	5.02 ± 0.01	5.51 ± 0.01
	Pb^{2+}	6.04 ± 0.01	4.86 ± 0.02

a Solutions contained 75% methanol and 25% water, I = 0.100 mol dm^{-3} ($NaClO_4$) and measurements were made at 298.2 K.

b Ligands are fully deprotonated.

c The units of K_1 and K_2 are dm^3 mol^{-1}.

d Upper limit based on the formation of 5% of the 1:1 species. As these species are not detected at a significant concentration their stability constant must be less than this value.

values were obtained using MacSpecies, a Macintosh compatible version of the program Spe.[1] They represent the maximum values of the stability constants for which ≤5% of the complexes concerned would have formed at the pH where precipitation began to occur.

The log of the stability constants for the various metal complexes of the acid, PTA, were derived from experimental data approximately within the pH ranges of 8.0 - 10.0, 7.8 - 10.2, 4.3 - 7.5 and 3.9 - 6.0, for Ca^{2+}, Zn^{2+}, Cd^{2+} and Pb^{2+} respectively. The pH range of experimental data points used to fit the stability constants was found to move to a higher pH and broaden in range, with decreasing amount of total metal ion concentration. Speciation plots for the acid PTA and Ca^{2+}, Zn^{2+}, Cd^{2+} and Pb^{2+} systems are shown in Figures 4.4 - 4.7 respectively. As can be seen from these plots, significant amounts of the complexes had formed, within the pH ranges used, to enable accurate determination of their stability constants

The stability constants, K_1 and K_2, for the 1:1 and 2:1 complexes formed between the thiazoline acid, PTA, and Zn^{2+} are 35 and 15 times larger than the corresponding Ca^{2+} complexes respectively. Such a trend is as expected, since for metal ions of the same ionic charge, the smaller the ionic radius of the ion, the greater the stability of the complexes formed.[9] Based on the Irving-Williams series,[10] it is expected that a Cu^{2+} complex would have a higher stability constant than the equivalent Zn^{2+} complex formed with the same ligand. This is because Cu^{2+} is able to form complexes of a distorted octahedral configuration, which maximises its ligand field stabilisation energy. Therefore it could be predicted that the value of the log of the stability constant, log K_1, for a 1:1 Cu^{2+} complex formed by PTA would be greater than the log K_1 for a 1:1 Zn^{2+} complex, 7.63, yet less than the upper limit calculated for the 1:1 Cu^{2+} complex, 12.3 (Table 4.1). This approximated range provides a better indication of the affinity of PTA for Cu^{2+} than the upper estimate of log K_1 for Cu^{2+}, 12.3, alone.

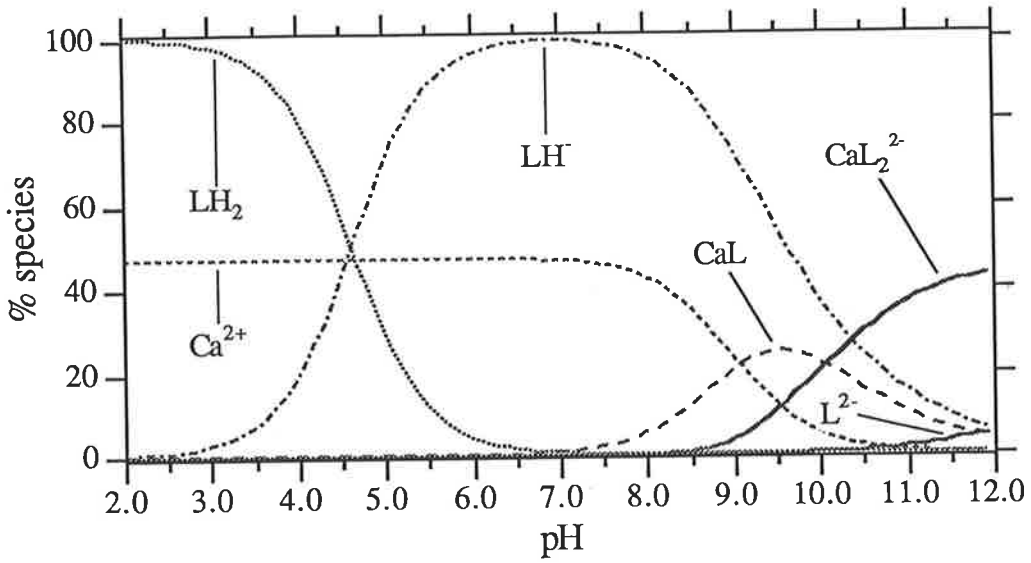


Figure 4.4 Species plot for the Ca^{2+} and thiazoline acid, PTA, system, determined using the appropriate values from Table 4.1. The total concentrations of PTA and Ca^{2+} are 0.001 mol dm^{-3} and 0.00047 mol dm^{-3} respectively. The discontinuity of the spectra is caused by the digitisation of the printer.

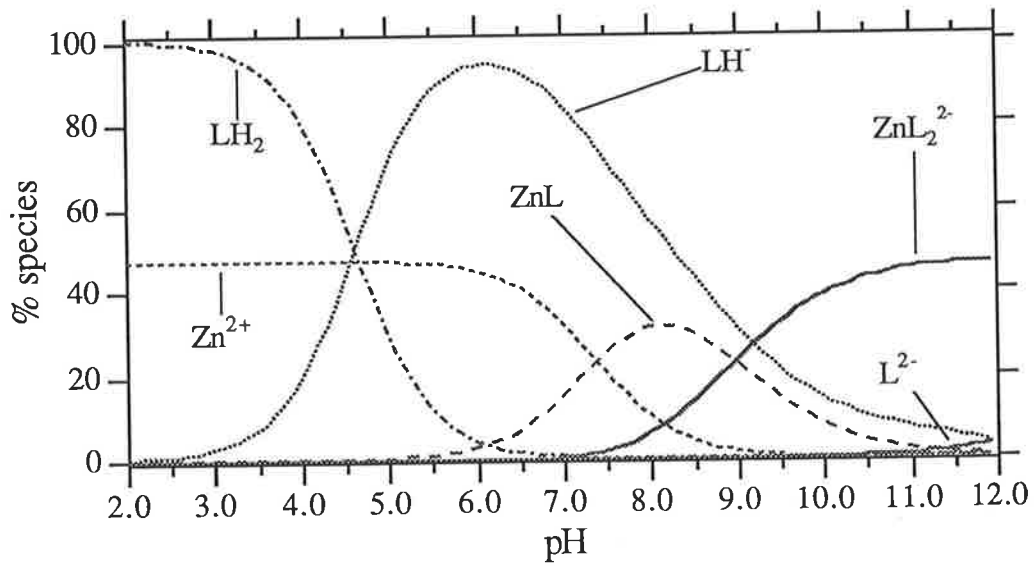


Figure 4.5 Species plot for the Zn^{2+} and thiazoline acid, PTA, system, determined using the appropriate values from Table 4.1. The total concentrations of PTA and Zn^{2+} are 0.001 mol dm^{-3} and 0.00047 mol dm^{-3} respectively.

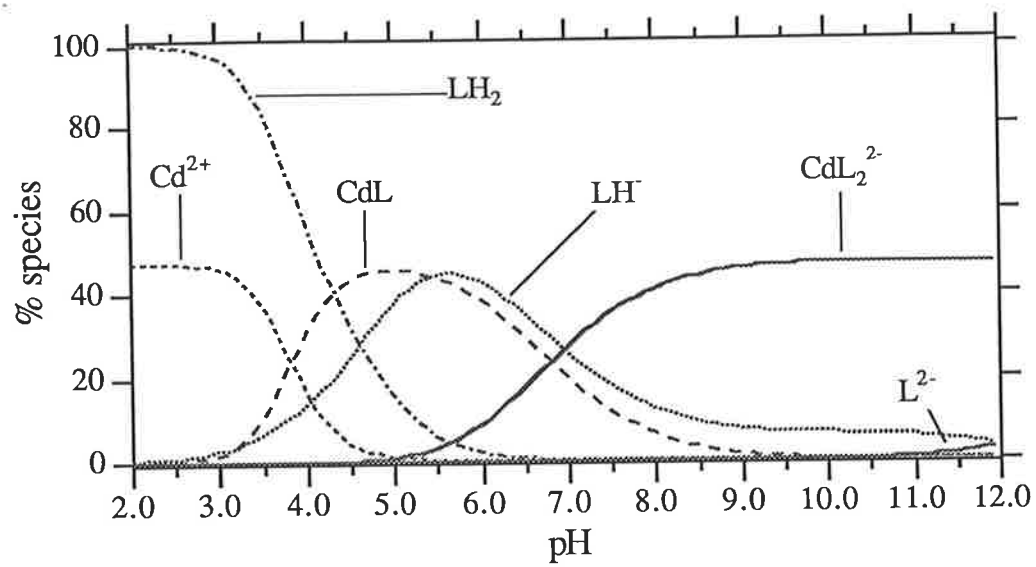


Figure 4.6 Species plot for the Cd^{2+} and thiazoline acid, PTA, system, determined using the appropriate values from Table 4.1. The total concentrations of PTA and Cd^{2+} are 0.001 mol dm^{-3} and 0.00047 mol dm^{-3} respectively.

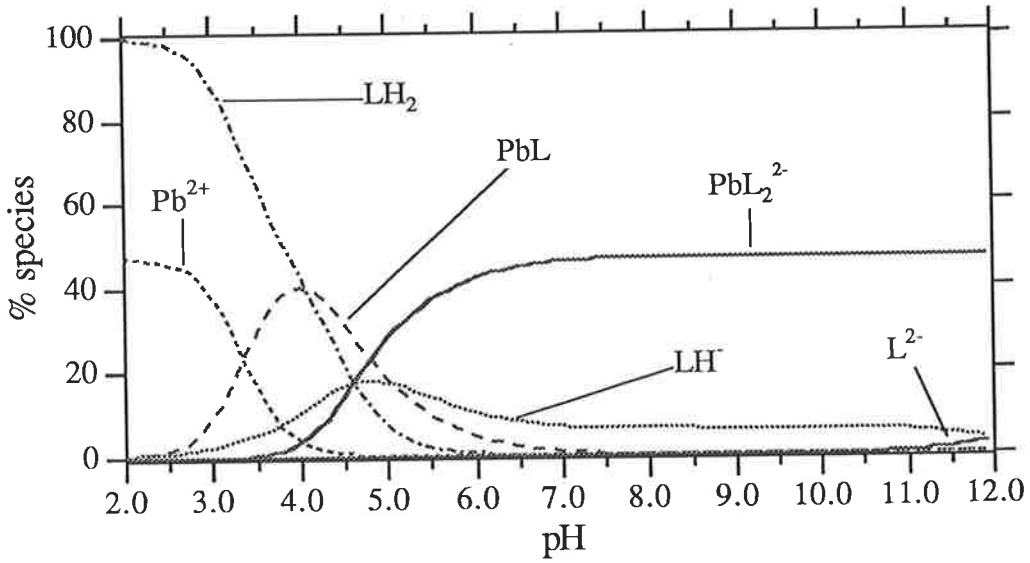


Figure 4.7 Species plot for the Pb^{2+} and thiazoline acid, PTA, system, determined using the appropriate values from Table 4.1. The total concentrations of PTA and Pb^{2+} are 0.001 mol dm^{-3} and 0.00047 mol dm^{-3} respectively.

The comparatively high stability constants of the 1:1 and 2:1 complexes of PTA with Pb^{2+} and Cd^{2+} suggests that a terdentate species may be forming between PTA and these metal ions, with chelation occurring probably through the phenolic oxygen, either the nitrogen or sulfur of the heterocyclic ring and one of the carboxylate oxygens. Whereas, PTA probably forms bidentate complexes with the smaller metal ions tested, with the chelation sites possibly being the phenolic oxygen and either of the ring nitrogen or sulfur. The increase of stability of a complex with increase in the denticity of the ligand is known as the chelate effect.[9,11] The ability for Pb^{2+} and Cd^{2+} to form terdentate complex species with PTA may be due to their relatively large atomic sizes, having ionic radii for a six coordinate species of 119 pm and 95 pm respectively. Zinc (II), in comparison, has an ionic radius of 74 pm for a six coordinate species.[12] Hence, PTA could bind to the larger Pb^{2+} and Cd^{2+} ions through its phenolic oxygen, a carboxylate oxygen and either the nitrogen or sulphur on the thiazoline ring. Binding to the nitrogen is more probable, since when a given electron acceptor (acid) binds to a strong electron donor (hard base) the likelihood for it to bind further to hard bases is increased[9] and the thiazoline nitrogen is the harder base of the two electron donors on the thiazoline ring.

From Table 4.1, it can also be seen that the 2:1 complexes formed between the acid and the metal ions tested have much lower stability constants than the corresponding 1:1 complexes. This reduced stability can be attributed not only to the statistical effect,[9,11] but also to the unfavourable formation of negatively charged species.

The values of the stability constants for the metal complexes of PTME were derived from experimental data within the pH ranges of 4.0 - 7.9 and 4.5 - 7.7, for Zn^{2+} and Pb^{2+} respectively. Similarly, the values of the stability constants for the metal complexes of the thiazole ethyl ester, PTEE, were derived from experimental data within the pH ranges of 6.0 - 7.5 and 5.0 - 8.0, for Zn^{2+} and Pb^{2+} respectively. As with PTA, the actual pH range of experimental data points used to fit the stability constants was

found to move to a higher pH and broaden in range, with decreasing amount of total metal ion concentration. Speciation plots for PTME and Zn^{2+} and Pb^{2+} systems are shown in Figures 4.8 and 4.9 respectively. Speciation plots for PTEE and Zn^{2+} and Pb^{2+} systems are shown in Figures 4.10 and 4.11 respectively. Once again, it can be seen from these plots, that significant amounts of the complexes had formed, within the pH ranges used, to permit accurate determination of their stability constants .

For both the thiazoline methyl ester, PTME, and the thiazole ethyl ester, PTEE, the stability constants of the 2:1 complexes formed with Zn^{2+} are larger than those of the 1:1 complexes. This suggests that the coordination number of Zn^{2+} may undergo a change from six coordinate to four coordinate on forming a 2:1 complex with these ligands, thus favouring a tetrahedral geometry. Such an occurrence is not observed for the 2:1 complex of the acid with Zn^{2+}, most probably because of the unfavourable increase in ligand charge interactions, which would occur on formation of the smaller tetrahedral geometry.

The 1:1 and 2:1 complexes of the thiazole ester, PTEE, with Zn^{2+} were found to have lower stability constant values than those of the acid, PTA, and its methyl ester, PTME. Molecular modelling[7] (section 8.2.4) found the distance between the phenolic oxygen and the nitrogen of the thiazoline acid, PTA, and the thiazole, PTEE, to be 255 pm and 266 pm respectively (see Figure 4.12). It is believed that the larger 'bite' distance between the phenolic oxygen and the nitrogen atom of the thiazole ring compared with that of the thiazolines is responsible for the reduced stability of the thiazole Zn^{2+} complexes. Also, the increased stability of the 1:1 Zn^{2+} complex of the acid, PTA, compared to that of the corresponding methyl ester, PTME, is probably due to the favourable formation of a neutral complex between the fully deprotonated acid and Zn^{2+}.

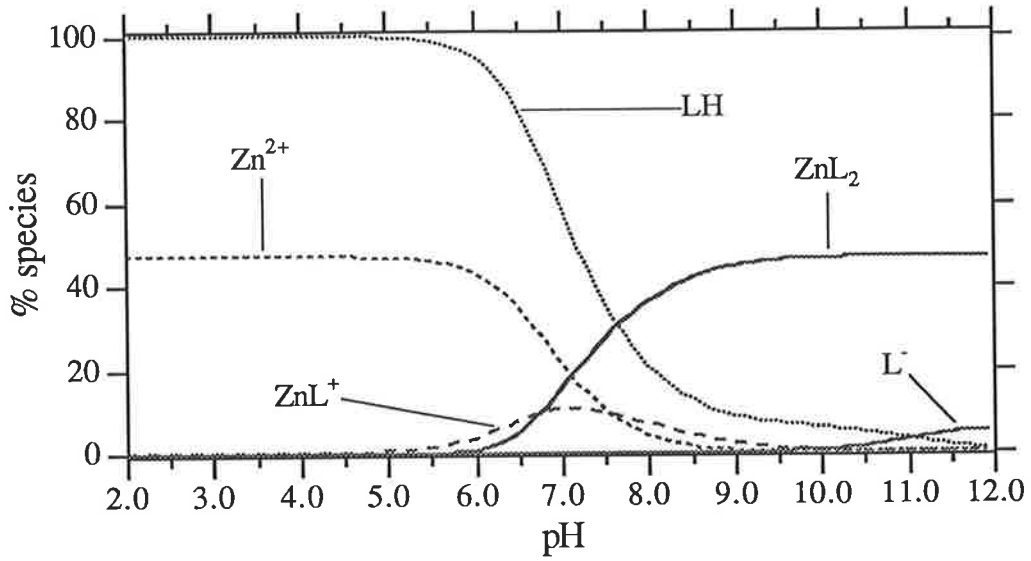


Figure 4.8 Species plot for the Zn^{2+} and thiazoline methyl ester, PTME, system, determined using the appropriate values from Table 4.1. The total concentrations of PTME and Zn^{2+} are 0.001 mol dm^{-3} and 0.00047 mol dm^{-3} respectively.

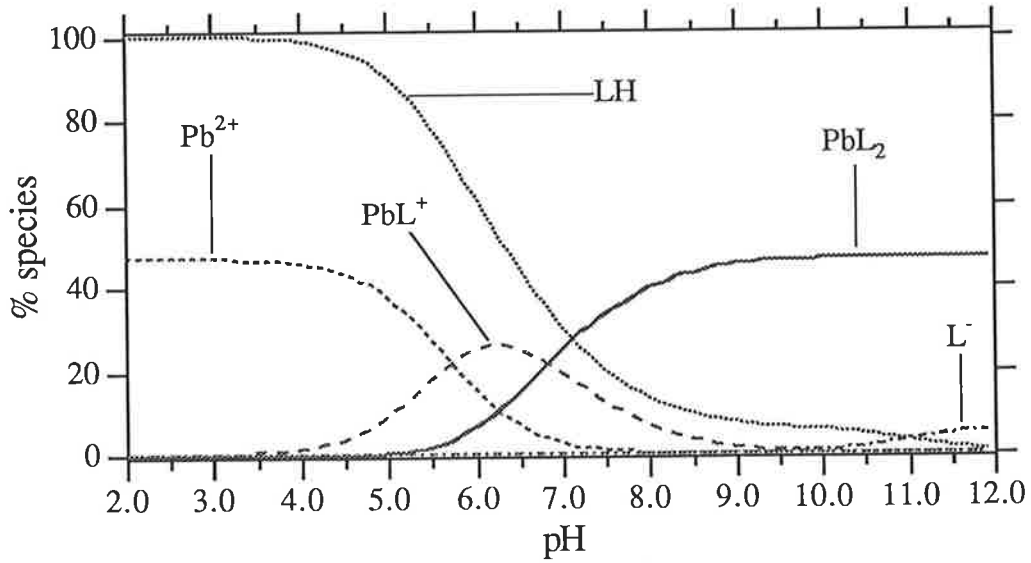


Figure 4.9 Species plot for the Pb^{2+} and thiazoline methyl ester, PTME, system, determined using the appropriate values from Table 4.1. The total concentrations of PTME and Pb^{2+} are 0.001 mol dm^{-3} and 0.00047 mol dm^{-3} respectively.

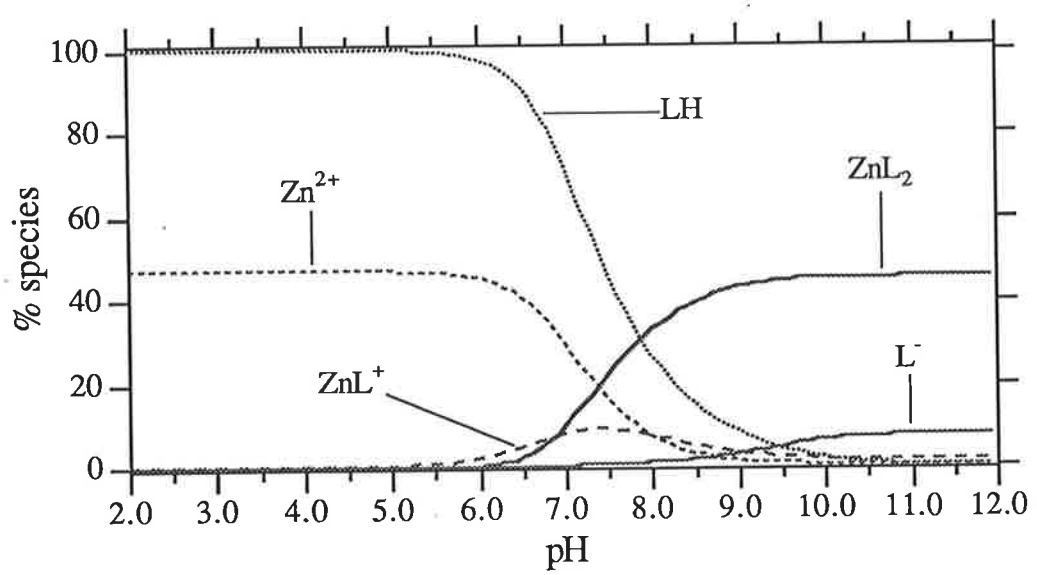


Figure 4.10 Species plot for the Zn^{2+} and thiazole ethyl ester, PTEE, system, determined using the appropriate values from Table 4.1. The total concentrations of PTEE and Zn^{2+} are 0.001 mol dm^{-3} and 0.00047 mol dm^{-3} respectively.

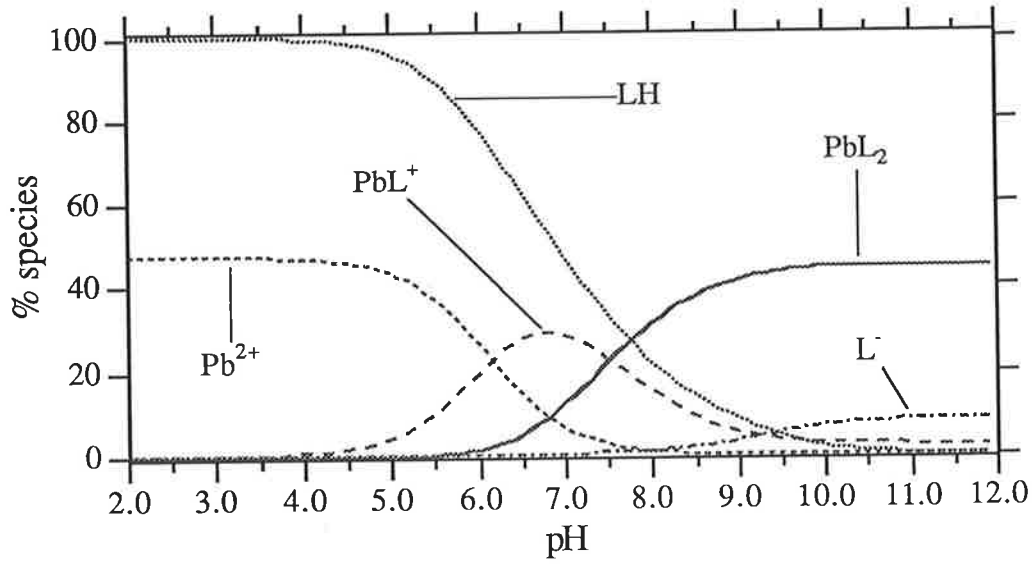


Figure 4.11 Species plot for the Pb^{2+} and thiazole ethyl ester, PTEE, system, determined using the appropriate values from Table 4.1. The total concentrations of PTEE and Pb^{2+} are 0.001 mol dm^{-3} and 0.00047 mol dm^{-3} respectively.

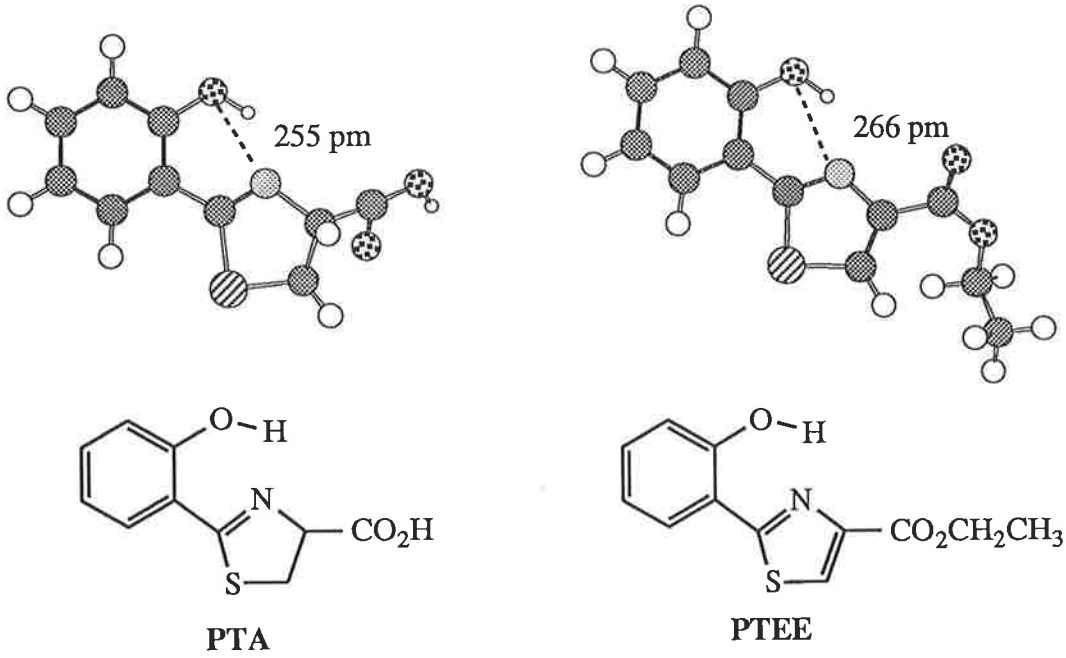


Figure 4.12 Molecular representation of the thiazoline, PTA, and the thiazole, PTEE,.

The stability constant values of the Pb^{2+} complexes of the esters, PTME and PTEE, are relatively low compared with those of the acid, PTA, suggesting that terdentate species are not formed between Pb^{2+} and these ligands. The reduced stability of the 2:1 complexes compared with those of the 1:1 complexes, indicates that no coordination number change occurs for Pb^{2+} on coordination to the esters, PTME and PTEE. Therefore these complexes are likely to be octahedral. However, like PTA, the stability constant values of the 1:1 complexes of the esters, PTME and PTEE, with Pb^{2+} are higher than those for the corresponding Zn^{2+} complexes. Thus all three ligands tested PTA, PTME and PTEE show a higher binding affinity to Pb^{2+} than Zn^{2+}.

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5 Fluorescence

5.1 Introduction

5.1.1 Principles of Fluorescence

Luminescence is the emission of light from a molecule as it relaxes from an excited electronic state to its ground state. This form of relaxation is known as radiative decay and usually occurs when the available forms of radiationless decay, such as the transfer of excess energy in the excited state into vibrational, rotational and thermal energy, are not sufficient enough to relax the molecule back to its ground state. There are two main forms of luminescence, these being fluorescence and phosphorescence. The Jablonski diagram[1] (Figure 5.1) shows the difference between the two processes.

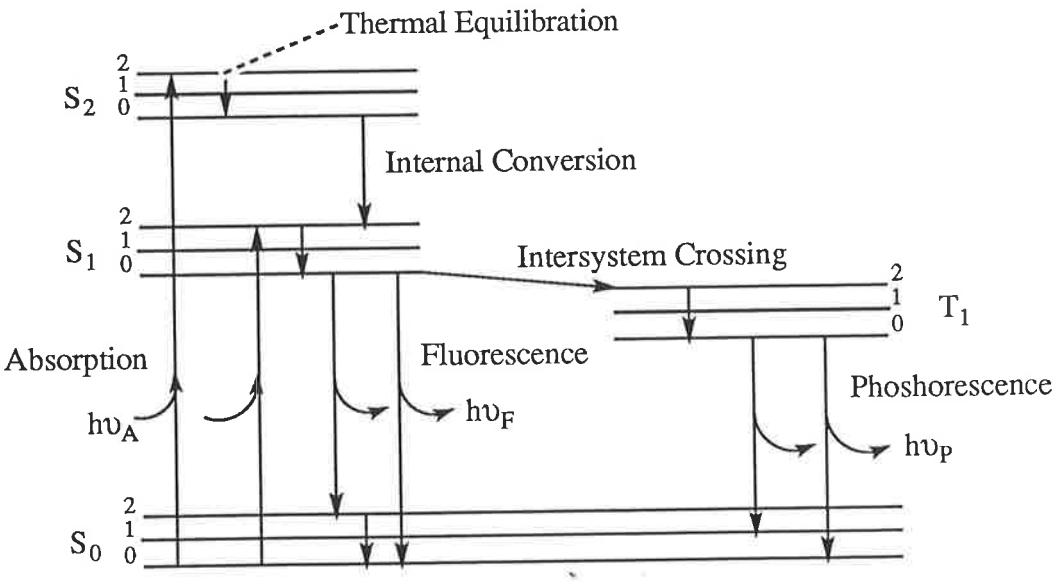

Figure 5.1 Jablonski Diagram

Energy level diagram for the absorption and emission of light.
S_0, S_1, S_2 represent the ground state and the first and second excited electronic states respectively.
T_1 is the first triplet state.
0, 1, 2 are the vibrational energy levels within an electronic state.

On absorption of light, a molecule is excited from its ground state to one of the various vibrational levels of higher electronic energy, depending on the energy of the light absorbed. This process of excitation occurs in approximately 10^{-15} seconds. Following excitation, the molecules, if not already in the lowest vibrational level of the excited electronic state, undergo thermal equilibration through vibrational relaxation. That is, the excited molecule will vibrate, releasing excess energy in the form of infrared quanta or kinetic energy by collision with other molecules, until it has relaxed to the lowest vibrational level of the excited electronic state in which the molecule exists. Thermal equilibration takes around 10^{-12} seconds.[1,2]

Once in the lowest vibrational level of an excited electronic state, a molecule can relax further to lower electronic energy levels through internal conversion. If vibrational levels of two electronic energy states overlap, internal conversion can occur through vibrational relaxation and thermal equilibration to the lowest vibrational level of the lower electronic energy state. If vibrational levels of sequential electronic energy levels do not overlap, but the energy gap between them is small, then internal conversion can occur by a process known as tunnelling.[2]

However, if the energy gap between neighbouring electronic energy states is relatively large, relaxation of an excited molecule to lower energy levels can not occur through internal conversion. In this circumstance, an excited molecule may relax to a lower energy level through radiative decay, releasing excess energy in the form of ultraviolet or visible light. This emission of light is known as fluorescence and occurs over a period of approximately 10^{-8} seconds, which is referred to as the fluorescence lifetime.[1,2]

For most compounds, their excited electronic states are usually close in energy, with the greatest energy gap mainly occurring between the ground electronic state and

the first excited energy level of a molecule. Thus, since internal conversion and thermal equilibration occur within 10^{-12} seconds, fluorescence mostly arises from a transition from the lowest vibrational level of the first excited electronic energy state to any of the vibrational levels of the molecular ground electronic state. The molecule then undergoes thermal equilibration to the lowest vibrational level of the ground state.

As absorption of light occurs from the lowest vibrational level of a molecule's ground state to any of the vibrational levels of an excited electronic state of a molecule, the absorption spectrum of a molecule represents the differences in vibrational energy levels of the excited electronic state. Similarly, since fluorescence occurs by relaxation of a molecule from the lowest vibrational level of its first excited electronic state, to any vibrational level of its ground state, the emission spectrum of a molecule represents differences in vibrational energy levels of the molecule's ground electronic state. Since there is little difference in the vibrational energy levels of a molecules various electronic energy states, the fluorescence emission spectrum of a molecule usually approximates to a mirror image of that molecule's absorption spectrum. However, a molecule's emission spectrum is usually shifted to longer wavelengths than its absorption spectrum due to energy losses through internal conversion and other such processes. This shift to longer wavelengths is referred to as a Stoke's shift.[1,2]

If the lifetime of the excited electronic state of the molecule is of long enough duration, the molecule may undergo intersystem crossing. This involves a spin state change as the molecule goes from its first excited singlet state to its first triplet state, which is usually of lower energy due to less electronic repulsion.[2] This process is found to occur when there is overlap of the vibrational energy levels of the two electronic states. Relaxation of the molecule from its triplet electronic state to its ground state is spin forbidden and is quite slow, with the excess energy being released, once again, in the form of light. The emission of light from this transition is known as phosphorescence and occurs at longer wavelengths than fluorescence. Phosphorescent

emissions tend to occur for comparatively long periods of time, once the emission source has been removed, whereas fluorescence ceases almost immediately. This is because triplet states have relatively long lifetimes from around 10^{-5} up to a few seconds.[1,2]

In the triplet state, a molecule may undergo excitation back to its first electronic singlet state by thermal excitation or transfer of energy through collision with another excited molecule in its triplet state. Relaxation of the molecule may then occur through radiative decay. This form of radiative decay is referred to as delayed fluorescence and is of a frequency equal to that of fluorescence, but is characterised by a decay time similar to that of phosphorescence.[2] Delayed fluorescence is a relatively uncommon form of radiative decay.

5.1.2 Characteristics of Fluorescent Organic Compounds

Not all compounds are fluorescent. For organic compounds, Bridges[3] has described some of the structural specifications required for a compound to be fluorescent. Firstly, the organic compounds require an extensive system of conjugated double bonds. It has been found that organic compounds whose conjugation exists in ring systems are more fluorescent than those with conjugated double bonds in a non-cyclic chain arrangement. The fluorescence of an organic molecule is again increased if multiple ring systems are arranged linearly as compared with any other arrangement.

Secondly the geometrical arrangement of the molecule is important for fluorescence. For instance, the conjugated system needs to be planar to maximise the delocalisation of the π electrons. If this planarity is destroyed, for example by steric hindrance, the delocalisation of the π electrons will be partially inhibited, which can result in a reduction or loss of fluorescence. A molecule also needs to be of rigid

structure for it to be fluorescent otherwise the absorbed energy may be lost through radiationless decay as described in section 5.1.1.

Substituents directly attached to part of the conjugated system of an organic molecule can also affect the fluorescence of the molecule as they can influence the delocalisation of the π electrons. For instance, electron donating substituents enhance the delocalisation of the π electrons and hence enhance fluorescence.[3] However, electron withdrawing substituents reduce the delocalisation of π electrons, therefore reducing fluorescence.[3] Also, substituents directly attached to or in close proximity of a conjugated system may affect the fluorescence of a molecule due to the substituent's bulkiness breaking down the planarity of the conjugated system and the delocalisation of π electrons.

It appears that whether or not an organic compound is fluorescent, is very dependent upon the rigidity or reduced vibrational degrees of freedom of a compound. This is why aliphatic compounds, with their non-rigid molecular skeletons and their many degrees of vibrational freedom are mostly non-fluorescent. Rigidity of organic compounds is achieved through the conjugation of double bonds and formation of ring systems. This is why many aromatic organic compounds are fluorescent. Also, the more extensive the conjugation in a compound, the greater is the delocalisation of the π electrons within the compound and the more fluorescent the compound becomes.

5.1.3 Applications of Fluorescence

Since modern photomultipliers are very sensitive, fluorescent materials may be measured at concentrations as low as 10^{-10} mol dm^{-3}.[4] Thus, fluorescence spectroscopy is a highly selective and sensitive analytical tool as compared to other colorimetric methods which, at best, measure concentrations down to approximately 10^{-7} mol dm^{-3}.[4]

Fluorescence spectroscopic techniques are widely used in medical and biological fields for the detection and measurement of biochemically important molecules.[4,5] Along with the biochemical usefulness of fluorescence measurements, the technique known as fluorescence microscopy has proven to be a very useful analytical tool in many areas of research ranging from material science to industrial and forensic science.[5] Due to its high sensitivity, fluorescence microscopy is used widely in detecting and studying the distribution of substances present in minute quantities, within various samples from living cells to coal.[6]

5.1.4 Fluorescent Probe Specifications for the Detection of Zinc (II)

In addition to the previously mentioned general structural requirements of fluorescent organic compounds, there exists a number of other properties desirable for compounds to be useful as fluorescent probes for the detection and measurement of readily available Zn^{2+} in biological systems. Along with the required specificity of the fluorescent probe or ligand for Zn^{2+}, it also needs to produce a highly fluorescent species once chelated to Zn^{2+}, for it to be a useful probe. Normally a fluorescent organic ligand will lose its tendency to fluoresce when it forms a complex with divalent metal ions from the first transition series. This is because these metal ions have partially filled d orbitals and can absorb the excess excited energy of the ligand by excitation of a d electron to a higher energy state. This process is known as a d-d transition and quenches the fluorescence of the attached ligand. Zinc (II) with its filled d orbitals, can not absorb the excess energy of an excited ligand coordinated to it through a d-d transition. Therefore, if a ligand has a tendency to fluoresce, its Zn^{2+} complex tends to be fluorescent as well. In most cases, a ligand's fluorescence is enhanced on coordinating metal ions such as Zn^{2+}, as the formation of a complex tends to lock the ligand in a geometry of greater rigidity, reducing the number of degrees of vibrational freedom of the ligand. This is provided that on forming a complex, the

delocalisation of the π electrons of the ligand is not adversely affected, for example, by the breakdown of planarity of the conjugated system of the ligand.

Another probe specification is that the fluorescent properties of the unbound ligand must be significantly different from that of the Zn^{2+} complex to enable differentiation between the two. For a ligand to be useful as a fluorescent probe for Zn^{2+}, it is desirable to be able to measure the fluorescence of the ligand's Zn^{2+} complex without interference from any uncomplexed ligand which may be present. Thus, it is preferable that the uncoordinated and Zn^{2+} coordinated ligand have differing emission spectra. Ideally there should be minimal fluorescence of the free ligand at an emission maxima of the Zn^{2+} complexed ligand, therefore enabling precise measurement. The optimum situation is where the Zn^{2+} complexed ligand is highly fluorescent and the unbound ligand is non-fluorescent, or at least poorly fluorescent, with emission maxima somewhat different to those of it's Zn^{2+} complex.

Similarly, if the ligand has a tendency to form complexes with other metal ions present in a sample, then these complexes formed need to have differing emission spectra from that of the Zn^{2+} complexed ligand. For the ligand to be a useful fluorescent probe for Zn^{2+}, no interference from other metal complexes formed by the ligand should occur during fluorescence measurements of the ligand's Zn^{2+} complex. Once again, the optimum situation would be where metal complexes of the ligand other than those with Zn^{2+} are non-fluorescent or at least poorly fluorescent with emission maxima different from those of its Zn^{2+} complex.

As seen earlier, (section 3.2), the thiazole derivatives, PTA, PTME and PTEE, are not Zn^{2+} specific and chelate to other metal ions as well as the Zn^{2+} ion. The fluorescence spectra of the thiazole derivatives, PTA, PTME and PTEE, were measured along with that of the various metal complexes, which they form. Comparison of these

results was expected to give more information on these ligands suitability as fluorescent probes for measuring Zn^{2+}.

5.2 Results and Discussion

This research is concerned with developing fluorescent probes, suitable for detecting and measuring readily exchangeable intracellular Zn^{2+} within biological systems. Therefore, fluorescence measurements of PTA, PTME and PTEE and their various metal complexes were conducted on sample solutions whose conditions mimicked, as closely as possible, those within a biological cell. Hence, for the reasons discussed in section 3.2, a solvent system comprising of 75% methanol and 25% water was used to prepare sample solutions. All sample solutions had an ionic strength of 0.100 mol dm^{-3}, controlled by sodium perchlorate.

The concentration of the thiazole derivatives used in sample solutions was based on that of PTME which, when in the presence of excess Zn^{2+}, produced a significantly strong fluorescence. This concentration of PTME was 5.0×10^{-6} mol dm^{-3} and was that of the thiazole derivatives used in all sample solutions prepared. This allowed for a direct comparison of the results obtained.

The chosen concentration of metal ions added to the sample solutions was based on the minimum concentration of Zn^{2+} required to be added to a 5×10^{-6} mol dm^{-3} solution of PTME, to ensure complete complex formation by PTME. The value of this concentration was calculated using MacSpecies[7] and values of the stability constants for the complexes formed between PTME and Zn^{2+}, determined previously (section 4.2). Thus the concentration of Zn^{2+} required was 2.00×10^{-3} mol dm^{-3}. Hence, 2.00×10^{-3} mol dm^{-3} of test metal ions were added to the relevant sample solutions.

To buffer the sample solutions to a pH of approximately 7.0, 1.00×10^{-3} mol dm^{-3} NaPIPES was added. Accurate measurement of the pH of the sample solutions found those which contained the metal ions Ca^{2+}, Co^{2+}, Ni^{2+}, Cu^{2+}, Zn^{2+}, or Cd^{2+} had pH values in the range 6.9 - 7.1. However, solutions which contained the metal ions Al^{3+} or Pb^{2+} had pH values of 3.80 ± 0.05 and 5.6 ± 0.5 respectively. Hence the effect of the buffer in these solutions was overridden by the formation of acidic hydrated species in solution by these metal ions. Increasing the concentration of the buffer, in an attempt to raise the pH of these solutions, created solubility problems. Therefore the solutions were worked with at these pH values.

On standing, over a period of a day or so, sample solutions which contained Al^{3+}, Cu^{2+}, Zn^{2+} and Pb^{2+}, produced a precipitate of an hydroxide species. Not only did this alter the total concentration of metal ion in solution, but it also affected the pH of the solutions. To avoid this situation, solutions were prepared and their spectra recorded within eight hours. This tended to eliminate the problem of precipitation occurring in these sample solutions, except for those which contained Pb^{2+}. For the solutions which contained Pb^{2+}, formation of a precipitate was unavoidable and the solutions were centrifuged, to remove the precipitate, before their various emission spectra were recorded. Therefore, the total Pb^{2+} concentration within the sample solutions used was not accurately known.

The fluorescence emission spectra in the visible region of the thiazole derivatives, PTA, PTME and PTEE, were measured under the conditions described in section 8.2.5. Also measured were the emission spectra of the metal complexes which PTA, PTME and PTEE form (section 3.2). A fluorescence emission with a maximum relative intensity of greater than 100 was taken to be significant. This way, the ratio of noise, caused by such factors as light scattering, to signal was minimal and the fluorescence could be taken as real. It was found that all three uncomplexed thiazole

derivatives were non-fluorescent in the visible region. Under the conditions used, each thiazole derivative produced an emission with a maximum relative intensity below 10.

