

Circular Dichroism Studies of Complexation of Antibiotic (+)- Cephadrine With Cr(III), Co(II) and Ni(II) in Methanol: Water (80:20) Solution

Nasir AHMAD, Javed SAFDER, Christy MUNIR

*Department of Chemistry,
Quaid-i-Azam University, Islamabad-PAKISTAN*

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Circular dichroism (CD) studies have been utilized to investigate complexation of antibiotic (+)-cephadrine with Cr(III), Co(II) and Ni(II) ions in methanol: water (80:20 v/v) solution. The optimum conditions of pH, stoichiometry and time for achieving equilibrium for maximum complexation in these systems are determined. The CD spectra of these complex ions consist of 2-3 Cotton bands between 16,129-25,994 cm^{-1} with differential absorptivity 0.0046-0.428 $\text{deg. L mol}^{-1} \text{ cm}^{-1}$ and bands are assigned to electronic transitions. The formation constants of these complexes in mixed solvent are reported

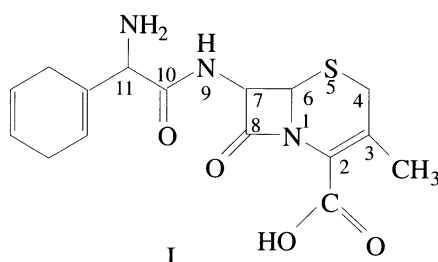
Key words: CD studies, (+)-cephadrine, Chromium (III)-(+)-ceph. complex, Cobalt (II)-(+)-ceph. complex, Nickel (II)-(+)-ceph. complex, Stability constants.

Introduction

A number of drugs, particularly antibiotics, are optically active and contain potential donor atoms. These drugs form complexes with metal ions and at the same time induce optical activity due to vicinal effect in complexes. A number of complexes of chiral antibiotics like (+)- cycloserine, (+)-penicillins, (+)-ampicillin, (+)-cephalexin and tetracyclines have already been reported ¹⁻⁶. However, most of these complexes are insoluble in water as well as in organic solvents, thus rendering them unsuitable for antibacterial studies. In biological systems soluble complexes are more effective against bacteria due to their fast absorption in blood plasma. Therefore, studies on soluble complexes of drugs furnish useful information about their mode of action in biological systems. In recent years, CD studies on complexation of Mg(II), Ca(II) and Cu(II) ions with (+)-tetracyclines in aqueous solution have been reported⁷. The complex forming ability of (+)-cycloserine with metal ions like VO(IV), Co(II) and Ni(II) at pH 4 have been reported from our laboratory⁸. Further, it has been observed that (+)-ampicillin forms water soluble complexes $[M((+) - amp)_2(H_2O)_2]^{2+}$ (M=Co(II), Ni(II) and Cu(II)) between pH 6.5-8.0⁹. The CD spectra were used to investigate complexation in these systems.

A recently developed antibiotic (+)-cephadrine (I) belongs to second generation cephalosporin, β -lactam drugs¹⁰. This antibiotic has great potential for coordination with metal ions since it contains a

number of donor atoms at appropriate position in the molecule. To the best of our knowledge, complexes of (+)-cephardine with transition metal ions under reference have not been reported.



As a part of our continued research, we have investigated the complexation (in situ) behaviour of (+)-cephardine with some transition metal ions in methanol: water (80:20 v/v) solution systems. In aqueous solution, this ligand forms insoluble complexes. Therefore, complexation studies were carried out in a mixed solvent system.

In the present paper, CD has been utilized to investigate the complexation potential of (+)-cephardine with Cr(III), Co(II) and Ni(II) in $MeO : H_2O$ solution. In this solvent system, complex ions $[Cr((+) - ceph.)_2(H_2O)_2]^{3+}$, $[Co((+) - ceph.)_2(H_2O)_2]^{2+}$ and $[Ni((+) - ceph.)_3]^{2+}$ are formed between pH 5.1-6.80 and fairly strong CD bands between 16,129-25,944 cm^{-1} are developed. The stability constants ($\log \beta$) of these complex ions are determined.

