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Gallium-71, Carbon-13 and Hydrogen-1 NMR Studies of the Interactions of Pyridoxal 5'-Phosphate Isonicotinyl Hydrazone with Gallium in Aqueous Solution

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Ga(III) binding by the new ligand, pyridoxal 5'-phosphate isonicotinyl hydrazone (PPL-INH) has been investigated by ^{71}Ga, ^{13}C and ^{1}H NMR. PPL-INH forms a strong complex with gallium. ^{71}Ga NMR studies are based on the observation of the $Ga(OD)_4^-$ signal, which allows the determination of the 2:1 stoichiometry for the complex. The complexation sites are shown by ^{13}C NMR. The complex is water-soluble from pH 6 to pH 11, a deprotonated form of the ligand being stabilized by gallium.

This work initiates the study of pharmacologic properties of a family of complexes of gallium with hydrazones.

Introduction

Interest in the biological aspects of gallium chemistry has increased rapidly during these last few years. Gallium-67, currently used in nuclear medicine is actually considered as one of the best antitumour scanning agents [1–6]. Under the $Ga(NO_3)_3$ form, gallium presents significant antitumour activity currently under clinical and pharmacokinetic investigation [7, 8]. Research into the mechanism of ^{67}Ga localization has proceeded along two lines: *in vitro* studies of the uptake of gallium by normal and malignant cells as well as NMR studies on the aqueous chemistry of gallium [14–16]. These latter ones concern mainly gallium citrate and more recently, interactions of gallium with various buffers and chelating agents [16]. Our intention is to extend this approach to other chelating agents. We are interested in several families of ligands and, in the first place, in hydrazones obtained by the action of pyridoxal phosphate on hydrazines or hydrazides which are known for their IMAO properties [17] or as an antituberculous drug [18].

We present here the results of ^{71}Ga, ^{13}C and ^{1}H NMR studies of interactions of Ga^{3+} with pyridoxal 5'-phosphate isonicotinyl hydrazone (L = PPL-INH) obtained with pyridoxal phosphate (PPL) and isonicotinic acid hydrazide (INH) (Fig. 1).

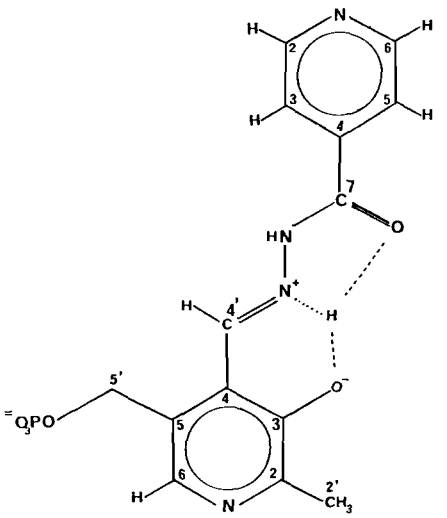

Fig. 1. Pyridoxal 5'-phosphate isonicotinyl hydrazone.

Experimental

Materials

$Ga(NO_3)_3 \cdot 8H_2O$ (Merck), isonicotinic acid hydrazide (Interchim), pyridoxal 5'-phosphate (Merck) and D_2O (Merck) were used as commercially supplied. In D_2O solution the pDc (pH + 0.40) was adjusted with DCl and NaOD, using a Tacusel 2G8N pH-meter.

Preparation of PPL-INH

PPL-INH has been prepared by mixing water–methanolic (50–50) solutions of PPL and INH and raising the pH to 8.0 by addition of NaOH. The solution is then stirred and gently refluxed during 24 hours. The resulting product is recrystallized by evaporation of the solvent. The I.R. spectrum shows

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an intense band at 1650 cm^{-1} characteristic of the >C=N– moiety [19].

NMR Spectra

All spectra were measured at ambient probe temperature (300 K). The ^{13}C NMR spectra with an without proton decoupling were obtained on a P.F.T. Brucker WH 90 spectrometer working at 22.62 MHz. Number of scans varied from 5000 to 30 000, spectral band-width of 6 KHz, 8 K memory, repetition rate of 3 s and impulsion close to 60°. A gated decoupling technique was used to obtain the proton coupled spectra. The timing sequence is: proton irradiation 2.0 s, waiting time 0.2 s, impulsion at the ^{13}C frequencies 8 μs, acquisition time 0.8 s, rest time 0.3 s with total time of 3.3 s. All the chemical shifts δC-i are given in ppm relative to TMS.

^{1}H NMR spectra were obtained on the same apparatus at 90 MHz. All the chemical shifts δH_i are given in ppm relative to TMS.

^{71}Ga NMR spectra were also obtained with the same apparatus working at the ^{13}C frequency of 22.62 MHz, with drop in field intensity, and by F.T. 5 000 scans were accumulated with a pulse RF of 20 μs. ^{71}Ga resonance in $Ga(OD)_4^-$ occurs at 548 Hz from the external $GaCl_4^-$ reference. A 1.0 *M* solution of gallium was prepared by dissolving the appropriate amount of $Ga(NO_3)_3 \cdot 8H_2O$ in D_2O. Solutions containing the given concentrations of the ligand were prepared by dissolving the appropriate amount of the solid ligand in 1.0 *M* gallium solution.