As discussed in section 5.1.4, complexes involving transition metal ions which have partially filled d orbitals are usually non-fluorescent, due to the likelihood of d-d transitions occurring. This is where the excited energy of the ligand is absorbed by the metal ion by exciting one of its electrons into a vacant d orbital, then the excess energy is released through radiationless modes of relaxation. Therefore, the Co^{2+}, Ni^{2+} and Cu^{2+} complexes of the thiazole derivatives, PTA, PTME and PTEE were expected to be non-fluorescent. To test this, the emission spectrum of the Co^{2+}, Ni^{2+} and Cu^{2+} complexes of the thiazoline acid, PTA, were recorded. As expected, no fluorescence emissions for any of these complexes were observed. The emission spectra of the complexes of PTME and PTEE with these transition metal ions were not recorded.

For comparison of the fluorescence of a ligand and one of its metal ion complexes, emission spectra were produced and recorded by irradiating solutions of the ligand and of the metal complex with monochromatic light of a wavelength equivalent to an absorption isosbestic wavelength of the ligand and its complex (Table 5.1). Values of these isosbestic wavelengths were determined from their corresponding ultraviolet absorption spectra. At the isosbestic wavelength, the number of photons being absorbed by the unbound ligand and its complex would be equal. Therefore, any enhancement in fluorescence observed for the complex compared with the unbound ligand could be attributed to the formation of a complex of enhanced fluorescence and not to a difference in the amount of light being absorbed.

None of the three thiazole derivatives showed any significant fluorescence when solutions of the unbound ligands were irradiated with light at any of the wavelengths used. Of the metal ion complexes formed by the thiazole derivatives with the series of metal ions tested, it was found that the thiazoline acid, PTA, showed an enhanced

Table 5.1 Maximum emission intensities of solutions[a,b] of the fluorescent metal complexes formed by the thiazolines, PTA and PTME, and the thiazole, PTEE.[c]

Ligand	Metal Ion	Excitation Wavelength[d] (nm)	Maximum Emission Intensity[e]	Emission Maxima Wavelength[f] (nm)
PTA	Al^{3+}	327	> 1000	428.0
		350	> 1000	428.0
	Zn^{2+}	328	253	448.5
		358	595	448.5
	Cd^{2+}	327	193	448.5
		357	480	448.5
PTME	Al^{3+}	324	> 1000	428.0
		346	> 1000	427.0
	Zn^{2+}	329	230	447.0
		358	498	446.5
PTEE	Zn^{2+}	346	200	452.0
		368	362	452.0

a Solutions contained 5.00 x 10^{-6} mol dm^{-3} ligand, 2.00 x 10^{-3} mol dm^{-3} metal perchlorate 1.00 x 10^{-3} mol dm^{-3} NaPIPES and 0.100 mol dm^{-3} $NaClO_4$, in 75% methanol and 25% water.

b The pH of the solutions was buffered to within the range 6.7 - 7.3, except for solutions containing Al^{3+}, which had a pH of 3.80 ± 0.05.

c Measurements were recorded on solutions with I = 0.100 mol dm^{-3} ($NaClO_4$) and at 298.2 K.

d The first excitation wavelength listed for each complex is equivalent to an absorption isosbestic wavelength between the ligand and its complex. The second excitation wavelength for each complex is equivalent to an absorption maxima of the complex.

e Errors in the emission intensity values are ± 1.

f Errors in the wavelength at which the maximum emission intensity occurred are ± 0.5.

fluorescence when it was chelated to Al^{3+}, Zn^{2+} and Cd^{2+} (Table 5.1). However, the Ca^{2+} and Pb^{2+} complexes of the thiazoline acid, PTA, did not fluoresce. Similarly, the complexes formed by the thiazoline methyl ester, PTME, with Al^{3+} and Zn^{2+} were much more fluorescent than the unbound ester itself. However, its Cd^{2+} and Pb^{2+} complexes did not fluoresce significantly. A typical fluorescence enhancement produced by these thiazole derivatives when complexed to metal ions such as Zn^{2+} can be seen in Figure 5.2. This shows the fluorescence enhancement observed when the thiazoline acid, PTA, is complexed to Zn^{2+}. The other fluorescence spectra measured of PTA, PTME and PTEE and their respective fluorescent metal complexes are presented in Appendix B.

Like the thiazolines, PTA and PTME, the thiazole ethyl ester, PTEE, produced an enhanced fluorescence when it was complexed with Zn^{2+}. However, when bound to Cd^{2+} or Pb^{2+}, PTEE did not fluoresce. The values of the maximum emission intensities and the wavelengths at which they occurred, for the fluorescent complexes of the thiazole derivatives, PTA, PTME and PTEE, are listed in Table 5.1.

As can be seen from Table 5.1, the metal complexes of PTA with Al^{3+}, Zn^{2+} and Cd^{2+} all have an absorption isosbestic wavelength with uncomplexed PTA of around 328 nm. Therefore, an equivalent number of photons is being absorbed by these complexes at 328 nm. This implies that any differences in the relative intensities of fluorescence between these complexes, when excited with light of 328 nm, is attributable to differences in the fluorescent nature of these complexes. Hence, the Al^{3+} complex of PTA is much more fluorescent than the Zn^{2+} or Cd^{2+} complexes. Also, the Zn^{2+} complex of PTA is more fluorescent than the Cd^{2+} complex. The Al^{3+}, Zn^{2+} and Cd^{2+} complexes of PTA produce fluorescence with maximum relative intensities of greater than 1000, 253 and 193 respectively, when excited with light of a wavelength of 327 to 328 nm.

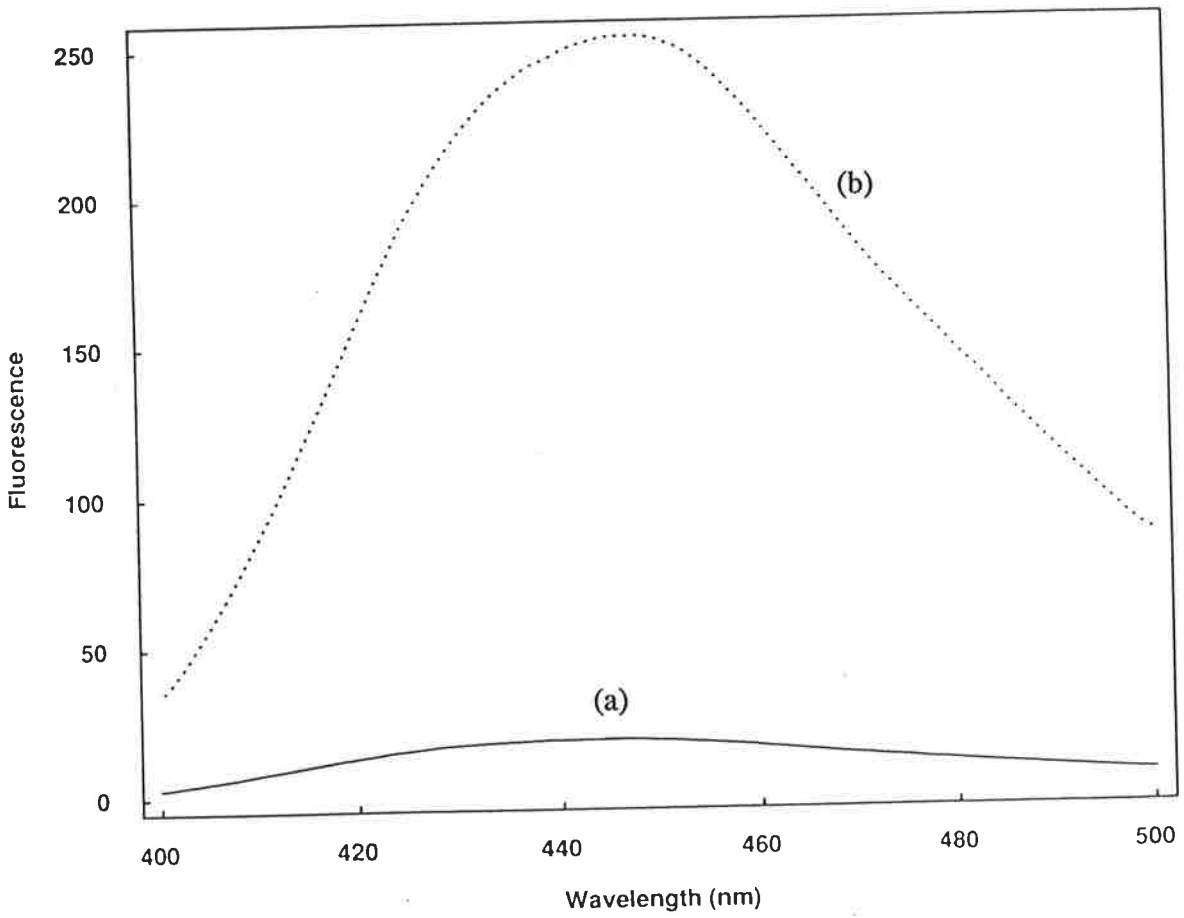

Figure 5.2 Comparison of the fluorescence emission spectra of the thiazoline acid, PTA, and it Zn^{2+} complex. Spectra were produced by irradiating solutions which contained either the acid or its Zn^{2+} complex with monochromatic light of 328 nm, which is equivalent to an absorption isosbestic point of the two species. The solutions contained (a) 5.00 x 10^{-6} mol dm^{-3} PTA, 1.00 x 10^{-3} mol dm^{-3} NaPIPES and 0.100 mol dm^{-3} $NaClO_4$, and (b) 5.00 x 10^{-6} mol dm^{-3} PTA, 2.00 x 10^{-3} mol dm^{-3} $Zn(ClO_4)_2$, 1.00 x 10^{-3} mol dm^{-3} NaPIPES and 0.100 mol dm^{-3} $NaClO_4$ in 75% CH_3OH and 25% H_2O at 298.2 K.

Similarly, the Al^{3+} and Zn^{2+} complexes of PTME have similar isosbestic wavelengths with the uncomplexed PTME (Table 5.1). Therefore, these complexes absorb an equivalent quantity of light at these wavelengths. Comparison of the relevant intensity of the fluorescence produced by the Al^{3+} and Zn^{2+} complexes of PTME, when irradiated at these wavelengths, shows that the Al^{3+} complex of PTME is more fluorescent than its Zn^{2+} complex. The Al^{3+} complex of PTME produces a fluorescence with a maximum relative intensity of over 1000, when irradiated with light of wavelength 324 nm, whilst the Zn^{2+} complex of PTME produces a fluorescence with a maximum relative intensity of 230, when irradiated with light of wavelength 329 nm.

To compare the differences in fluorescence of the Zn^{2+} complexes formed by the three thiazole derivatives, further emission spectra of these complexes were recorded. For this comparative study, the excitation wavelengths used were equivalent to an absorption isosbestic wavelength between the Zn^{2+} complex of concern and either of the two other Zn^{2+} complexes to which it was to be compared. These isosbestic wavelengths were determined by comparing the relevant ultraviolet absorption spectra of the Zn^{2+} complexes. In Table 5.2 is listed the maximum relative intensities obtained when the fluorescence of the Zn^{2+} complexes of the thiazole derivatives, PTA, PTME and PTEE, were compared.

From Table 5.2 it can be seen that the Zn^{2+} complex of the thiazoline acid, PTA, was more fluorescent than the Zn^{2+} complex of the thiazoline methyl ester PTME. Both the Zn^{2+} complexes of PTA and PTME had maximum relative fluorescence intensities of 525 and 305 respectively, when they were excited with light of a wavelength of 373 nm, which is equivalent to an isosbestic wavelength between the two complexes. The Zn^{2+} complex of PTA was also more fluorescent than the Zn^{2+} complex of PTEE, each having maximum relative emission intensities of 423 and 390 respectively. Of the two esters, PTME and PTEE, the thiazole, PTEE, produced a more

Table 5.2 Comparison of the fluorescence intensities of the metal complexes of the thiazole derivatives, PTA, PTME and PTEE.[a,b,c]

Metal Ion M	Ligand 1[d] L1	Ligand 2[d] L2	Excitation Wavelength[e] (nm)	Maximum Emission Intensity of ML1[f,g]	Maximum Emission Intensity of ML2[f,g]
Zn^{2+}	PTA	PTME	373	525	305
	PTA	PTEE	378	423	390
	PTME	PTEE	378	248	390
Al^{3+}	PTA	PTME	329	423	305

a Solutions contained a) 5.00 x 10^{-6} mol dm^{-3} ligand, 2.00 x 10^{-3} mol dm^{-3} zinc perchlorate 1.00 x 10^{-3} mol dm^{-3} NaPIPES and 0.100 mol dm^{-3} $NaClO_4$, or b) 1.00 x 10^{-6} mol dm^{-3} ligand, 2.00 x 10^{-3} mol dm^{-3} aluminium perchlorate 1.00 x 10^{-3} mol dm^{-3} NaPIPES and 0.100 mol dm^{-3} $NaClO_4$, in 75% methanol and 25% water.

b The pH of the solutions containing $Zn^{2+}$was buffered to 6.7 ± 0.1 and solutions containing Al^{3+}, were buffered to a pH of 3.80 ± 0.05.

c Measurements were recorded on solutions with I = 0.100 mol dm^{-3} ($NaClO_4$) and at 298.2 K.

d Comparing the maximum relative fluorescence intensities of the metal complex of Ligand 1 with that of the metal complex of Ligand 2.

e The excitation wavelength is the same as an absorption isosbestic wavelength between the metal complexes of Ligand 1 and Ligand 2.

f Errors in the emission intensity values are ± 1.

g Values of the wavelengths at which emission maxima of the complexes occurred were equal to those listed in Table 5.1.

fluorescent species when complexed to Zn^{2+} than the thiazoline, PTME. On comparison, these Zn^{2+} complexes have maximum relative emission intensities of 390 and 248, for the thiazole, PTEE, and the thiazoline, PTME, respectively, when excited with light of 378 nm, which is the same as an isosbestic wavelength between the two.

A similar comparison of the Al^{3+} complexes of the thiazoline acid PTA and the thiazoline ester PTME was also carried out, to determine which of these were more fluorescent. However, to make such a comparison the concentration of ligand, in the sample solutions used, was reduced so that the resulting fluorescence of the Al^{3+} complex formed did not exceed the measurable limits of the spectrometer. Therefore sample solutions used for this comparison, had a total concentration of ligand which was reduced to 1.00×10^{-6} mol dm^{-3}, a fifth of that used in all other fluorescence measurements. The concentration of all other compounds added to these sample solutions remained the same as those used previously in other fluorescence measurements. The maximum relative emission intensities obtained from this comparative study of the Al^{3+} complexes of PTA and PTME are listed in Table 5.2. From Table 5.2, it can be seen that when irradiating solutions of the Al^{3+} complexes of PTA or PTME with monochromatic light of 329 nm, an absorption isosbestic wavelength of the two complexes, the Al^{3+} complex of PTA was more fluorescent than the Al^{3+} complex of PTME. The maximum relative emission intensities of the acid and the ester Al^{3+} complexes were 423 and 305 respectively.

As shown above, the thiazoline acid produces the most fluorescent species when complexed to Zn^{2+} and Al^{3+} ions, compared with those formed by the thiazoline ester, PTME, and the thiazole, PTEE. Also, in comparing the two esters, the thiazole, PTEE, forms a more fluorescent species when complexed to Zn^{2+} than the thiazoline, PTME,. Why the thiazoline acid forms the most fluorescent complexes out of the three thiazole derivatives is not fully known. A possible reason is that the carboxylate anion of the

acid, PTA, may be in close enough proximity to the metal ion, coordinated to PTA, to undergo an electrostatic interaction with the metal ion, thus 'locking' complexed PTA into a more rigid conformation than those of the esters, PTME and PTEE. It is not believed the carboxylate anion coordinates to a metal ion bound to PTA, such as Zn^{2+}, as this would produce a terdentate species which would be expected to have higher stability constants, *K*, than those of the corresponding ester, PTME. Derivations of values of the stability constants, *K*, for complexes formed between Zn^{2+} and the acid, PTA, or the corresponding ester, PTME, showed this not to be the case, as the values obtained were similar (section 4.2).

None of the three thiazole derivatives produced a fluorescent species when complexed to Pb^{2+}. This may be due to the relatively large size of the Pb^{2+} ion, which has an ionic radius of 119 pm for a six coordinate species as compared with ionic radii of 53.5 pm, 74 pm and 95 pm for six coordinate Al^{3+}, Zn^{2+} and Cd^{2+} ions.[8] This larger size of a Pb^{2+} ion possibly breaks down the planarity of the conjugated systems of the thiazole derivatives on complex formation, thus restricting the delocalisation of the π electrons and therefore destroying the possibility for the complexes to fluoresce.

In summary, none of the three thiazole derivatives are significantly fluorescent when not complexed to any metal ion. The thiazoline acid, PTA, produces the most fluorescent species of the three ligands, when complexed to Zn^{2+} or Al^{3+}. Of these two complexes of PTA, the Al^{3+} complex is far more fluorescent than the Zn^{2+} complex. The acid, PTA, also produces a fluorescent species when complexed to Cd^{2+}, however, this species is the least fluorescent of the three fluorescent complexes formed by PTA. The complexes formed between PTA and Ca^{2+}, Co^{2+}, Ni^{2+}, Cu^{2+} or Pb^{2+} were non-fluorescent.

The thiazoline methyl ester, PTME, is strongly fluorescent when complexed to Al^{3+} or Zn^{2+}. Of these complexes, the Al^{3+} complex of PTME was much more

fluorescent than the Zn^{2+} complex. Both the Al^{3+} and Zn^{2+} complexes of PTME are less fluorescent than the corresponding Al^{3+} and Zn^{2+} complexes of PTA. Of the three thiazole derivatives, PTA, PTME and PTEE, the Zn^{2+} complex of PTME was the least fluorescent. None of the other metal complexes formed by PTME were fluorescent.

The thiazole, PTEE, only produced a strongly fluorescent species when chelated to Zn^{2+}. This Zn^{2+} complex of PTEE was more fluorescent than that of PTME, however, it was less fluorescent than the Zn^{2+} complex of PTA.

5.3 Bibliography

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(7) PC MODEL 4.41

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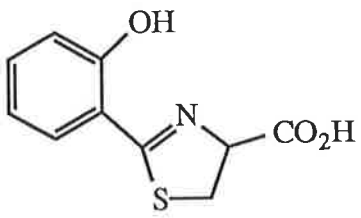
6 Further Development of Zinc (II) Probes

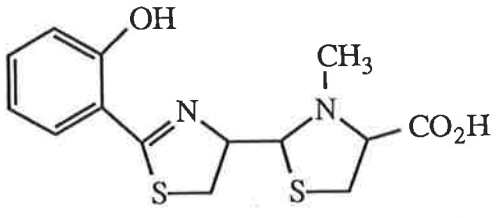
6.1 Development of More Trial Ligands

6.1.1 Design of Ligands

In chapter 2, an initial series of compounds based on the structure of the thiazoline acid, PTA, were synthesised as trial ligands, which were to be tested to determine their suitability as fluorescent probes for biological Zn^{2+}. The acid, PTA, was selected as the structure to base ligand design on as it is a substructure of a naturally occurring fluorescent Zn^{2+} chelator, pyochelin, (2). As well as synthesising the acid, PTA, and the corresponding methyl ester, PTME, other compounds were initially synthesised whose structures were similar to that of the acid, but where the phenolic hydroxy group had been removed or replaced by a methoxy substituent. Also synthesised were the corresponding fully aromatic thiazole compounds. Investigations into the chelation tendency of these compounds with Zn^{2+} showed that only those which had an ortho hydroxy substituent on the phenyl ring chelated to Zn^{2+}. Further studies were then conducted on these phenolic compounds to determine their suitability as probes for Zn^{2+} in biological systems.



PTA



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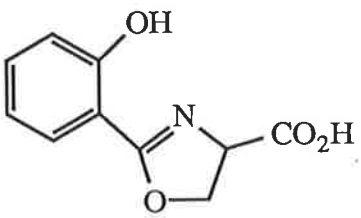
It is not known whether the nitrogen or the sulfur atom of the heterocyclic ring is involved in the chelation to Zn^{2+}. As discussed previously, the ring nitrogen of the

thiazole, PTEE, and thiazolines, PTA and PTME, was considered to be the more likely heteroatom that is involved in coordination to Zn^{2+}. Preparing compounds whose structures are similar to that of PTA, but where either of the ring nitrogen or sulfur of PTA had been substituted with other heteroatoms, or replaced with carbon, should shed some light on the role of the heteroatoms and increase the number of Zn^{2+} probes for evaluation. Assessment of the Zn^{2+} chelation properties of these new compounds would provide information on the effect heteroatom substitution has on the binding tendencies of these compounds and on the necessity of the nitrogen and sulfur in the thiazoline, PTA, for Zn^{2+} chelation.



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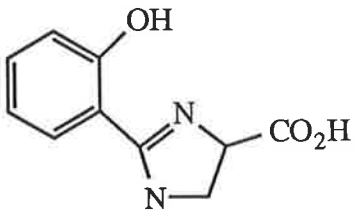

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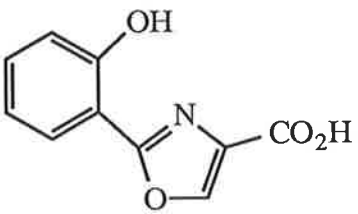

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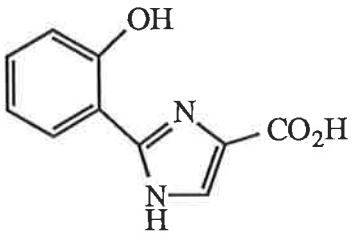

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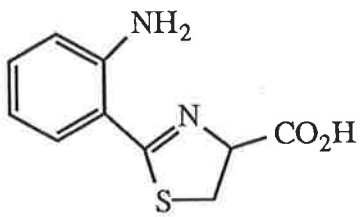
Because the ring nitrogen of the thiazole derivatives, PTA, PTME and PTEE, was believed to be involved in Zn^{2+} coordination, it was initially decided to retain the ring nitrogen in any further trial compounds developed. Thus, studies were centred on synthesising compounds whose structures were similar to that of PTA, but where the ring sulfur had been replaced by other heteroatoms. Suitable targets were thus the oxazoline, (15), and the imidazoline, (16), as well as the corresponding fully aromatic oxazole, (17),

and the imidazole, (18). Preference was given to preparing the oxazoline compound, (15), and the oxazole, (17), rather than the imidazoline and imidazole compounds, (16) and (18), as the chelating ability of the oxazole derivatives should provide greater information on whether the sulfur or the nitrogen of the thiazoline, PTA, is involved in Zn^{2+} chelation. In the event there was not time to synthesise the imidazoline and imidazole structures, (16) and (18).

The investigations carried out on the original series of thiazole and thiazoline compounds showed that an ortho hydroxy substituent was required for Zn^{2+} chelation. According to the theory of hard and soft acid and bases,[1,2] the Zn^{2+} ion is a borderline acid and has predominant features of both hard and soft groupings. Instead of binding to hard base oxygen donor atoms, the Zn^{2+} ion prefers to bind to nitrogen donor atoms, which in terms of hard and soft bases are more borderline in nature.[1,2] Thus a Zn^{2+} ion should bind to the nitrogen of an aniline in preference to the harder oxygen donor atom of a phenol. Therefore, it was thought that the equivalent aniline, (19), of the phenol substituted thiazoline, PTA, could have a greater binding ability to Zn^{2+} ions. Also, such replacement of the hydroxy group on PTA with an amino group may improve the selectivity and specificity of the ligand for Zn^{2+} ions, as hard acid metal ions, such as Al^{3+}, which bind to the phenol thiazoline, PTA, would most likely not bind to the corresponding softer base aniline substituted thiazoline, (19).



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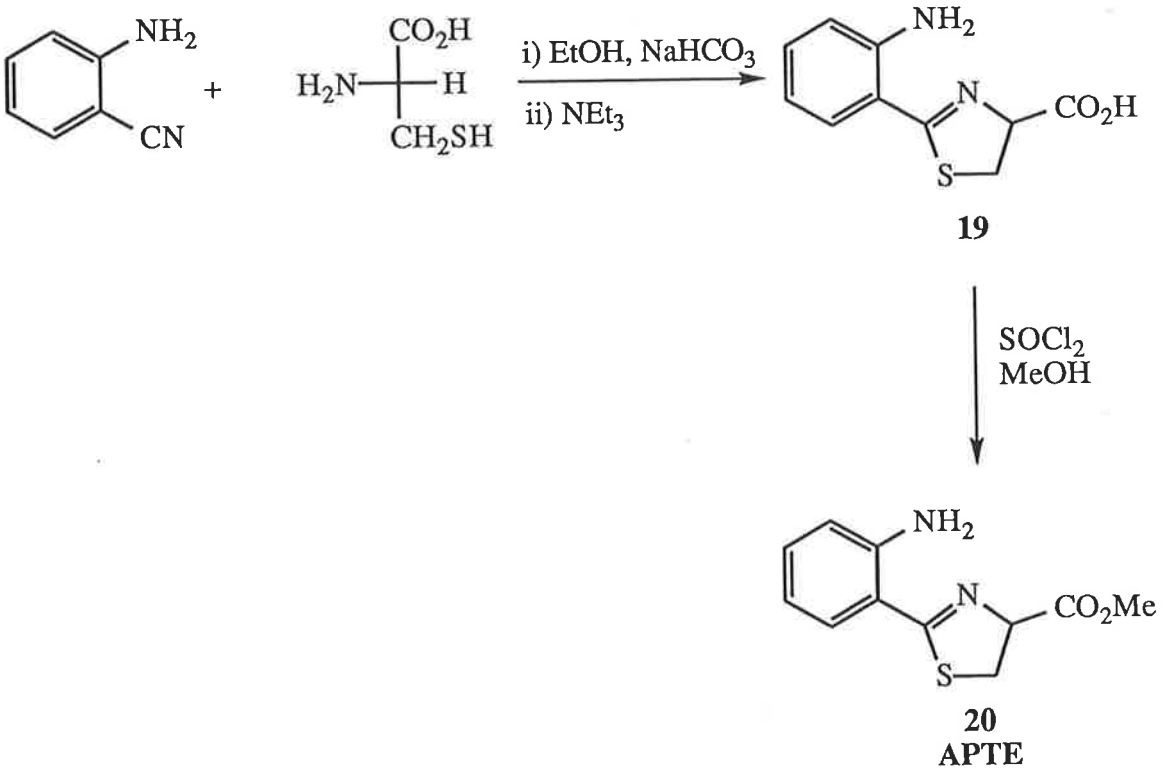
Thus, the oxazole derivatives, (15) and (17), and the aniline substituted thiazoline, (19), were the initial synthetic targets for this section of the work.

6.1.2 Synthesis of Further Ligands



Scheme 6.1

The method reported by Mathur and coworkers[3] was employed to prepare the amino thiazoline, (19), using chemistry similar to that used to prepare the thiazolines (sec 2.2.2). Readily available 2-aminobenzonitrile was refluxed with L-cysteine hydrochloride in a basic ethanol solution (Scheme 6.1). On completion of the reaction, the cooled reaction mixture was acidified, however, the acid, (19), did not precipitate out. An oily yellow residue was obtained by extraction of the reaction mixture with ethyl acetate. On treating this residue with thionyl chloride in anhydrous methanol, the corresponding methyl ester, (20), which was purified by squat chromatography followed by distillation, was

obtained as a yellow oil in high overall yield of 80%. The mass spectrum of the product showed a molecular ion at m/z 236, consistent with the ester, (20). A further peak at m/z 117 represented the characteristic loss of the ester group. The infrared spectrum of the compound showed two peaks at 3472 and 3376 cm^{-1} due to N-H stretches of the amino group. The presence of the amino group was also confirmed by ^1H n.m.r. spectroscopy, which showed a broad exchangeable peak at 6.19 ppm. The ^1H n.m.r. also showed an apparent triplet at 5.34 ppm and two apparent doublets at 3.52 ppm and 3.51 ppm for the H4 and H5 protons of the thiazoline ring respectively. From this point on the ester, (20), will be referred to as APTE.

21
POEE

Scheme 6.2

The ethyl ester, (21), of the oxazole, (17), was prepared by reacting salicylamide with ethyl bromopyruvate (Scheme 6.2). The reaction proceeded at a slower rate than that of the thiazole, PTEE, due to the difference in reactivity of the amide compared to the thioamide. After two days of heating, the reaction mixture was worked up and separated by squat chromatography to obtain the oxazole, (21), in a low yield of 15%, with over 60% of the starting amide being recovered. The product was identified from its mass spectrum, which showed a molecular ion at m/z 233. Also the ^1H n.m.r. spectrum of the product showed a broad singlet at 10.72 ppm for the hydroxy proton, a quartet at 4.41 ppm and a triplet at 1.41 ppm for the ethyl protons of the ester and a singlet at 8.25 ppm for the H5 proton of the oxazole ring. Although the yield was low, enough of the oxazole, (21),

henceforth referred to as POEE, was obtained to carry out the desired coordination and fluorescence analysis, so no optimisation of the yield from this preparation was attempted.

6.2 Investigations into the Suitability of APTE and POEE as Fluorescent Zinc (II) Probes

6.2.1 Measurement of Zinc (II) Specificity

The chelation properties of these two compounds, APTE and POEE, were determined using ultraviolet absorption spectroscopy as described in chapter 3. The sample solutions for the ultraviolet absorption measurements were prepared in a similar manner as those for PTA, PTME and PTEE (section 3.2). This allowed for a direct comparison of the results obtained for POEE and APTE with those of PTA, PTME and PTEE. Preliminary measurements of methanolic solutions of POEE or APTE with Na^+, showed these compounds did not coordinate Na^+. Hence, sodium perchlorate could be used as the inert electrolyte to control the ionic strength of the solutions.

The preparation of the sample solutions used for ultraviolet absorption spectroscopy measurements and the method of measurement are described in section 8.2.2. All sample solutions had an ionic strength of 0.100 mol dm^{-3} controlled by sodium perchlorate and were buffered to a pH of approximately 7.0 with 1.00×10^{-3} mol dm^{-3} NaPIPES. As with previous measurements (section 3.2), problems were created when attempting to buffer solutions containing Al^{3+}, Cu^{2+}or Pb^{2+} to a pH of 7.0, due to these ions forming highly acidic hydrated species in solution. The resulting values of the pH of the solutions containing these ions were 3.72 ± 0.01, 5.33 ± 0.02 and 5.88 ± 0.03 respectively. All other solutions were of a pH which lay within the range 6.9 to 7.1. To avoid precipitation of hydroxide species, all solutions were prepared and measurements recorded within eight

hours. Due to unavoidable precipitation of an hydroxide species, solutions of APTE and POEE which contained Pb^{2+} ions needed to be filtered through a millipore filter before measurement.

The metal ions which were coordinated by APTE and the wavelengths at which absorption maxima occurred for APTE and its complexes are listed in Table 6.1. From the spectra obtained, it was apparent that APTE coordinated Co^{2+}, Ni^{2+}, Cu^{2+}, Zn^{2+}, Cd^{2+} and Pb^{2+}. Figure 6.1 displays the absorption spectra of APTE when measured in the presence and absence of Zn^{2+}. The change in the absorption spectrum of APTE on addition of Zn^{2+} is typical to that observed when the spectrum of APTE was recorded in the presence of any of the metal ions to which APTE coordinated. The spectra of APTE recorded whilst in the presence of other metal ions from the cation series are presented in Appendix A. No significant coordination between the thiazoline, APTE, and the metal ions Mg^{2+}, Al^{3+}, K^{+} and Ca^{2+} was observed. The ultraviolet absorption spectrum of APTE did not alter on addition of these metal ions.

As can be seen from Figure 6.1 and from the data in Table 6.1, when APTE coordinated a metal ion, its absorption maxima underwent a hypsochromic shift. Therefore coordinated APTE absorbs radiation of a higher energy. This suggests that, when APTE coordinates a metal ion, the conjugation of the π and non-bonding electrons within APTE is broken down in such a manner as to increase the separation between the electronic energy levels of APTE. Therefore, to excite an electron into a higher energy level, coordinated APTE needs to absorb radiation of higher energy and hence shorter wavelength. This is unlike the thiazole derivatives, PTA, PTME and PTEE, whose absorption maxima underwent a bathochromic shift when they were coordinated to metal ions.

Table 6.1 Ultraviolet absorption maxima of the thiazoline, APTE, and the oxazole, POEE, and their metal complexes.[a,b]

Ligand[c]	Metal Ion[d]	Absorption Maxima between 250 - 450 nm[e] (nm)
APTE		346; 258
	Co^{2+}	340; 285
	Ni^{2+}	250
	Cu^{2+}	256
	Zn^{2+}	285
	Cd^{2+}	340; 285
	Pb^{2+}	No definite maxima
POEE		306; 270; 258; 254
	Co^{2+}	347; 314; 272; 260
	Ni^{2+}	352; 266
	Cu^{2+}	351; 266
	Zn^{2+}	346; 268
	Cd^{2+}	348; 308; 272; 260
	Pb^{2+}	350; 263

a All solutions contained 1.00×10^{-3} mol dm^{-3} NaPIPES, 0.100 mol dm^{-3} $NaClO_4$ in 75% methanol and 25% water. Spectra were recorded at 298.2 K.

b The pH of the solutions was buffered to within the range 6.9 - 7.1, except for those containing Al^{3+}, Cu^{2+} or Pb^{2+}, whose pH were 3.72 ± 0.01, 5.33 ± 0.02 and 5.88 ± 0.03 respectively.

c The concentration of the ligand in solution was 2×10^{-5} mol dm^{-3}.

d The concentration of metal ions in solution was 4×10^{-3} mol dm^{-3}.

e Errors in the absorption maxima values are $\pm$ 1 nm.

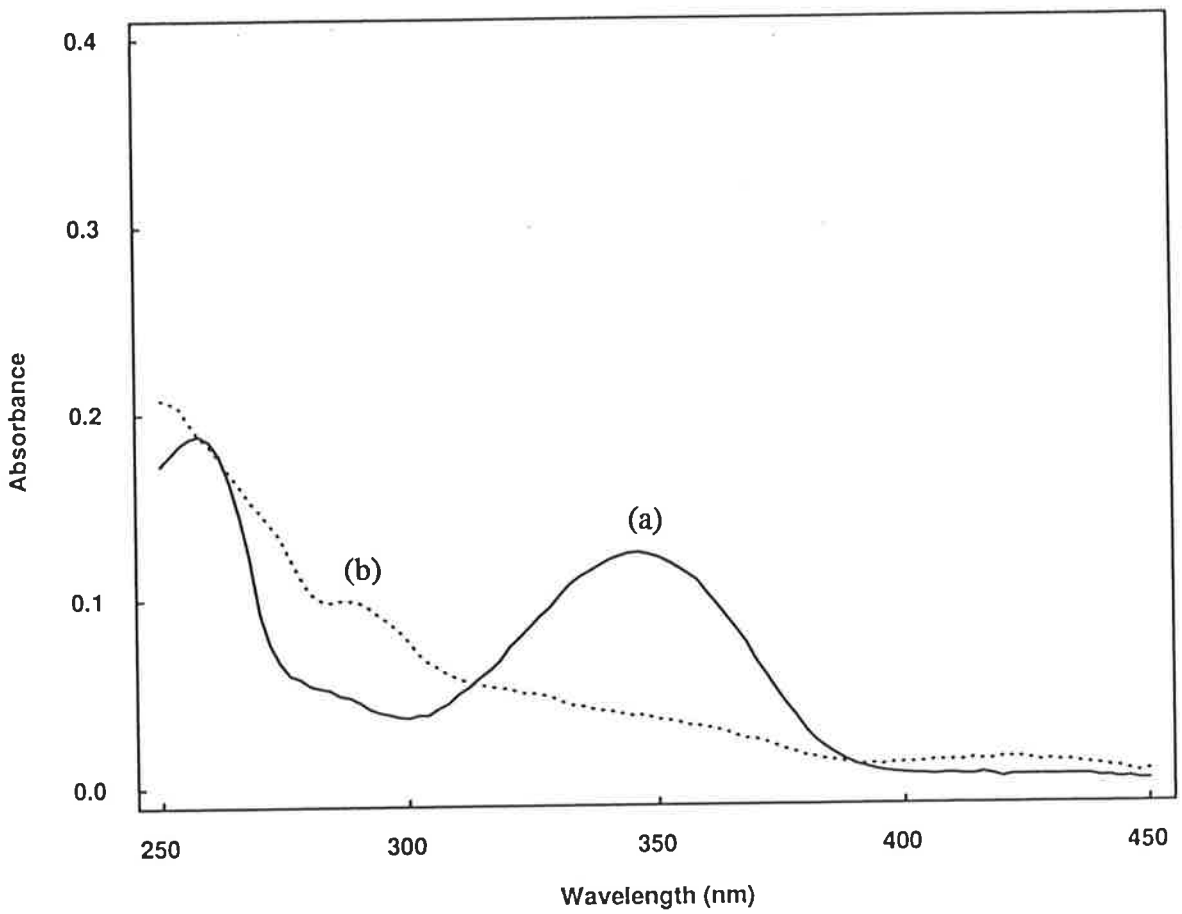


Figure 6.1 Comparison of the ultraviolet absorption spectra of APTE measured in the absence and presence of Zn^{2+}. The solutions contained (a) 2.0 x 10^{-5} mol dm^{-3} APTE, 1.00 x 10^{-3} mol dm^{-3} NaPIPES and 0.100 mol dm^{-3} $NaClO_4$ and (b) 2 x 10^{-5} mol dm^{-3} APTE, 4 x 10^{-3} mol dm^{-3} $Zn(ClO_4)_2$, 1 x 10^{-3} mol dm^{-3} NaPIPES and 0.100 mol dm^{-3} $NaClO_4$ in 75% CH_3OH and 25% H_2O. Spectra were recorded at 298.2 K.

A similar comparison of the ultraviolet absorption spectra of POEE was carried out. The metal ions which were coordinated by POEE and the wavelengths, at which absorption maxima of POEE and its metal complexes occurred, are listed in Table 6.1. The oxazole, POEE, coordinated the transition metal ions, Co^{2+}, Ni^{2+}, Cu^{2+} and Zn^{2+}, as well as the heavy metals, Cd^{2+} and Pb^{2+}. The oxazole, POEE, did not coordinate Mg^{2+}, Al^{3+}, K^{+} or Ca^{2+}. Figure 6.2 displays a comparison of the absorption spectrum of POEE when measured in the presence and absence of Zn^{2+}. The shift in absorption maxima of POEE when recorded in the presence of Zn^{2+} ions is typical of that which was observed when the absorption spectrum of POEE was recorded whilst in the presence of any one of the metal ions, which it coordinated. The spectra of POEE and also those of APTE measured in the presence of the other metal ions from the cation series selected are presented in Appendix A.

Unlike the thiazoline, APTE, the absorption maxima of the oxazole, POEE, underwent a bathochromic shift when the oxazole formed a complex. This indicates that the energy gap between successive electronic energy levels is decreased on complex formation, suggesting the delocalisation of π and non-bonding electrons is enhanced on coordination to the metal ions tested.

Both the oxazole, POEE, and the thiazoline, APTE, showed no tendency to coordinate to the biologically prevalent metal ions, Na^{+}, Mg^{2+}, K^{+} and Ca^{2+}. This result is similar to that obtained for the thiazole, PTEE, and the thiazoline, PTME, whose structures are similar to those of POEE and APTE respectively. APTE coordinated similar metal ions as PTME, except for Al^{3+}. Thus, the amino phenyl substituted thiazoline, APTE, showed only slightly greater selectivity in comparison to the corresponding hydroxy phenyl substituted thiazoline, PTME. This difference can be explained by considering the theory of hard and soft acids and bases,[1,2] as the metal ion, Al^{3+}, is a hard acid and prefers to bind

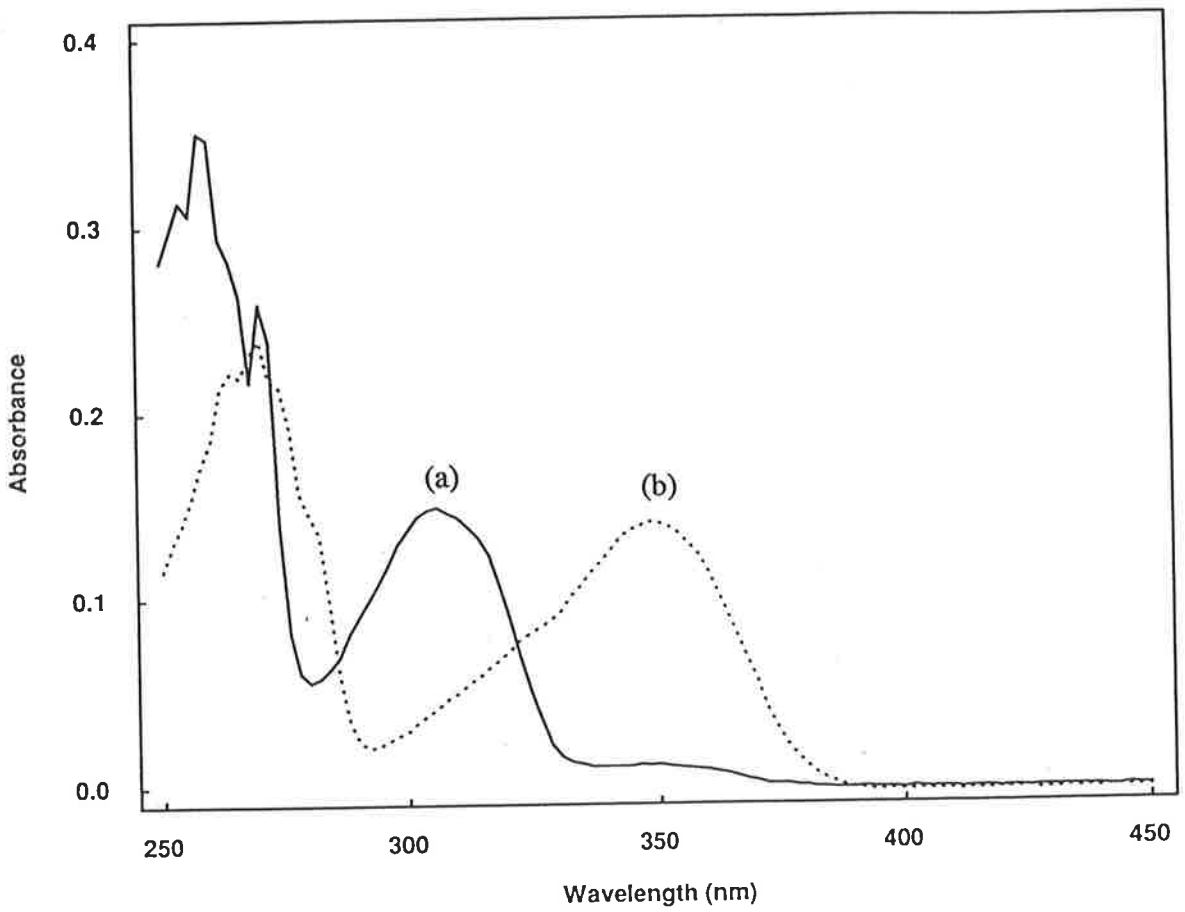


Figure 6.2 Comparison of the ultraviolet absorption spectra of POEE measured in the absence and presence of Zn^{2+}. The solutions contained (a) 2.00 x 10^{-5} mol dm^{-3} POEE, 1.00 x 10^{-3} mol dm^{-3} NaPIPES and 0.100 mol dm^{-3} $NaClO_4$ and (b) 2.00 x 10^{-5} mol dm^{-3} POEE, 4.00 x 10^{-3} mol dm^{-3} $Zn(ClO_4)_2$, 1.00 x 10^{-3} mol dm^{-3} NaPIPES and 0.100 mol dm^{-3} $NaClO_4$ in 75% CH_3OH and 25% H_2O. Spectra were recorded at 298.2 K.

to hard bases, such as oxygen donor atoms, rather than the softer base nitrogen donor atoms. Therefore, it is not surprising that on substitution of the hydroxy group on the phenyl ring of PTME with an amino group to form APTE, the ability to coordinate Al^{3+} is lost.