Experimental

All reagents and solvents of analytical reagent grade (Merck or Fluka) were used without further purification. The antibiotic (+)-cephardine (as free base) was obtained from Squibb (Pakistan) Ltd. Karachi. The drug was stored in a refrigerator at 4°C. Its purity was checked from time to time by infra-red and specific rotation measurements. Methanol was distilled before its use and mixed solvent was prepared from double distilled water.

Solutions

(i) Stock solution of (+)-cephardine:

Accurately weighed 0.8735 g (0.0025 mol) of the drug was dissolved in about 50 ml of mixed solvent with thorough shaking till a clear solution was obtained. This was diluted to 100 ml with the solvent. Solutions of appropriate concentrations were obtained by diluting the stock solution. Since the solutions of (+)-ceph. are unstable, fresh solutions were prepared each time.

(ii) Metal salt solutions:

These were obtained by dissolving appropriate amounts of hydrated metal chlorides in the mixed solvent and diluted to 100 mL so as to constitute 0.01-0.025 mol L^{-1} solutions. Solutions of requisite concentrations were prepared by diluting appropriate volumes of the stock solutions with mixed solvent. 3.512g (0.25 mol L^{-1}) of $NaClO_4$ was dissolved in the solvent and diluted to 100 mL.

(iii) Solutions for complexation studies:

To study the effect of pH on complexation, a series of solutions was prepared by mixing 1.0 mL of metal ion (0.025 mol L^{-1}) and 3.0 mL of ligand solution (0.025 mol L^{-1}). pH of these solutions was varied

between 1.50-8.50 or up to the values when precipitation occurred. The pH of these solutions was adjusted with dilute solution of (0.05 mol L^{-1}) NaOH. The samples were kept in dark for 8.0 hours and their CD spectra were recorded. The differential optical density ($\Delta O.D.$) at Cotton band maxima was plotted as a function of pH (Figure 1). From these plots the optimum pH for each metal-ligand system was selected. The appropriate pH for each system was maintained during subsequent studies.

The method of continuous variation of molar ratios was applied to determine the metal to ligand ratio of complexes formed in solution. Keeping sum of moles of metal ions and ligand constant, a number of sample solutions were prepared by mixing gradually increasing volumes of metal ion solution and decreasing volume of ligand solution. These solutions were brought to optimum pH with dilute solution of NaOH. These solutions were kept in dark for 8.0 hours and their CD spectra were recorded. A plot of $\Delta O.D$ at CD band maxima vs mole fraction of the metal ions was prepared (Figure 2). The curves drawn by a computer programme were extrapolated and from the point of intersection of two straight lines, stoichiometry of the complexes was determined.

A number of sample solutions were prepared by mixing 1.00 mL of the stock metal ion solution and 0.66-6.66 mL of ligand stock solution. Ionic concentration of background electrolyte $NaClO_4$ was maintained at 0.025 mol L^{-1} in these samples. The samples were adjusted to appropriate pH and kept in dark for 8 hours before recording their CD spectra. It was observed that Cotton band intensity gradually increased with increasing concentration of the ligand and became constant above certain limits. From the maximum $\Delta O.D.$, $\Delta\epsilon$ was calculated for each system. These values of $\Delta\epsilon$ were subsequently used to estimate equilibrium concentrations of the metal complex, free metal ion and free ligand in a number of systems and finally stability constants were determined.

Instrumentation

Absorption spectra in the region 200-700 nm were measured on Shimadzu model UV 265 recording spectrophotometer using a pair of 1 cm quartz cuvettes. pH measurements were carried out on F-8 Horbia pH meter equipped with a combination electrode system. Circular dichroism (CD) spectra, of the solutions in 200-700 nm region were measured on Jasco-20A spectropolarimeter using 1 cm cylindrical quartz cell. The base lines were recorded using pure (+)-cephradine solution at appropriate pH in $MeOH : H_2O(80 : 20)$.

