CHAPTER-II

EXPERIMENTAL TECHNIQUES

In this chapter, the analytical procedures and physico-chemical techniques employed in the present investigation are presented. The solvents were distilled prior to use and double distilled water was used for the preparation and chemical analyses. All the chemicals used were of AR grade received from S.D fine chemicals. The hydrazine hydrate, 99-100 % was used as such as received.

Chloroform, CHCl₃
Carbontetrachloride, CCl₄
Ethylenediaminetetraaceticacid, \(H_4edta\)
Ethylenediaminetetraaceticacid- disodium salt, \(Na_2H_2edta\)
Hydrazine hydrate 99 – 100 %, \(N_2H_4.H_2O\)
Hydrochloric acid, HCl
Nitric acid, HNO₃
Potassium iodate, KIO₃
Lanthanum oxide, La₂O₃
Cerium oxide, Ce₂O₃
Praseodymium oxide, Pr₆O₁₁
Neodymium oxide, Nd₂O₃
Samarium oxide, Sm₂O₃
Gadolinium oxide, Gd₂O₃
Terbium oxide, Tb₄O₇
Europium oxide, Eu₂O₃
Dysprosium oxide, Dy₂O₃
Magnesium nitrate hexahydrate, Mg(NO₃)₂·6H₂O
Manganese carbonate, MnCO₃
Cobalt nitrate hexahydrate, Co(NO$_3$)$_2$.6H$_2$O
Nickel nitrate hexahydrate, Ni(NO$_3$)$_2$.6H$_2$O
Cupric nitrate trihydrate, Cu(NO$_3$)$_2$.3H$_2$O
Zinc nitrate hexahydrate, Zn(NO$_3$)$_2$.6H$_2$O
Cadmium nitrate tetrahydrate, Cd(NO$_3$)$_3$.4H$_2$O
Ferric nitrate nanohydrate, Fe(NO$_3$)$_3$.9H$_2$O
Hexamine
Eriochrom Black-T
Xylenol orange
Buffer solution,
Anhydrous sodium sulphate, Agar-Agar
Aqueous ammonia, ethyl alcohol, distilled water,
NaCl, peptone, beef extract, yeast extract, sucrose

**Analytical methods**

The analytical methods used include quantitative estimation of hydrazine and metal ions such as manganese, cobalt, nickel, zinc, cadmium, lanthanum, cerium, praseodymium, neodymium, samarium, europium, gadolinium, terbium and dysprosium.

**Estimation of hydrazine**

The hydrazine content in the complexes was determined volumetrically using a standard potassium iodate (0.025 M) solution under Andrew’s conditions$^{294}$

\[
\text{IO}_3^- + \text{N}_2\text{H}_4 + 2\text{H}^+ + \text{Cl}^- \rightarrow \text{ICl} + \text{N}_2 + 3\text{H}_2\text{O}
\]

1 ml of 0.025 M KIO$_3$ = 0.0008013 g of hydrazine

In a typical experiment about 100 mg of the sample was dissolved in a mixture of 30 ml of concentrated HCl and 20 ml of distilled water and 5 ml of CCl$_4$ or CHCl$_3$ was added. The mixture contained in a stoppered bottle was titrated against standard KIO$_3$ solution. The solution was shaken well after the
addition of each ml of KIO₃ solution. Towards the end point the KIO₃ solution was added drop by drop. The end point is the disappearance of violet colour in the organic layer.

**Estimation of metal ions**

The metal content in the complexes was determined by *edta* complexometric titrations, after decomposing a known weight of the complex with nitric or hydrochloric acid. A standard (0.01 M) solution of disodium *edta* was prepared by directly weighing out the disodium salt of *edta* (3.7225 g per litre of the solution) and dissolving in distilled water. The solution was standardised by titration with standard zinc sulphate solution.

**Manganese, Magnesium, Zinc and Cadmium**

Manganese and magnesium were estimated by titrating against standard Na₂H₂*edta* (0.01 M) solution using Eriochrome Black-T as indicator. The solution was maintained at pH = 10 using a buffer solution containing ammonium chloride and ammonia. The end point was the colour change from red to blue.

**Nickel**

In the case of nickel, a known excess of Na₂H₂*edta* solution of known strength was added, followed by a few drops of Eriochrome Black-T indicator solution and the buffer solution (pH = 10). The excess *edta* was titrated against standard zinc sulphate solution until the colour changed from blue to wine red.