The oxazole, POEE, chelated to the same metal ions as the equivalent thiazole, PTEE, with both systems giving rise to similar ultraviolet shifts. Thus, it is possible that the sulfur in the thiazole, PTEE, is not utilised for coordination to any of the metal ions coordinated by PTEE, since replacement of the sulfur in PTEE with an oxygen donor atom to produce the oxazole, POEE, does not create any differences in selectivity for chelation between the two. Therefore, it appears that the nitrogen of the thiazole, PTEE, and the oxazole, POEE, is a more likely coordination site. Further support for this conclusion can be obtained by measuring the stability of the complexes formed by the oxazole, POEE, and comparing the results with those of the thiazole, PTEE.

Neither the thiazole, PTEE, nor the oxazole, POEE, displayed any tendency to chelate to the Al^{3+} ion. Even though the oxazole, POEE, contained an extra oxygen donor atom, which Al^{3+} ions show a preference in coordinating to, no indication of complex formation was observed. This suggests that the 'bite' distance between the phenolic hydroxy group and the nitrogen, or oxygen, of the heterocyclic ring may be too large for chelation to the relatively small Al^{3+} ion. Molecular modelling studies[4] have shown the distance between the phenolic oxygen and ring nitrogen to be 260 pm (Figure 6.3). Although smaller than that of the corresponding thiazole, PTEE, 266 pm, this distance is still greater than that of the thiazoline, PTA, which is 255 pm. Hence, this supports the conclusion drawn previously in section 3.2 that the larger 'bite' distance of the fully aromatic thiazole, as compared to the thiazoline, is responsible for the greater selectivity of these compounds for coordinating metal ions of relatively large size.

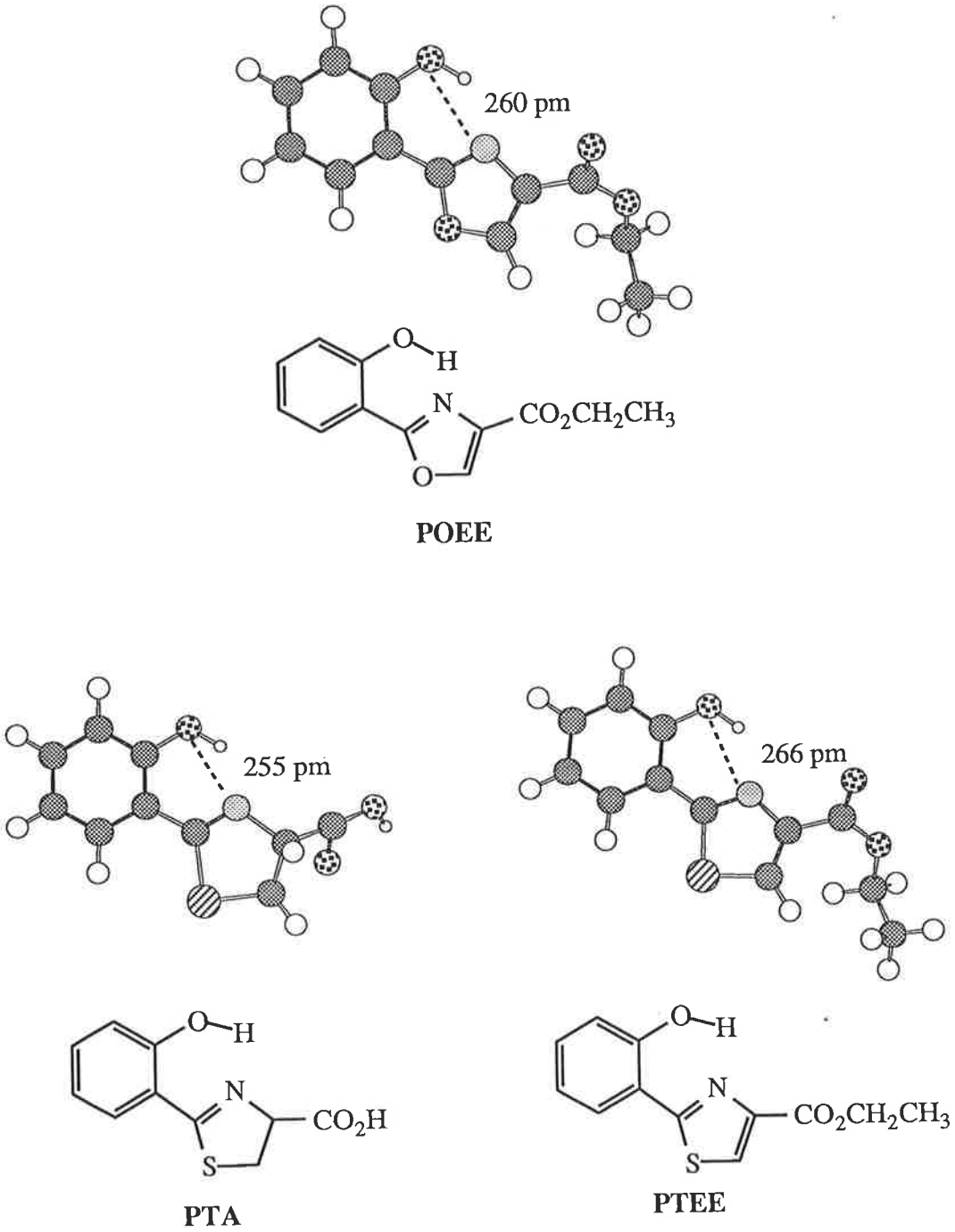


Figure 6.3 Molecular representation of POEE, PTA and PTEE.

The thiazole derivatives, PTA, PTME and PTEE, the thiazoline, APTE, and the oxazole, POEE, showed very similar chelation abilities. To gain more information about any differences in complex formation properties which these new ligands may have, studies on the stability of the complexes formed by APTE and POEE needed to be carried out. Results obtained should aid in providing direction into producing a Zn^{2+} selective ligand.

6.2.2 Acid Dissociation and Stability Constant Determination of APTE and POEE and their Complexes

The potentiometric titration technique and the conditions used previously (chapter 4) to determine acid dissociation and stability constant values for PTA, PTME and PTEE and their respective complexes, were used to measure those for APTE and POEE. In this way, results obtained for APTE and POEE could be directly compared with those for PTA, PTME and PTEE obtained previously. The main interest of this research was to investigate whether the structural differences of APTE and POEE, as compared to PTME and PTEE respectively, cause any variance in the stability of complexes formed with Zn^{2+}.

Acid dissociation constants for the acidic protons of APTE and POEE were determined by titrating an acidic solution of each compound with a strong base, whilst monitoring changes in the e.m.f. of the solution throughout the titration. The pK_a values of the acidic protons were then derived from the best fit of the experimental data recorded to equations 4.4 and 4.5. The stability constants of Zn^{2+} complexes formed by the ligands APTE and POEE were determined by similarly titrating an acidic solution of the ligand, to which had been added a known amount of Zn^{2+} ions, with a strong base, whilst monitoring the e.m.f. of the solution throughout the titration. Values for the log of the stability constants of Zn^{2+} complexes formed were then derived from the best fit of the experimental data recorded to equations 4.9 and 4.10.

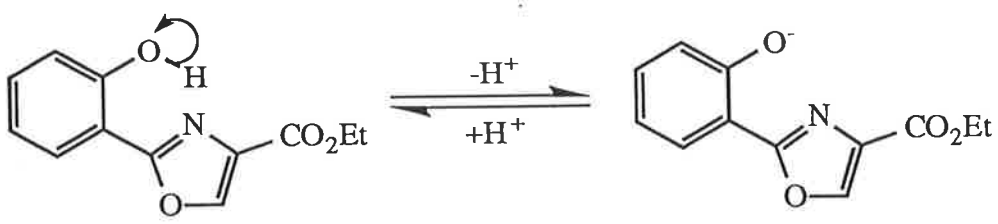
$$LH \rightleftharpoons L + H \qquad K_a = \frac{[L]\ [H]}{[LH]} \qquad (4.4)$$

$$pK_a = -\log K_a \qquad (4.5)$$

$$M + L \rightleftharpoons ML \qquad K_1 = \frac{[ML]}{[M]\ [L]} \qquad (4.9)$$

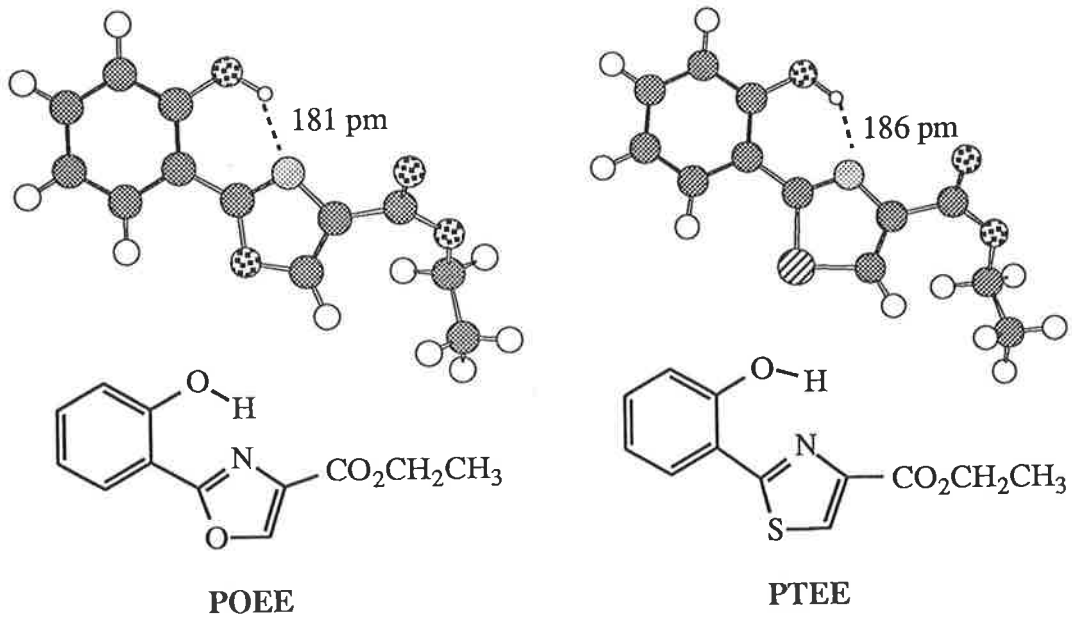
$$ML + L \rightleftharpoons ML_2 \qquad K_2 = \frac{[ML_2]}{[ML]\ [L]} \qquad (4.10)$$

From the titrations carried out on POEE, a value for the pK_a of the acidic proton in POEE, represented in Scheme 6.3, was calculated as being 10.57 ± 0.02. Therefore, the phenolic hydrogen of POEE is less acidic than that of the corresponding thiazole, PTEE, which has a pK_a value of 9.43 ± 0.01. This suggests the acidic proton of POEE is bound more strongly to the oxazole than that of PTEE is bound to the thiazole. A possible reason for this is that the hydrogen bonding between the phenolic hydrogen and the heteroatoms of the ring is stronger for POEE than PTEE. Molecular modelling studies[4] have shown that the distance between the phenolic hydrogen and ring nitrogen of POEE is 181 pm, when both rings lie in the same plane (Figure 6.4). This distance is smaller than that of the corresponding thiazole, PTEE, which has a distance of 186 pm separating these atoms, when both rings lie in the same plane. This smaller separation in POEE may give rise to the formation of a stronger hydrogen bond between the phenolic hydrogen and ring nitrogen than that in PTEE. Hence, the phenolic proton of POEE would be less acidic than that of PTEE.



POEE

Scheme 6.3



POEE **PTEE**

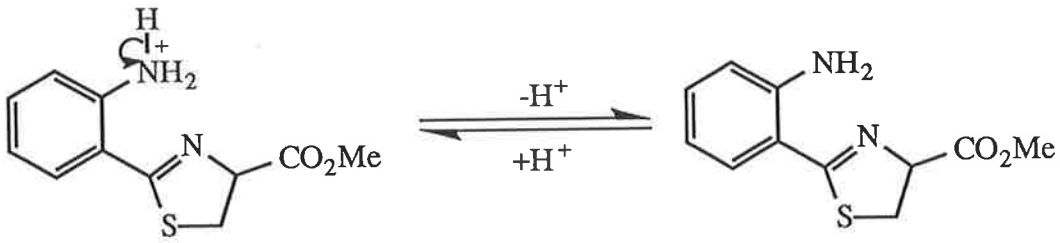
Figure 6.4 Molecular representation of POEE and PTEE.

The value of the pK_a for the protonated nitrogen of oxazole itself has been reported as being 0.8.[5] The pK_a of the protonated nitrogen in POEE was expected to be of approximately the same acidity as that of oxazole. Therefore, due to the low acidity of the protonated nitrogen in POEE and the accuracy limitations of the electrode used during the potentiometric titrations, a value for the pK_a of the protonated nitrogen in POEE could not be derived.

The log values of stepwise stability constants, K_1 and K_2, defined in equations 4.9 and 4.10, for the 1:1 and 2:1 complexes formed between POEE and Zn^{2+} were 6.62 ± 0.01 and 7.32 ± 0.01. These values are larger than the corresponding values obtained for the 1:1 and 2:1 complexes formed between PTEE and Zn^{2+}, which were 5.02 ± 0.01 and 5.51 ± 0.01, respectively. Therefore, the oxazole, POEE, forms more stable complexes with Zn^{2+} than the thiazole, PTEE. This suggests that either there is an increased electron availability in the oxazole, POEE, for coordination to Zn^{2+} or possibly that the 'bite' distance between the phenolic oxygen and the heterocyclic ring of the oxazole, POEE, compared to that of the thiazole, PTEE, is more favourable for Zn^{2+} chelation. However, the possibility of the ring oxygen of POEE being a chelation site for Zn^{2+}, instead of the ring nitrogen, can not be ruled out. Thus, the differences in the stability of the Zn^{2+} of POEE and PTEE may be due to differing donor atoms.

No definite conclusions to justify the differences in the stability of Zn^{2+} complexes can be made without knowing how Zn^{2+} is chelated by POEE and by PTEE. Attempts were made to obtain crystal structures of the Zn^{2+} and other metal complexes of POEE and PTEE as well as the other ligands, PTA, PTME and APTE. Although crystals of the complexes were grown successfully, they were quite unstable and would decompose before X-ray spectra could be obtained. Further attempts to obtain X-ray crystal structures are being carried out.

The experimental data obtained from the potentiometric titrations carried out on APTE could not be successfully fitted to obtain values for the pK_a for the deprotonation of the protonated amino nitrogen of APTE, represented in Scheme 6.4, nor for the stability constants for the Zn^{2+} complexes formed by APTE. The value of the pK_a for protonated aniline has been reported in the literature as 4.65 ± 0.03 at 298 K and ionic



APTE

Scheme 6.4

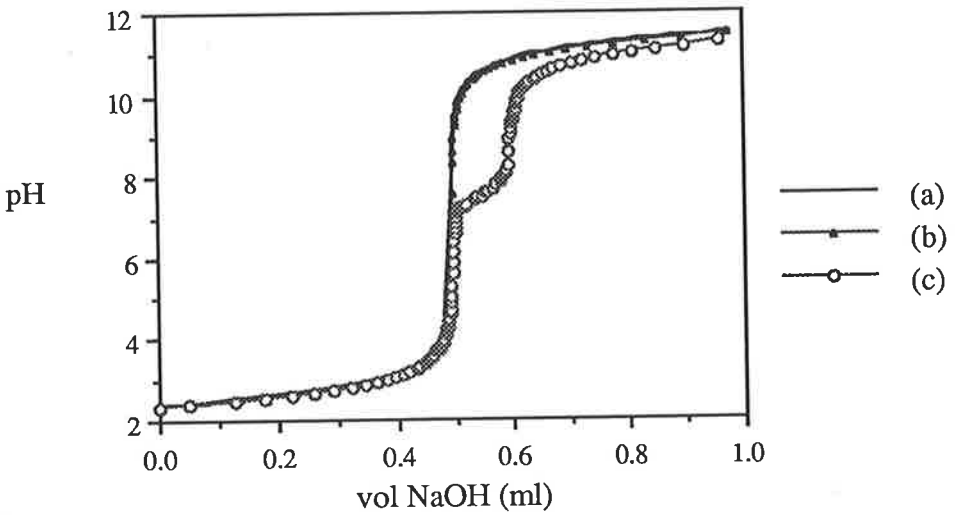


Figure 6.5 Experimental titration profiles of

a) 5.00×10^{-3} mol dm^{-3} $HClO_4$

b) 1.00×10^{-3} mol dm^{-3} APTE and 5.00×10^{-3} mol dm^{-3} $HClO_4$

c) 9.00×10^{-4} mol dm^{-3} $Zn(ClO_4)_2$, 1.00×10^{-3} mol dm^{-3} APTE and 5×10^{-3} mol dm^{-3} $HClO_4$

in 75% CH_3OH, 25% H_2O and 0.100 mol dm^{-3} $NaClO_4$ at 298.2 K against 0.100 mol dm^{-3} NaOH.

The curves, running through the experimental points collected, represent the best fit of the data over the pH range of the titration.

The curves (a) and (b) are superimposed.

strength 0.1 when measured in an aqueous solution.[6] Given the differing conditions being used in this study and that the aniline of APTE had an electron withdrawing thiazole group attached in an ortho position to the amine substituent, the amine of APTE was expected to be less basic than that of aniline. The titration profiles of a titration of a strong acid and APTE against a strong base for a pK_a determination, (b), and that of a titration of a strong acid against a strong base for an electrode calibration, (a), are displayed in Figure 6.5. On comparison of the two titration profiles, no deviation of the titration curve (b) from (a), which represents the point where deprotonation of an acidic proton of APTE occurred, was observed. This suggests that the deprotonation of the protonated amine of APTE occurs below the accurately detectable limits of the electrode used in the titrations, and that the value of the pK_a for this process is possibly around 2.

In Figure 6.5, the curve (c) represents the titration profile for a titration of a strong acid with APTE and Zn^{2+} ions present in solution, against a strong base. Precipitation of an hydroxide species occurred in titrations such as these at approximately pH 5.7. Experimental data from these titrations collected above the pH where precipitation began, could not be fitted to equations 4.9 and 4.10, as accurate total concentrations of species in the titration solution were not known. Therefore, only experimental data collected from such titrations below pH 5.7 could be used to derive values for the stability constants of Zn^{2+} complexes formed by APTE. No deviation of the titration profile (c) from that of (b), which represents the point where complex formation occurs, was observed below pH 5.7. Therefore, derivation of values for the stability constants of Zn^{2+} complexes formed by APTE could not be carried out with the experimental data collected below pH 5.7. Using the computer program MacSpecies an upper limit was estimated for the log of the stability constant, log K_1, for the 1:1 Zn^{2+} complex formed by APTE. The estimated value obtained was log $K_1 < 2.1$. This limit was based on formation of up to 5% of the 1:1 species at pH 5.7, which is the minimum formation limit for accurate detection. As the 1:1 species is not

detected at a significant concentration, the actual value of log K_1 must be less than 2.1. Thus, the aniline substituted thiazoline, APTE, forms less stable Zn^{2+} complexes than the corresponding phenol substituted thiazoline, PTME. A possible cause for the reduced stability of the Zn^{2+} complex of APTE as compared to PTME is the reduced availability of the electrons on the amino phenyl substituent as compared to the phenoxide substituent.

Although APTE forms complexes of low stability with Zn^{2+}, it may still be useful as a biological probe for Zn^{2+}. As discussed previously, it is desirable to develop a biological Zn^{2+} probe which can be used to detect and measure the readily exchangeable Zn^{2+} within a cell. Therefore a Zn^{2+} specific ligand needed to be developed which would readily bind to the freely available Zn^{2+} within a cell, but did not compete for the Zn^{2+} which was already tightly bound to various proteins and enzymes within the cell. The actual affinity for Zn^{2+} required of the ligand probe was not fully known. Thus by synthesising a range of ligands with varying affinities for Zn^{2+} ions, tests on cells could be undertaken to determine which of the ligands had the optimum properties to carry out the tasks required. Therefore, either APTE or POEE may prove to be useful as a biological probe for Zn^{2+}. However, as the proposed mode of detecting and measuring the Zn^{2+} complexed probe within a cell was with fluorescence spectroscopy, it needed to be determined whether the Zn^{2+} complexes formed by APTE and POEE were fluorescent.

6.2.3 Fluorescence Measurements on APTE and POEE

The fluorescence of APTE and POEE were examined along with that of the metal complexes which APTE and POEE form. Since fluorescence quenching can occur through d-d transitions in complexes of APTE and POEE with the transition metal ions Co^{2+}, Ni^{2+} and Cu^{2+}, fluorescence measurements on these complexes were not conducted. Therefore,

fluorescence investigations were made on solutions of APTE and POEE and their corresponding Zn^{2+}, Cd^{2+} and Pb^{2+} complexes.

To carry out the fluorescence measurements on APTE and POEE, the conditions under which measurements were made and the method of measurement were the same as those used for the fluorescence measurements made on PTA, PTME and PTEE (section 5.2). This way, a direct comparison of the results obtained for APTE and POEE could be made with those obtained previously. The preparation of the sample solutions, the method of measurement and analysis of data are outlined in section 8.2.5. All sample solutions had an ionic strength of 0.100 mol dm^{-3} controlled by sodium perchlorate and were buffered with 1.00×10^{-3} mol dm^{-3} NaPIPES. The solutions which contained Zn^{2+} or Cd^{2+} were found to have a pH of 6.8 ± 0.02 and 7.09 ± 0.02 respectively. Similar problems to those which occurred previously (section 5.2) were experienced when trying to buffer the solutions containing Pb^{2+} to a pH of 7.0. The resulting value of the pH of the solutions which contained Pb^{2+} were 5.6 ± 0.5. To avoid problems with the precipitation of hydroxide species, all solutions were prepared and the spectra collected within eight hours. Due to unavoidable precipitation of hydroxide species, solutions of APTE and POEE which contained Pb^{2+} ions were filtered through a millipore filter before measurement. Therefore the total concentration of Pb^{2+} ions within the sample solutions used was not accurately known.

The emission spectra in the visible region of APTE and POEE were collected by irradiating solutions of each compound with monochromatic light of a wavelength equivalent to an ultraviolet absorption maximum of the compounds. From the spectra obtained, APTE was shown to be poorly fluorescent having a relative emission intensity maximum of 83 ± 1 out of 1000. The oxazole, POEE, was found to be relatively non-fluorescent having an emission maximum with a relative intensity of 25 ± 1. A species was

taken as being significantly fluorescent if an emission maximum with a relative intensity of over 100 was observed.

For comparison of the fluorescence of a ligand and one of its metal complexes, emission spectra of the ligand and of the metal complex were obtained by irradiating sample solutions of each species with monochromatic light of a wavelength equivalent to an absorption isosbestic wavelength of the ligand and its complex. Values of these isosbestic wavelengths were determined from the corresponding ultraviolet absorption spectra. This way, the number of photons being absorbed by the unbound ligand and its complex were equivalent. Therefore any change in fluorescence observed for the complex compared with the unbound ligand could be attributed to the formation of a complex of enhanced fluorescence and not to a differential in the amount of light being absorbed.

To examine the maximum fluorescence of the metal complexes of APTE and POEE, in the visible region, fluorescence emission spectra of the complexes were recorded by irradiating relevant sample solutions with monochromatic light of a wavelength equivalent to an ultraviolet absorption maximum wavelength of the complex, which was nearest the visible region. Spectra obtained were compared with those of the unbound ligands collected by irradiating sample solutions of the unbound ligands at similar wavelengths.

Values of the maximum relative intensities of fluorescence emissions of the Zn^{2+} and Cd^{2+} complexes formed by APTE and POEE are listed in Table 6.2. The Pb^{2+} complexes of APTE and POEE were non-fluorescent. Therefore, the corresponding data of these Pb^{2+} complexes have been omitted from Table 6.2. The other fluorescence spectra measured of APTE and POEE and their respective fluorescent metal complexes are presented in Appendix B.

Table 6.2 Maximum emission intensities of solutions[a,b] of the fluorescent metal ion complexes formed by the thiazole APTE and the oxazole POEE.[c]

Ligand	Metal Ion	Excitation Wavelength[d] (nm)	Maximum Emission Intensity[e]	Emission Maxima Wavelength[f] (nm)
APTE	Zn^{2+}	312	25	405.5
		285	30	441.5
	Cd^{2+}	320	92	459.5
		281	130	458.5
POEE	Zn^{2+}	321	155	411.0
		346	352	410.0
	Cd^{2+}	318	64	416.0
		348	160	414.5

a Solutions contained 5.00×10^{-6} mol dm^{-3} ligand, 2.00×10^{-3} mol dm^{-3} metal perchlorate 1.00×10^{-3} mol dm^{-3} NaPIPES and 0.100 mol dm^{-3} $NaClO_4$, in 75% methanol and 25% water.

b The pH of the solutions which contained Zn^{2+} or Cd^{2+} were 6.8 ± 0.02 and 7.09 ± 0.02 respectively.

c Measurements were recorded on solutions with I = 0.100 mol dm^{-3} ($NaClO_4$) and at 298.2 K.

d The first excitation wavelength for each complex is equivalent to an absorption isosbestic wavelength between the ligand and its complex. The second excitation wavelength for each complex is equivalent to an absorption maxima of the complex.

e Errors in the emission intensity values are $\pm$ 1.

f Errors in the wavelength at which the maximum emission intensity occurred are $\pm$ 0.5.

From Table 6.2, it can be seen that the thiazoline, APTE, produces a relatively non-fluorescent species when coordinated to Zn^{2+}. Unbound APTE produced a stronger fluorescent emission than its Zn^{2+} complex when irradiated with monochromatic light of a wavelength equivalent to either an absorption isosbestic wavelength of the ligand and its Zn^{2+} complex or an absorption maxima wavelength of the Zn^{2+} complex. Values for the relative intensities of the maximum emission produced by unbound APTE and the wavelengths at which they occurred are 29 ± 1 at 433.0 ± 0.5 nm and 67 ± 1 at 435.0 ± 0.5 nm, when irradiated with monochromatic light of wavelength 312 nm or 285 nm respectively. Even though APTE absorbs less light at 285 nm than its Zn^{2+} complex (see Figure 6.1), APTE produced a stronger fluorescence emission than its Zn^{2+} complex, when irradiated with light at this wavelength. This implies that the delocalisation of the π electrons in APTE is restricted when APTE coordinates to Zn^{2+}, suggesting that the conjugation between the π bonds of the phenyl and heterocyclic rings in APTE is broken down on Zn^{2+} chelation.

The Cd^{2+} complex formed by APTE is also poorly fluorescent. However, it did produce stronger fluorescence emissions when irradiated with light of the wavelengths listed in Table 6.2 than APTE itself. The corresponding relative intensities of maximum emission for APTE and the wavelengths at which they occurred are 43 ± 1 at 433.5 ± 0.5 nm and 67 ± 1 at 436.0 ± 0.5 nm, when irradiated with monochromatic light of wavelength 320 nm or 281 nm respectively. Therefore, it appears that neither the thiazoline, APTE, nor any of the metal complexes it forms are strongly fluorescent.

Although APTE and its complexes appeared to be poorly fluorescent, a comparison of the fluorescence of the Zn^{2+} complexes of APTE and PTME was carried out. To do this emission spectra of the complexes were recorded by irradiating solutions of each complex

with monochromatic light of a wavelength equivalent to an absorption isosbestic wavelength of the two complexes, which was determined by comparing the corresponding ultraviolet absorption spectra. The relative intensities of the maximum fluorescence emissions obtained for the Zn^{2+} complexes of APTE and PTME and the wavelengths at which these occurred were 38 ± 1 at 400.0 ± 0.5 nm and 106 ± 1 at 445.0 ± 0.5 nm respectively. Hence, the Zn^{2+} complex of the ortho hydroxy phenyl substituted thiazoline, PTME, is more fluorescent than that of the corresponding ortho amino phenyl substituted thiazoline, APTE.

The Zn^{2+} complex of the oxazole, POEE, produced significant fluorescence when irradiated with light of the wavelengths listed in Table 6.2. The values of the maximum emission intensities obtained for the Zn^{2+} complex of POEE show that this species is not strongly fluorescent. Similarly, the Cd^{2+} complex of POEE, also produced fluorescence emissions when irradiated at the wavelengths listed in Table 6.2, however, these emissions were quite weak. Unbound POEE itself, did not produce any significant fluorescence emissions when irradiated with light at any of the wavelengths used. Thus, both the Cd^{2+} and Zn^{2+} complexes formed by POEE are more fluorescent than unbound POEE.

As can be seen from Table 6.2, the isosbestic wavelength between unbound POEE and its Zn^{2+} complex occurs at approximately the same wavelength as that between POEE and its Cd^{2+} complex, that is 321 nm and 318 nm respectively. This suggests that both the Zn^{2+} and the Cd^{2+} complexes of POEE absorb approximately an equivalent number of photons at these wavelengths. Thus, by comparing the relative intensities of the maximum fluorescence produced by these complexes, when irradiated with monochromatic light at these wavelengths, it can be seen that the Zn^{2+} complex formed by POEE is more fluorescent than the corresponding Cd^{2+} complex.

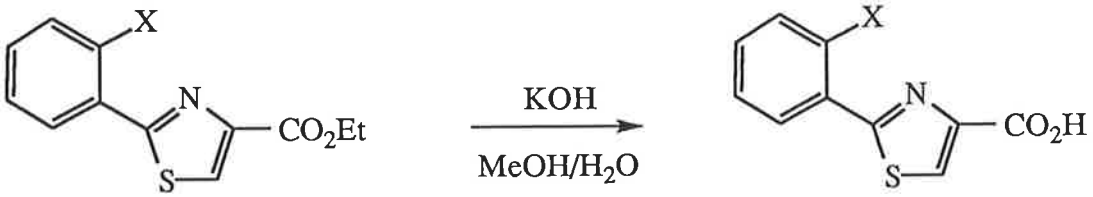
A comparison of the fluorescence of the Zn^{2+} complexes of the oxazole, POEE, and the corresponding thiazole, PTEE, was carried out. The emission spectra of each complex was recorded whilst irradiating a solution of the complex with monochromatic light of a wavelength equivalent to an absorption isosbestic wavelength of the two complexes, which was 363.0 ± 0.5 nm. Values of the relative intensities for the maximum emissions produced by the Zn^{2+} complexes of POEE and PTEE and the wavelengths at which they occurred were 213 ± 1 at 410.0 ± 0.5 nm and 371 ± 1 at 453.0 ± 0.5 nm respectively. Hence the thiazole, PTEE, is more fluorescent when coordinated to Zn^{2+} than the oxazole, POEE. Since PTEE produces a more fluorescent species when complexed to Zn^{2+} than POEE, this suggests that the delocalisation of the π and non-bonding electrons in POEE may be restricted on chelation to Zn^{2+}, unlike those in PTEE.

Both ligands APTE and POEE are weakly fluorescent. APTE does not produce strongly fluorescent complexed species with the metal ions tested. However on coordinating to Zn^{2+} POEE produces a moderately fluorescent species. The Cd^{2+} complex of POEE is poorly fluorescent.

6.2.4 Summary

In summary, the oxazole, POEE, coordinates the same metal ions as the corresponding thiazole, PTEE, which were Co^{2+}, Ni^{2+}, Cu^{2+}, Zn^{2+}, Cd^{2+} and Pb^{2+}. It was found that the phenolic hydrogen of POEE, which had a pK_a of 10.57 ± 0.02, was less acidic than that of PTEE, which had a pK_a of 9.43 ± 0.01. Also, the oxazole, POEE, formed more stable complexes with Zn^{2+} than the thiazole, PTEE. The oxazole, POEE was also found to be non-fluorescent, only forming a moderately fluorescent species when coordinated to Zn^{2+} and a poorly fluorescent species when coordinated to Cd^{2+}. The Zn^{2+} complex of POEE was less fluorescent than the Zn^{2+} complex of PTEE.

For PTEE or POEE to be used as probes for monitoring loosely or unbound Zn^{2+} within biological cells a study of the chelation and fluorescence of the corresponding acids of PTEE and POEE is required, since biological cells contain esterases which hydrolyse ester functional groups. The thiazole, PTEE, was hydrolysed to the corresponding acid (aeruginoic acid), (9a), using potassium hydroxide[7] (Scheme 6.5). Ultraviolet spectroscopy measurements showed that the acid, (9a), also chelates Zn^{2+}. Also, when a methanolic solution of the acid, (9a), was observed under ultraviolet light of 364 nm, no fluorescence was observed. However, when a methanolic solution of the acid, (9a), to which zinc perchlorate had been added, was observed under ultraviolet light of 364 nm the solution fluoresced a bright blue. Thus, the Zn^{2+} complex of (9a) is strongly fluorescent in the visible region, whereas unbound (9a) is not. A study into the selectivity of aeruginoic acid, (9a), for Zn^{2+} and the fluorescence of the complexes formed needs to be carried out, as does one on the corresponding acid of the oxazole, POEE.



PTEE **9a**

Scheme 6.5

The ortho amino phenyl substituted thiazoline, APTE, showed slightly more selectivity than the ortho hydroxy phenyl substituted thiazoline, PTME. Unlike PTME, APTE did not coordinate Al^{3+}, however, APTE did coordinate all the other metal ions which PTME coordinated. The acidity of the protonated aniline in APTE was too low to be determined accurately, also, under the conditions used, values for the stability constants of

the Zn^{2+} complexes formed by APTE could not be determined accurately. From an estimate of the upper limit of the log of the stability constant for a 1:1 complex between APTE and Zn^{2+}, it appears that this Zn^{2+} complex of APTE is much less stable than the corresponding Zn^{2+} complex of PTME. Neither APTE itself nor any of the metal complexes it formed were strongly fluorescent. Therefore, APTE does not appear to be useful as a fluorescent probe for Zn^{2+} or any other metal ion.

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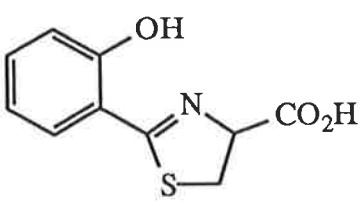
7 Summary and Conclusions

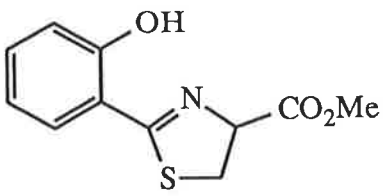
This thesis describes the development of Zn^{2+} selective fluorescent ligands which could be used as probes to detect and measure readily exchangeable Zn^{2+} within living cells. Also of interest was the production of ligands which would be suitable as fluorescent probes for Al^{3+} and Pb^{2+} in biological systems.

A series of ligands were synthesised based on a substructure of pyochelin, a naturally occurring fluorescent Zn^{2+} chelator. 2-(2-Hydroxyphenyl)-thiazoline-4-carboxylic acid, PTA, was prepared along with the corresponding methyl ester, PTME, and the ethyl ester of the thiazole, PTEE. Also synthesised were the analogous unsubstituted and methoxy substituted phenyl thiazoline and thiazole esters. Initial investigations into the binding properties of these compounds with Zn^{2+} showed the ortho hydroxy substituent was necessary for Zn^{2+} coordination. The absence of significant coordination of Zn^{2+} to the methoxy substituted phenyl thiazole derivatives suggests that the phenolic hydrogen may be displaced by Zn^{2+} upon coordination.

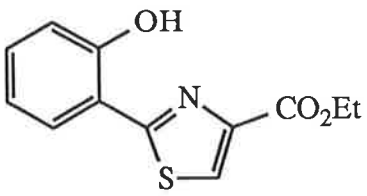
Further synthesis was undertaken to produce the amino substituted phenyl thiazoline ester, APTE, in order to compare the Zn^{2+} coordination ability of an amino substituent to a hydroxy substituent. The ortho hydroxy substituted phenyl oxazole, POEE, was also synthesised in order to determine any differences in chelation of Zn^{2+} by an oxazole and a thiazole. Studies carried out using ultraviolet absorption spectroscopy showed these compounds also coordinated Zn^{2+}.



PTA



PTME



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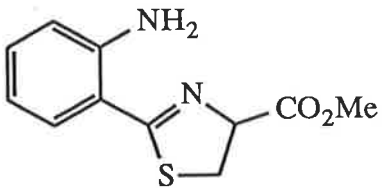
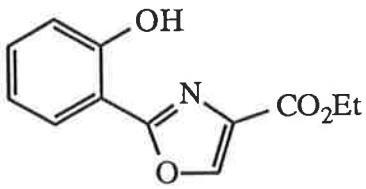


APTE



POEE

The coordination and fluorescence properties of APTE and POEE compounds along with the *o*-hydroxy substituted thiazole derivatives, PTA, PTME and PTEE, were measured to evaluate their suitability as fluorescent probes for Zn^{2+} or other metal ions.

A series of metal ions were chosen to test the coordination and fluorescence properties of the synthesised compounds. Selection was based on whether the metal ion is prevalent in biological systems, if it has similar binding properties to Zn^{2+}, or whether development of a probe selective for that metal ion was of interest. Therefore the series of metal ions which were selected was Na^{+}, Mg^{2+}, Al^{3+}, K^{+}, Ca^{2+}, Co^{2+}, Ni^{2+}, Cu^{2+}, Zn^{2+}, Cd^{2+} and Pb^{2+}.

The coordination of these metal ions by the ligands, PTA, PTME, PTEE, POEE, and APTE, which were previously shown to coordinate Zn^{2+}, was investigated using ultraviolet absorption spectroscopy. All five ligands were found to coordinate the transition metal ions, Co^{2+}, Ni^{2+} and Cu^{2+}. This was not surprising as these ions, being from the first transition series and those nearest Zn^{2+} in the periodic table, tend to coordinate to the

same ligands as Zn^{2+}. The five ligands also coordinated the heavy metal ions Cd^{2+}, which also has similar chelation properties to Zn^{2+}, and Pb^{2+}. Coordination by the five ligands to the biologically prevalent metal ions, Na^{+}, Mg^{2+}, K^{+} and Ca^{2+} was not observed except for the acid, PTA, with Ca^{2+}. Under the conditions used, the acid moiety of PTA was predominantly in an anionic carboxylate form. Since Ca^{2+} binds readily to oxygen donor atoms and no coordination of Ca^{2+} occurred with the ester, PTME, it is likely that Ca^{2+} is coordinated by PTA through the carboxylate group.

The acid, PTA, and the corresponding ester, PTME, both coordinate Al^{3+}, however, the other three ligands did not. The lack of coordination of the amino phenyl thiazoline, APTE, to Al^{3+} is most probably due to Al^{3+} being a hard acid which prefers to coordinate to hard bases, such as oxygen donor atoms and therefore coordination of Al^{3+} by the softer, nitrogen donor atom of the amino substituent of APTE is unlikely. The thiazole, PTEE, has been shown, using computer aided molecular modelling techniques, to have a larger 'bite' distance between the phenolic oxygen and the heteroatoms in the ring system compared to the corresponding thiazoline, PTME. Hence this 'bite' distance in PTEE may be too large for chelation of the relatively small Al^{3+} ion. This reasoning also explains the lack of coordination of POEE to Al^{3+}.

The stability constants of complexes formed between the ligands PTA, PTME, PTEE, POEE and APTE with various metal ions were determined using a potentiometric titration technique. Of the five ligands, both the acid, PTA, and the corresponding methyl ester, PTME, formed the most stable complexes with Zn^{2+}. The Zn^{2+} complexes formed by thiazole, PTEE, and the oxazole, POEE, were less stable than those of PTME. This could be attributed to the larger 'bite' distance between the phenolic oxygen and the nitrogen of the heterocyclic ring of these compounds, as compared to that of PTME. However, the Zn^{2+} complexes formed by POEE were more stable than the corresponding

thiazole, PTEE. It is not clear whether this difference is due to the differences in the 'bite' sizes of these ligands or to differing donor atoms. Also, the stepwise stability constants for the bis complexes of the three ligands PTME, PTEE, and POEE, with Zn^{2+} were all greater in value than those of the corresponding mono complexes. This indicates that on forming the bis complex with these ligands, Zn^{2+} undergoes a change in its coordination number preferring a tetrahedral geometry in favour of an octahedral one. This change in coordination number of the metal ion is not observed when PTA chelates to Zn^{2+}. The amino substituted phenyl thiazoline, APTE, formed weak complexes with Zn^{2+}, whose stability constants were too low to be measured accurately. Once again, this indicates the necessity of an ortho hydroxy substituent on the phenyl ring of these substituted thiazole derivatives, in order to form stable Zn^{2+} complexes.