Results and Discussion

When aqueous solutions of the metal ions and (+)-cephradine ((+)-ceph.) were mixed in 1:3 ratio, either precipitation occurred immediately or there was no indication of complexation. In strongly acidic solutions (pH 1-2), the two components remained in solution without appearance of CD bands in visible region indicating the absence of any interaction between the metal ions and (+)-ceph. The chiral ligand as well as metal salts are fairly soluble in $MeOH : H_2O$ (80:20) mixed solvent. Interaction of Cr(III), Co(II) and Ni(II) ions with (+)-ceph. in $MeOH : H_2O$ solution was indicated by appearance of fairly strong Cotton bands in the crystal field region. The Cr(III)-(+)-ceph. complex in $MeOH : H_2O$ has a broad, positive Cotton band at 18,797 cm^{-1} while Co(II)-(+)-ceph. and Ni(II)-(+)-ceph. have CD bands at 17,852 cm^{-1} and 16,129 cm^{-1} , respectively. Subsequent studies were carried out at wavelength of their CD band maxima.

The complexation in these systems is dependent upon pH. In Cr(III)-(+)-ceph., the ellipticity of CD band rapidly increased as pH of the system was raised to 5.2 and then sharply decreased beyond pH 5.2. Precipitation occurred near pH 7 in this system. In Co(II)-(+)-ceph., the ellipticity gradually increased

with variation in pH upto 7.5. Precipitation of the complex occurred beyond pH 7.5. In case of Ni(II)-(+)-ceph., ellipticity of Cotton band increased with pH upto 7.0. A slight decrease in ellipticity was observed at higher pH but it remained constant between pH 7.5-8.0. As pH of the system was raised to 8.5, immediate precipitation occurred. Therefore, further studies on Cr(III)-(+)-ceph., Co(II)-(+)-ceph. and Ni(II)-(+)-ceph. systems were carried out at pH 5.1, 6.8, and 6.2 respectively.

The complexation in these systems is affected with time. In Cr(III)-(+)-ceph. system a couplet of oppositely signed CD bands was initially observed upon mixing the components. The ellipticity of negative band rapidly increased with time while relatively less increase in ellipticity of positive band was observed. In case of Co(II)-(+)-ceph. and Ni(II)-(+)-ceph., ellipticity of all CD bands gradually increased with time. After about eight hours, ellipticity of Cotton bands became constant in all of these systems. Therefore, CD spectra of these systems were recorded after keeping the samples for eight hours in the dark.

Composition of the complexes formed in MeOH: H_2O mixed solvent was determined by varying mole fractions of the metal ions and (+)-ceph. maintained at appropriate pH and measuring the ellipticity at CD band maxima as shown in Figure 2. It is apparent from these plots that two molecules of (+)-ceph. are bonded with each of Cr(III) and Co(II) while three molecules of the ligand are coordinated with Ni(II) ion.

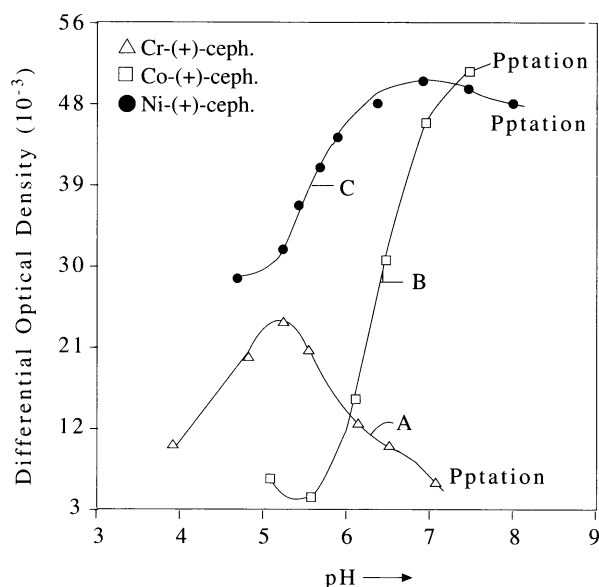


Figure 1. Effect of pH upon differential optical density at Cotton band maxima of Metal-(+)-cephradine systems. $[M^{n+}] = 6.25 \times 10^{-3} M$ $[L] = 1.88 \times 10^{-2} M$