**Cobalt**

A known volume of the solution was pipetted out into a titration flask. The pH of the solution was increased by adding hexamine, which acts as a buffer of pH = 6. A few drops of xylenol orange were added as indicator. The resultant solution was titrated with Na₂H₂*edta* solution until the colour changed from red to yellow.
Lanthanides

A known amount of the complex was decomposed with concentrated nitric acid and the solution was evaporated to dryness. This process was repeated for three times. The final residue, the lanthanide nitrate was dissolved in 30 ml of distilled water. To this solution a few drops of xylenol orange and hexamine powder were added till the solution became red. The resultant solution was titrated with standard Na₂H₂edta solution until the colour changed from red to yellow.

Physico-Chemical Techniques

The instrumental techniques employed in the present investigation include melting point measurements, CHN analyses conductance and magnetic susceptibility measurements, the spectroscopic techniques like electronic and infrared spectra and thermal analysis techniques like differential thermal analysis (DTA), thermo gravimetry (TG) and differential thermal gravimetry (DTG). The powder and single X-ray studies were also employed in the present study.

Melting point

Melting points were determined on metler FP5 instrument and are uncorrected.

Elemental Analysis

C, H, N analyses were done on a perkin-Elmer (model 1240 CHN analyzer).

Molar conductance

Conductance measurement were carried out at room temperature in conductivity water by a century digital conductivity meter model CC 601 and dip type cell with a smooth platinum electrode.
Magnetic susceptibility measurements

Magnetic susceptibility measurements were carried out by Gouy balance at room temperature using powdered samples of the complexes. Mercurytetrathiocyanatocobaltate(II), Hg[Co(NCS)₄], was used as the calibrant. Diamagnetic corrections were applied by summing up the Pascal’s constants for the diamagnetic contributions of various atoms of the molecule. The effective magnetic moment, \( \mu_{\text{eff}} \) was calculated using relation \( \mu_{\text{eff}} = 2.839 \sqrt{\chi'_{\text{m}} \times T} \). Where \( \chi'_{\text{m}} \) = corrected molar susceptibility, \( T \) = absolute temperature.

Spectral Techniques

Infrared spectra

The infrared spectra of the solid samples in the range 4000-200 cm\(^{-1}\) were recorded on a Perkin-Elmer 597/1650 spectrophotometer using KBr pellets.

Electronic spectra

Absorption spectra of the samples in aqueous solutions were recorded on a Schimadzu 160A / 240A UV – visible recording spectrophotometer.

Thermal analysis

Thermal analysis is a group of techniques in which a physical property of a substance is measured as a function of temperature whilst the substance is subjected to a controlled temperature (heating or cooling). This includes differential thermal analysis, thermogravimetry, differential thermal gravimetry, differential scanning calorimetry, thermomechanical analysis, thermomagnetometry, thermosonometry and so on. In many cases, the use of a single thermal analysis technique may not provide sufficient information and hence the use of other thermal analysis techniques either by independent or simultaneous measurements for complementary information becomes necessary. The TG and DTA experiments are mainly used in the present investigation.
The important factors affecting both TG and DTA curves have been discussed in greater detail in the literature\textsuperscript{295}. Some of the important ones are: nature and shape of the material used as a container, the sample weight and its history, the particle size, packing density of the sample, sensitivity of the thermocouple and its location, the heating rate employed, the surrounding atmosphere, etc.

**Differential thermal analysis**

The sample and an inert material are heated at a controlled heating rate and the temperature difference between them is recorded as a function of furnace temperature. The experiments were done in static or dynamic air. The heating rate employed was 10 °C min\textsuperscript{-1}. About 2-10 mg of well-packed powdered samples were used.

**Thermogravimetry**

Here, the mass of the substance under consideration is measured as a function of temperature whilst the substance is subjected to a controlled temperature (heating, cooling or constant temperature).

The TG experiments were carried out using a Philips General V2.2A Dupont 9900 Thermal Analyser, Shimadzu Japan DT-40 thermal analyzer or Perkin-Elmer TGS-2 Thermogravimetric System. Simultaneous TG-DTG was also carried out in TGD-5000RH, Thermo balance of ULVAC-RICO, Japan and Delta series TGA 7 or using a STA 1500 Thermal Analyzer. The heating rate employed was 10 °C min\textsuperscript{-1} in static or dynamic air. About 2-15 mg of the sample was used for each experiment.

**X-ray powder diffraction**

The X-ray diffraction patterns of the sample were obtained using a Philips X-ray Diffractometer with vertical goniometer, model PW 1050/70 using CuK\textalpha/CoK\textalpha radiation with nickel/ iron filter.
X-ray single Crystal study

Details of crystal data collection and refinement parameters for the complexes was performed on a BRUKER SMART APEX CCD type X-ray diffractometer system using graphite- monochromated MoKα radiation (\(\lambda = 0.7107 \text{Å}\)). The structures were solved by Patterson method\(^{298}\). The non-hydrogen atoms were refined with anisotropic thermal parameters by the full-matrix least squares on F\(^2\) method. All calculations were carried out using the SHELXL-97 program\(^{299}\) on PDP11/44 and Pentium MMX/166 computers.