Values for the stability constants of the Pb^{2+} complexes formed by the ligands PTA, PTME and PTEE were also determined. From the values obtained it could be seen that each of these ligands formed complexes of greater stability with Pb^{2+} than with Zn^{2+}. For the Pb^{2+} complexes of the acid, PTA, the derived values of the stability constants were sufficiently high to suggest that a terdentate species is formed between this ligand and Pb^{2+}. Values of the stability constants were also determined for the complexes formed by the acid, PTA, with Ca^{2+} and Cd^{2+}. The values determined for the Cd^{2+} complexes formed by PTA were greater than those of the Zn^{2+} complexes formed by PTA, however, those of the Ca^{2+} complexes were smaller. Hence, the Zn^{2+} complexes of PTA are less stable than its Cd^{2+} complexes, but more stable than its Ca^{2+} complexes. As with Pb^{2+}, the relatively large values of the stability constants of the Cd^{2+} complexes formed by PTA suggest these complexes are terdentate as well.

The fluorescence of the complexes formed by the ligands PTA, PTME, PTEE, POEE and APTE was examined. All five ligands produced little fluorescence when not

chelated to any metal ions. APTE did not produce a strongly fluorescent complex when chelated to Zn^{2+}, nor any other metal ion tested. The Zn^{2+} complexes of the other ligands, PTA, PTME, PTEE and POEE, were all significantly fluorescent, producing moderately high fluorescent emissions upon irradiation with ultraviolet light. Of the Zn^{2+} chelated species, the thiazoline acid, PTA, was the most fluorescent and the thiazole ester, PTEE, was slightly less fluorescent.

Of the other metal complexes formed by these ligands, the Al^{3+} complexes of PTA and PTME were highly fluorescent. These Al^{3+} complexes produced the most intense fluorescence of all the complexes of PTA and PTME investigated. The acid, PTA, also produced quite a strongly fluorescent complex when chelated to Cd^{2+}. The Cd^{2+} complexes of the ligands PTME, PTEE, POEE and APTE were only weakly fluorescent. The Pb^{2+} complexes of the five ligands were all non-fluorescent as was the Ca^{2+} complex of PTA.

Overall, on assessing the value of these ligands as probes for Zn^{2+} or other metal ions, it appears that the thiazole, PTEE, and the oxazole, POEE, are the most suitable of the five ligands for use as Zn^{2+} probes, due to the greater selectivity shown by these two ligands for Zn^{2+}. PTEE and POEE chelated to the fewest metal ions from the series tested. Both PTEE and POEE form stable complexes with Zn^{2+} which are strongly fluorescent. Neither PTEE nor POEE chelated to the biologically prevalent metal ions Na^{+}, Mg^{2+}, K^{+} or Ca^{2+}, making them suitable for use in biological systems. The other metal ion complexes formed by PTEE and POEE were either poorly or non-fluorescent.

Since an aim of this research was to design a probe for monitoring readily exchangeable Zn^{2+} within biological cells and these cells contain esterases which hydrolyse ester functional groups on compounds to the corresponding acids, a study of the chelation

and fluorescence of the corresponding acids of PTEE and POEE was required to fully assess these ligands suitability as fluorescent probes for exchangeable Zn^{2+} in biological systems. Initial tests using ultraviolet spectroscopy showed that the corresponding acid of PTEE forms a strongly fluorescent complex with Zn^{2+}. Further investigation into the selectivity of this acid for Zn^{2+} and the fluorescence of the complexes formed needs to be carried out, as does the study on the corresponding acid of the oxazole, POEE.

The acid, PTA, and the corresponding methyl ester, PTME, also appear to be suitable for use as probes for Zn^{2+}. They both form stable fluorescent complexes with Zn^{2+}, however the acid, PTA, also chelates Ca^{2+}, which makes this ligand less appropriate for use in biological systems. The complexes formed by PTA with Ca^{2+} are less stable than those with Zn^{2+}, therefore, PTA should show a preference for chelation to Zn^{2+} instead of Ca^{2+}. Also, the Ca^{2+} complexes formed by PTA are non-fluorescent and, if formed, would not interfere with fluorescence measurements of the Zn^{2+} complex. However, the concentration of Ca^{2+} ions present in biological cells is far greater than the concentration of Zn^{2+}. The high concentration of Ca^{2+} compared to Zn^{2+} may override the preference of PTA for binding to Zn^{2+} and preclude the formation of any Zn^{2+} complex unless a relatively high concentration of PTA was present, to chelate all the Ca^{2+} and labile Zn^{2+} in the cell. Therefore, competitive binding studies of PTA with Ca^{2+} and Zn^{2+} would be useful to further assess the suitability of this ligand as a probe for biological Zn^{2+}.

Both PTA and PTME form highly fluorescent complexes with Al^{3+} and may be suitable as fluorescent probes for Al^{3+}. Even though these ligands also form fluorescent complexes with Zn^{2+}, the Al^{3+} complexes formed are over four times more fluorescent than the Zn^{2+} complexes and have emission maxima which differ by 20 nm to the Zn^{2+} complexes. Therefore, these ligands could be used as fluorescent probes for Al^{3+} with

minimal interference from Zn^{2+}. Also, when using these ligands as probes for Al^{3+}, it is possible to determine and account for the effect of any Zn^{2+} present with the aid of another probe specific for Zn^{2+}.

Due to the high acidity of hydrated Al^{3+} and the insolubility of aluminium hydroxide, the stability constants of the Al^{3+} complexes formed by PTA and PTME could not be accurately measured under the conditions used in this study. Therefore, the stability of the Al^{3+} complexes formed by PTA and PTME needs to be determined using different methods, to further assess the applicability of these ligands as Al^{3+} probes.

The amino substituted phenyl thiazoline, APTE, formed relatively weak complexes with Zn^{2+} which were poorly fluorescent. This ligand would not be useful as a fluorescent probe for measuring biological Zn^{2+}. Of the other complexes formed by APTE, none of them were strongly fluorescent. Thus, APTE is not a suitable ligand for use as a fluorescent probe for the heavy metal ions Cd^{2+} or Pb^{2+} either.

Although the ligands PTA, PTME, PTEE and POEE formed complexes with Pb^{2+}, which for the case of PTA, PTME and PTEE were highly stable in comparison to other metal complexes formed by these ligands, none of these complexes were fluorescent. Therefore, none of these ligands are suitable for use as fluorescent probes for Pb^{2+}. However, these ligands may be used as a basis from which fluorescent probes suitable for Pb^{2+} could be designed.

This study produced four ligands, PTA, PTME, PTEE and POEE, which meet the necessary requirements for use as fluorescent biological probes for Zn^{2+}. *In vivo* tests are now required to further assess the applicability of these ligands for measuring exchangeable Zn^{2+} within biological cells. All four of these ligands form complexes with Zn^{2+} which

have absorption maxima that lie within the range 340 to 370 nm and emission maxima that lie within the range 410 to 460 nm. As previously discussed in chapter one, an aim of this research is to develop fluorescent probes for biological Zn^{2+} which form complexes that have absorption maxima of around 488 nm and emission maxima that lie well into the visible region, so that they can be readily excited and visualised with a standard confocal microscope which is equipped with a laser of 488 nm. Although the ligands PTA, PTME, PTEE and POEE are not suitable for this use, they provide a basis from which other ligands, which form Zn^{2+} complexes that have the required absorption and emission maxima, may be developed. For instance, by introducing a naphthalene ring rather than a phenyl ring into the substituted thiazoline structure of PTA, a Zn^{2+} chelating ligand would be produced, which, due to the greater conjugation within the naphthalene ring, would have an absorption maximum of higher wavelength.

8 Experimental

8.1 Synthetic Methods

8.1.1 General

Infrared spectra were recorded on a Hitachi 270 - 30 infrared spectrometer, as nujol mulls, liquid films or solutions, where indicated. Mass spectra were recorded on an AEI MS3074 spectrometer operating at 70 eV. Fast atom bombardment (FAB) mass spectra were recorded on a Vacuum Generators ZAB 2HF mass spectrometer. Ultraviolet/Visible spectra were recorded on a PYE Unicam SP8/100 UV/VIS spectrophotometer. All ultraviolet spectra recorded were of solutions in methanol, unless otherwise stated.

^{1}H N.m.r. spectra were recorded at 60 MHz on a Varian T-60 spectrometer. High field ^{1}H n.m.r. and ^{13}C n.m.r. were recorded at 300 MHz on a Brucker ACP 300 spectrometer. All ^{1}H n.m.r. spectra were recorded as solutions in carbon tetrachloride, deuterochloroform or deuterium oxide, where indicated, using tetramethylsilane as an internal standard. All chemical shifts are quoted as δ in parts per million and coupling constants, J, are given in Hertz. All multiplicities are abbreviated: s, singlet; d, doublet; t, triplet; q, quartet; m, multiplet; br, broad.

Melting points were recorded on a Kofler hot-stage apparatus equipped with a Reichert microscope, and are uncorrected. Microanalyses were performed by the Chemistry Department, University of Otago, Dunedin.

Analytical thin layer chromatography (tlc) was carried out using Merk Kieselgel $60F_{254}$ on aluminium backed plates. Flash chromatography[1] was performed using

Amicon Matrix silica, with a pore diameter of 60 Å. Silica squat chromatography[2] was carried out using Merk Kieselgel HF_{254}.

All solvents were purified and dried using standard laboratory procedures.[3] Light petroleum refers to the fraction with boiling range 66° - 69°C. Tetrahydrofuran is abbreviated to THF, diethylether to ether and hexamethylphosphoramide to HMPA. All organic extracts were dried over anhydrous magnesium sulfate or sodium sulfate unless otherwise specified.

8.1.2 Synthesis

Salicylonitrile

Salicylaldehyde (5.00 g, 41 mmol), hydroxylamine hydrochloride (3.04 g, 47 mmol) and sodium formate (5.00 g, excess) were refluxed in formic acid (75 ml) for 1 h. The cooled solution was diluted with water (75 ml) and extracted with ether (4 x 50 ml). The combined ether extracts were washed with water (1 x 50 ml), dried (magnesium sulfate) and the solvent was evaporated. The oily residue was purified via squat chromatography (ethyl acetate / light petroleum) to obtain two fractions. The first and minor fraction was *o*-hydroxybenzaldehyde oxime, (6), (0.79 g, 14%), m.p. 55-57°C (lit.[4] 57°C). υ_{max} (Nujol) 3372 (OH), 1620 (C=N), 1576, 1496 (C=C arom.) cm^{-1}. Mass spectrum m/z 137 ($M^{+\bullet}$), 119 (M - H_2O), 91 (100%), 64.

The second and major fraction was salicylonitrile (3.81 g, 78%), m.p. 93-95°C (lit.[5] 98°C). υ_{max} (Nujol) 3284 (OH), 2228 (CN), 1604, 1506 (C=C arom), 750, 734 (*o*-disub. arom) cm^{-1}. Mass spectrum m/z 119 ($M^{+\bullet}$), 91 (M - CO, 100%), 64 (C_5H_4).

2-(2-Hydroxyphenyl)-2-thiazoline-4-carboxylic acid (3a)

Salicylonitrile (0.30 g, 2.5 mmol), L-cysteine hydrochloride (0.40 g, 25 mmol) and sodium bicarbonate (0.21 g, 2.5 mmol) were refluxed in absolute ethanol (2 ml) for

30 min. The solution was cooled and the pH was adjusted to approximately 9 with triethylamine, then the reaction mixture was refluxed for a further 12 h. The resulting mixture was diluted with enough water to just dissolve the sodium bicarbonate and acidified to pH 3.9 with acetic acid. The acid, (3a), precipitated out as a yellow powder, which was collected and washed with ethanol and then with water (0.51 g, 91%), m.p. 266-267°C (lit.[6] 269°C). υ_{max} (Nujol) 2800 - 2500 (OH), 1736 (C=O), 1618 (C=N), 1604, 1506 (C=C arom.), 748, 726 (*o*-disub. arom.) cm^{-1}. λ_{max} (MeOH) 315, 256, 250, 214 nm. ^1H n.m.r. δ (300 MHz, D_2O) 7.59-6.95, m, 4H, ArH; 5.23, dd, *J* 9.2, 7.4 Hz, 1H, H4; 3.78, dd, *J* 7.4, 11.3 Hz, 1H, $H5_a$; 3.60, dd, *J* 9.2, 11.3 Hz, 1H, $H5_b$. Mass spectrum m/z 223 ($M^{+\bullet}$), 178 (M - CO_2H, 100%), 119, 91, 64.

Methyl 2-(2-hydroxyphenyl)-2-thiazoline-4-carboxylate (8a)

The acid, (3a), (2.01 g, 9.0 mmol) was added to a stirred solution of thionyl chloride (1.5 ml, 20.6 mmol) in dry methanol and then refluxed for 1.5 h. A saturated solution of sodium bicarbonate was added to the cooled solution which was then extracted with ether (3 x 30 ml). The combined extracts were washed with water (1 x 30 ml), dried (magnesium sulfate) and the ether was removed by evaporation. The residue was purified by squat chromatography (ether / light petroleum) to obtain the ester, (8a), as a pale yellow oil (1.94 g, 91%), b.p. 140-143°C / 0.5 mm Hg (Found: C, 55.44; H, 4.87; N, 6.11. $C_{11}H_{11}NO_3S$ requires C, 55.68; H, 4.67; N, 5.90%). υ_{max} (neat) 3300-2400 (OH), 1744 (C=O), 1622 (C=N), 1592, 1492 (C=C arom.) cm^{-1}. λ_{max} (MeOH) 317, 252, 214 nm. ^1H n.m.r. δ (300 MHz, $CDCl_3$) 9.24, br, 1H, OH; 7.40, d, *J* 7.5 Hz, 1H, H6'; 7.37, dd, *J* 8.3, 7.5 Hz, 1H, H4'; 7.01, d, *J* 8.3 Hz, 1H, H3'; 6.88, t, *J* 7.5 Hz, 1H, H5'; 5.34, dd, *J* 9.3, 8.1 Hz, 1H, H4; 3.81, s, 3H, OCH_3; 3.67, dd, *J* 11.1, 8.1 Hz, H5a; 3.58, dd, *J* 11.1, 9.3 Hz, H5b. Mass spectrum m/z 237 ($M^{+\bullet}$), 178 (M - CO_2Me, 100%), 119, 91.

Methyl 2-phenyl-2-thiazoline-4-carboxylate (8b)

Benzonitrile (1.00 g, 9.7 mmol), L-cysteine hydrochloride (1.58 g, 10 mmol) and sodium bicarbonate (0.84 g, 10 mmol) were refluxed in absolute ethanol (10 ml) under nitrogen for 30 min. The solution was cooled, and the pH was adjusted to approximately 9 with triethylamine, then the solution was refluxed for a further 12 h. The resulting mixture was diluted with just enough water to dissolve the sodium bicarbonate and acidified with concentrated hydrochloric acid, until the pH of the solution was approximately 1. The solvent was evaporated under reduced pressure, leaving a white solid which was relatively insoluble in most organic solvents. The solid residue was stirred in dry methanol (30 ml) and thionyl chloride (2.0 ml, 27.5 mmol) was added. The solution was refluxed under nitrogen for 4 h. The cooled solution was treated with a saturated sodium bicarbonate solution and extracted with dichloromethane (3 x 30 ml). The organic extracts were washed with water (1 x 30 ml), dried (sodium sulfate) and the solvent was evaporated. The crude product was purified by flash chromatography (dichloromethane) and recrystallised (methanol) to obtain the ester, (8b), as an off white solid (0.98 g, 46%), m.p. 62-66°C (Found: C, 59.57; H, 4.94; N, 6.38. $C_{11}H_{11}NO_2S$ requires C, 59.70; H, 5.01; N, 6.32%). υ_{max} (CH_2Cl_2) 1744 (C=O), 1602, 1494 (C=C arom.) cm^{-1}. λ_{max} (MeOH) 207, 243 nm. ^1H n.m.r. δ (300 MHz, $CDCl_3$) 7.85, d, *J* 6.9 Hz, 2H, H2', H6'; 7.45, dd, *J* 6.9, 7.0 Hz, 2H, H3'. H5'; 7.41, t, *J* 7.0 Hz, 1H, H4'; 5.30, t, *J* 9.1 Hz, 1H, H4; 3.84, s, 3H, OCH_3; 3.73, dd, *J* 8.9, 11.2 Hz, 1H, H5a; 3.64, dd, *J* 9.5, 11.2 Hz, 1H, H5b. Mass spectrum m/z 221 ($M^{+\bullet}$), 162 (M - CO_2Me, 100%).

o-Methoxybenzaldehyde

Sodium (0.23 g, 10 mmol) was dissolved in anhydrous ethanol (10 ml) and salicylaldehyde (1.1 ml, 10 mmol) was added, followed by methyl iodide (0.70 ml, 11 mmol). The mixture was stirred under reflux for 1.5 days, then cooled and diluted with water (5 ml). The solution was extracted with ether (3 x 5 ml) and the extracts were washed firstly with 10% sodium hydroxide (5 ml) then with water (5 ml) and dried

(magnesium sulfate). The solvent was evaporated and the crude product was purified by squat chromatography (light petroleum / ethyl acetate) yielding *o*-methoxybenzaldehyde (0.84 g, 62%), m.p. 37-39°C (lit.[5] 37-38°C). Mass spectrum m/z 136 ($M^{+\bullet}$, 100%), 135 (M - H), 104.

o-Methoxybenzamide

Sodium hydride (0.75 g, 22 mmol, 70% in mineral oil) was slowly added to a stirred solution of salicylamide (2.75 g, 20 mmol) in anhydrous THF (40 ml), followed by methyl iodide (5 ml, 80 mmol). The solution was allowed to stir at room temperature, under nitrogen, for 1 day, then poured into water (60 ml) and extracted with ether (3 x 20 ml). The extracts were washed with water (1 x 20 ml), dried (sodium sulfate) and the solvent was evaporated. The product was recrystallised from water to yield *o*-methoxybenzamide (2.46 g, 81%), m.p. 127-128°C (lit.[5] 129°C). υ_{max} (Nujol) 3416, 3184 (NH_2), 1626 (C=O), 1598, 1488 (C=C arom.), 1106 (C-O) cm^{-1}. ^1H n.m.r. δ (60 MHz, $CDCl_3$) 7.0 -8.0, m, 4H, arom: 3.97, s, 3H, OCH_3. Mass spectrum m/z 151 ($M^{+\bullet}$), 135 (M - NH_2).

o-Methoxybenzonitrile

Method 1

o-Methoxybenzaldehyde (0.65 g, 4.8 mmol), hydroxylamine hydrochloride (0.33 g, 4.8 mmol) and sodium formate (0.63 g, excess) were refluxed in formic acid (10 ml) for 1 h. The cooled reaction mixture was diluted with water (20 ml) and extracted with ether (3 x 15 ml). The combined ether extracts were washed with water (1 x 20 ml), dried (magnesium sulfate) and the solvent was evaporated. The oily residue was purified via squat chromatography (ether / light petroleum) to obtain the nitrile (0.53 g, 84 %), b.p. 146°C / 20 mm Hg (lit.[5] 146°C / 20 mm Hg). υ_{max} (neat) 2224 (CN), 1598, 1496 (C=C arom.), 756, 730 (*o*-disub. arom.) cm^{-1}. Mass spectrum m/z 133 ($M^{+\bullet}$, 100%).

Method 2

o-Methoxybenzamide (1.03g, 6.8 mmol) and phosphonitrilic chloride (0.77g, 2.2 mmol) were refluxed in chlorobenzene (30 ml) overnight, under anhydrous conditions. The cooled solution was filtered and the white precipitate was washed with ether. The solvent was evaporated from the combined filtrate and washings and the residue was purified by squat chromatography (ethyl acetate / light petroleum) to obtain the nitrile (0.66g, 73%). The spectral data obtained were the same as that quoted above.

Methyl 2-(2-methoxyphenyl)-2-thiazoline-4-carboxylate (8c)

o-Methoxybenzonitrile (0.37 g, 2.8 mmol), L-cysteine hydrochloride (0.44 g, 2.8 mmol) and sodium bicarbonate (0.3 g, 3.6 mmol) were refluxed in absolute ethanol (10 ml) for 30 min. The reaction mixture was cooled and triethylamine was added dropwise until the pH of the solution was approximately 9. The reaction mixture was then refluxed for a further day. The resulting mixture was cooled and just enough water was added to dissolve the sodium bicarbonate. The solution was acidified to pH 1 with concentrated hydrochloric acid and extracted with ethyl acetate (4 x 10 ml). The extracts were dried (sodium sulfate) and the solvent removed to obtain the crude acid, (3c), (0.36 g, 54%) b.p. 120 - 122°C / 0.045 mm Hg. Mass spectrum m/z 237 ($M^{+\bullet}$), 192 (M - CO_2H), 133. The crude acid, (3c), was dissolved in methanol (15 ml) and thionyl chloride (0.60 ml, 8.2 mmol) was slowly added. The solution was stirred under reflux for 1 h, cooled, poured into saturated sodium bicarbonate (30 ml) and extracted with ether (3 x 30 ml). The combined extracts were washed with water (1 x 20 ml), dried (sodium sulfate) and the solvent was evaporated. The resulting yellow oil was purified by flash chromatography (light petroleum / ethyl acetate). The major fraction was collected and distilled to yield the ester, (8c), as a yellow oil (0.19 g, 50%), b.p. 120 - 122°C / 0.045 mm Hg. Although spectral data confirmed the structure of the ester, microanalysis of the sample was unsuccessful. υ_{max} (neat) 1744 (C=O), 1654 (C=N), 1598, 1486 (C=C arom.), 1114 (C-O) cm^{-1}. λ_{max} (MeOH) 289, 237, 211 nm. ^1H n.m.r. δ (300 MHz, $CDCl_3$) 7.47 - 6.93, m, 4H, ArH; 5.17, t, *J* 9.3 Hz, 1H, H4; 3.90,

s, 3H, OCH_3; 3.82, s, 3H, CO_2CH_3; 3.60, dd, *J* 11.2, 9.3 Hz, 1H, $H5_a$; 3.53, dd, *J* 11.2, 9.4 Hz, 1H, $H5_b$. Mass spectrum m/z 251 ($M^{+\bullet}$), 192 (M-CO_2Me, 100%), 133.

Thiobenzamide (10b)

Benzamide (0.34 g, 2.81 mmol) was stirred in anhydrous THF (20 ml) in a dry 2-necked round bottom flask, equipped with a mechanical stirrer and submerged into the water bath of a sonicator to just above the solvent level. Phosphorus pentasulphide (0.44 g, 1 mmol) was added to the solution which was sonicated for 30 min with stirring, after which more phosphorus pentasulphide (0.44 g, 1 mmol) was added to the solution followed by further sonication for 90 min. The reaction mixture was filtered and the precipitate washed with dichloromethane. The solvent was removed from the combined filtrate and washings leaving a yellow oil which was purified by flash chromatography (dichloromethane) to give thiobenzamide as a light yellow powder (0.32 g, 83%), m.p. 119-120°C (lit.[7] 116-118°C). υ_{max} (Nujol) 3364 (NH), 1622 (C=C arom), 1460 (C=S) cm^{-1}. Mass spectrum m/z 137 ($M^{+\bullet}$), 121 (M - NH_2), 104 (M - SH), 77 (C_6H_5), 51 (C_4H_3).

Lawesson's reagent (11)

Phosphorus pentasulphide (14.00 g, 31.5 mmol) was stirred in anisole (40 ml, 370 mmol) under an inert atmosphere and the solution was heated for 6 h at 155°C. The yellow precipitate was collected, under a stream of nitrogen, from the cooled reaction mixture and washed with chloroform (50 ml) and ether (50 ml), then dried under vacuum, in a desiccator over phosphorus pentoxide, yielding Lawesson's reagent (11.79 g, 93%), m.p. 222-226°C (lit.[8] 226-228°C). Mass spectrum m/z 404 ($M^{+\bullet}$), 202.

o-Hydroxythiobenzamide (10a)

Lawesson's reagent (4.05 g, 10 mmol) was slowly added to a stirred solution of salicylamide (2.74 g, 20 mmol) in HMPA (20 ml), whilst heating at 70°C and the stirring was continued at this temperature for 16 h. Water (80 ml) was added to the cooled

solution and the solution was extracted with ether until no more product was being extracted, as shown by tlc. The extracts were dried (sodium sulfate) and the solvent was evaporated. The residue was purified by flash chromatography (dichloromethane) giving three fractions of which the third fraction was the starting material, salicylamide (0.28g, 20%). The first fraction was 2,3-dihydro-2-(4-methoxyphenyl)-4H-1,3,2-benzoxaphosphorine-4-thione 2-sulfide, (12), which was recrystallised from toluene (0.5 g, 8%), m.p. 130 - 134°C (Found: C, 51.99; H, 3.82; N, 4.36. $C_{14}H_{13}NO_2PS_2$ requires C, 52.33; H, 3.76; N, 4.36%). υ_{max} (neat) 2836, 1596, 1504 (C=C arom.), 1474 (C=S), 1462 (P-Ph), 1264 (P=S), 1208 (P-O-Aryl) cm^{-1}. ^1H n.m.r. δ (300 MHz, $CDCl_3$) 6.9 - 8.7, m, 8H, ArH; 3.85, s, 3H, OCH_3; 1.76, br, NH. ^{13}C n.m.r. δ (300 MHz, $CDCl_3$) 193.8, C=S; 164.0; 148.4; 135.8; 133.9; 133.7; 124.8; 123.0; 121.2; 114.2; 55.6, OCH_3. Mass spectrum m/z 321 ($M^{+\bullet}$, 100%), 288 (M - SH).

The second fraction was the thioamide, (10a), (1.11 g, 36%), m.p. 117-118°C (lit.[9] 118°C). υ_{max} (CH_2Cl_2) 3380 (NH_2), 1606, 1504 (C=C arom.), 1254 (C=S) cm^{-1}. Mass spectrum m/z 153 ($M^{+\bullet}$), 136, 108.

o-Methoxythiobenzamide

Lawesson's reagent (2.02 g, 5 mmol) was added to a stirred solution of *o*-methoxybenzamide (1.51 g, 9.9 mmol) in HMPA (10 ml) whilst heating the solution at 80°C. The reaction mixture was stirred for 3 h, whilst heating at 90°C, then cooled and poured into water (20 ml). The solution was extracted with ether until tlc analysis of the extracts showed no presence of the product. The combined extracts were dried (sodium sulfate) and the solvent was evaporated, leaving a crude yellow oil, which was purified by squat chromatography (light petroleum / ethyl acetate) obtaining the thioamide, (10c), as a yellow powder (1.27 g, 77 %), m.p. 92 - 96°C. υ_{max} (CH_2Cl_2) 3480, 3348 (NH_2), 2840, 1590, 1484 (C=C arom.), 1470, 1260 (C=S) cm^{-1}. Mass spectrum m/z 167 ($M^{+\bullet}$), 134 (M - SH, 100%), 151 (M - NH_2).

Ethyl 2-(2-hydroxyphenyl)-thiazole-4-carboxylate (13a)

o-Hydroxythiobenzamide (0.93 g, 6.07 mmol) in absolute ethanol (14 ml) was added to a stirred solution of ethyl bromopyruvate (0.85 ml, 6.77 mmol) in absolute ethanol (28 ml) and the reaction mixture was heated to 60°C and stirred for 1 h. To the cooled solution was added aqueous sodium carbonate (50 ml, 10%) and the solution was extracted with ethyl acetate (3 x 40 ml). The extracts were washed with water (40 ml), dried (sodium sulfate) and the solvent was evaporated under reduced pressure. The crude crystalline product was decolourised with charcoal and recrystallised (methanol / water) to give the thiazole, (13a), as a white crystalline powder (0.95 g, 62%), m.p. 107-110°C (lit.[10] 107°C). υ_{max} (CH_2Cl_2) 3688 (OH), 1730 (C=O), 1622 (C=N), 1582, 1552, 1480 (C=C arom.) cm^{-1}. λ_{max} (MeOH) 323, 276, 215 nm. ^1H n.m.r. δ (300 MHz, $CDCl_3$) 11.93, s, 1H, OH; 8.10, s, 1H, H5; 7.62, dd, *J* 7.6, 1.5 Hz, 1H, H6'; 7.36, ddd, *J* 8.5, 7.6, 1.5 Hz, 1H, H4'; 7.09, dd, *J* 8.5, 1.0 Hz, 1H, H3'; 6.93, td, *J* 7.6, 1.0 Hz, 1H, H5'; 4.43, q, *J* 7.06 Hz, 2H, $OC\underline{H}_2CH_3$; 1.42,, t, *J* 7.06 Hz, 3H, $OCH_2C\underline{H}_3$. Mass spectrum m/z 249 ($M^{+\bullet}$), 203 (100%), 175.

Ethyl 2-phenylthiazole-4-carboxylate (13b)

Thiobenzamide (0.12 g, 0.9 mmol) in absolute ethanol (4 ml) was added dropwise to a stirred solution of ethyl bromopyruvate (0.13 ml, 1.0 mmol) in absolute ethanol (5 ml). The solution was heated to 70°C and stirred for 1.5 h. The cooled reaction mixture was poured into a sodium carbonate solution (10 ml, 10%) and extracted with ethyl acetate (3 x 10 ml). The combined organic extracts were washed with water (1 x 15 ml), dried (sodium sulfate) and the solvent was evaporated. The product was purified by squat chromatography (ethyl acetate / light petroleum) and distilled to yield the ester (0.18 g, 87%), m.p. 44-49°C, b.p. 118-120°C / 0.03 mm Hg (Found: C, 61.51; H, 4.72; N, 5.96. $C_{12}H_{11}NO_2S$ requires C, 61.78; H, 4.75; N, 6.00%). υ_{max} (neat) 1728 (C=O), 1580, 1498, 1470 (C=C arom.) cm^{-1}. λ_{max} (CH_2Cl_2) 286, 230 nm. ^1H n.m.r. δ (300 MHz, $CDCl_3$) 8.16, s, 1H, H5; 8.01, m, 2H, H2', H6'; 7.45, m, 3H, H3', H4',

H5'; 4.52, q, *J* 7.0 Hz, 2H, $OC\underline{H}_2CH_3$; 1.43, t, *J* 7.0 Hz, 3H, $OCH_2C\underline{H}_3$. Mass spectrum m/z 233 ($M^{+\bullet}$), 188 (M - C_2H_5O, 100%), 160 (188 - CO).

Ethyl (2-methoxyphenyl)-thiazole-4-carboxylate (13c)

o-Methoxythiobenzamide (0.95 g, 5.7 mmol) in absolute ethanol (15 ml) was added dropwise to a stirred solution of ethyl bromopyruvate (0.8 ml, 6.4 mmol) in absolute ethanol (26 ml) and the resulting solution was heated at 70°C for 4 h. An aqueous solution of sodium carbonate (50 ml, 10%) was added to the cooled reaction mixture and the mixture was extracted with ethyl acetate (4 x 20 ml). The extracts were dried (sodium sulfate), the solvent was removed and the product residue was purified by squat chromatography (light petroleum / ethyl acetate) and recrystallised (methanol / water) to yield the thiazole, (13c), (1.30 g, 87%), m.p. 78 - 81°C (Found: C, 59.58; H, 5.06; N, 5.44. $C_{13}H_{13}NO_3S$ requires C, 59.30; H, 4.98; N, 5.32%). υ_{max} (CH_2Cl_2) 2840, 1728 (C=O), 1598, 1584, 1500 (C=C arom.) cm^{-1}. λ_{max} (MeOH) 310, 286, 277, 228, 209 nm. ^1H n.m.r. δ (300 MHz, $CDCl_3$) 8.18, s, 1H, H5; 7.37 - 6.93, m, 4H, ArH; 4.42, q, *J* 7.1 Hz, $OC\underline{H}_2CH_3$; 3.93, s, 3H, OCH_3; 1.40, t, *J* 7.1 Hz, OCH_2CH_3. Mass spectrum m/z 263 ($M^{+\bullet}$), 133 (100%).

Methyl 2-(2-aminophenyl)-2-thiazoline-4-carboxylate (20)

2-Aminobenzonitrile (0.40 g, 2.5 mmol), L-cysteine hydrochloride (0.30 g, 2.5 mmol) and sodium bicarbonate (0.21 g, 2.5 mmol) were refluxed in absolute ethanol for 30 min. The reaction mixture was cooled and triethylamine was added dropwise until the pH of the solution was approximately 9. The reaction mixture was then refluxed for a further 12 h. The cooled mixture was diluted with water until all the solid residues had dissolved, then acidified with acetic acid to pH 4. The solution was then extracted with ethyl acetate (4 x 10 ml) and the combined extracts were washed with water (10 ml), dried (sodium sulfate). The solvent was removed under reduced pressure leaving a yellow oily residue. This residue was dissolved in anhydrous methanol (20 ml) and a solution of thionyl chloride (0.55 ml, 7.54 mmol) in methanol (5 ml) was slowly added

with stirring. After the addition the reaction mixture was refluxed for 4 h. The cooled solution was neutralised with saturated aqueous sodium bicarbonate solution and extracted with ethyl acetate (4 x 20 ml). The combined extracts were dried (sodium sulfate) and the solvent was removed under reduced pressure. The yellow residue was purified by squat chromatography (light petroleum / ethyl acetate) and the major fraction was distilled, yielding the thiazoline ester, (20), as a yellow oil (0.47 g, 80%), b.p. 125 - 128°C / 0.15 mm Hg (Found: C, 56.20; H, 5.36; N, 12.03. $C_{11}H_{12}N_2O_2S$ requires C, 55.91; H, 5.12; N, 11.86%). υ_{max} (neat) 3472, 3376 (N-H), 1732 (C=O), 1632 (C=N), 1590, 1496 (C=C arom.) cm^{-1}. ^1H n.m.r. δ (300 MHz, $CDCl_3$) 7.42, dd, *J* 8.0 1.5 Hz, 1H, H6'; 7.17, ddd, *J* 8.0, 7.8, 1.5 Hz, 1H, H4'; 6.69, dd, *J* 7.8, 1.1 Hz, 1H, H3'; 6.63, td, *J* 8.0, 1.1 Hz, 1H, H5'; 6.19, br, 2H, NH_2; 5.34, t, *J* 8.8 Hz, 1H, H4; 3.80, s, 3H, CO_2CH_3; 3.52, d, *J* 9.0 Hz, 1H, H5a; 3.51, d, *J* 9.0 Hz, 1H, H5b. Mass spectrum m/z 236 ($M^{+\bullet}$), 177, 118, 91.

Ethyl 2-(2-hydroxyphenyl)-oxazole-4-carboxylate (21)

Salicylamide (1.00 g, 7.29 mmol), dissolved in absolute ethanol (20 ml) was added dropwise to a stirred solution of ethyl bromopyruvate (1.0 ml, 7.9 mmol) in absolute ethanol (30 ml). The solution was heated to 70°C for 2 days. To the cooled reaction mixture was added aqueous sodium carbonate (20 ml, 10%). The mixture was extracted with ethyl acetate (3 x 20 ml) and the combined extracts were washed with water (20 ml), dried (sodium sulfate) and the solvent was evaporated under reduced pressure leaving a yellow oily residue. The residue was purified by squat chromatography (light petroleum / ethyl acetate), from which two main fractions were obtained. The first main fraction was the oxazole, (21), which was recrystallised from ethanol/water (0.25 g, 15 %), m.p. 82 - 83°C (Found: C, 61.80; H, 4.78; N, 6.02. $C_{12}H_{11}NO_4$ requires C, 61.80; H, 4.75; N, 6.01%). λ_{max} (MeOH) 212, 256, 261, 273, 309 nm. ^1H n.m.r. δ (300 MHz, $CDCl_3$) 10.72, br, s, 1H, OH; 8.25, s, 1H, H5; 7.84, dd, *J* 7.9, 1.6 Hz, 1H, H6'; 7.41, td, *J* 8.0, 1.6 Hz, 1H, H4'; 7.09, dd, *J* 8.0, 1.1 Hz, 1H, H3'; 6.98, td, *J* 7.9, 1.1 Hz, 1H, H5'; 4.41, q, *J* 7.1 Hz, 2H, $OC\underline{H}_2CH_3$;

1.41, t, *J* 7.1 Hz, 3H, $OCH_2C\underline{H}_3$. Mass spectrum m/z 233 ($M^{+\bullet}$, 100%), 205 (M - CO), 187 (M - C_2H_6O).

Further elution of the column gave a second major fraction which was the starting material, salicylamide (0.58g, 58%), m.p. 139-140°C (lit.[4] 140°C).

2-(2-Hydroxyphenyl)-thiazole-4-carboxylic acid (9a)

A solution of ethyl 2-(2-hydroxyphenyl)-thiazole-4-carboxylate, (13a), (0.10 g, 0.40 mmol) and potassium hydroxide (0.16 g, mmol) in a mixture of methanol (3 ml) and water (1ml) was heated at 70°C for 3 h, with stirring. The cooled solution was poured into water (15 ml) and extracted with ether (1 x 15 ml). The aqueous layer was then acidified with hydrochloric acid (10%). A white precipitate formed which was extracted with ethyl acetate (3 x 10 ml). The combined ethyl acetate extracts were washed with water (1 x 10 ml), dried (magnesium sulfate) and the solvent was evaporated off under reduced pressure. The remaining white powder was recrystallised (methanol) to yield the acid, (9a), as white crystalline needles (0.062 g, 70%), m.p. 269 - 270°C dec. (lit.[10] 271 - 272°C dec.).

8.2 Physical Methods

8.2.1 General

Solutions were prepared from a stock solvent containing 25% water and 75% methanol, by volume. All analytical solutions were composed of this solvent mix, unless otherwise stated. Deionised water purified with a Milli Q - Reagent system was used in the preparation of solutions and stock solvent. Freshly opened analytical grade methanol (Ajax Chemicals), containing $\leq 0.1\%$ water, was used for solution preparation without

further purification. Stock solvent was degassed by bubbling high purity nitrogen through it before use.

The ligands (PTA, PTME, PTEE, APTE and POEE) were prepared, purified and their purity was assessed by the methods described in section 8.1.2. The pure ligands were stored over anhydrous phosphorus pentoxide.

The sodium perchlorate salt used was of analytical grade and was dried over anhydrous phosphorus pentoxide, under vacuum. Prestandardised 0.100 mol dm^{-3} aqueous solutions of the required metal perchlorates were used for addition of the metal ions to the ligand solutions.

Standard aqueous stock solutions of 1.00 mol dm^{-3} sodium hydroxide and 0.100 mol dm^{-3} hydrochloric acid were prepared from Convol® ampoules. These were then diluted to prepare the solutions required for titration.

Solutions requiring perchloric acid were prepared from dilution of an aqueous 5.00×10^{-2} mol dm^{-3} perchloric acid stock solution. This solution was prepared using analytical grade 70% aqueous perchloric acid. The perchloric acid solutions were standardised by titration with 0.100 mol dm^{-3} sodium hydroxide.

Solutions used in ultraviolet and fluorescence spectra measurements were buffered with the sodium salt of piperazine-N,N'-bis-(2-ethanesulfonic acid), NaPIPES, which has a pK_a of 6.8 at 20°C. Accurate measurement of the pH of the solutions was carried out using a calibrated Orion 8172 Sureflow Ross pH electrode, connected to an Orion SA720 potentiometer. Three separate pH measurements were taken of each solution and the results were averaged.

8.2.2 Ultraviolet Absorption Spectra Measurements

Ultraviolet absorption spectra of the ligands (PTA, PTME, PTEE, APTE and POEE) and their various metal complexes were recorded using a Zeiss DMR10 Ultraviolet/Visible Spectrophotometer. All sample solutions were thermostatted to 298.2 ± 0.1 K, by circulation of water through the cell compartment of the spectrometer, using a Julabo P water pump. The measurement and the recording of ultraviolet absorption spectra were computer controlled through an IBM compatible personal computer.

The solutions used for ultraviolet absorption spectra measurements all contained 0.100 mol dm^{-3} sodium perchlorate and 1.00 x 10^{-3} mol dm^{-3} NaPIPES. Solutions used to measure the absorption spectra of the ligands also contained 2.00 x 10^{-5} mol dm^{-3} of ligand. To measure the absorption spectra of the metal complexes formed by the ligands, solutions containing 2.00 x 10^{-5} mol dm^{-3} ligand and 4.00 x 10^{-3} mol dm^{-3} metal perchlorate were used. Baseline measurements were run for all spectra of sample solutions, using reference solutions containing either 0.100 mol dm^{-3} sodium perchlorate and 1.00 x 10^{-3} mol dm^{-3} NaPIPES or these and 4 x 10^{-3} mol dm^{-3} of the metal perchlorate salt, for baselines of the absorption spectra of the ligands alone and those of the ligand in the presence of metal ions respectively. These solutions used for absorption spectra measurements were prepared from stock solutions which contained 0.100 mol dm^{-3} sodium perchlorate; 0.100 mol dm^{-3} sodium perchlorate and 1.00 x 10^{-2} mol dm^{-3} NaPIPES; 0.100 mol dm^{-3} sodium perchlorate and 1.00 x 10^{-4} mol dm^{-3} ligand; 0.100 mol dm^{-3} sodium perchlorate and 1.00 x 10^{-2} mol dm^{-3} metal perchlorate.

All absorption spectra of the solutions were recorded over the wavelength range of 500 - 200 nm, sampling every 2 nm with 3.2 seconds between each measurement. The bandwidth of the spectrometer was set to 0.4 nm and its slit height was set to 12 mm. Data from two individual scans each of the absorption spectrum of every sample solution and its corresponding baseline reference solution were recorded. The

absorption spectrum of a ligand alone or its metal complex was then produced by averaging data collected from the two scans of its sample solution and subtracting its corresponding averaged baseline data. Plots of the emission spectra were then produced on a SUN 3/60 work station using the computer package ACE.

8.2.3 Potentiometric Titrations

Potentiometric titrations were performed using a Metrohm E665 Dosimat autoburette, an Orion SA720 potentiometer and an Orion 8172 Sure flow Ross pH electrode. Titrations were controlled and data collected using an IBM compatible program, AUTOTIT7. Data were collected in millivolt units. All titrations were carried out in a 10 cm^3 titration vessel, which was thermostatted to 298.2 ± 0.1 K, by circulation of water through an outer jacket of the vessel using a Julabo P water pump.