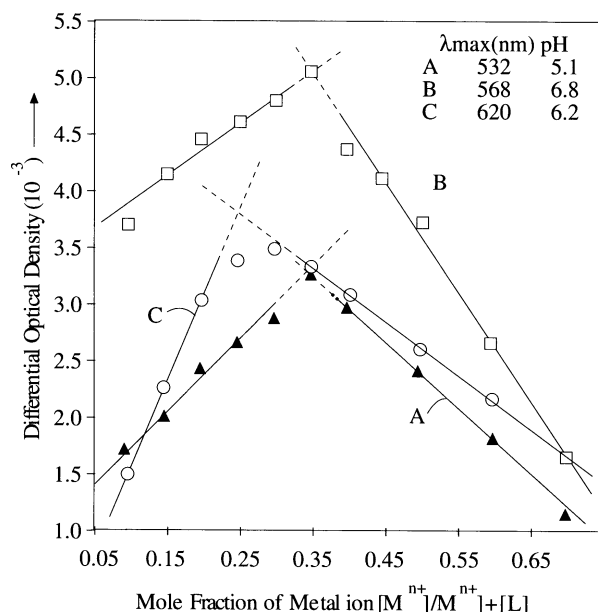
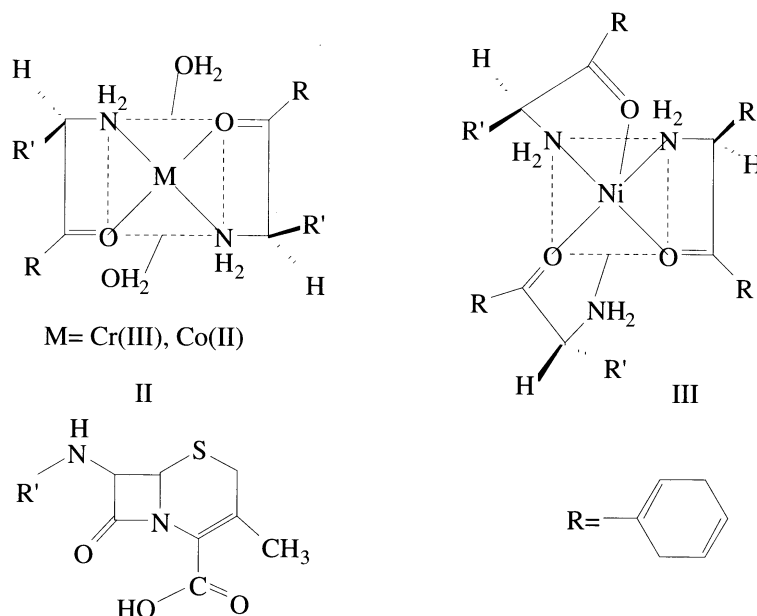


Figure 2. Mole ratio plot for determining stoichiometry of metal complexes of (+)-cephradine in methanol: water solution. (A).Cr(III)-(+)-ceph. (B).Co(II)-(+)-ceph. (C). Ni(II)-(+)-ceph., $[M^{n+}] = [L] = 2.5 \times 10^{-2} M$

An equilibrium is achieved amongst the complex ion formed, free metal ion and the ligand upon mixing solutions of the metal ion and (+)-ceph. Keeping metal ion constant, a gradual increase in concentration of the ligand will shift the equilibrium towards right resulting in the increase of ellipticity of Cotton band. If the ligand is in large excess, practically all of metal ion will be complexed and concentration of the free metal ion may be assumed as negligible. Further increase of ligand in the system should have little effect upon ellipticity of Cotton bands. This method was used to determine molar differential absorptivity ($\Delta\epsilon$) of CD bands in these systems. The calculated differential absorptivity values were used to estimate equilibrium concentrations of the complex ions, free metal ion and free ligand in a number of systems of varying concentrations and their overall stability constants were determined. The stability constants decrease in the

order as $[\text{Ni}((+)\text{-ceph.})_3]^{2+} > [\text{Cr}((+)\text{-ceph.})_2(\text{H}_2\text{O})_2]^{3+} > [\text{Co}((+)\text{-ceph.})_2(\text{H}_2\text{O})_2]^{2+}$. $[\text{Ni}((+)\text{-ceph.})_3]^{2+}$ is more stable than other two complexes.

