Chapter III

Experimental methods and techniques

The details of general experimental techniques, analytical procedures, materials employed along with purification of solvents and procedures adopted for testing the antimicrobial activity are described in this chapter. Details of the preparation of the individual Mannich bases, and their complexes are given in the relevant chapters.

3.1 Purification of chemicals

Common solvents like ethanol, chloroform used at various stages of this work were purified as per the procedures given in the literature\textsuperscript{205,206}. The solvents used for cyclic voltammetric work like dimethylsulphoxide (DMSO) and acetonitrile were purified and stored in an inert atmosphere.

Acetamide, Benzamide, Piperidine, Morpholine and Salicylaldehyde were AR E.Merck products and used after purification.

3.2 Preparation of supporting electrolyte

The supporting electrolyte, tetramethylammonium perchlorate (TMAP), (Me\textsubscript{4}N)ClO\textsubscript{4} was prepared by mixing an aqueous solution of tetramethylammonium hydroxide (25mmol) with aqueous perchloric acid (2.1 mL, 70%). Tetramethylammonium perchlorate that got precipitated was filtered and washed thoroughly with ethyl acetate and dried. The sample was recrystallised from 3\% aqueous perchloric acid.
3.3 Analytical methods for characterization

3.3.1 Elemental analysis

The carbon, hydrogen and nitrogen contents of the complexes were estimated through microanalysers (performed) at Sophisticated Analytical Instruments Facility (SAIF), Indian Institute of Technology (IIT), Chennai, and Central Drug Research Institute (CDRI), Lucknow. In the microanalysis, sample weighing about 10 mg concealed in a tin capsule was injected into the instrument and the computerized elemental percentages were obtained. EDTA was used for calibrating the instrument.

3.3.2 Estimation of metal content

The metal content of the complex was estimated gravimetrically as their oxides by fusion with Analar ammonium oxalate. In a typical analysis, about 0.2 g of the dried complex was accurately weighed in a previously prepared silica crucible. Analar ammonium oxalate roughly 3 parts by weight of the complex was added and the mixture was ignited slowly at first and then vigorously using Meker burner for 2h. It was then cooled in a desiccator and weighed. The procedure was repeated till the final oxide weight was constant. From this, the percentage of metal in the complex was calculated.

3.4 Instrumental methods of characterization

The instrumental techniques used to elucidate the structure of the complexes are outlined below.
3.4.1 Infrared spectra

The IR spectra of all the ligands and the metal complexes were recorded on a Perkin-Elmer 783 FT-IR spectrometer in 4000-200 cm⁻¹ range using KBr as disc. IR spectra were also recorded at Pondicherry University on a Jasco FT-IR 5300 instrument (KBr pellet). Generally a comparison of the infrared spectrum of the ligand and that of its complex will be of much help to find out the atom or atoms through which the ligand is attached to the metal ion.

3.4.2 Electronic absorption spectra

The electronic spectra are often very helpful in the evaluation of results furnished by other methods of structural investigation. The UV-Visible spectra of the ligand and the complexes were taken in the range 200-1100nm on a Perkin Elmer Lambda EZ201 UV-VIS spectrophotometer. The spectra of the complexes were recorded using DMSO or DMF (spectral grade) as the solvent. The electronic spectral measurements were used for assigning the stereochemistry of metal ions in the complexes based on the positions and number of d-d transition peaks.

3.4.3 Magnetic susceptibility measurements

The magnetic susceptibility measurements of the colored complexes of Cu(II), Co(II) and Ni(II) ions were carried out in order to find out the effective magnetic moment per each metal atom in the complexes. The magnetic susceptibility values of the complexes were calculated using the formula,

\[ 10^6 \kappa = \alpha + \beta F''/W \]

Where,
\( F'' \) = corrected force in mg

\( \alpha = 0.029 \times \text{volume correction} \)

\( \beta = \text{calibrant constant} \)

\( W = \text{weight of the substance in gram} \)

\( \kappa = \text{gram susceptibility} \)

The number of unpaired electrons ‘n’ possessed by the metal ion can be determined from the effective magnetic moment, \( \mu_{\text{eff}} \) of the metal ion using the formula,

\[
\mu_{\text{eff}} = 2.83 (XmT)^{1/2} \text{ BM}
\]

Where,

\( Xm = \text{Molar susceptibility; } T = \text{Absolute temperature} \)

From the calculated number of unpaired electrons it is possible to infer the valence state of the metal ion in a complex. The data help us to find out whether the complex is a high spin or a low spin complex.

The weight of the clean, empty, dry Gouy tube was taken in the absence and in the presence of magnetic field. To evaluate the value of calibrant constant \( \beta \), the tube was filled with \( \text{Hg}[\text{Co(CNS)}_4 ] /\text{CuSO}_4 .2\text{H}_2\text{O} \) up to the mark and the weight was taken in the absence and in the presence of magnetic field.
The calibrant was removed and the Gouy tube was washed with water and acetone and dried. Then the tube was filled with various complexes and the weights were taken. Finally, the weight of the Gouy tube with water filled up to the mark was noted in order to find out the volume of the sample.

Using the above formula, the effective magnetic moment, $\mu_{\text{eff}}$ of the metal ions was calculated after making proper diamagnetic corrections using Pascal’s constants.

### 3.4.4 Conductivity measurements

Conductance measurements of the complexes in solution were made mainly to verify the ionic formulation of the complexes i.e. to see whether the anions of the metal salts remain inside or outside the coordination sphere of the central metal atom. The molar conductivity of the metal complexes was measured on a Systronic conductivity bridge with a dip type cell, using $10^{-3}$ M solution of complexes in DMSO. The cell constant of the conductivity cell used was 1.03 cm$^{-1}$.