Throughout a titration, a fine stream of nitrogen, which had been humidified with 0.100 mol dm^{-3} sodium perchlorate solution, was bubbled through the titration solution, to remove carbon dioxide from the system. The titration solution was stirred magnetically to aid mixing.

The titrant used for all titrations was 0.100 ± 0.001 mol dm^{-3} sodium hydroxide. This was housed in a dark glass vessel and protected from carbon dioxide by a drying tube containing soda lime. The titrant was standardised weekly by titration of a 5.00 x 10^{-3} mol dm^{-3} hydrochloric acid solution. At least three of these titrations were performed and the concentration of sodium hydroxide was taken as the average of these. The error in this value was calculated as the greatest deviation of titration values obtained from the mean value.

The reference electrode was filled with 0.100 mol dm^{-3} sodium perchlorate. This solution was changed weekly and the electrode was allowed to stand for at least 12 hours

before use, each time fresh solution was added. The electrode's response was calibrated after each solution change and every second day thereafter. Calibration was carried out by titrating a solution containing $(4.91 \pm 0.02) \times 10^{-3}$ mol dm^{-3} perchloric acid and 0.100 mol dm^{-3} sodium perchlorate. From the data collected values for E_0 and pK_W were determined.

Acid dissociation constants for the acidic protons on each ligand were determined by titration of an acidic solution containing the fully protonated ligand with standardised 0.100 mol dm^{-3} sodium hydroxide. The ligand solution was composed of 1.00×10^{-3} mol dm^{-3} ligand, 4.91×10^{-3} mol dm^{-3} perchloric acid and 0.100 mol dm^{-3} sodium perchlorate. At least three such titrations were carried out. The acid dissociation constants were determined from corresponding values calculated from the data of at least three separate runs.

The titrations for the determination of the stability constants for complexes formed between these ligands and the metals tested, were carried out in a similar manner to those for determining acid dissociation constants. However, varying amounts of metal ion solution was added to the ligand solution prior to the commencement of a titration. Ligand to metal ion ratios of 1.1 : 1, 2.1 : 1 and 3.1 : 1 were selected for the titrations. This required addition of 0.090, 0.047, 0.032 cm^3 of the 0.100 mol dm^{-3} metal perchlorate solution to 10 cm^{-3} of the ligand titration solution respectively. At least one stability constant titration of each ligand to metal ion ratio was run for each particular metal ion. The stability constants were determined using corresponding values calculated from at least three separate runs, of which at least two of the varied metal ion concentrations were represented.

Data, recorded in millivolts, were later converted to pH units using equation 8.1, derived from the Nernst Equation, for the purpose of plotting titration profiles. Plots of titration profiles were produced using Cricket Graph. Derivations of acid dissociation

and stability constant values from experimental data, were carried out on an Apple Macintosh Classic II, using the program MiniQuad,[11] which fits data by minimising the square sum of the differences between the measured and calculated data of all components in solution. The fit of a single run was considered to be statistically acceptable if all residuals were within two standard deviations of the mean and they approximated a Gaussian distribution.[11]

$$pH = \frac{E_o - E}{59.15} \tag{8.1}$$

The values of the stability constants, K, and their corresponding errors, σ, were calculated using methods as described by Bevington.[12] The value of a directly measured stability constant was determined as the weighted mean, K_1, of statistically significant best fit stability constants, K_{1i}, for i=1...N of N separate runs and their corresponding errors, σ_{1i}, (eq.8.2).

$$K_1 = \frac{\Sigma(K_{1i} / \sigma_{1i}^2)}{\Sigma(1 / \sigma_{1i}^2)} \tag{8.2}$$

Its associated error, σ_1, was derived from the uncertainties, σ_{1i}, (eq. 8.3).[12]

$$\sigma_1 = \sqrt{\frac{1}{\Sigma(1 / \sigma_{1i}^2)}} \tag{8.3}$$

The values of derived stability constants, K_2, were calculated using measured best fit overall stability constants, K_{1i} and β_{2i}, for i = 1...N of N statistically significant separate runs (eq 8.4).

$$K_2 = \frac{\Sigma(\beta_{2i} / K_{1i})}{N} \tag{8.4}$$

Its associated error, σ_2, was determined by the propagation of errors, $\sigma\beta_{2i}$ and σ_{1i}, associated with β_{2i} and K_{1i} respectively (eq 8.5).[12]

$$\sigma_2 = \frac{\Sigma\sqrt{(\beta_{2i} / K_{1i})^2 ((\sigma_{\beta 2i} / \beta_{2i})^2 + (\sigma_{1i} / K_{1i})^2)}}{N} \qquad (8.5)$$

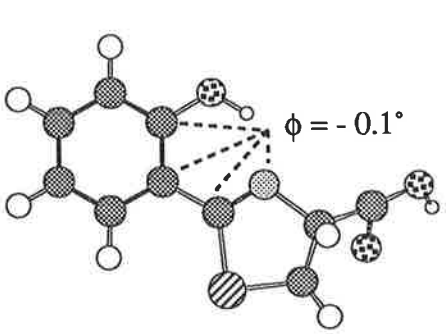
Similarly, acid dissociation constant values and their associated errors were determined by substituting the appropriate measured values into equations (8.2) and (8.3) for directly measured acid dissociation constants or into equations (8.4) and (8.5) for derived acid dissociation constants. Speciation diagrams were produced using Igor® wavemetrics package.

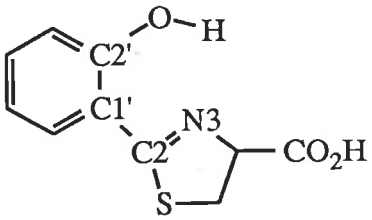
8.2.4 Molecular Modelling

Molecular model representations of the thiazole derivatives, PTA and PTEE, and the oxazole, POEE, were produced using the Macintosh compatible modelling package, PC MODEL 4.41 with MMX forcefield.[13] The torsional angle between the atoms N3, C2, C1' and C2' was adjusted and fixed as close to zero as the program would allow (see Figure 8.1). The value of this torsional angle was -0.1° for the thiazoline acid, PTA, 0.56° for the thiazole ethyl ester, PTEE, and 0.32° for the oxazole, POEE. The distances between the ring nitrogen and the phenolic oxygen or hydrogen of these compounds were then calculated with their two rings being restricted in a planar arrangement.

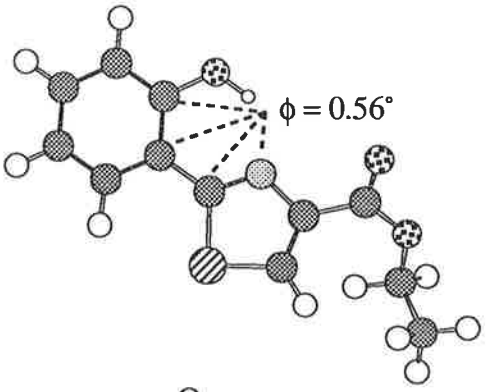
8.2.5 Fluorescent Emission Spectra Measurements

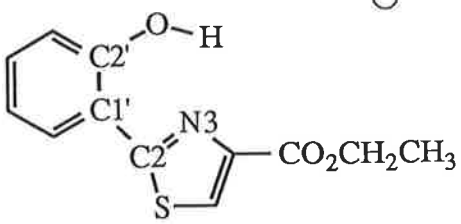
Fluorescence emission spectra of the free ligands, (PTA, PTME, PTEE, APTE and POEE), and their various metal complexes were measured using a Perkin Elmer Luminescence Spectrometer LS50B. All sample solutions were thermostatted to 298.2 ±



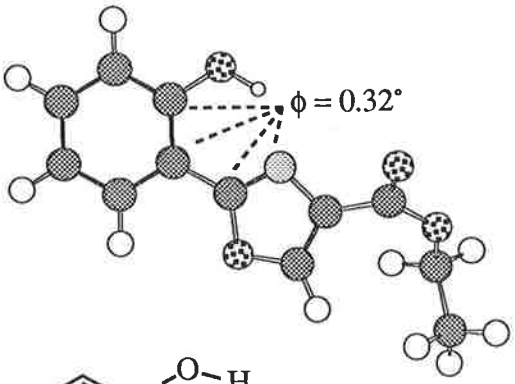


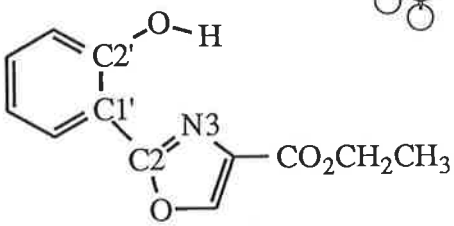
PTA





PTEE





POEE

Figure 8.1 Molecular model representation of the thiazoline acid, (PTA), the thiazole ethyl ester, (PTEE), and the oxazole, (POEE) showing the torsional angle between each of their two rings.

0.2 K, by circulation of temperature controlled water through the cell compartment of the spectrometer, using a Grant Technical Specification Thermostatic Bath/Circulator Type ZD. Excitation of solutions and the recording of emission spectra were carried out using the computer application, Fluorescence Data Manager, FLDM, an LS50B instrument control program by Perkin Elmer, and a personal computer, which was directly connected to the spectrometer.

All solutions used for fluorescence measurements contained 0.100 mol dm^{-3} sodium perchlorate and 1.00 x 10^{-3} mol dm^{-3} NaPIPES. For emission spectra of the free ligands, solutions also containing 5.00 x 10^{-6} mol dm^{-3} of the respective ligand were used. To measure the emission spectra of the metal complexes of the ligands, solutions which contained 5.00 x 10^{-6} mol dm^{-3} ligand and 2.00 x 10^{-3} mol dm^{-3} metal perchlorate were used. Baseline measurements were run for all spectra of sample solutions, using reference solutions containing either 0.100 mol dm^{-3} sodium perchlorate and 1.00 x 10^{-3} mol dm^{-3} NaPIPES or these and 2.00 x 10^{-3} mol dm^{-3} of the metal perchlorate salt, for baselines of the ligands' emission spectra and those of the metal complexes emission spectra respectively. The solutions used for fluorescence measurements were prepared from stock solutions which contained 0.100 mol dm^{-3} sodium perchlorate; 0.100 mol dm^{-3} sodium perchlorate and 1.00 x 10^{-2} mol dm^{-3} NaPIPES; 0.100 mol dm^{-3} sodium perchlorate and 1.00 x 10^{-4} mol dm^{-3} ligand; 0.100 mol dm^{-3} sodium perchlorate and 1.00 x 10^{-2} mol dm^{-3} metal perchlorate.

All emission spectra of the solutions were recorded over the wavelength range of 400 - 500 nm, measuring emission at every 0.5 nm. The excitation and emission slit widths of the spectrometer were set to 7.5 mm. Emission spectra data were recorded as the average of two scans, each scan run at a speed of 240 seconds. Averaged baseline emission spectral data were subtracted from the corresponding averaged sample emission spectral data. Plots of the emission spectra were then produced on a SUN 3/60 work station using the computer package ACE.

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(13) MMX Forcefield is derived from the MM2(QCPE-395,1977) forcefield of Allinger, N. L., with the pi-VESCF routines taken from MMP1(QCPE-318), also by Allinger, N. L.

Appendix A Ultraviolet Absorption Spectra

This appendix contains the ultraviolet absorption spectra of the Zn^{2+} chelating ligands, PTA, PTME, PTEE, POEE and APTE, and their metal ion complexes. Each spectral plot displays a comparison of the ultraviolet absorption spectra of the ligand when measured in (a) the presence and (b) the absence of one of the metal ions from the series selected. Only the spectra of the ligands in the presence of coordinating metal ions, as shown by a shift in the absorption maxima of the ligand, have been included in this appendix. The spectra of the ligands when in the presence of non-coordinating metal ions have been omitted as they overlay the spectra of the free ligand itself. The solutions used to record the spectra of the ligands in the absence of metal ions contained 2.00 x 10^{-5} mol dm^{-3} ligand, 1.00 x 10^{-3} mol dm^{-3} NaPIPES and 0.100 mol dm^{-3} $NaClO_4$. The solutions used to record the spectra of the ligands in the presence of metal ions contained 2.00 x 10^{-5} mol dm^{-3} ligand, 4.00 x 10^{-3} mol dm^{-3} metal perchlorate, 1.00 x 10^{-3} mol dm^{-3} NaPIPES and 0.100 mol dm^{-3} $NaClO_4$. All solutions were made up in a solvent system comprising of 75% CH_3OH and 25% H_2O by volume and all spectra were recorded at 298.2 K.

A.1 PTA and Complexes

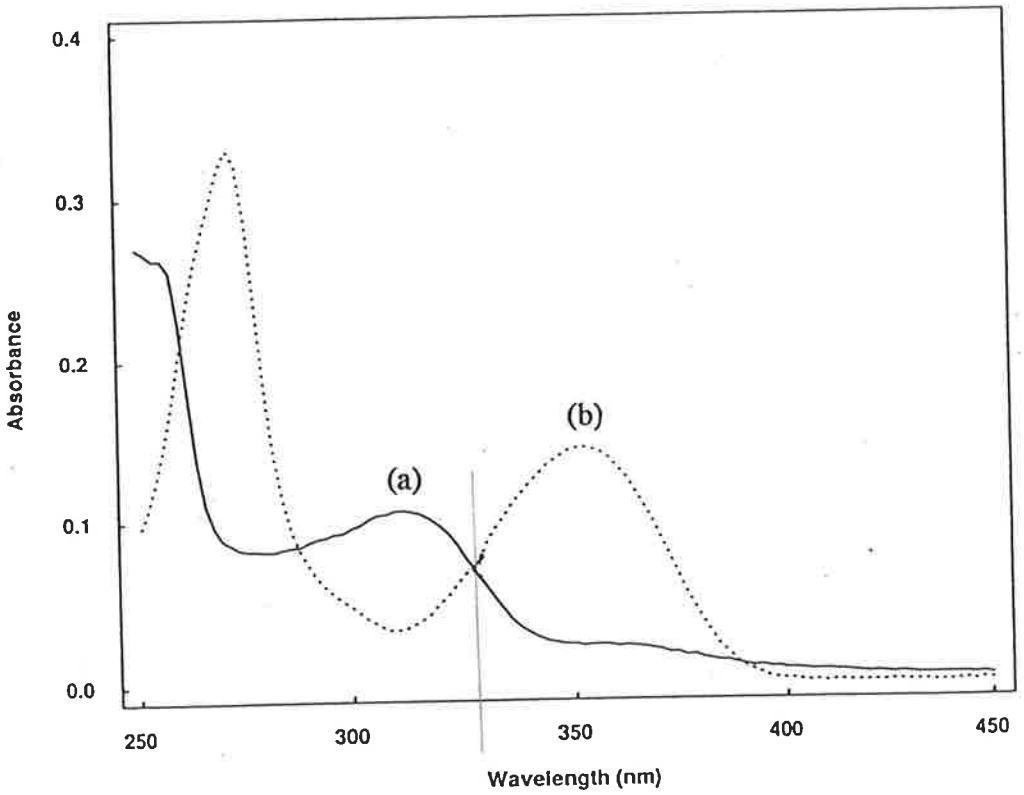


Figure A.1.1 Comparison of the ultraviolet absorption spectra of PTA measured in (a) the absence and (b) the presence of Al^{3+}.

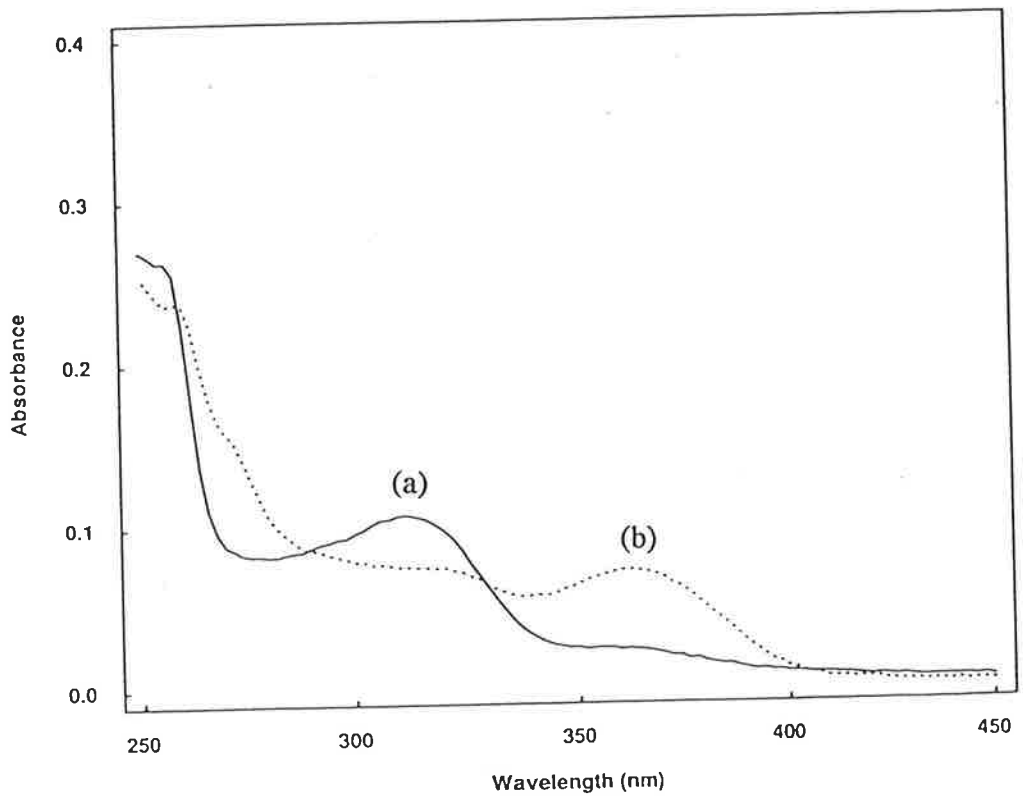


Figure A.1.2 Comparison of the ultraviolet absorption spectra of PTA measured in (a) the absence and (b) the presence of Ca^{2+}.

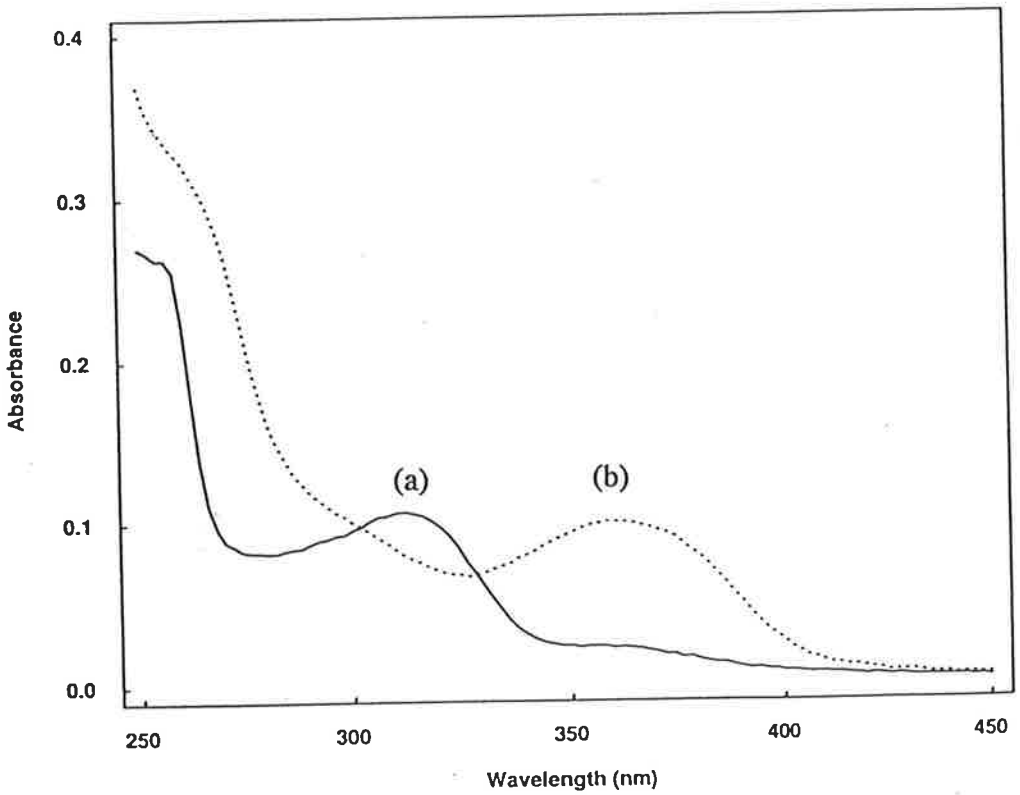


Figure A.1.3 Comparison of the ultraviolet absorption spectra of PTA measured in (a) the absence and (b) the presence of Co^{2+}.

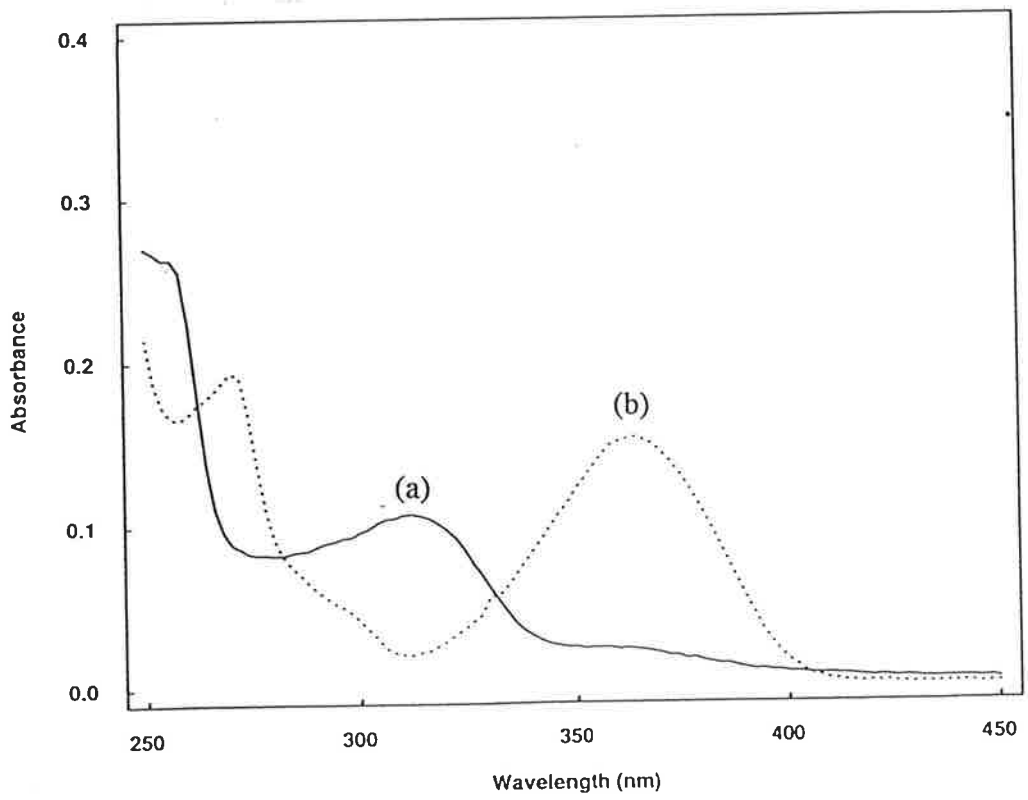


Figure A.1.4 Comparison of the ultraviolet absorption spectra of PTA measured in (a) the absence and (b) the presence of Ni^{2+}.

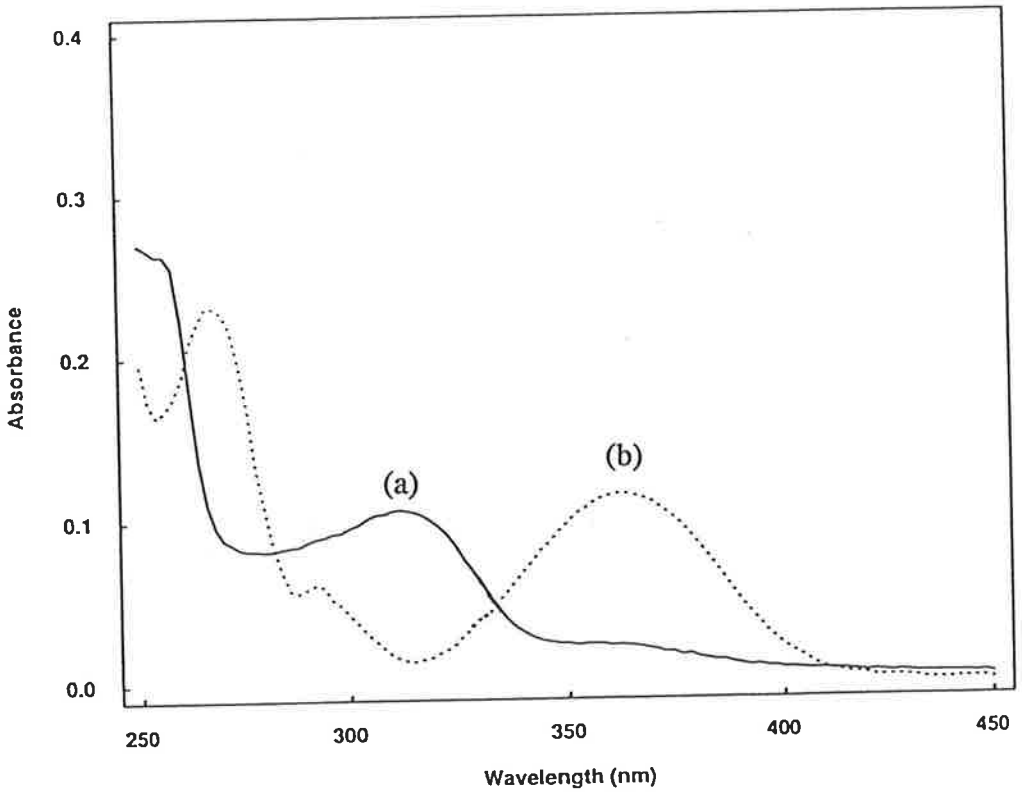


Figure A.1.5 Comparison of the ultraviolet absorption spectra of PTA measured in (a) the absence and (b) the presence of Cu^{2+}.

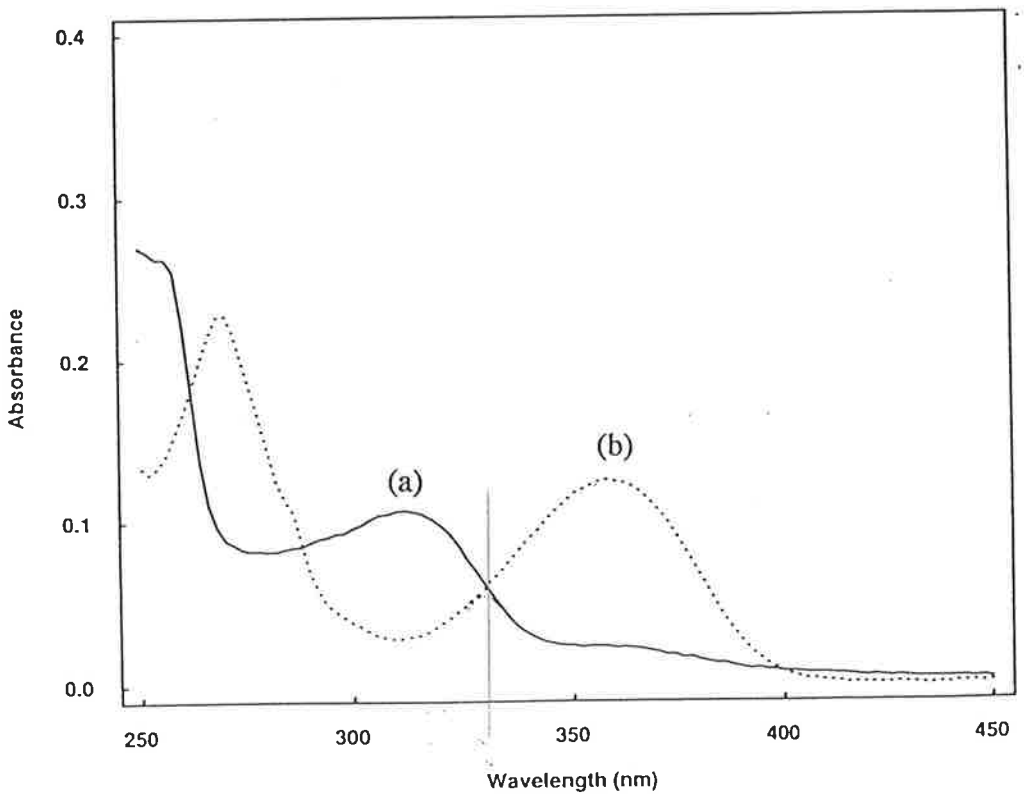


Figure A.1.6 Comparison of the ultraviolet absorption spectra of PTA measured in (a) the absence and (b) the presence of Zn^{2+}.

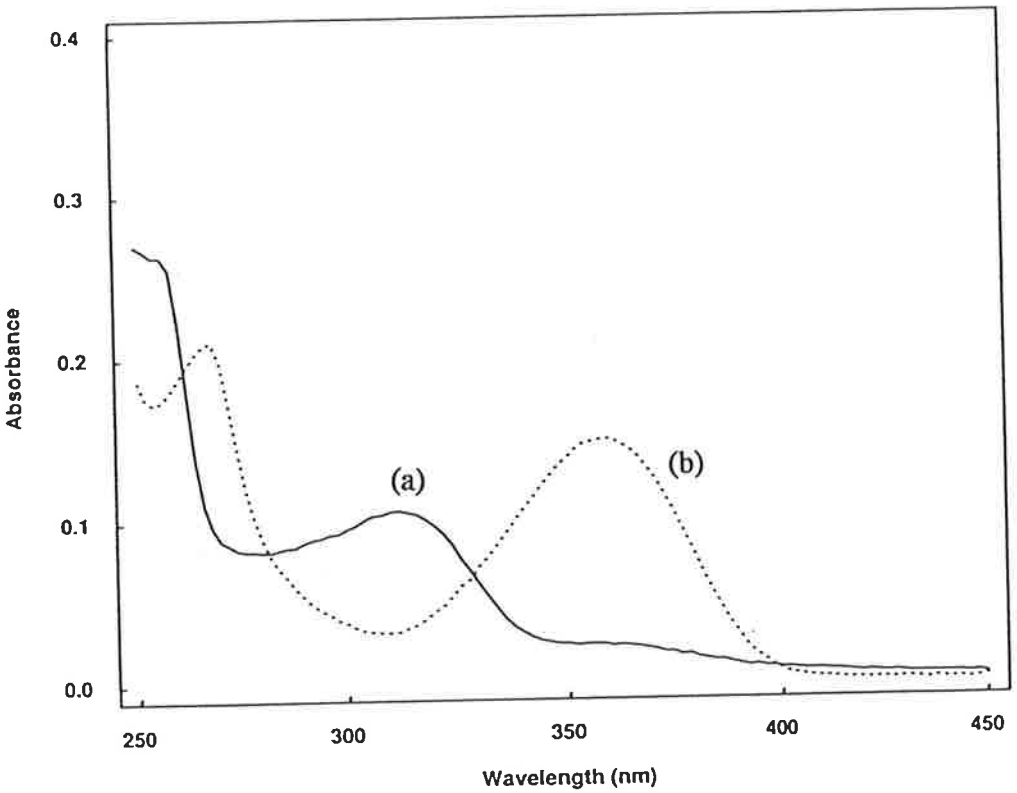


Figure A.1.7 Comparison of the ultraviolet absorption spectra of PTA measured in (a) the absence and (b) the presence of Cd^{2+}.

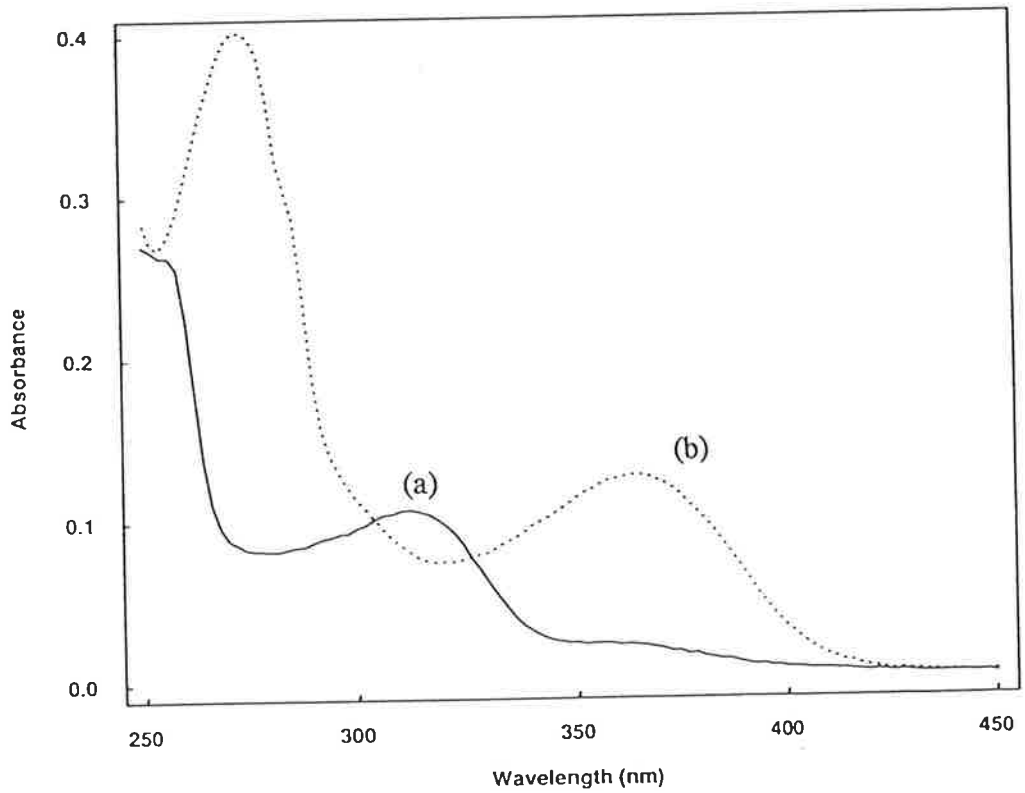


Figure A.1.8 Comparison of the ultraviolet absorption spectra of PTA measured in (a) the absence and (b) the presence of Pb^{2+}.

A.2 PTME and Complexes

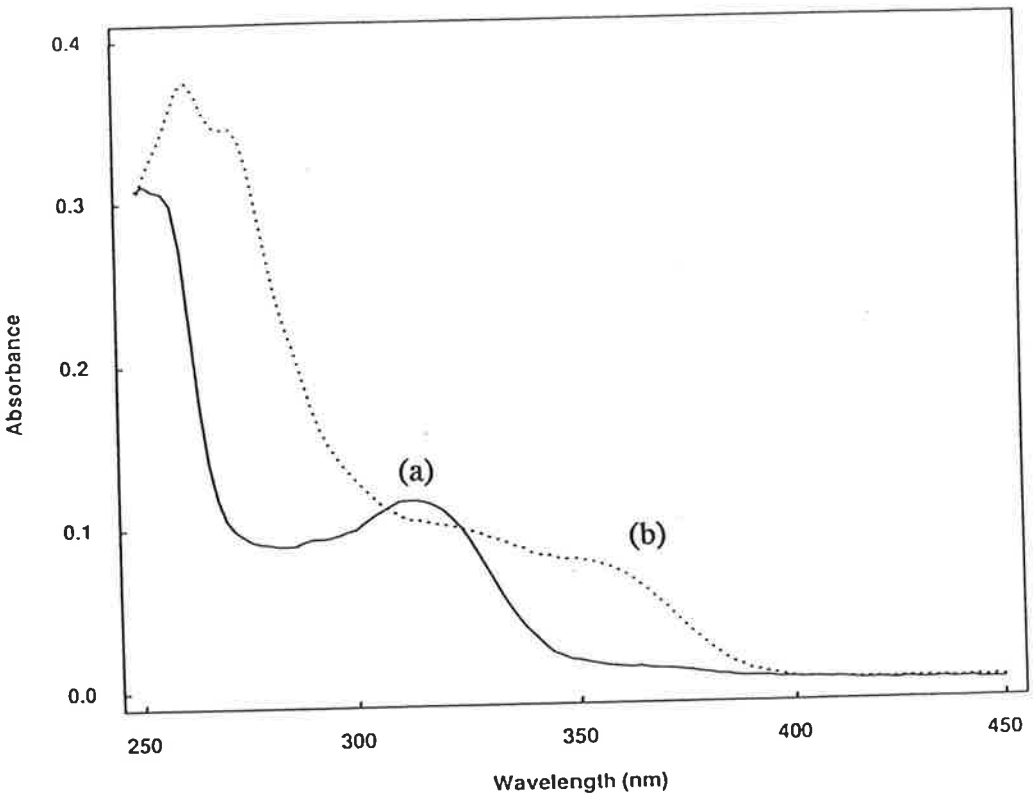


Figure A.2.1 Comparison of the ultraviolet absorption spectra of PTME measured in (a) the absence and (b) the presence of Al^{3+}.

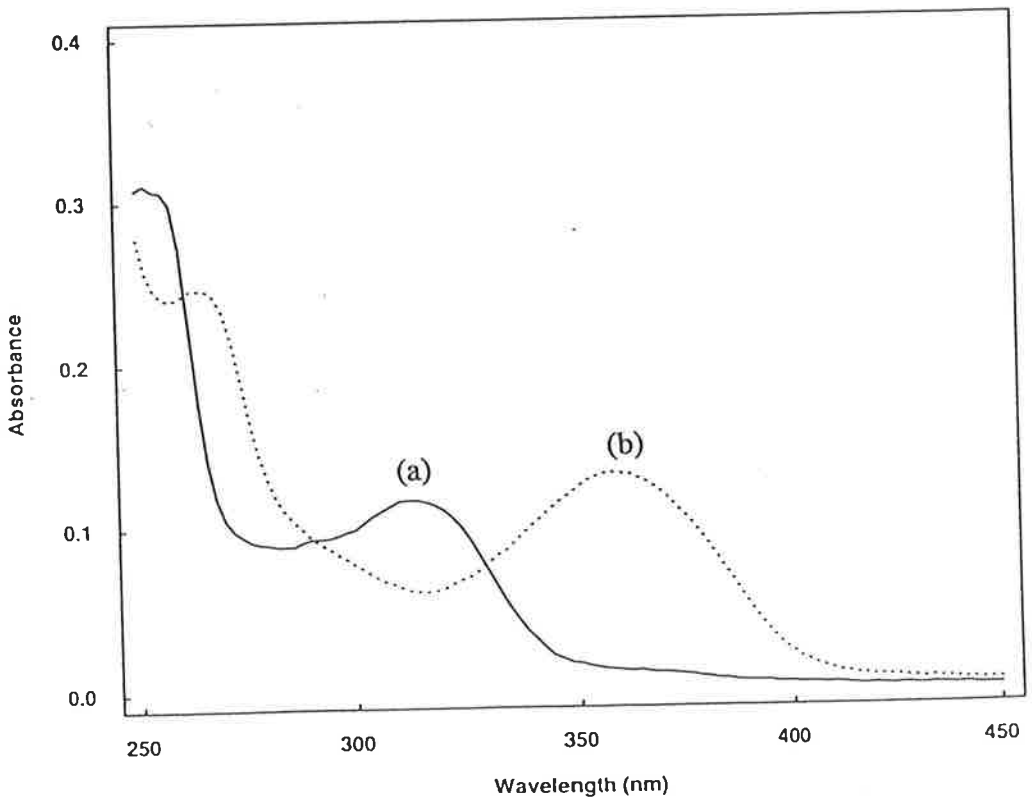


Figure A.2.2 Comparison of the ultraviolet absorption spectra of PTME measured in (a) the absence and (b) the presence of Co^{2+}.

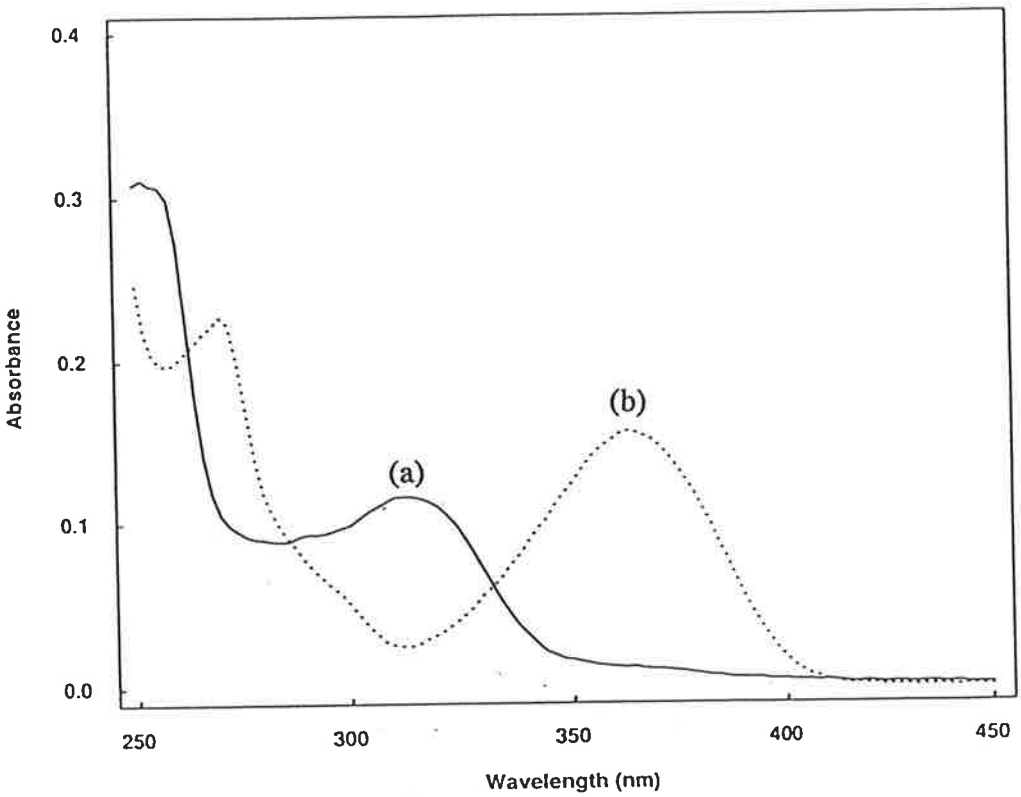


Figure A.2.3 Comparison of the ultraviolet absorption spectra of PTME measured in (a) the absence and (b) the presence of Ni^{2+}.