A number of donor atoms are present at various positions in the ligand. Two donor atoms S-5 and N-1 in (+)-ceph., present in a bicyclic ring, cannot effectively form a chelate with metal ion. However, bidentate chelation of the ligand through its S-5 and N-9 or N-1 and carboxylic oxygen attached to C-2 or primary amine at C-11 and oxygen attached to C-10 or N-9 is possible. It may act as a tridentate ligand through donor atoms N-9, primary amine attached to C-11 and carbonyl oxygen at C-8 or S-5. However, the molecule will be highly strained if coordinated with a metal ion as a tridentate ligand. Optical activity of (+)-ceph. arises from a number of asymmetric atoms such as C-6, C-7 and C-11. The presence of a chiral centre in the chelate has a profound effect upon optical activity of the complex and fairly strong CD bands are observed in the crystal field region. In the present series of complexes, fairly strong and well defined CD bands indicate that at least bidentate chelation incorporating an asymmetric atom, has occurred in these systems. It is therefore proposed, that the metal ions are bonded through $-\text{NH}_2$ of C-11 and carbonyl oxygen of C-10 of the ligand. In this bonding situation, asymmetric carbon C-11 is a part of the chelate. Since Cr(III) and Co(II) are bound with two molecules of the ligand, remaining two coordination sites are occupied by water molecules resulting octahedral species in solution as shown in II. Further, $[\text{Ni}((+)\text{-ceph.})_3]^{2+}$ should also be octahedral due to bidentate chelation of three ligands as III. In this bonding situation, Cr(III) and Co(II) complexes have D_{2h} or C_{2v} local symmetry while $[\text{Ni}((+)\text{-ceph.})_3]^{2+}$ has C_3 or C_2 symmetry. However, overall symmetry of these complexes cannot exceed C_1 due to the presence of an asymmetric carbon atom in the chelate.



The absorption spectrum of $[\text{Cr}((+)\text{-ceph.})_2(\text{H}_2\text{O})_2]^{3+}$ ion consist of a broad band at $19,011 \text{ cm}^{-1}$ ($\epsilon 59 \text{ L. mol}^{-1} \text{ cm}^{-1}$) while its CD shows three bands of alternating sign i.e; a stronger positive band I at $18,797 \text{ cm}^{-1}$ ($\Delta\epsilon 0.388 \text{ deg. L mol}^{-1} \text{ cm}^{-1}$), a very weak negative band II at $21,739 \text{ cm}^{-1}$ and another strong positive band III at $25,974 \text{ cm}^{-1}$ ($\Delta\epsilon 0.182 \text{ deg. L mol}^{-1} \text{ cm}^{-1}$).

Octahedral complexes of Cr(III) ion (d^3) have ${}^4A_{2g}$ ground state and three excited states ${}^4T_{1g}$, ${}^4T_{2g}$ and ${}^4T_{1g}$ (P). The absorption spectra of Cr(III) complexes in octahedral environment usually consist of three bands due to spin allowed ν_1 , ν_2 and ν_3 transitions. The degenerate states ${}^4T_{1g}$ and ${}^4T_{2g}$ further split to give unidimensional (A, B) levels in low symmetry complexes of this ion. The excitation of electrons to split

levels in this system gives rise to separate CD bands. The absorption band in $[\text{Cr}((+)\text{-ceph.})_2(\text{H}_2\text{O})_2]^{3+}$ at 19.011 cm^{-1} may be assigned to ν_1 transition. The positive CD band I in this system may be due to excitation of electrons to ${}^4A_2 \longrightarrow {}^4B_1$ and band II to ${}^4A_2 \longrightarrow {}^4B_2$, component of ${}^4T_{2g}$ state of octahedral symmetry. The CD band III in this complex may be assigned to ${}^4A_2 \longrightarrow {}^4B_2$ (components of ${}^4T_{1g}$ state) transition.