All measurements were corrected for the conductance of pure solvent by subtracting the conductance of pure solvent from that of the solution. Molar conductance in the solvent depends on the number of ions present in the solution, degree of dissociation, mobility of ions and temperature.

The values were compared with the standard values from the literature to find out the electrolytic nature of the complex such as undissociated, 1:1, 1:2, etc.
3.4.5  \(^1\)H and \(^{13}\)C-NMR spectra

\(^1\)H-NMR and \(^{13}\)C-NMR spectra of the samples were recorded employing TMS as internal reference and CDC\(_3/DMSO-d_6\) as solvent at ambient temperature.

The \(^1\)H-NMR study is of help in the structural elucidation of the ligands as well as in locating precisely the donor site or sites of a ligand. Usually \(^1\)H-NMR spectral studies on ligands and their zinc complexes are carried out to confirm the mode of coordination suggested from IR data. Comparing the spectrum of the free ligand with that of a complex in freshly prepared CDC\(_3/DMSO-d_6\) solution, generally, a deshielding effect on the hetero atom proton resonance is observed. The chemical shift towards lower fields is due to the electron withdrawal by a metal from the donor atom and the noticeable deshielding effect supports its coordination.

The \(^{13}\)C-NMR study is useful in the structural elucidation of the ligands. The number of sharp peaks (multiplets) in the proton noise decoupled spectra represents the number of carbons of the compound which are chemically non-equivalent.

3.4.6. FAB-mass spectra

The FAB-mass spectra of the ligands and the complexes were recorded on a JEOL SX 102/DA-6000 mass spectrometer/data system using Argon/Xenon (6 kV, 10 mA) as the FAB gas. The accelerating voltage was
10kV and the spectra were recorded at room temperature. m-Nitrobenzyl alcohol (NBA) was used as the matrix.

3.4.7 Cyclic voltammetry

Electrochemical measurements were carried out in electrochemical analyzer model BAS-50 Voltammograph. The three-electrode cell contained a reference Ag/AgCl electrode, Pt wire auxiliary electrode and glassy carbon working electrode. (Me₄N)ClO₄ was used as the supporting electrolyte. Dissolved oxygen was removed by flushing the solution with Analar nitrogen gas for 5-10 min. prior to each series of experiments. The purity of the supporting electrolyte and the solvents used were checked by scanning the cyclic voltammogram before adding the sample.

3.4.8 Thermal analysis

The water content (both lattice and coordinated) in the complexes was determined by simple thermogravimetric study. In a typical analysis, about 0.3 g of the metal complex was taken in a silica crucible and it was maintained at 110°C for 3 h in a hot air oven. It was then cooled in a desiccator and weighed. The weight loss was due to the number of lattice held water molecules. The same crucible was then heated at 160°C for further 3 h. It was then cooled and weighed, and the weight loss corresponded to the number of coordinated water molecules. Though the method at best was approximate gave good indication of the number of water molecules.
This technique also examines the various chemical changes like thermal decomposition, oxidation etc. and several processes like solvent and water desorption, evaporation, sublimation etc. which may take place with a consequent change in weight of the sample when heated at a desired heating rate with proper furnace atmosphere.

3.5 Antimicrobial study

3.5.1 Antibacterial activity

A standard nutrient agar was used as medium for testing the activity of microorganisms as antibacterial agents. For preparing agar media, about 38 g of the Mueller-Hinton agar (it is composed of casein acid hydrolyzed 17.5 g, beef heart infusion 2 g, starch 1.5 g and agar 17 g per litre) was dissolved in 1000 mL of distilled water in a clean conical flask. The pH of the solution was maintained at neutral. The solution was boiled to dissolve the medium completely and sterilized by autoclaving at 15 lbs pressure (121°C) for 15 min. After sterilization, 20 mL media was poured into the sterilized Petri plates. These Petri plates were kept at room temperature for sometime. After a few minutes, the medium solidified in plates. Then this was incubated for 12 h. After the incubation this was inoculated with microorganisms using sterile swabs. All these manipulations were carried out with utmost care under aseptic conditions. The antibacterial activity of the ligand and its complexes was studied by the well diffusion method using DMSO/DMF/pyridine as solvent. The test solutions were prepared by dissolving the compounds in DMSO/DMF/pyridine. In a typical procedure, a well was made on the agar medium inoculated with microorganisms. The well was filled with the test
solution using a micropipette and the plate was incubated at 37°C for 24 h. During this period, the test solution was diffused and the growth of the inoculated microorganisms such as Staphylococcus aureus, Pseudomonas aeruginosa, Bacillus subtilis and Proteus vulgaris was affected. The activity developed on the plate was determined by measuring the diameter of the inhibited zone in millimeters. The drug tetracycline was used as the standard.

3.5.2 Antifungal activity

A standard potato dextrose agar (PDA) was used as medium for antifungal activity by well diffusion method. For preparing this agar media, 200g of potato extract, 20 g of agar and 20 g of dextrose were dissolved in one litre of distilled water. The solution was boiled to dissolve the medium completely and sterilized by autoclaving at 15 lbs pressure (121°C) for 30 min. After sterilization, 20 mL of media was poured into the sterilised Petri plates and kept at room temperature for sometime. After a few minutes, the medium got solidified in the plates. DMSO/DMF/pyridine was used as solvent and the drug amphotericin as control.

In a typical procedure, a well was made on the agar medium inoculated with microorganisms. The well was filled with the test solution using a micropipette and the plates were inoculated at 37°C for 72 h. During this period, the test solution was diffused and affected growth of the inoculated microorganisms. A zone was developed on the plate and the inhibition zones were determined by measuring the diameter of the inhibited zone in millimeters.
REFERENCES