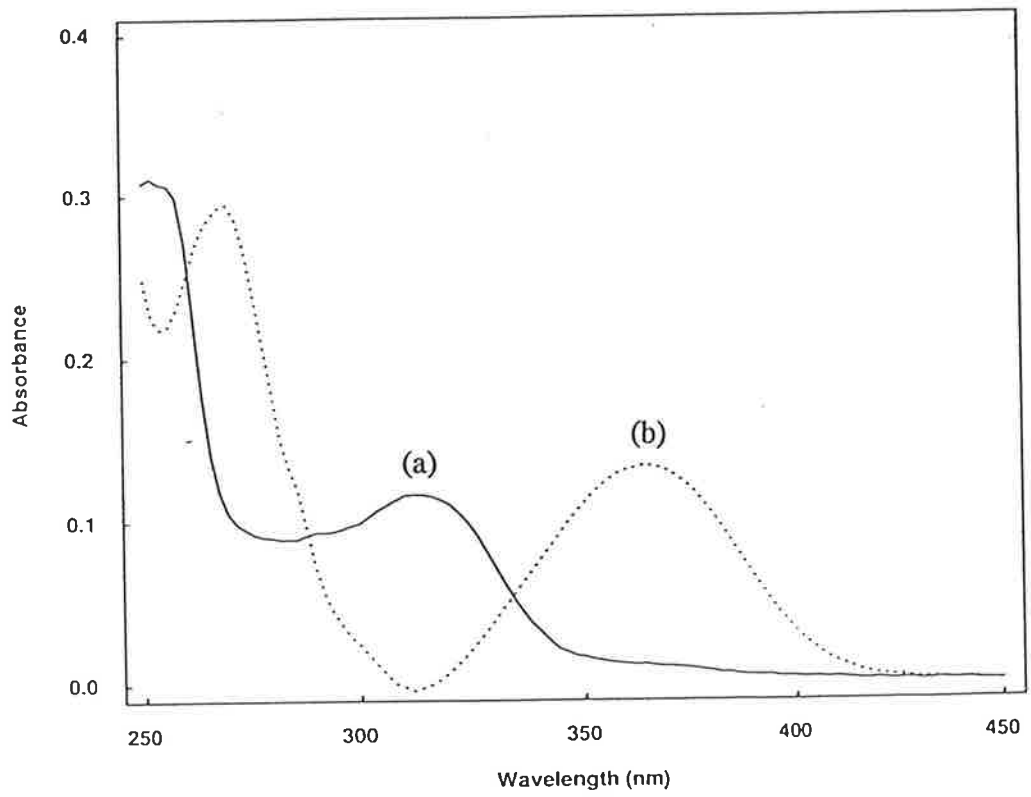


Figure A.2.4 Comparison of the ultraviolet absorption spectra of PTME measured in (a) the absence and (b) the presence of Cu^{2+}.

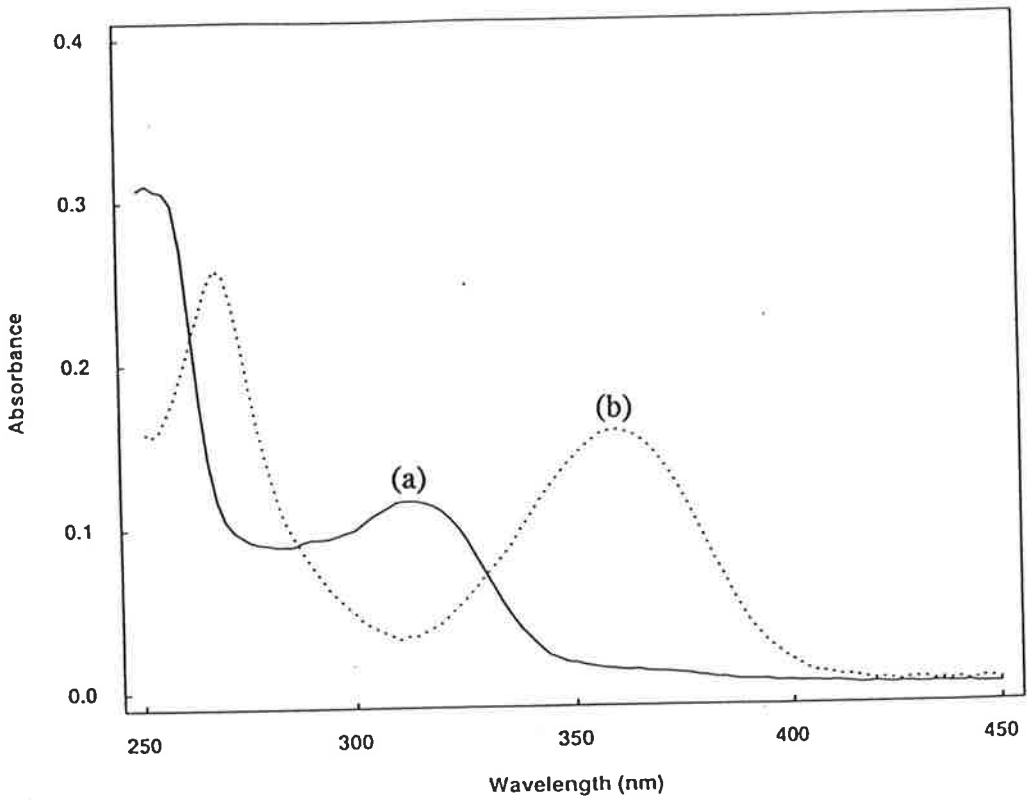


Figure A.2.5 Comparison of the ultraviolet absorption spectra of PTME measured in (a) the absence and (b) the presence of Zn^{2+}.

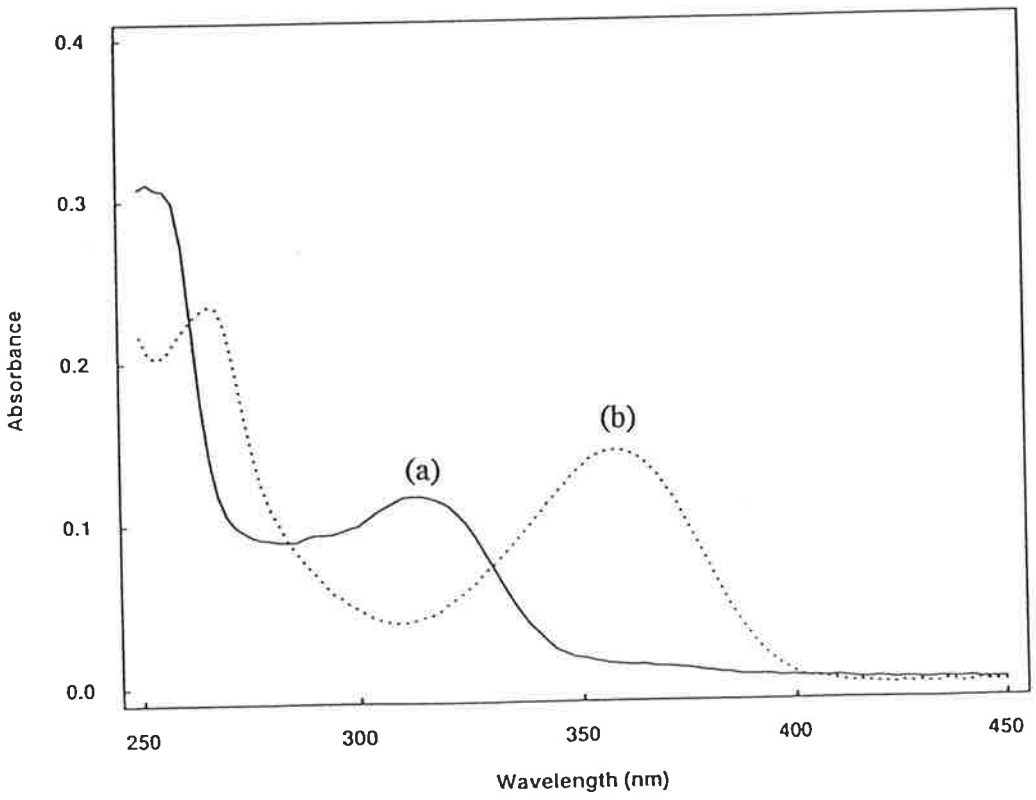


Figure A.2.6 Comparison of the ultraviolet absorption spectra of PTME measured in (a) the absence and (b) the presence of Cd^{2+}.

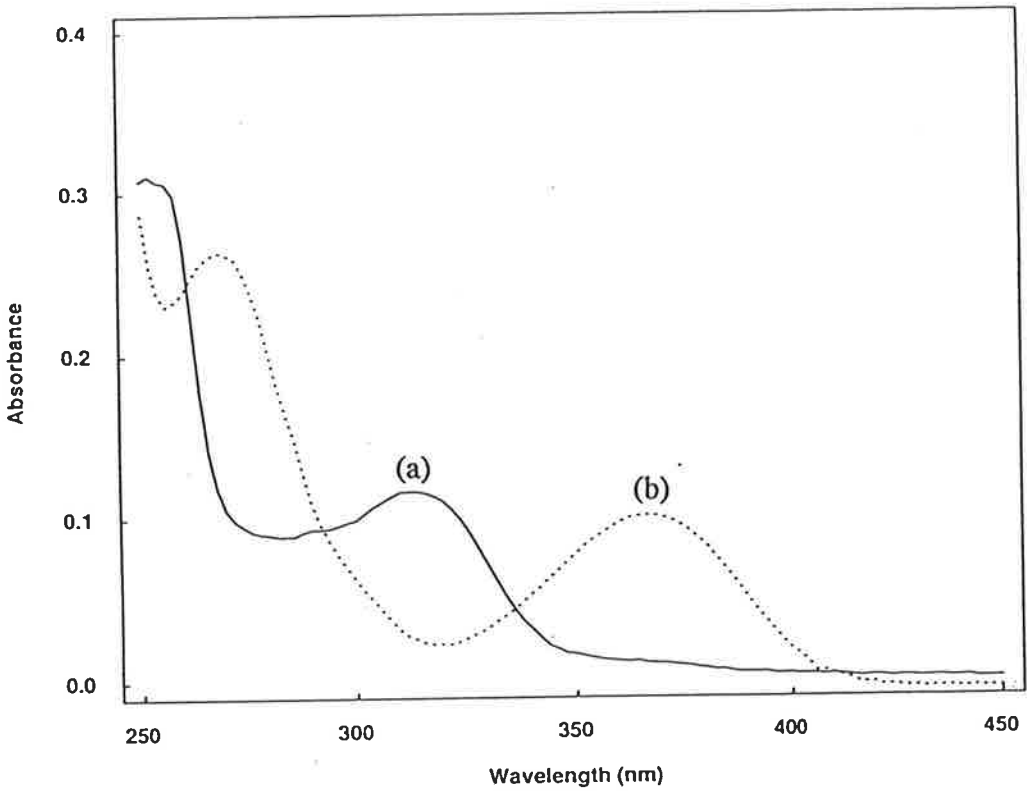


Figure A.2.7 Comparison of the ultraviolet absorption spectra of PTME measured in (a) the absence and (b) the presence of Pb^{2+}.

A.3 PTEE and Complexes

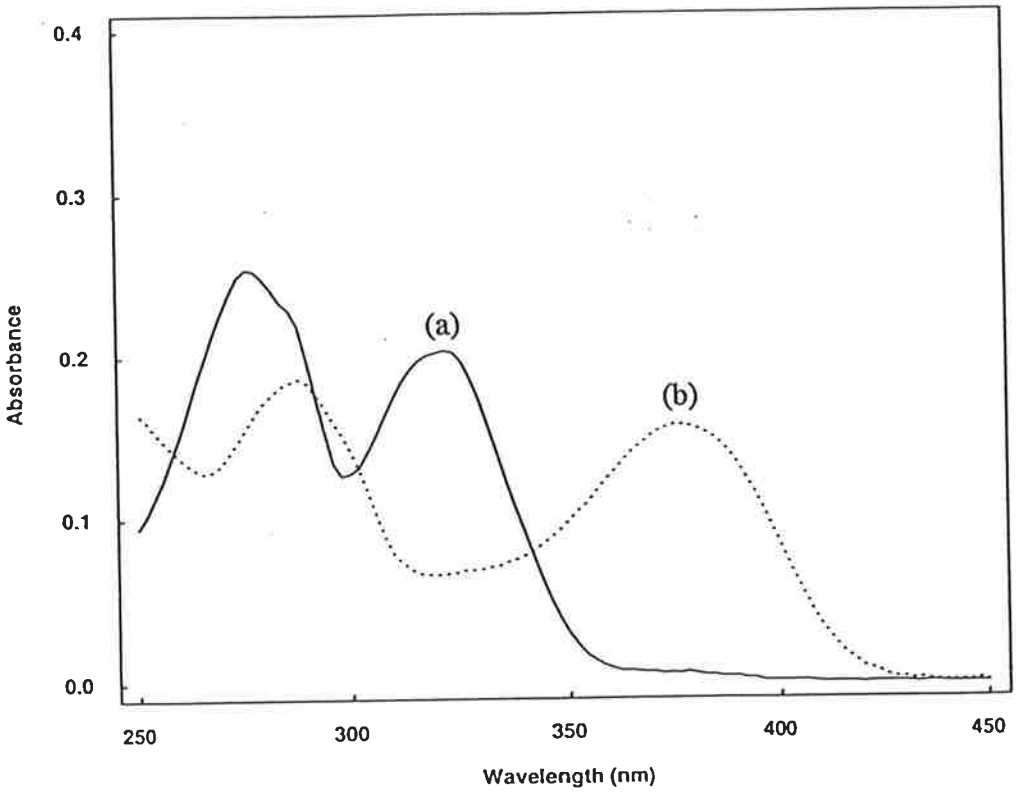


Figure A.3.1 Comparison of the ultraviolet absorption spectra of PTEE measured in (a) the absence and (b) the presence of Co^{2+}.

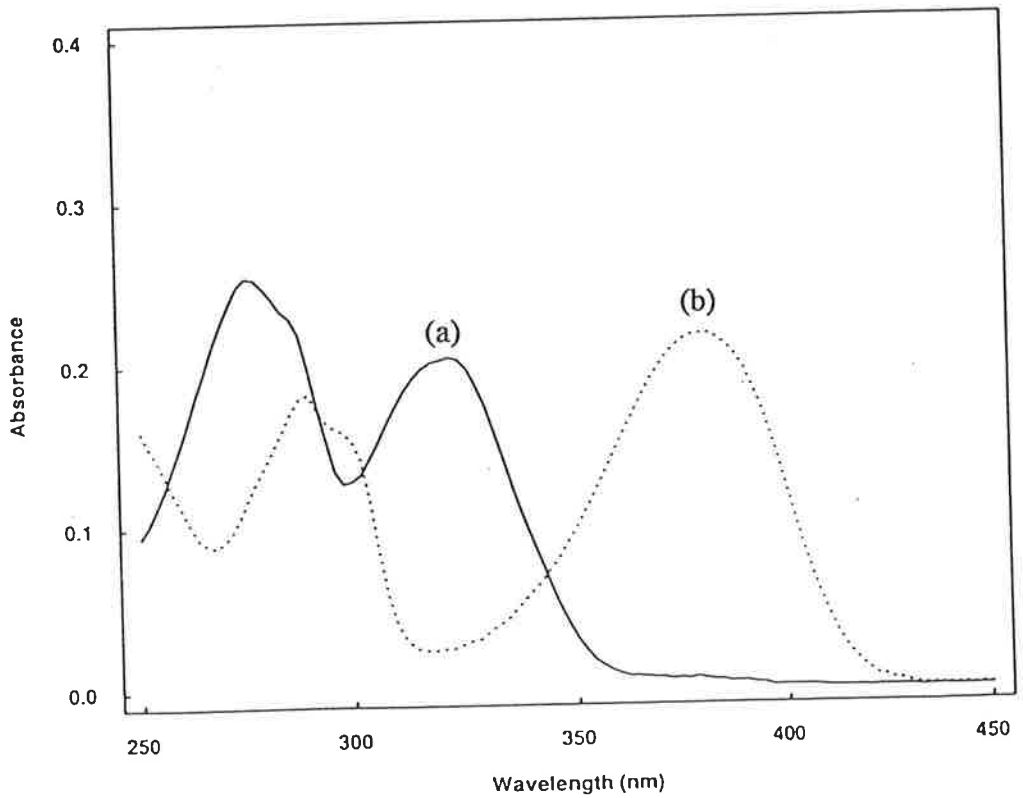


Figure A.3.2 Comparison of the ultraviolet absorption spectra of PTEE measured in (a) the absence and (b) the presence of Ni^{2+}.

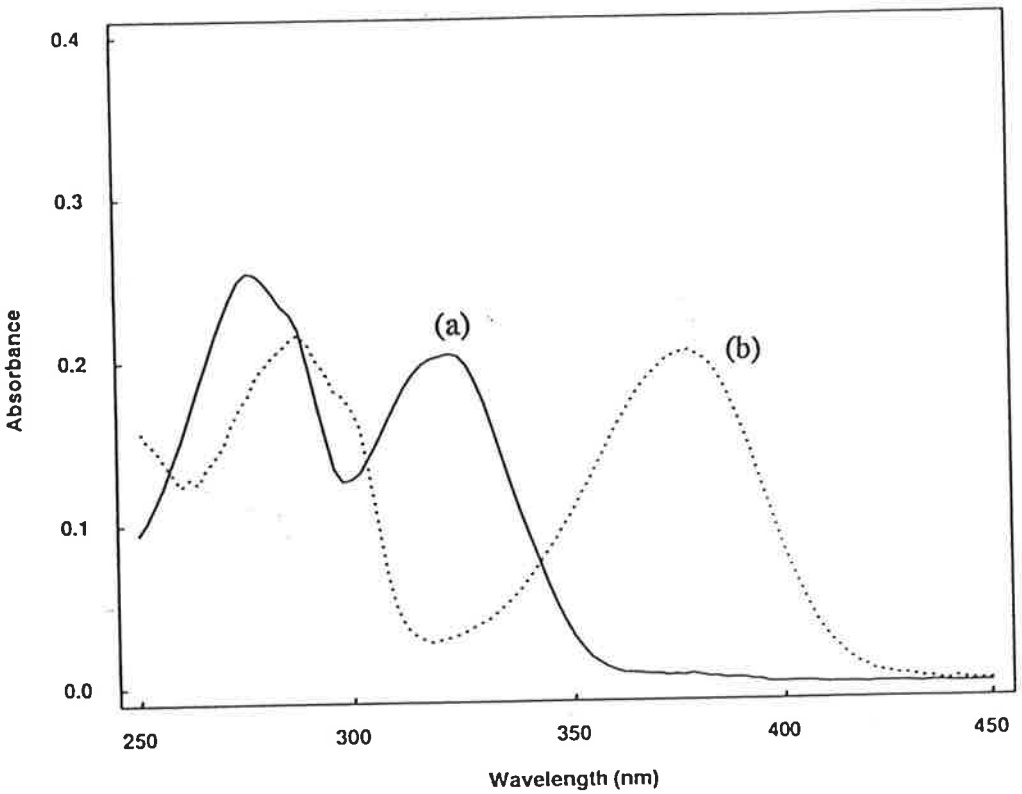


Figure A.3.3 Comparison of the ultraviolet absorption spectra of PTEE measured in (a) the absence and (b) the presence of Cu^{2+}.

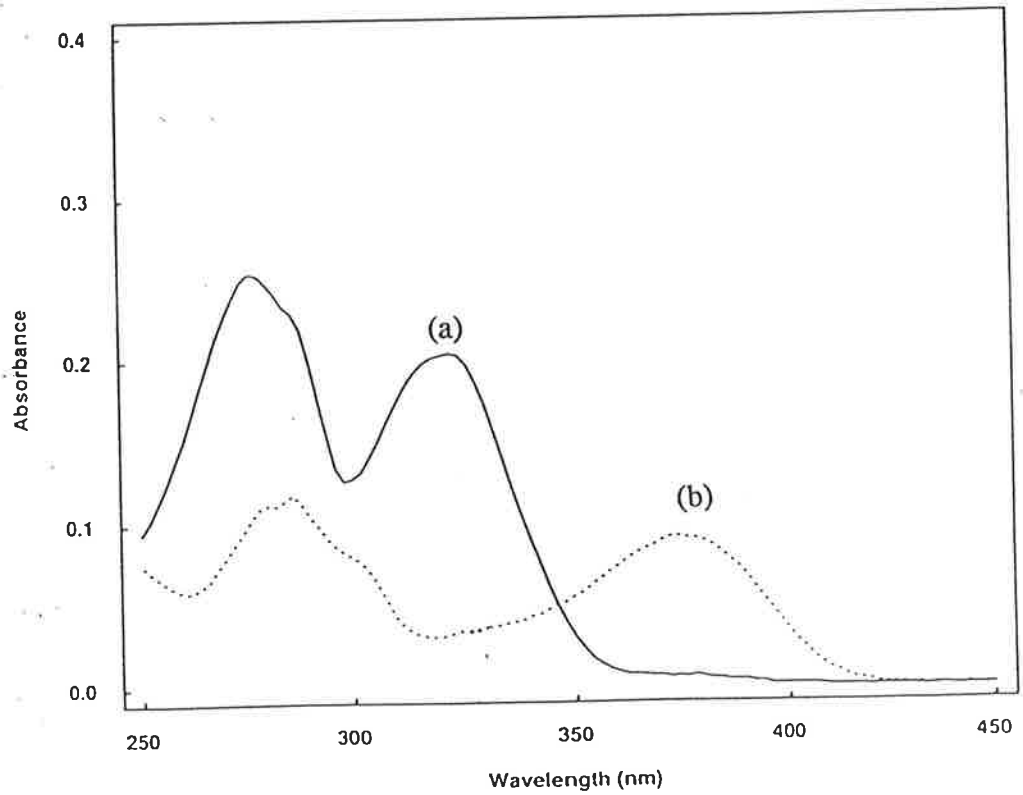


Figure A.3.4 Comparison of the ultraviolet absorption spectra of PTEE measured in (a) the absence and (b) the presence of Zn^{2+}.

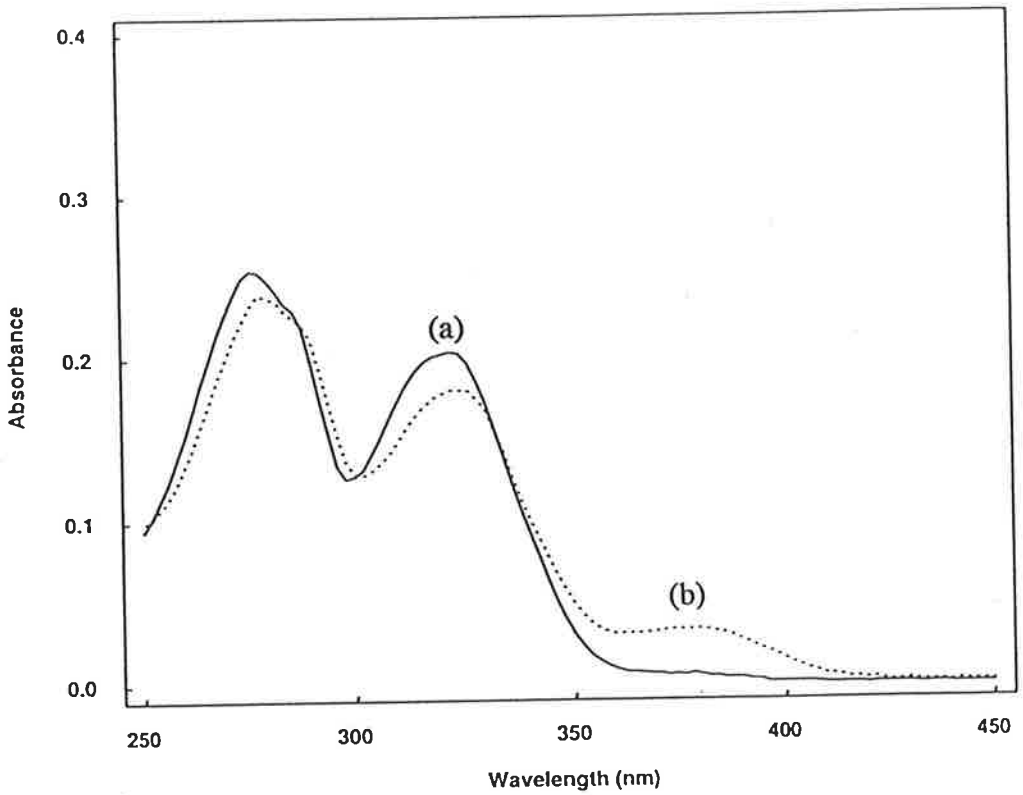


Figure A.3.5 Comparison of the ultraviolet absorption spectra of PTEE measured in (a) the absence and (b) the presence of Cd^{2+}.

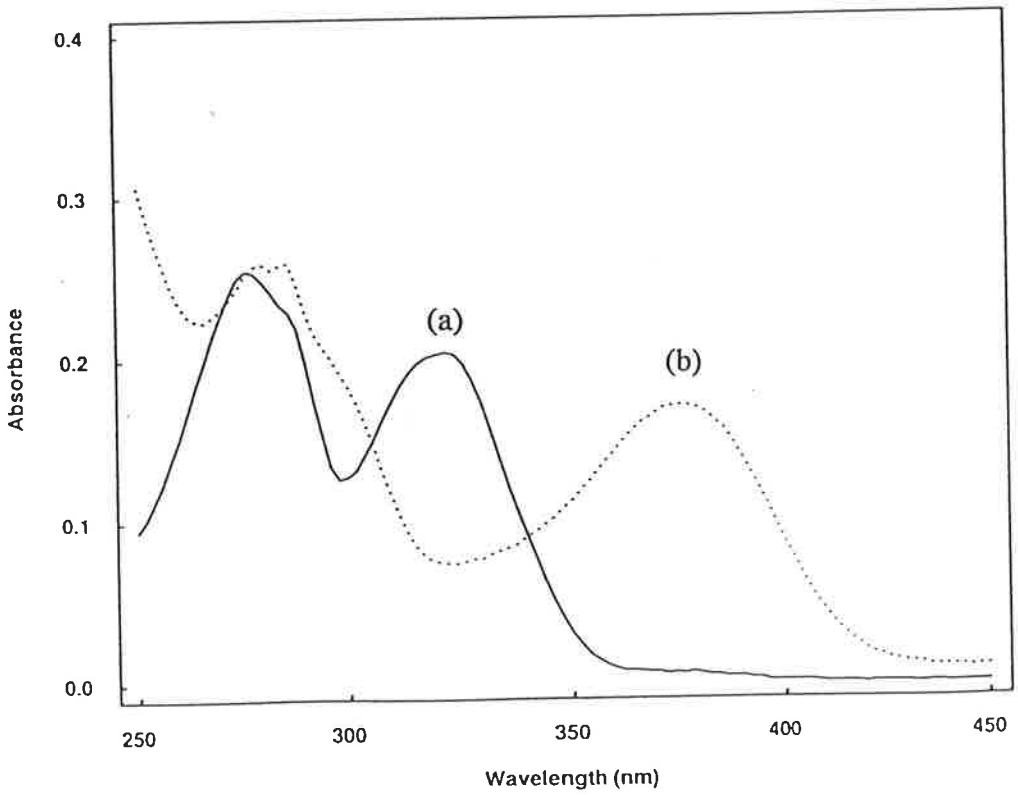


Figure A.3.6 Comparison of the ultraviolet absorption spectra of PTEE measured in (a) the absence and (b) the presence of Pb^{2+}.

A.4 POEE and Complexes

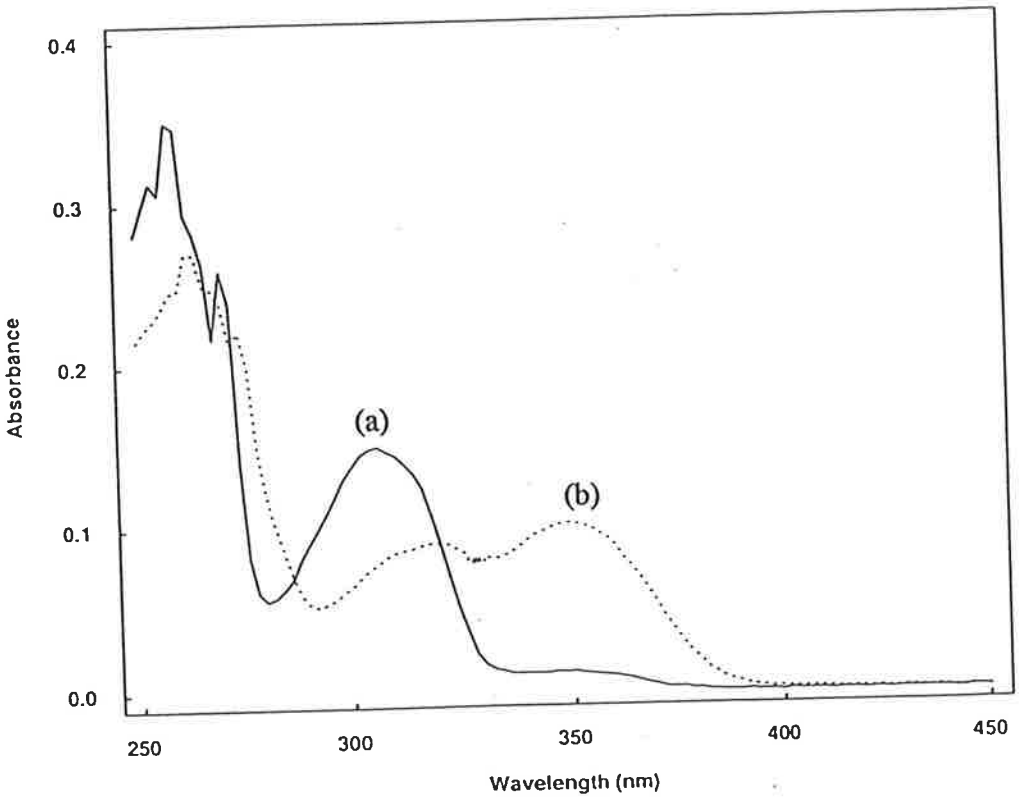


Figure A.4.1 Comparison of the ultraviolet absorption spectra of POEE measured in (a) the absence and (b) the presence of Co^{2+}.

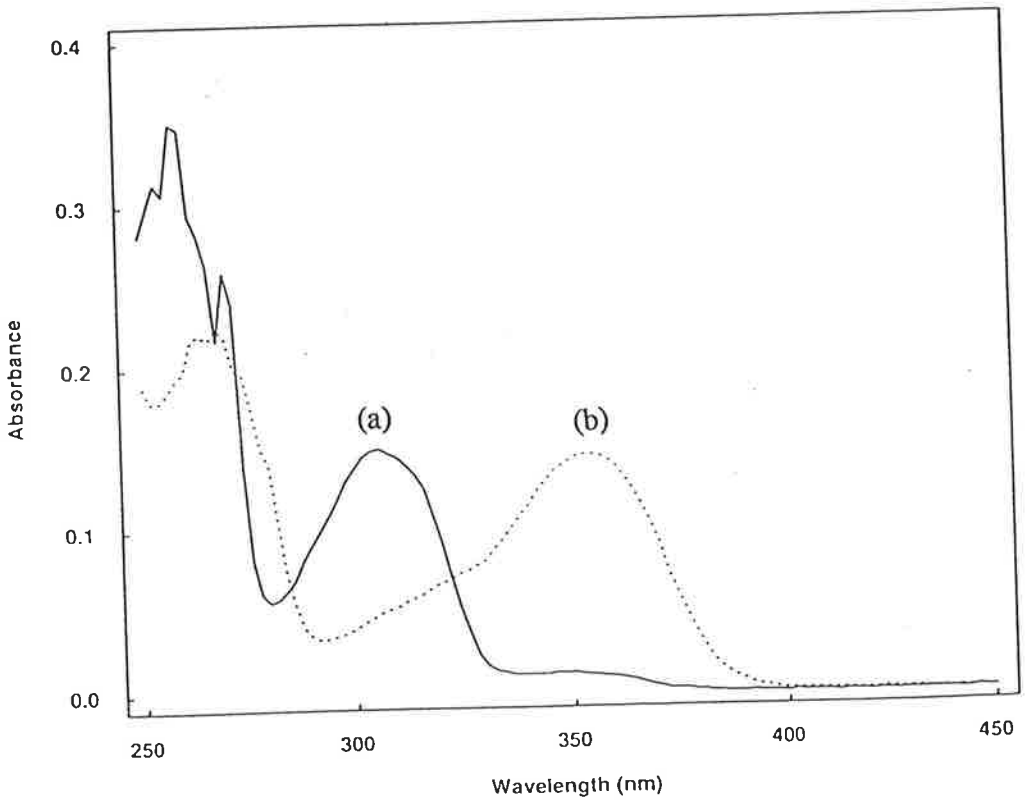


Figure A.4.2 Comparison of the ultraviolet absorption spectra of POEE measured in (a) the absence and (b) the presence of Ni^{2+}.

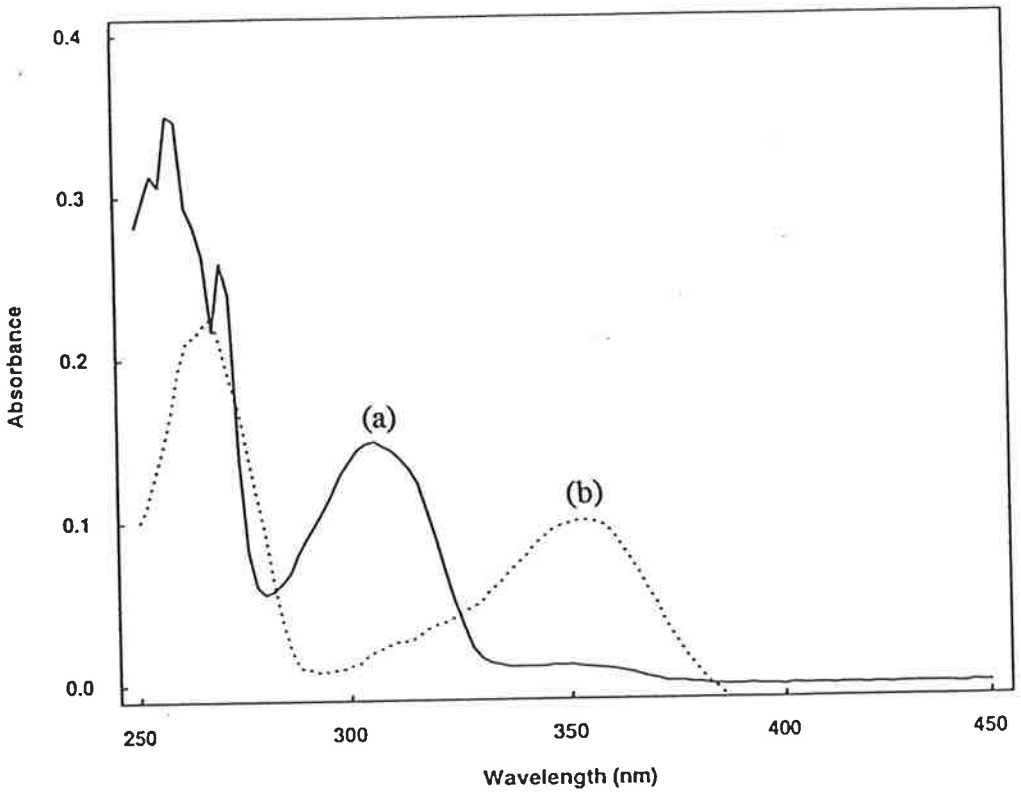


Figure A.4.3 Comparison of the ultraviolet absorption spectra of POEE measured in (a) the absence and (b) the presence of Cu^{2+}.

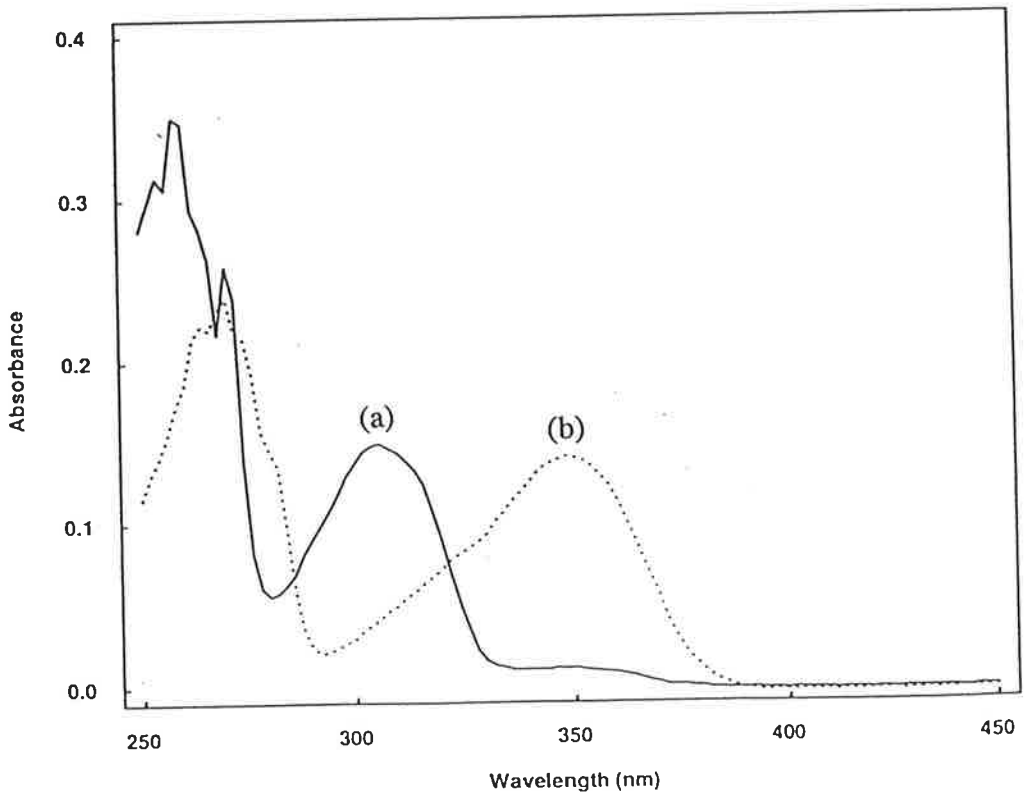


Figure A.4.4 Comparison of the ultraviolet absorption spectra of POEE measured in (a) the absence and (b) the presence of Zn^{2+}.

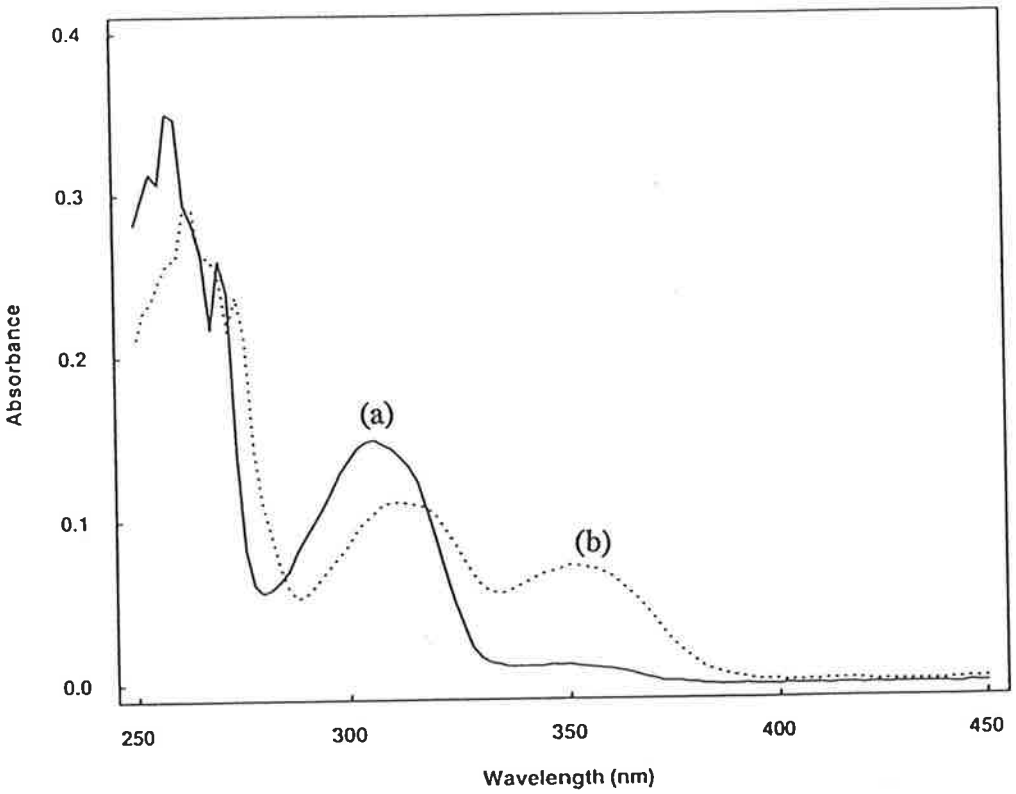


Figure A.4.5 Comparison of the ultraviolet absorption spectra of POEE measured in (a) the absence and (b) the presence of Cd^{2+}.