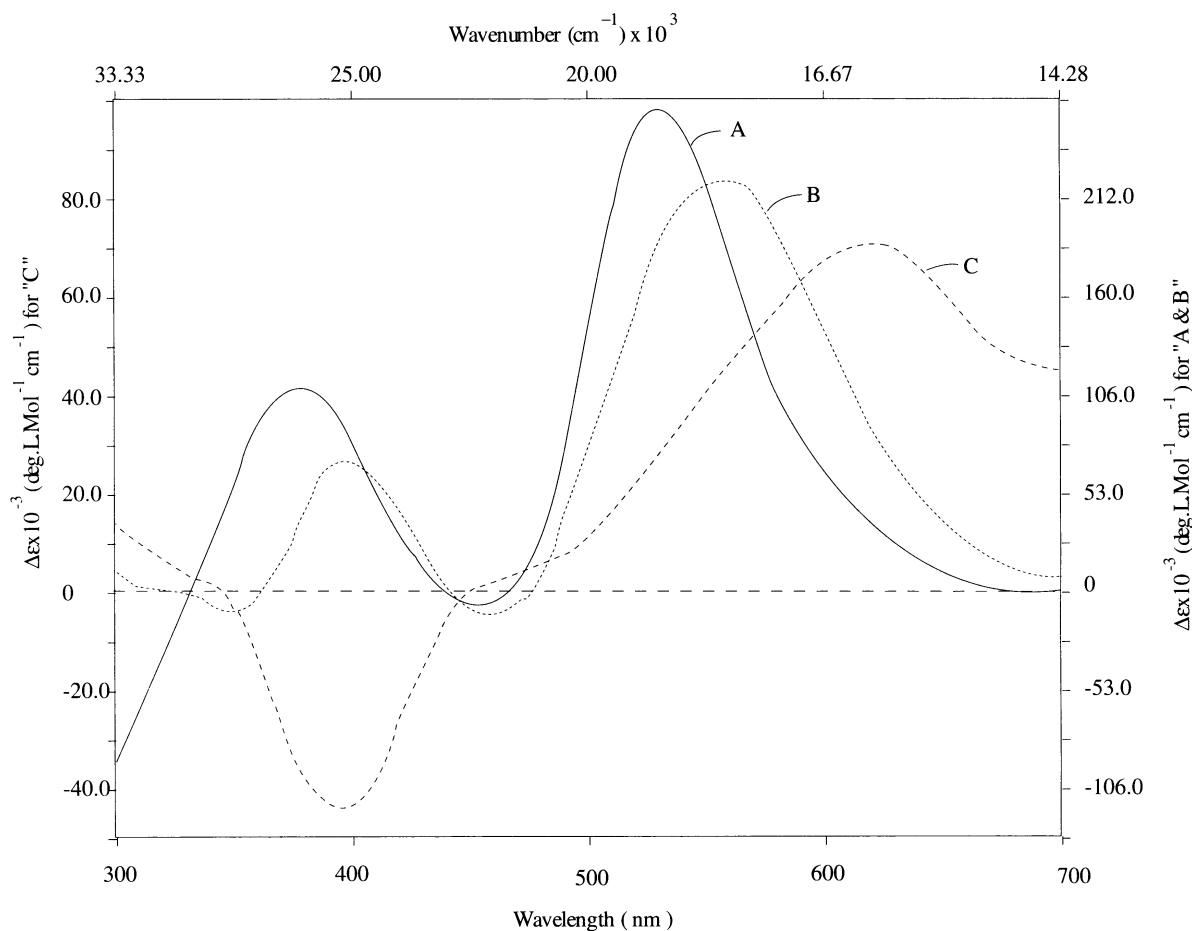


Figure 3. Circular Dichroism Spectra of: (A). $[\text{Cr}((+)\text{-ceph.})_2(\text{H}_2\text{O})_2]^{3+}$ (Conc. $4.20 \times 10^{-3} M$) (B). $[\text{Co}((+)\text{-ceph.})_2(\text{H}_2\text{O})_2]^{2+}$ (Conc. $3.16 \times 10^{-3} M$) (C). $[\text{Ni}((+)\text{-ceph.})_3]^{2+}$ (Conc. $2.5 \times 10^{-2} M$) formed in $\text{MeOH}:\text{H}_2\text{O}$ solution