Determination of antimicrobial activity

Cup- plate- agar- diffusion method

The anti bacterial activities of all the salts were studied by the usual cup-plate-agar-diffusion method\(^{296,297}\). The compounds were screened for their antibacterial activity against the following microorganisms: (a) gram positive staphylococcus aureus (S aureus) and bacillus subtilis (b) gram negative E coli and Pseudomonas aeruginosa. The cup-plate-agar-diffusion method comprises the following steps.

1. Preparation of media, sterilization, and tubing.
2. Sterilization of the cleaned glass apparatus.
3. Pouring of the seeded medium into sterilized petri dishes and cutting of the cups
4. Pouring of the dilute solution of the compounds into the tubs.
5. Incubation at a particular temperature.

The composition of the test media is the factor, which often exerts the greatest effect upon the drug activity. This is particularly true for the salts, since inhibitors of these compounds appear to be present in the common bacteriological culture medium. Efficient media of known chemical composition are available for many species such as S aureus and E.coli. In
addition to the composition of the test media, its pH is a factor which may directly or indirectly influence the activity of a compound. The pH of the test media taken for S aureus and E.coli was adjusted in the range 7.6 ± 0.1. The composition of the basal media used in the experiments was (i) sodium chloride = 6.0 g, (ii) peptone = 10.0 g, (iii) beef extract = 3.0 g, (iv) yeast extract = 2.0 g, (v) sucrose = 1.5 g, (vi) agar-agar = 3.0 % and (vii) distilled water = 1.0 litre.

**Method-I**

The measured quantity of culture of the test organism (0.5 ml) was added to each heated (nearly 55°C) agar-media tubes. The tubes were shaken well, and the inoculated media were poured on to the sterilized petri dishes and then allowed to set in a refrigerator maintained at 4-8 °C. The test solution of 500 µg/ml and 1000 µg/ml dilution of the respective ligands were prepared in distilled water.

Five cups of 5 mm diameter were cut in the culture media on the Petri dishes. A compound solution of particular dilution (500 µg/ml and 1000 µg/ml) was put in the outer four cups of one of the petri dishes, and the second solution was put in the four cups of other petri dishes. The central cups of all the petri dishes were filled with the controlled solution, and all the petri dishes were allowed to remain in the refrigerator maintained at 10 °C for 1 hour to allow diffusion of the solution. The patricides were then transferred to an incubator maintained at 35 °C and kept for nearly 30 hrs. The zones of inhibition formed were measured with calipers. The control of DMF and water (3:7, v/v) showed no activity. The activities of the compounds are represented by size of the diameter in mm. The antifungal activity of the compounds was screened by using filter paper disc diffusion method. The tests were carried out by taking 6 mm diameter filter paper discs against the fungi (A.niger and C.albicans ).
**Method-II**

The bacterial cultures were adjusted to 0.5 McFarland turbidity standards and inoculated onto Mueller Hintonagar (MHA, Oxoid) plates (diameter: 15 cm). A sterile cork borer was used to make a well (6 mm in diameter) on the MHA plates. The samples in distilled water at concentration of 200, 150, 100 and 50 mg/ml, and were applied in each of the wells in the culture plates previously seeded with the test organisms. The cultures were incubated at 37 °C for 24 hrs. A well was made in each of the culture plates and filled with 20 µl of 10 mg/ml of ciprofloxacin and streptomycin as positive controls, and sterile filter paper soaked in sterile glycerol served as a negative control. Antimicrobial activity was determined by measuring the zone of inhibition around each well (excluding the diameter of the well). For each extract, three replicate trials were conducted against each organism.

**Microorganisms**

The bacteria’s that were used for the bactericidal studies were *Staphyloccocuaureus*, *Eschereschiacoli*, *Pseudomonas aeruginosa*, and *Bacillus subtilis*. They were from the stock culture of the laboratory of the Sri.Ramakrishina Hospital, Coimbatore, Tamilnadu, India.

**Activity testing**

The antibacterial activity of the synthesized salts was determined by disc diffusion method. The bacteria were cultured in nutrient agar medium and used as inoculum for the study. Bacterial cells were swabbed onto nutrient medium in petri dishes. The test solutions were prepared in distilled water and applied to filter paper discs (Whatmann No 4, 5 mm diameter). These discs were placed on the already seeded plates and incubated at 37 °C for 24hrs. Ciprofloxacin were used as positive control.