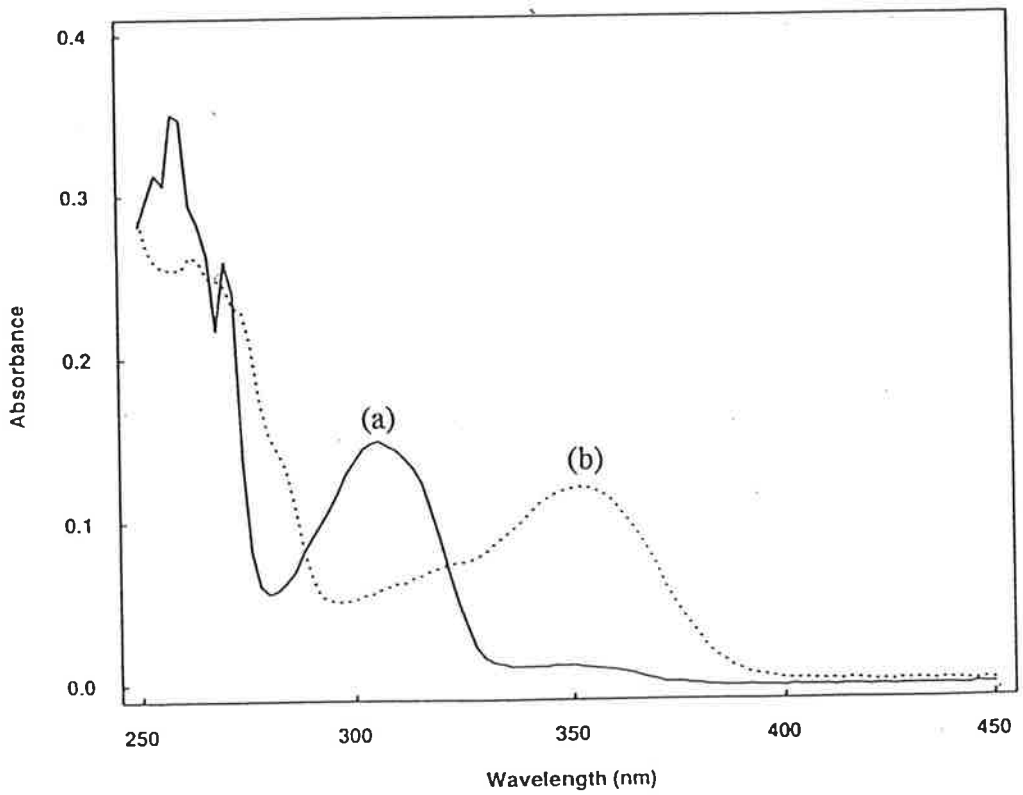


Figure A.4.6 Comparison of the ultraviolet absorption spectra of POEE measured in (a) the absence and (b) the presence of Pb^{2+}.

A.5 APTE and Complexes

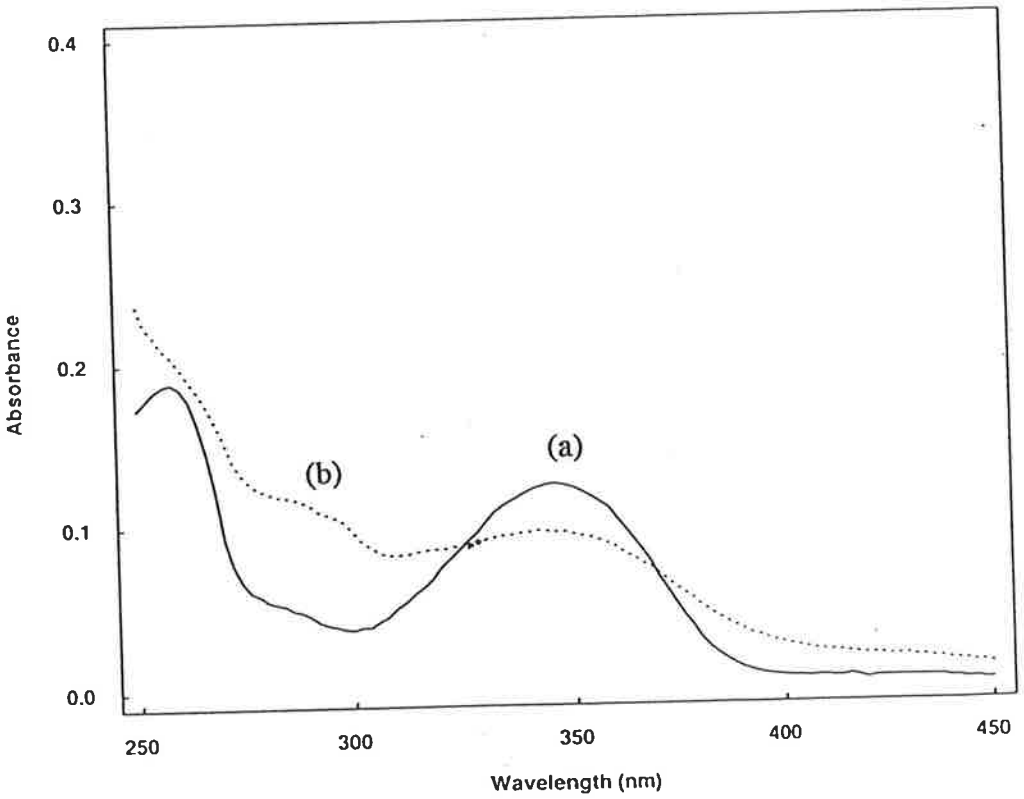


Figure A.5.1 Comparison of the ultraviolet absorption spectra of APTE measured in (a) the absence and (b) the presence of Co^{2+}.

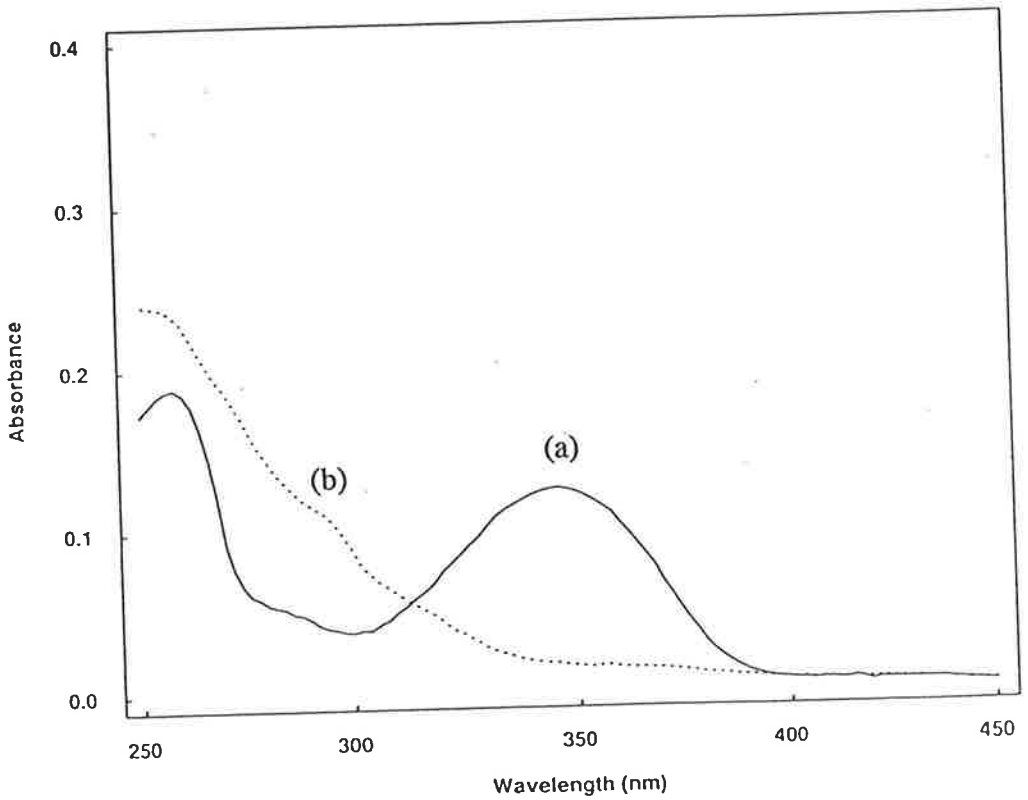


Figure A.5.2 Comparison of the ultraviolet absorption spectra of APTE measured in (a) the absence and (b) the presence of Ni^{2+}.

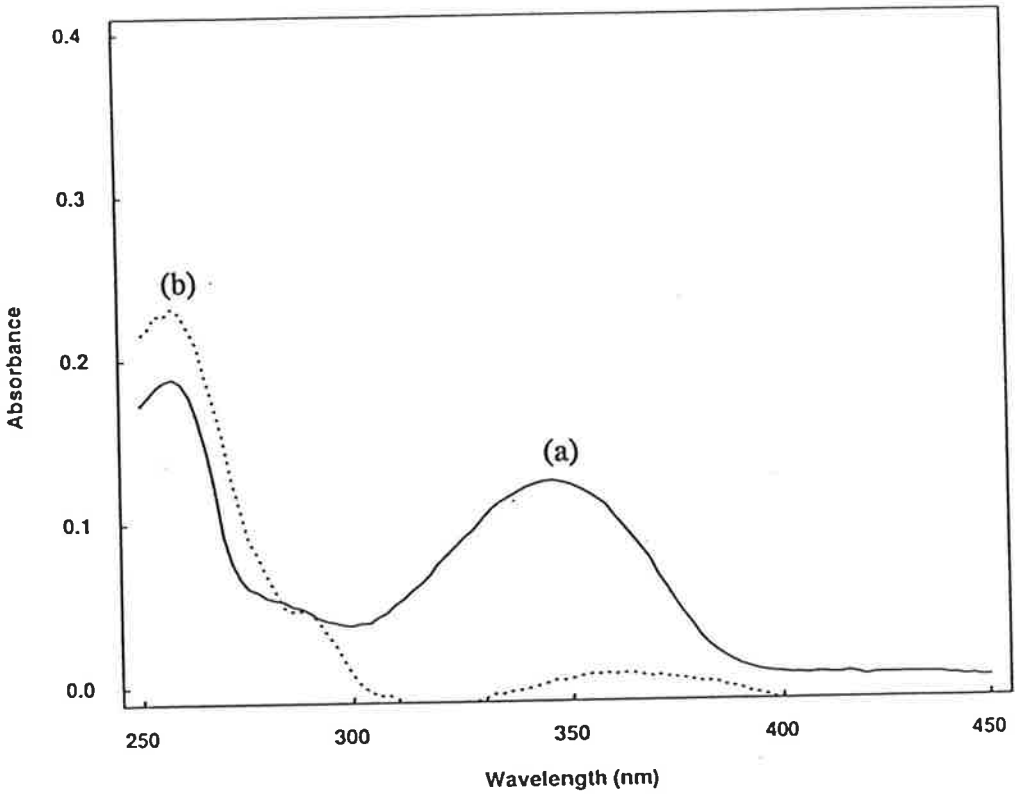


Figure A.5.3 Comparison of the ultraviolet absorption spectra of APTE measured in (a) the absence and (b) the presence of Cu^{2+}.

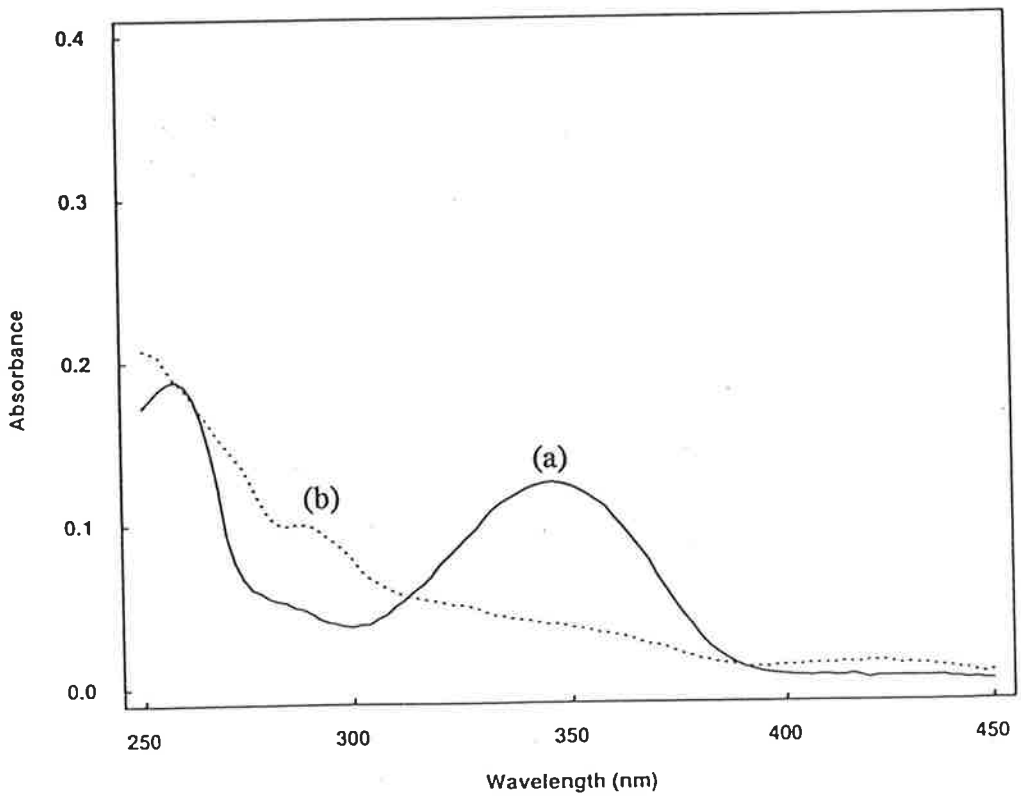


Figure A.5.4 Comparison of the ultraviolet absorption spectra of APTE measured in (a) the absence and (b) the presence of Zn^{2+}.

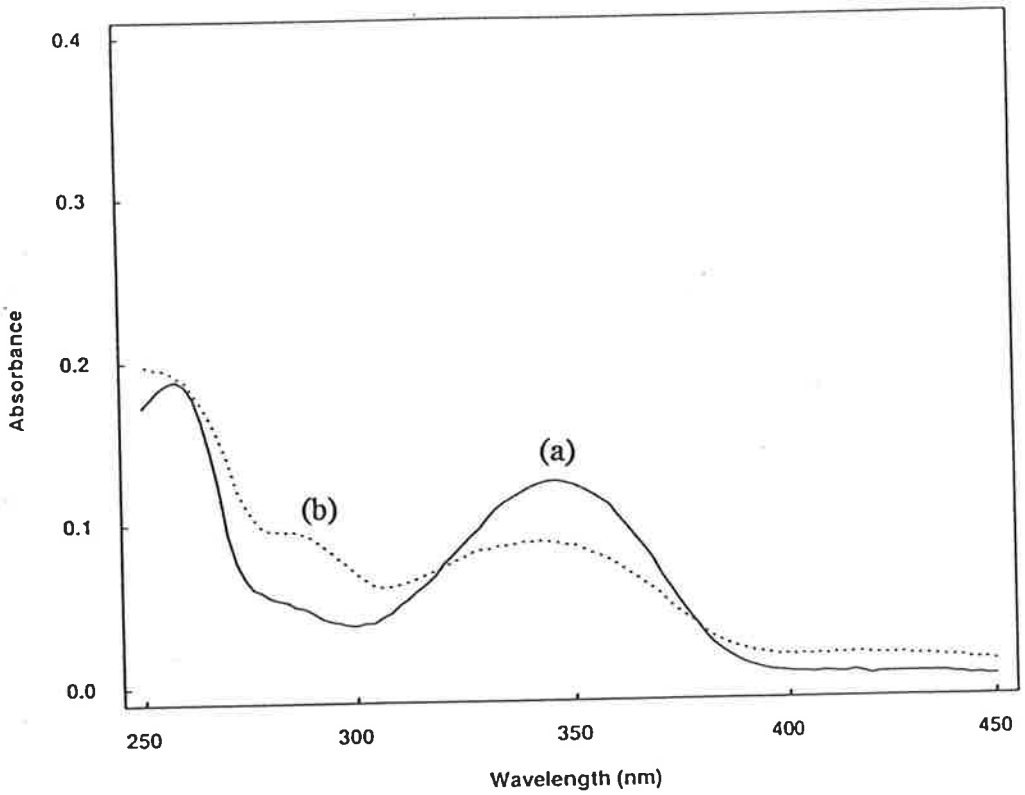


Figure A.5.5 Comparison of the ultraviolet absorption spectra of APTE measured in (a) the absence and (b) the presence of Cd^{2+}.

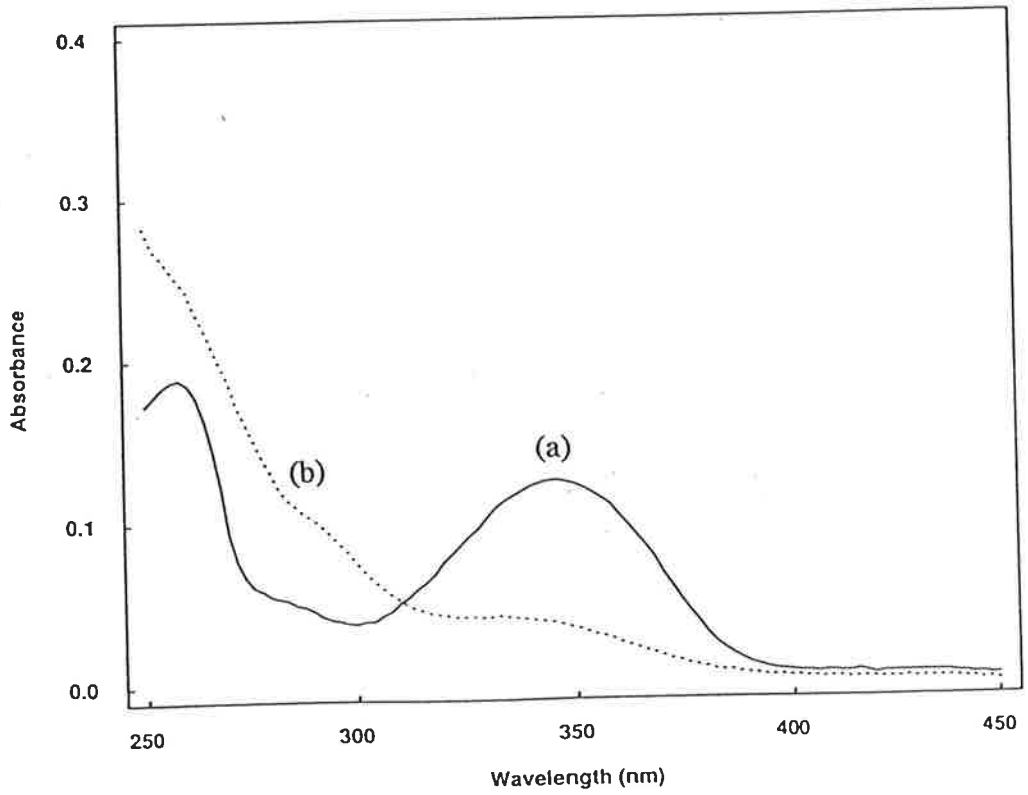


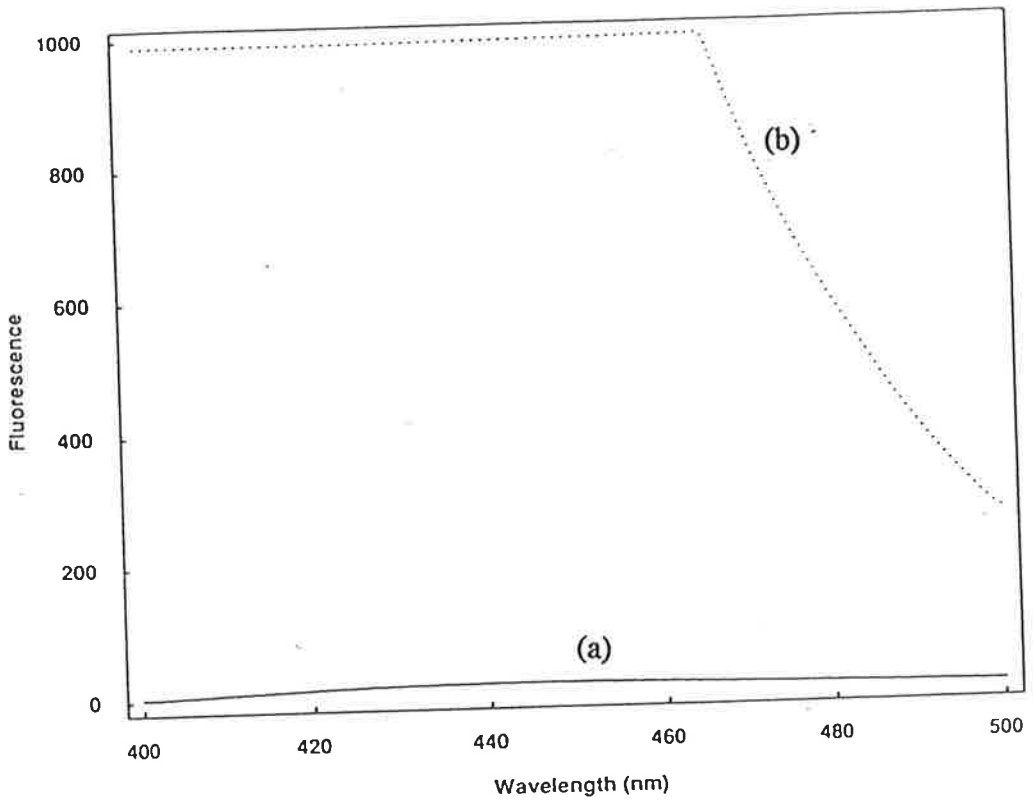
Figure A.5.6 Comparison of the ultraviolet absorption spectra of APTE measured in (a) the absence and (b) the presence of Pb^{2+}.

Appendix B Fluorescence Spectra

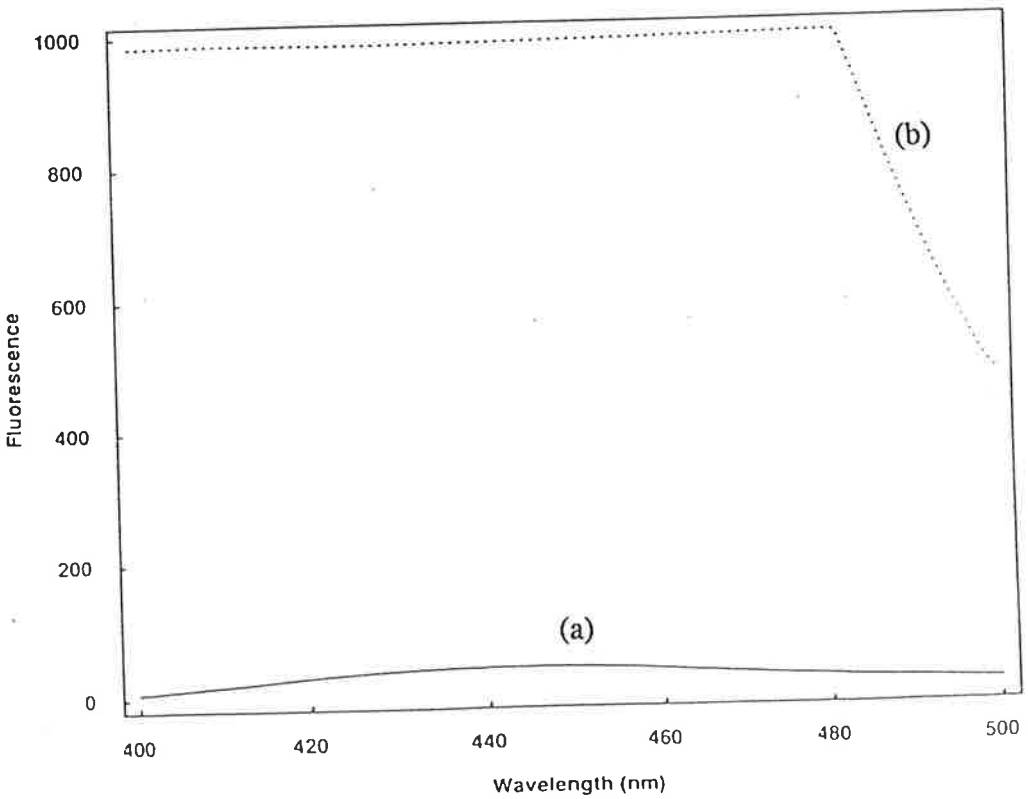
This appendix contains the fluorescence spectra of the Zn^{2+} chelating ligands, PTA, PTME, PTEE, POEE and APTE, and their metal ion complexes. Each spectral plot displays a comparison of the fluorescence spectra of (a) the ligand itself and (b) one of the metal complexes of the ligand. Only the spectra of the metal complexes of the five ligands which showed a significant fluorescence enhancement, as compared to the free ligands, have been included in this appendix. The spectra of the metal complexes of the ligands which were poorly or non-fluorescent have been omitted as they show little variation to the spectra of the free ligand itself. The solutions used to record the fluorescence spectra of the free ligands contained 5.00×10^{-6} mol dm^{-3} ligand, 1.00×10^{-3} mol dm^{-3} NaPIPES and 0.100 mol dm^{-3} $NaClO_4$. The solutions used to record the fluorescence spectra of the metal complexes of the ligands contained 5.00×10^{-6} mol dm^{-3} ligand, 2.00×10^{-3} mol dm^{-3} metal perchlorate, 1.00×10^{-3} mol dm^{-3} NaPIPES and 0.100 mol dm^{-3} $NaClO_4$ unless otherwise stated. All solutions were made up in a solvent system comprising of 75% CH_3OH and 25% H_2O by volume and all spectra were recorded at 298.2 K. Two fluorescence spectra of each metal complex were recorded by exciting the solutions with light equal to two different excitation wavelengths. These excitation wavelengths were the same as (i) an absorption isosbestic wavelength of the free ligand and the metal complex of the ligand and (ii) an absorption maxima of the metal complex of the ligand.

B.1 PTA and Complexes

Figure B.1.1 Comparison of the fluorescence spectra of (a) PTA and (b) its Al^{3+} complex

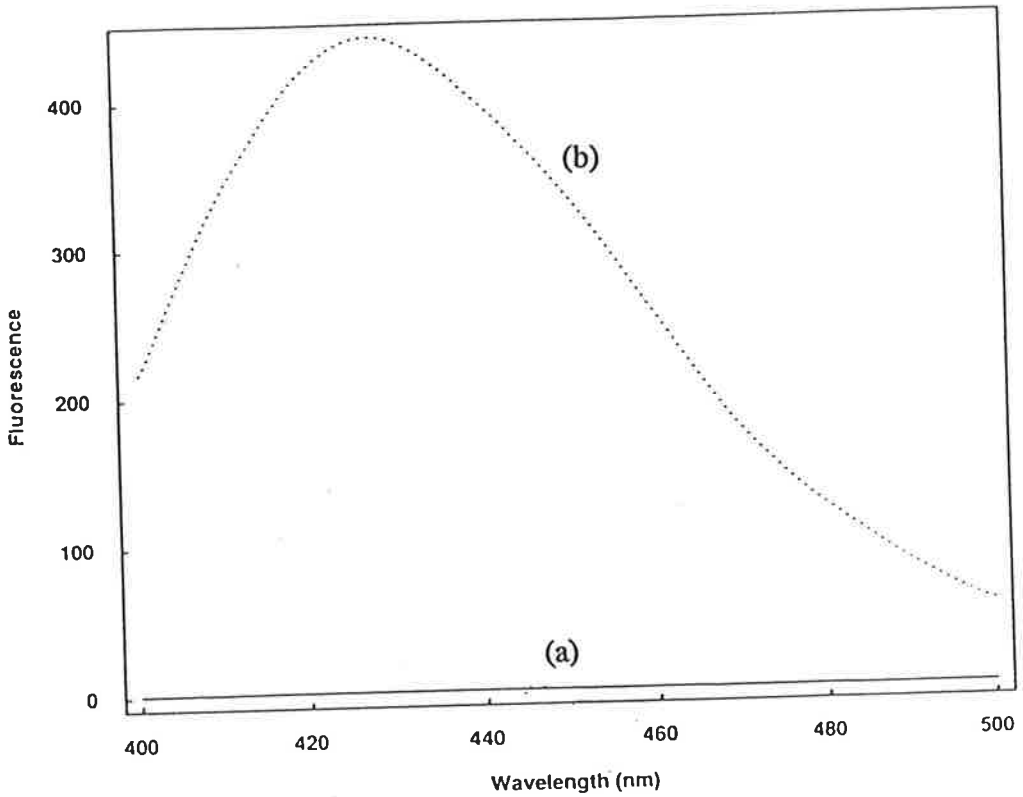


(i) Solutions were excited with light of 327 nm.

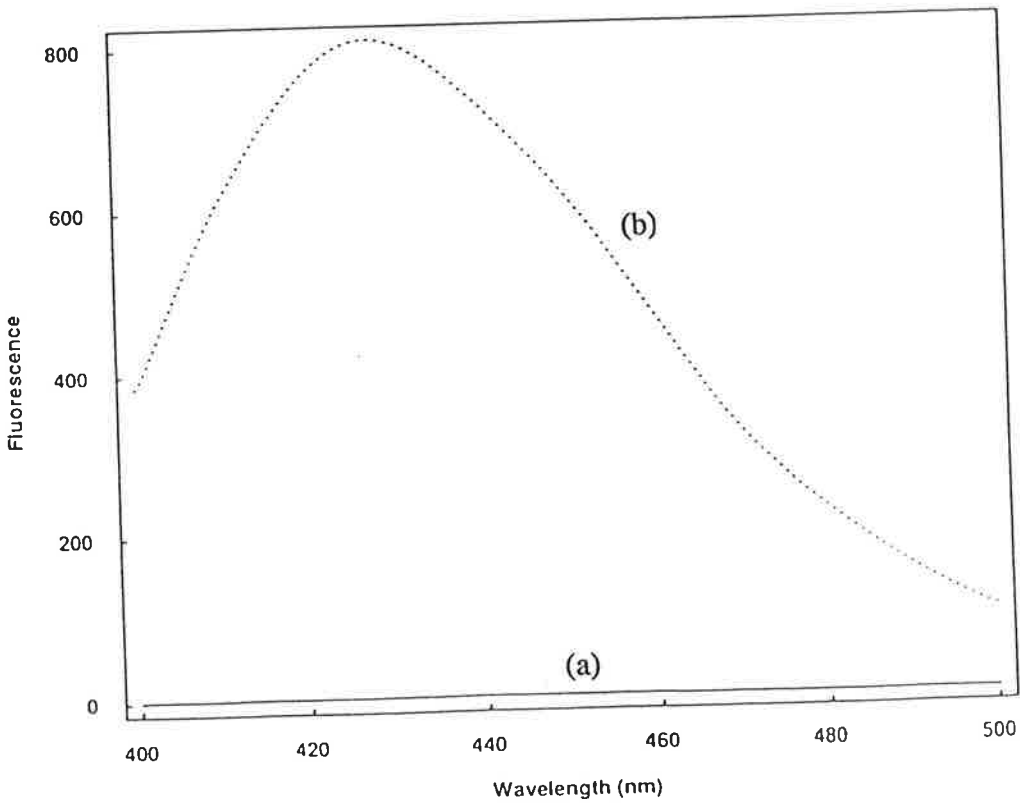


(ii) Solutions were excited with light of 350 nm.

Figure B.1.2 Comparison of the fluorescence spectra of (a) PTA and (b) its Al^{3+} complex where the concentration of PTA in each solution was 1.00×10^{-6} mol dm^{-3}.

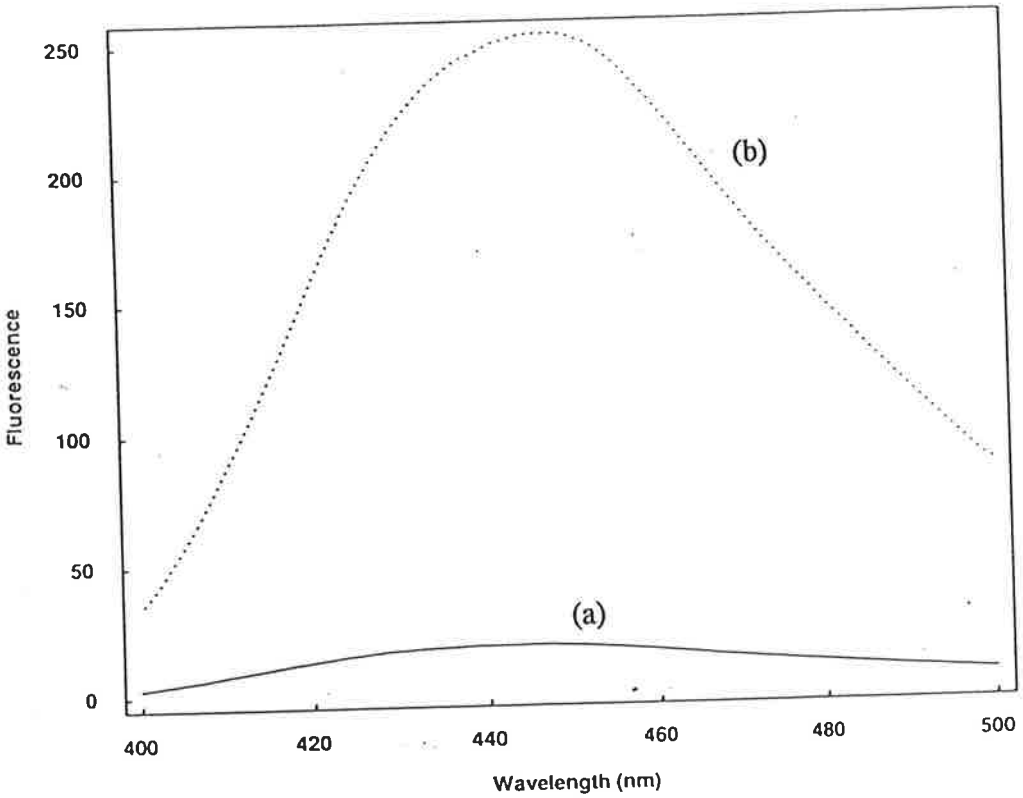


(i) Solutions were excited with light of 327 nm.

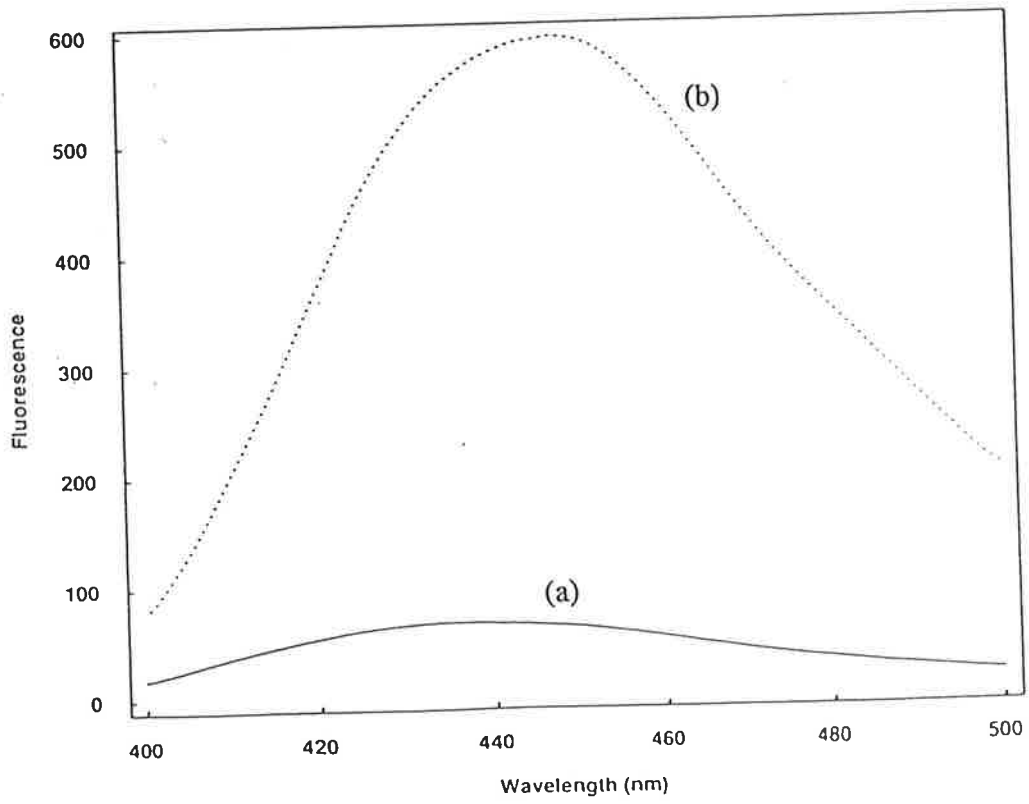


(ii) Solutions were excited with light of 350 nm.

Figure B.1.3 Comparison of the fluorescence spectra of (a) PTA and (b) its Zn^{2+} complex

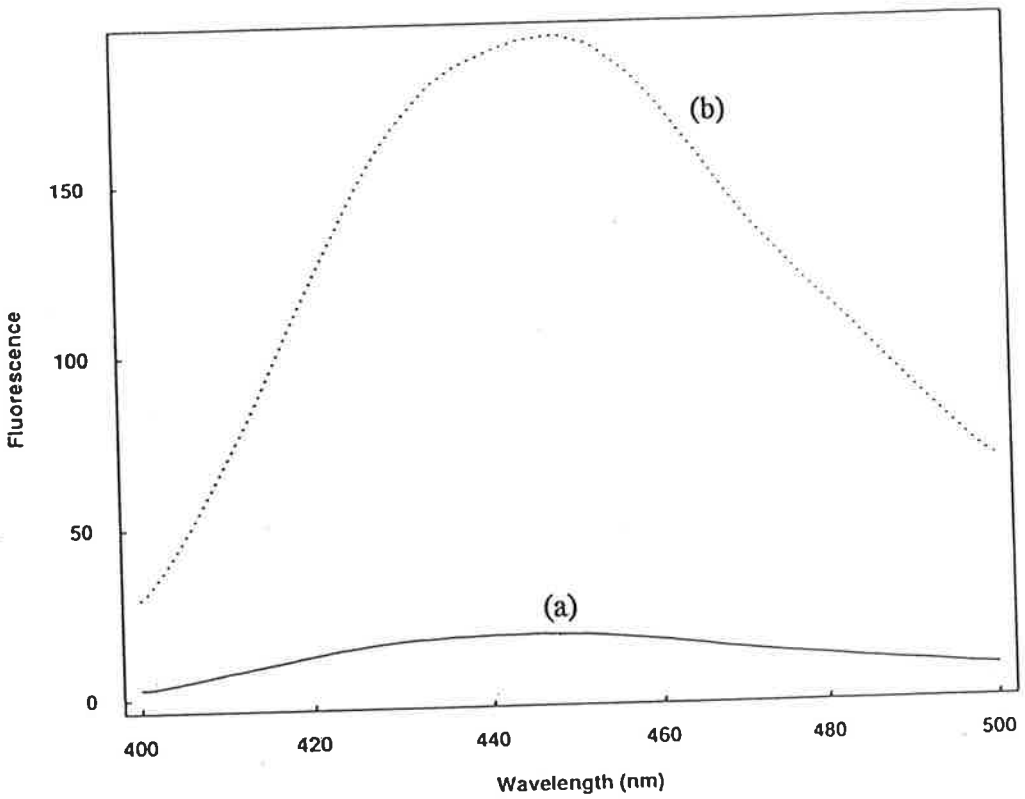


(i) Solutions were excited with light of 328 nm.

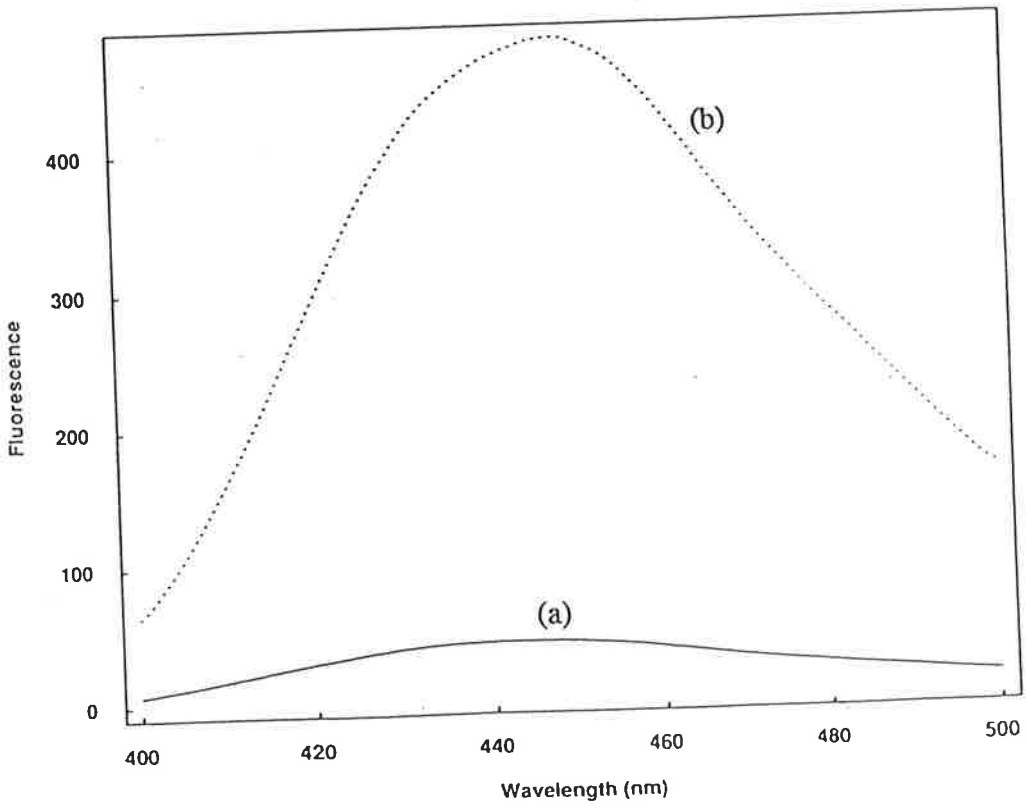


(ii) Solutions were excited with light of 358 nm.