The absorption spectrum of $[\text{Co}((+)\text{-ceph.})_2(\text{H}_2\text{O})_2]^{2+}$ ion formed in $\text{MeOH}:\text{H}_2\text{O}$ mixed solvent consists of a band at $25,575 \text{ cm}^{-1}$ (ϵ $347 \text{ L. mol}^{-1} \text{ cm}^{-1}$). The CD of this. Ion consists of a positive Cotton band at $17,857 \text{ cm}^{-1}$ ($\Delta\epsilon$ $0.064 \text{ deg. L mol}^{-1} \text{ cm}^{-1}$), a weaker negative Cotton band at $21,739 \text{ cm}^{-1}$ ($\Delta\epsilon$ $0.0046 \text{ deg. L mol}^{-1} \text{ cm}^{-1}$) and another positive band at $25,994 \text{ cm}^{-1}$ ($\Delta\epsilon$ $0.0233 \text{ deg. L. mol}^{-1} \text{ cm}^{-1}$). In octahedral environment, Co(II) , d^7 ion, excitation of electrons occurs from ${}^4T_{1g}$ (ground state) to ${}^4T_{2g}$, ${}^4A_{2g}$ and ${}^4T_{1g}$ (P) states. However, in this case, ${}^4T_{1g}$ and ${}^4T_{2g}$ states further split to give unidimensional levels due to lower symmetry. The CD band at $17,857 \text{ cm}^{-1}$ in $[\text{Co}((+)\text{-ceph.})_2(\text{H}_2\text{O})_2]^{2+}$ may be assigned to ${}^4A_2 \longrightarrow {}^4B_1$ while band at $21,739 \text{ cm}^{-1}$ to ${}^4A_2 \longrightarrow {}^4B_2$, components of ${}^4T_{1g}$ (P) state of octahedral symmetry. The positive Cotton band at $25,994 \text{ cm}^{-1}$ may be due to a low energy charge transfer (MLCT) transition. These band assignments agree well with already reported for $[\text{Co}((+)\text{-CCS})_3]^{2+}$ ($19,231 \text{ cm}^{-1}$ and $23,256 \text{ cm}^{-1}$) and for $[\text{Co}((+)\text{-amp.})_2(\text{H}_2\text{O})_2]^{2+}$ at $19,231 \text{ cm}^{-1}$ and $23,697 \text{ cm}^{-1}$ ^{8,9}.

The absorption spectrum of $[\text{Ni}((+)\text{-ceph.})_3]^{2+}$ ion consists of a broad band at $25,641 \text{ cm}^{-1}$ (ϵ 228

L. mol⁻¹ cm⁻¹) due to ν_3 . The CD of this complex ion consists of two positive Cotton bands at 16,129 cm⁻¹ ($\Delta\epsilon$ 0.422 deg. L. mol⁻¹ cm⁻¹) and 21,505 cm⁻¹ (shoulder) and a negative band at 25,000 cm⁻¹ ($\Delta\epsilon$ 0.30 deg. L. mol⁻¹ cm⁻¹). The sequence of highest spin energy levels for octahedral complexes of Ni(II) (d^8) is similar to that of Cr(III) (a d^3 system) and hence three bands due to spin allowed transitions are expected. Therefore, Cotton band at 16,129 cm⁻¹ in this system may be assigned to ${}^3A_2 \longrightarrow {}^3A_1$ (${}^3T_{1g}$), second band at 21,505 cm⁻¹ to ${}^3A_2 \longrightarrow {}^3A_1$ and third at 25,000 cm⁻¹ to ${}^3A_2 \longrightarrow {}^3B_1$ (${}^3A_1, {}^3B_1$ being components of ${}^3T_{1g}$ (P) state of Oh symmetry) transitions. These band assignments are comparable with Cotton bands observed at 18,868 cm⁻¹ and 22,723 cm⁻¹ for $[\text{Ni}((+)\text{-amp.})_2(\text{H}_2\text{O})_2]^{2+}$ in aqueous solution⁹.

It may be concluded from preceding discussion that (+)-cephradine forms soluble complexes with Cr(III), Co(II) and Ni(II) in a mixed solvent MeOH:H₂O (80:20 v/v). CD studies of these complex ion systems may be used to determine their stoichiometry, stability constants and for the assignment of bands to excitation of electrons to higher levels.

Table 1. Optimum conditions of pH, absorption and CD spectra of Metal-(+)-ceph. systems in methanol: water (80:20 v/v) solution

Complex ion	pH	Stability Constant (log β)	Absorption spectra		CD spectra		
			ν cm ⁻¹)	ϵ	ν (cm ⁻¹)	$\Delta\epsilon$	Band Assignment
$[\text{Cr}((+)\text{-ceph})_2(\text{H}_2\text{O})_2]^{3+}$	5.1	3.81 ± 0.25	19,011	59	18,797	+0.3878	${}^4A_2 \longrightarrow {}^4B_1$ (${}^4T_{2g}$)
					21,739	-0.0243	${}^4A_2 \longrightarrow {}^4B_2$ (${}^4T_{2g}$)
					25,974	+0.1818	${}^4A_2 \longrightarrow {}^4B_2$ (${}^4T_{1g}$)
$[\text{Co}((+)\text{-ceph})_2(\text{H}_2\text{O})_2]^{2+}$	6.8	2.36 ± 0.003	25,575	347	17,857	+0.0640	${}^4A_2 \longrightarrow {}^4B_1$ (${}^4T_{1g}$)
					21,739	-0.0046	${}^4A_2 \longrightarrow {}^4B_2$ (${}^4T_{1g}$)
					25,994	+0.0233	(MLCT)
$[\text{Ni}((+)\text{-ceph})_3]^{2+}$	6.2	6.86 ± 0.25	25,641	228	16,129	+0.4218	${}^3A_2 \longrightarrow {}^3A_1$ (${}^3T_{1g}$)
					21,505	+0.0469	${}^3A_2 \longrightarrow {}^3A_1$ (${}^3T_{1g}$)
					25,000	-0.2999	${}^3A_2 \longrightarrow {}^3B_1$ (${}^3T_{1g}$)

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References

1. C. Preti and G. Tosi, **Aust. J. Chem.**, **33**, 57 (1980).
2. (a). M. Asso, R. Panossian, M. Guiliano, **Spectros. Lett.** **17**, 271 (1984). (b). M. A. Chapp, M. Phil Dissert. Q. A. Univ. Islamabad (1992).
3. (a). A. M. S. Alam, A. K. Ghose, M. A. Hossain, **J. Bangladesh Chem. Soc.**, **1**, 17 (1988). (b). P. J. Martinez, P. Gutierrez, M. I. Martinez, J. Thomas, **An. R. Acad. Farm.**, **51**, 721 (1985). (c). P. Martinez, P. Gutierrez, M. I. Martinez and A. Cardoba., **An. Quim. Ser. B.**, **85**, 122 (1989).

4. (a). J. M. Moratal, J. Borrás, A. Donaise, M. J. Martínez, **Inorg. Chim. Acta.**, **162**, 113 (1989). (b). M. A. Chughtai, M. Phil. Dissert. Q. A. Univ. Islamabad (1990).
5. (a). M. Jelíkie-Stankov, D. Maleševs, D. Veselinović, and Z. Radović, **Polyhedron**, **10**, 455, (1991). (b). N. Ahmad, M. Jamil and C. Munir, **J. Chem. Soc. Pak.** **12**, 168 (1990).
6. (a). J. Magnan, D. Barthes, J. J. Giraud, **Ann. Pharm. Fr.** **44**, 467 (1986). (b). S. S. M. Hassan, M. M. Amer, S. A. Ahmad, **Mikrochim. Acta.**, **3**, 165 (1984). (c). T. K. Katakwar and M. S. Kacchawaha, **Hind. Antibiot. Bull.**, **26**, 9 (1984).
7. (a). L. Lambs, B. Becock-Le Reverend, H. Kozłowski and G. Berthan, **Inorg. Chem.** **27**, 3001 (1988). (b). M. Jezowska-Bojczuk, L. Lambs, H. Kozłowski and G. Berthan, **Inorg. Chem.** **32**, 428 (1993).
8. N. Ahmad, C. Munir, M. Jamil and S. Rauf, **J. Chem. Soc. Pak.** **15**, 190 (1993).
9. N. Ahmad, M. Jamil and C. Munir. **J. Chem. Soc. Pak.**, **17**, 149 (1995).
10. (a). J. E. Dolfini, H. E. Applegate, G. Bach, H. Basch, J. Bernstein, J. Schwartz and F. L. Weisenborn. **J. Med. Chem.** **14**, 117 (1971). (b). F.L. Weisenborn et. al. US. Pat. 3, 485, 819 (1969). (c). K. Florey Ed. in **Analytical Profiles of Drug Substances**. vol. 5, Academic Press, New York, pp 21-59 (1976).