Figure B.1.4 Comparison of the fluorescence spectra of (a) PTA and (b) its Cd^{2+} complex



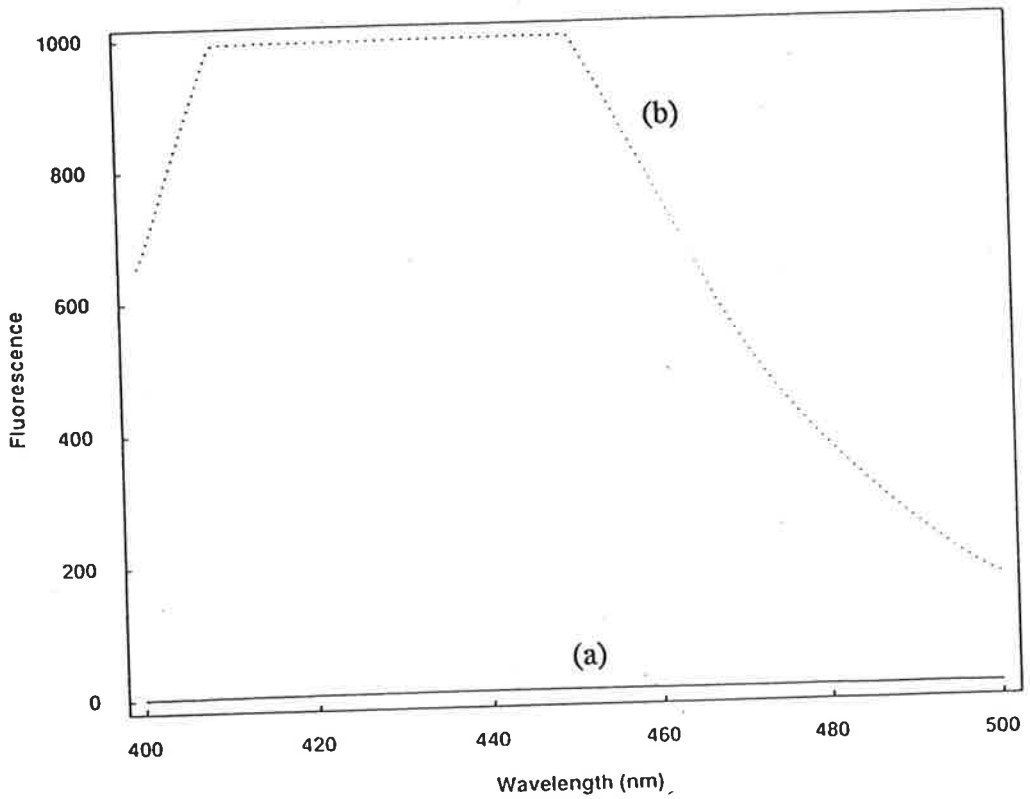
(i) Solutions were excited with light of 327 nm.



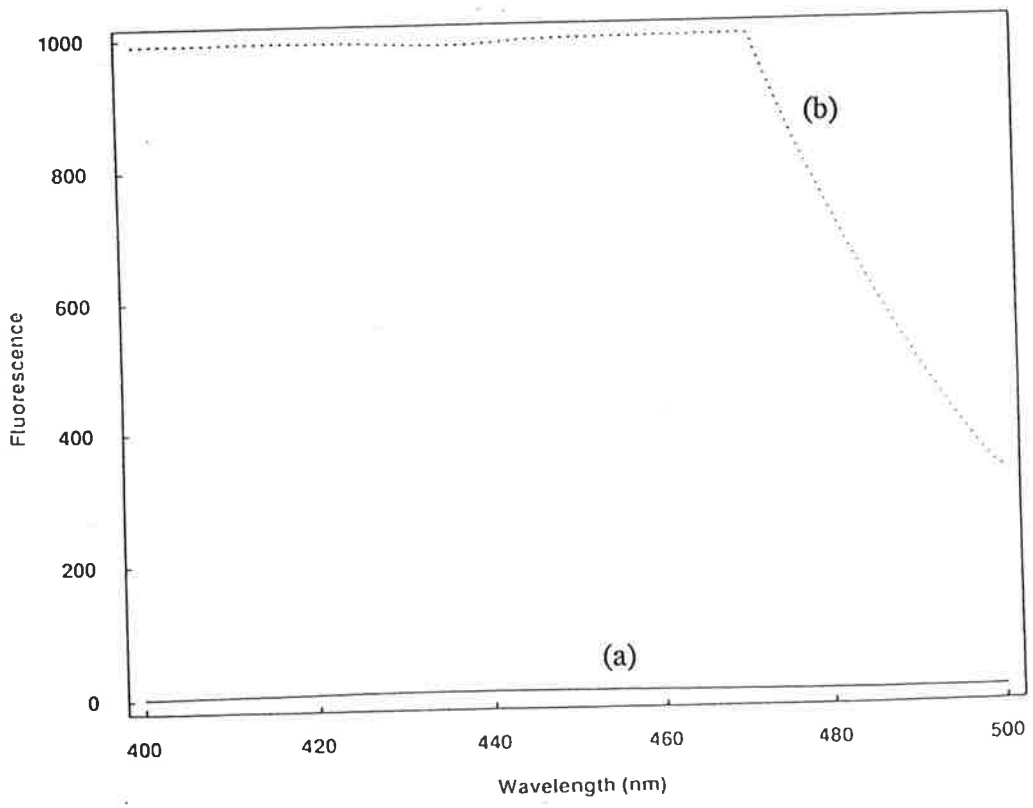
(ii) Solutions were excited with light of 357 nm.

B.2 PTME and Complexes

Figure B.2.1 Comparison of the fluorescence spectra of (a) PTME and its (b) Al^{3+} complex

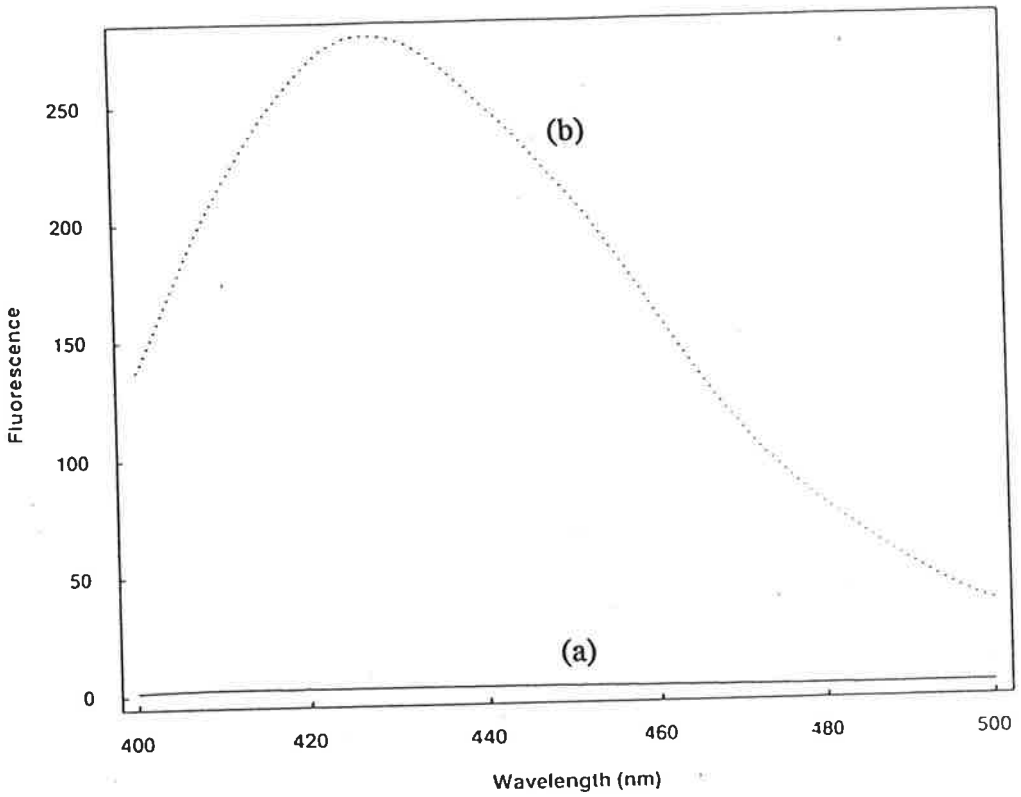


(i) Solutions were excited with light of 324 nm.

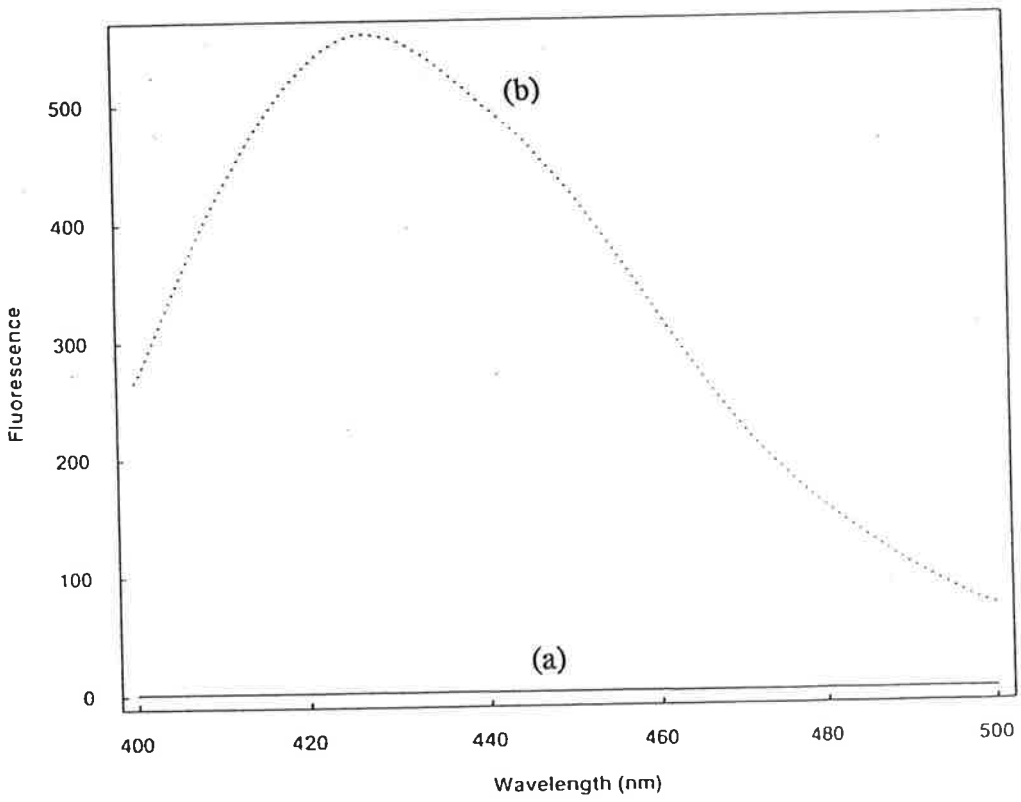


(ii) Solutions were excited with light of 346 nm.

Figure B.2.2 Comparison of the fluorescence spectra of (a) PTME and (b) its Al^{3+} complex where the concentration of PTME in each solution was 1.00×10^{-6} mol dm^{-3}.

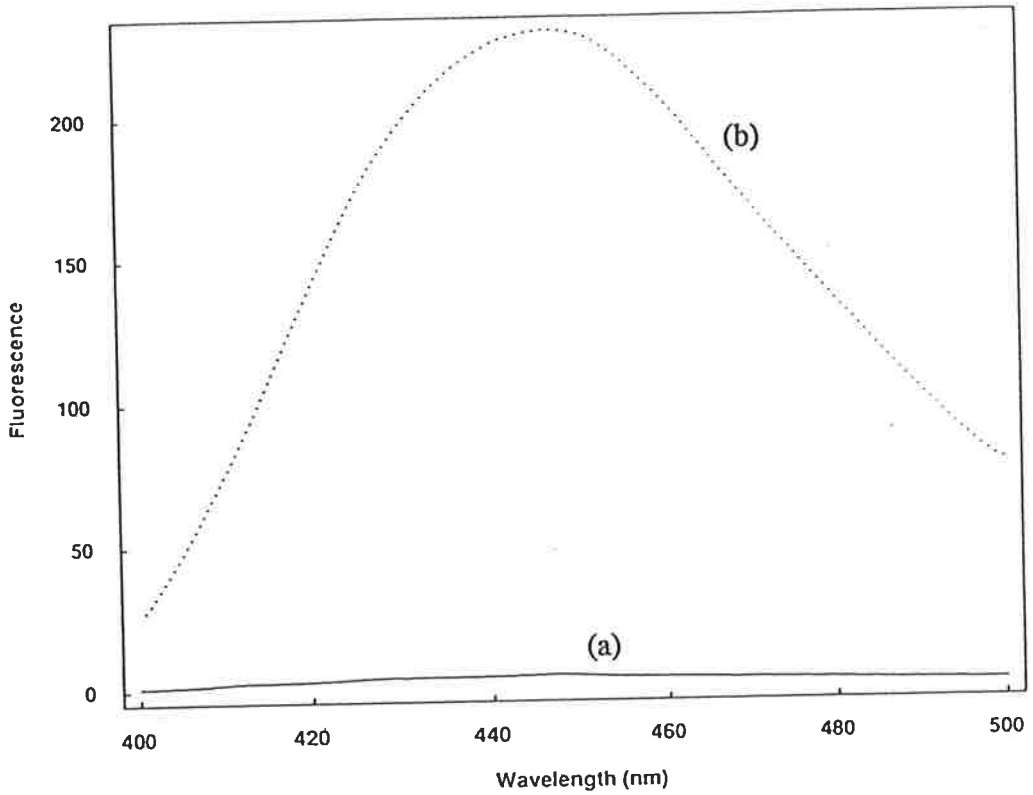


(i) Solutions were excited with light of 324 nm.

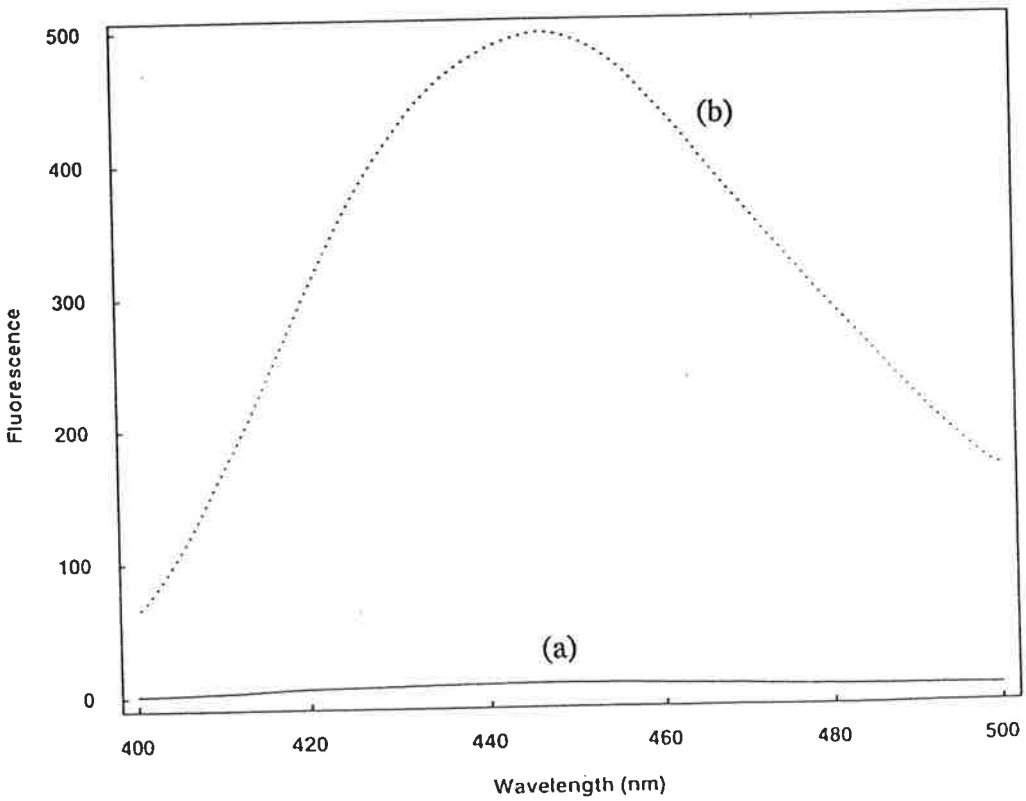


(ii) Solutions were excited with light of 346 nm.

Figure B.2.3 Comparison of the fluorescence spectra of (a) PTME and (b) its Zn^{2+} complex



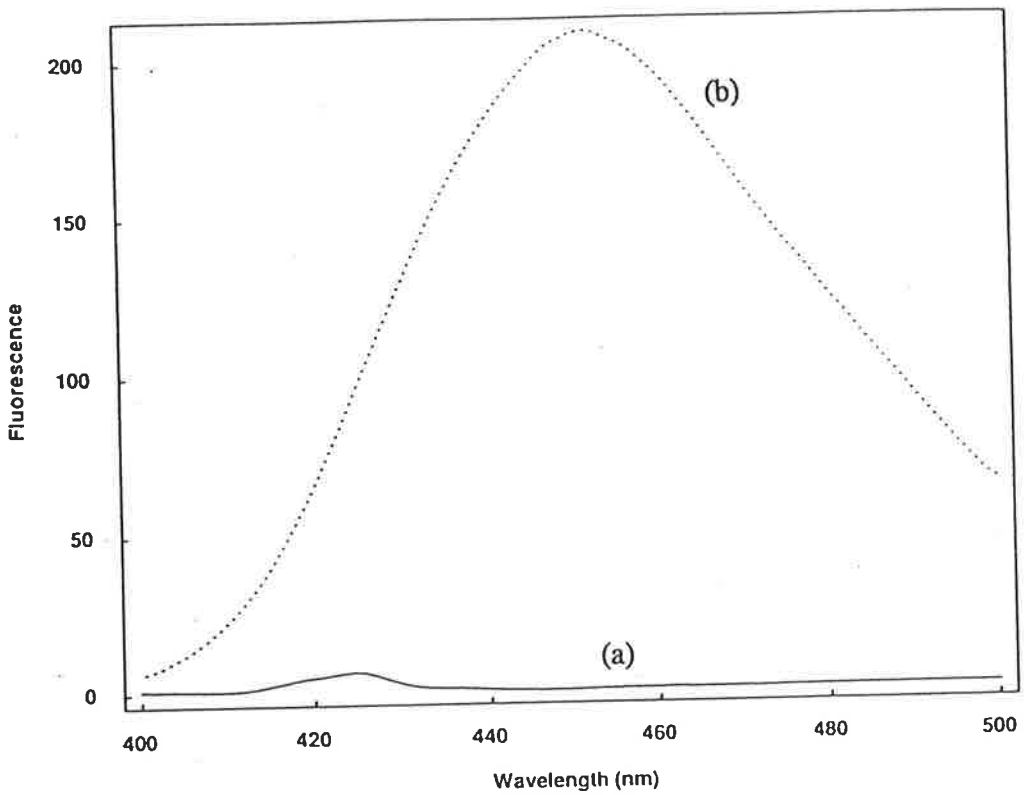
(i) Solutions were excited with light of 329 nm.



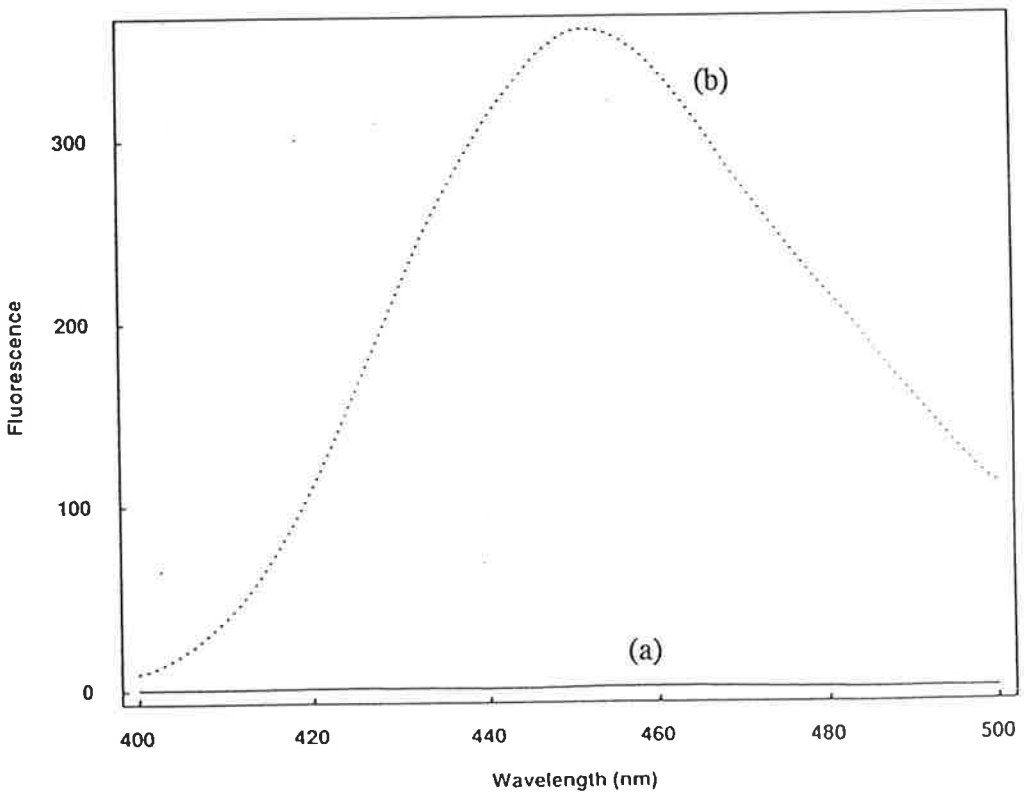
(ii) Solutions were excited with light of 358 nm.

B.3 PTEE and Complexes

Figure B.3.1 Comparison of the fluorescence spectra of (a) PTEE and (b) its Zn^{2+} complex



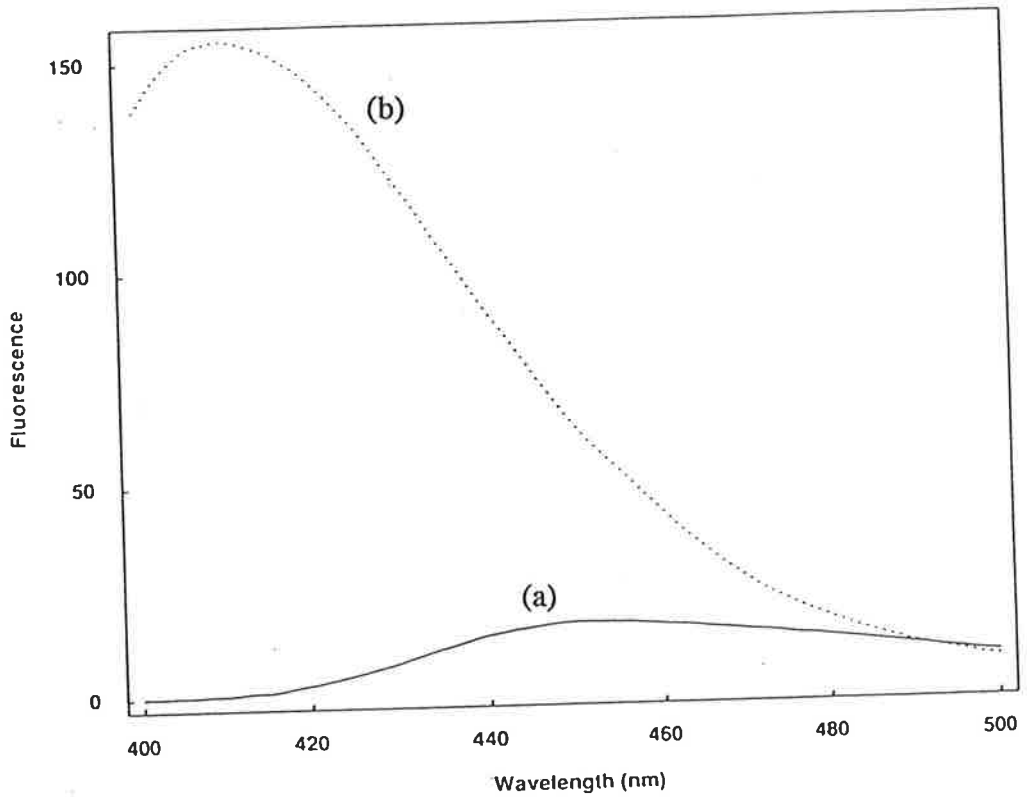
(i) Solutions were excited with light of 346 nm.



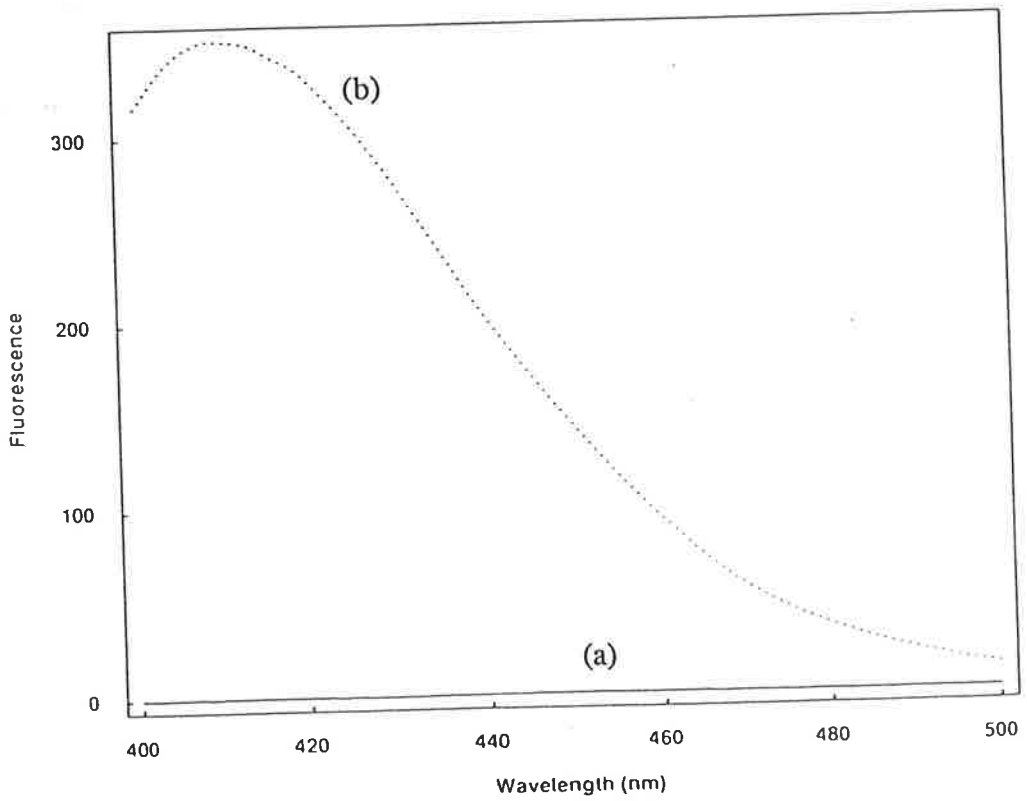
(ii) Solutions were excited with light of 368 nm.

B.4 POEE and Complexes

Figure B.4.1 Comparison of the fluorescence spectra of (a) POEE and (b) its Zn^{2+} complex

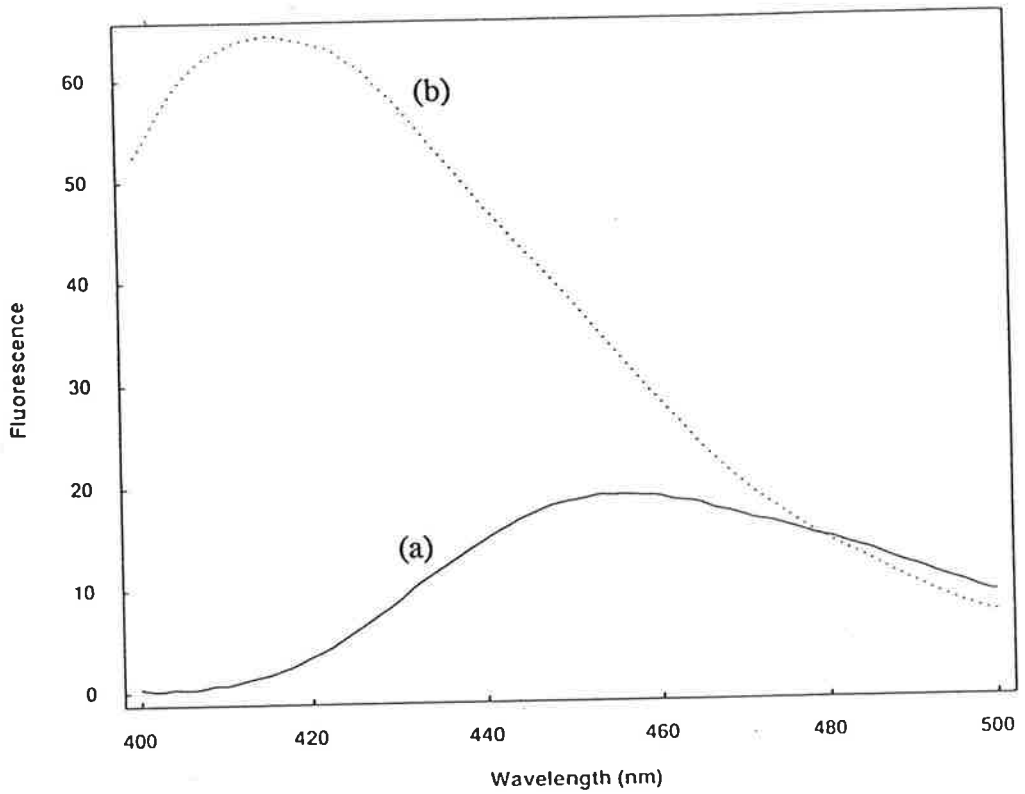


(i) Solutions were excited with light of 321 nm.

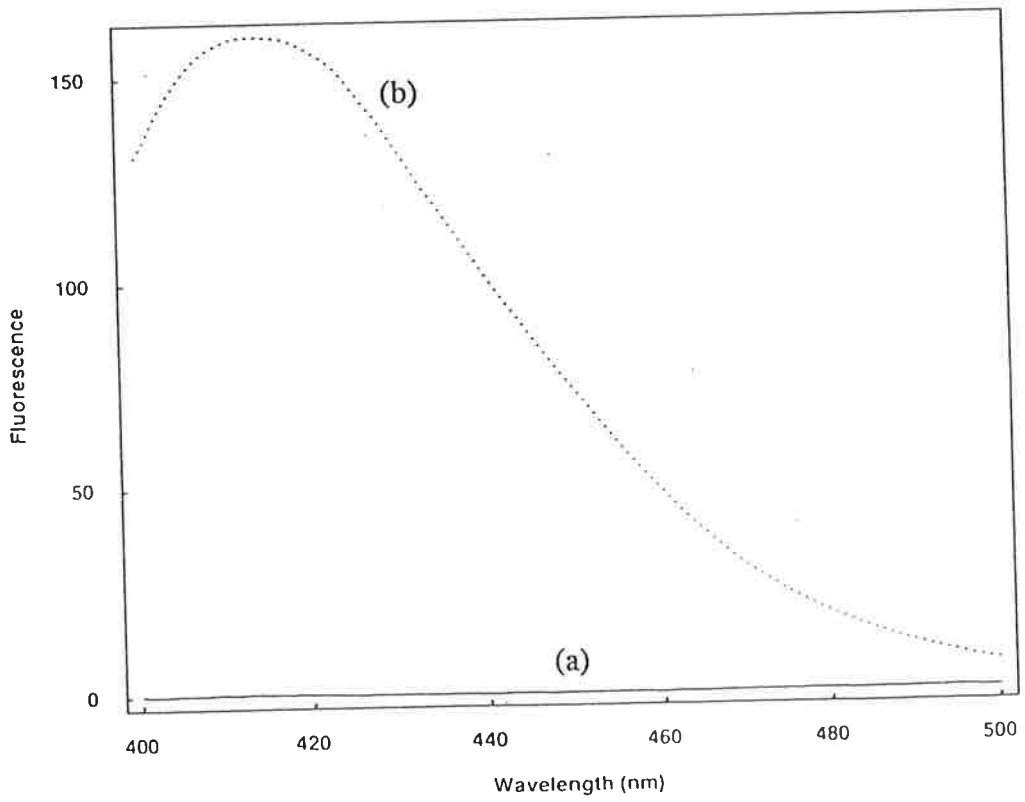


(ii) Solutions were excited with light of 346 nm.

Figure B.4.2 Comparison of the fluorescence spectra of (a) POEE and (b) its Cd^{2+} complex



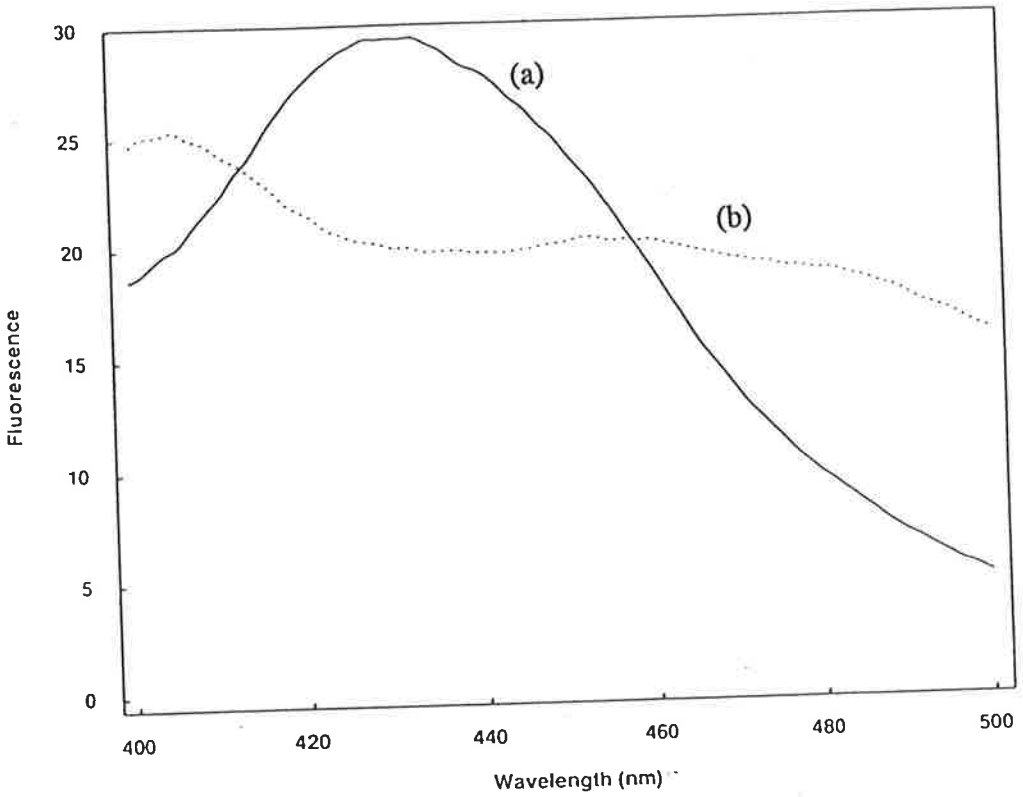
(i) Solutions were excited with light of 318 nm.



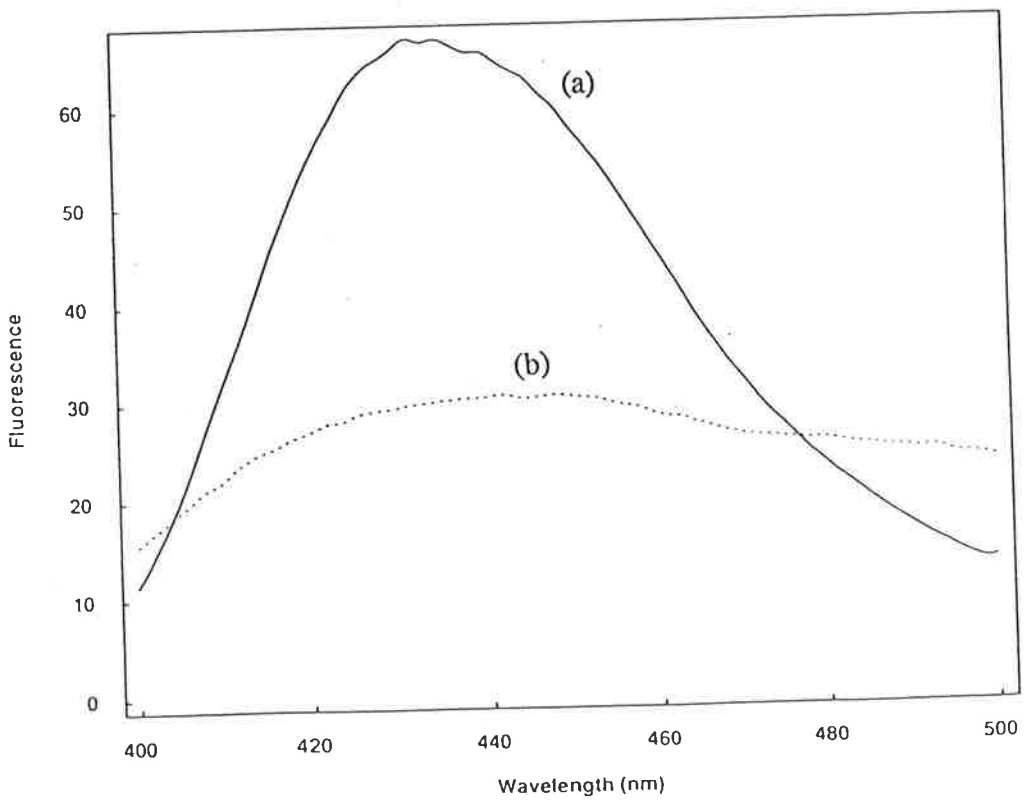
(ii) Solutions were excited with light of 348 nm.

B.5 APTE and Complexes

Figure B.5.1 Comparison of the fluorescence spectra of (a) APTE and (b) its Zn^{2+} complex

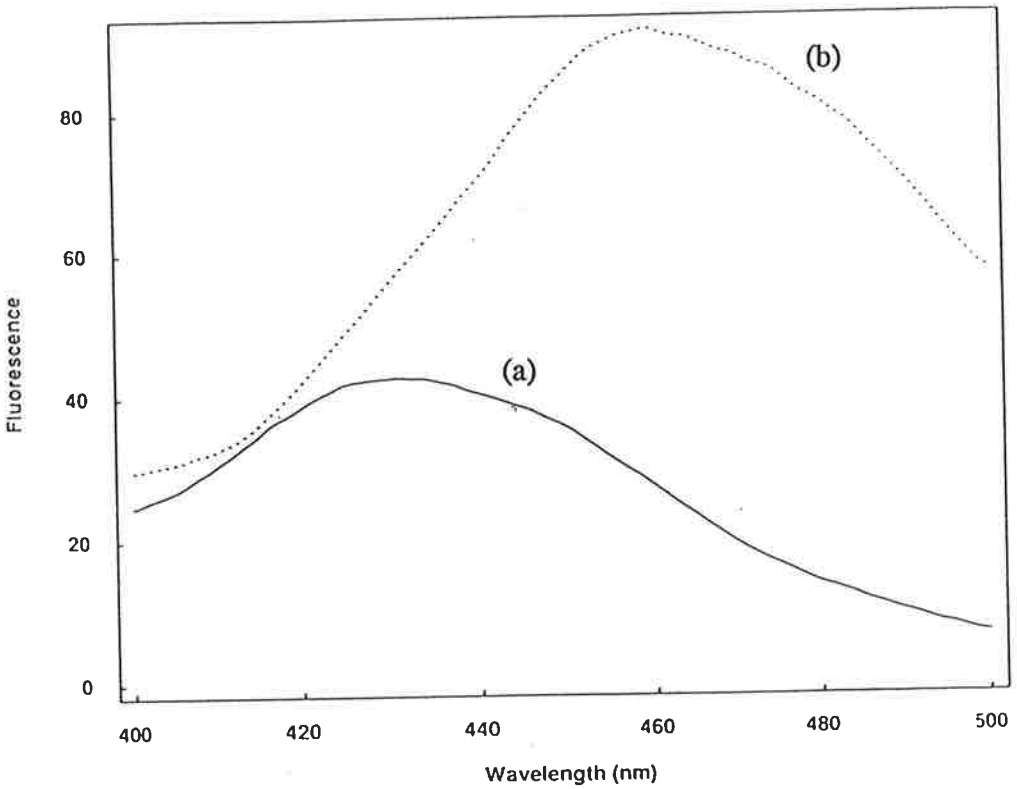


(i) Solutions were excited with light of 312 nm.

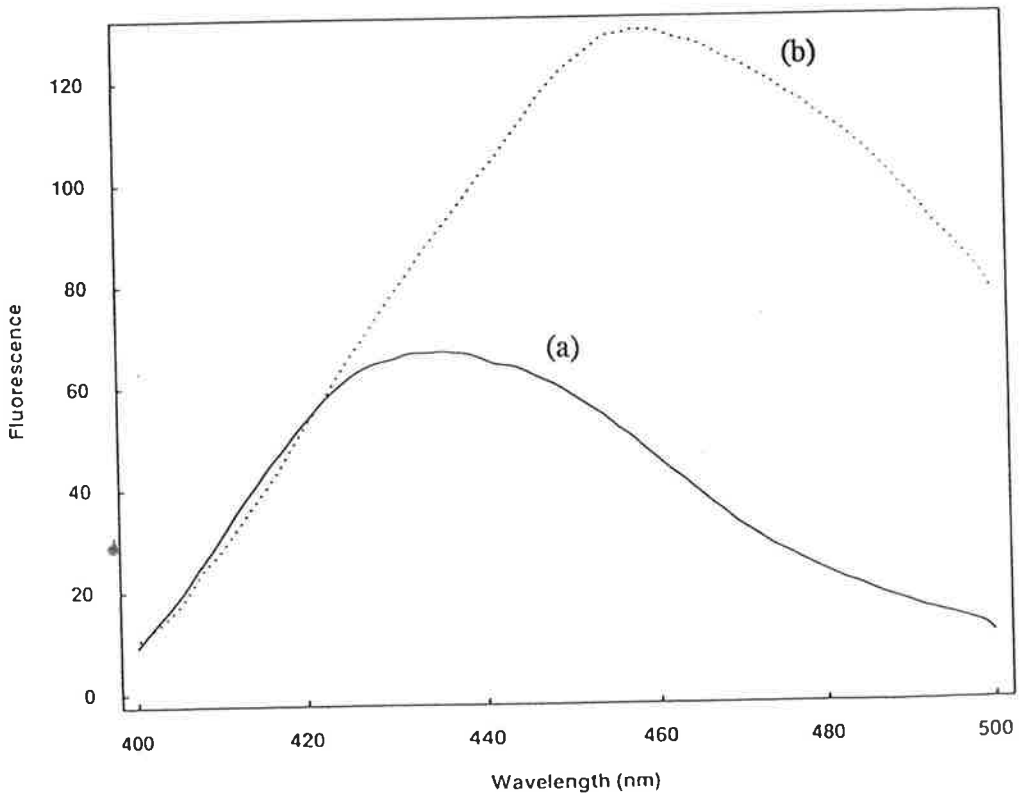


(ii) Solutions were excited with light of 285 nm.

Figure B.5.2 Comparison of the fluorescence spectra of (a) APTE and (b) its Cd^{2+} complex



(i) Solutions were excited with light of 320 nm.



(ii) Solutions were excited with light of 281 nm.