Results and Discussion

^{71}Ga NMR

The formation of complexes between PPL-INH and gallium nitrate has been studied by ^{71}Ga NMR. The resonance (spin $\frac{3}{2}$ nucleus) can be detected only when gallium is in a highly symmetrical environment, because of its quadrupole electric moment (0.11–0.15 e 10^{-24} cm^2). This parameter appears in the spin lattice relaxation time relation:

$$\frac{1}{T_1} = A \frac{2I+3}{I^2(2I-1)} \cdot \left(1 + \frac{\eta^2}{3}\right) \cdot \frac{(e^2qQ)^2}{h} \cdot \tau_C$$

T_1 depends more particularly on the quadrupolar moment and the asymmetrical parameter η of the molecular environment. For gallium nitrate solutions in D_2O it is possible to observe ^{71}Ga resonance in $Ga(D_2O)_6^{3+}$ in the case of acidic solutions and ^{71}Ga resonance in $Ga(OD)_4^-$ in the case of basic solutions.

Stability of PPL-INH in relation with pH allows us to undertake the study of basic solutions at pD 10.3 and to observe the resonance of $Ga(OD)_4^-$. Figure 2 shows spectra of a 1 *M* $Ga(NO_3)_3$ solution in D_2O at this pD, in which increasing amounts of ligand are added. The decrease of resonance intensity observed by adding hydrazone is in relation with the formation of a gallium complex and consequent drop symmetry. The accompanying broadening of the peak can be assigned to an increase in the viscosity of the solution by addition of ligand [20]. In Fig. 3 we have represented the variations of integrated intensities of the ^{71}Ga signal in $Ga(OD)_4^-$ in relation with the ratio PPL-INH: Ga^{3+} at pD 10.3 (a) and pD 11.5 (b). Curve (a) shows that the stoichiometry of the complex is 2 ligands for 1 Ga^{3+} and that gallium is in slow exchange between the free and complexed forms. Curve (b) shows us that 40% of the gallium is present in the $Ga(OD)_4^-$ form at pD 11.5, when in dissociation equilibrium with the 2:1 complex at this pD. This result is quite comparable with the one obtained by Chang *et al.* [16] for the complex of gallium with EDTA.

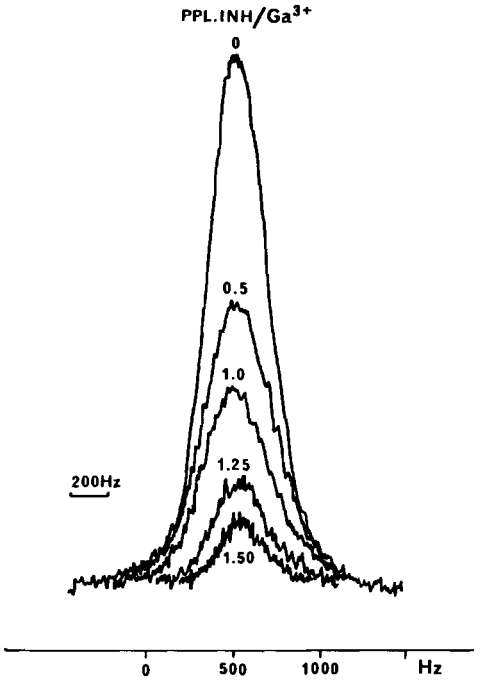

Fig. 2. ^{71}Ga NMR spectra of 1.0 *M* $Ga(NO_3)_3$ in D_2O (pD = 10.3) after addition of 0, 0.5, 1.0, 1.25 and 1.50 equivalents of PPL-INH.

^{13}C NMR spectra of 0.5 *M* PPL-INH solutions were obtained at several pD (Table I). The peaks were assigned with the help of known assignments of related species [21, 22], evolution of these shifts in relation with pD, multiplicity of peaks by $^{n}J_{C-H}$ coupling on spectra obtained by the gated-decoupling technique.

The δC-4'PPL value (≃ + 150 ppm) indicates hydrazone formation. This value is however far from the one observed for this carbon in the case of aldimines [21] (δ > 160 ppm). This observation is

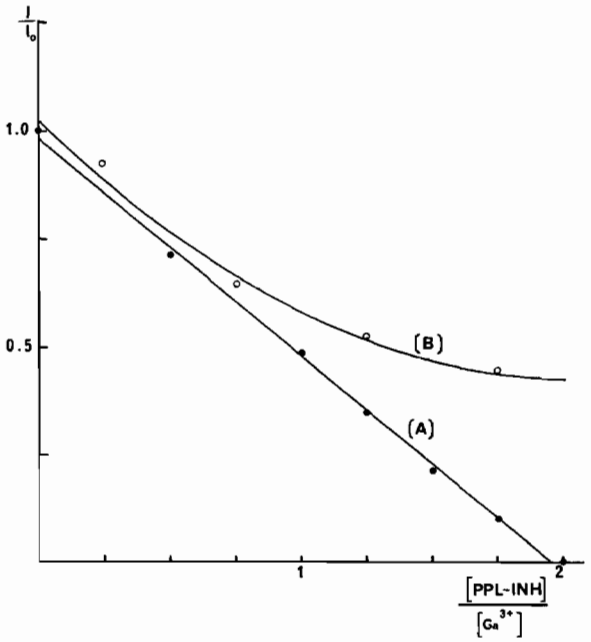

Fig. 3. The relative intensities of the $Ga(OD)_4^-$ resonance solutions in the presence of PPL-INH.

also available for the other carbons of PPL involved in this molecule. The C-2'PPL value (δ = +19.1 ppm) at pD 7.4 indicates that PPL pyridinium function is strongly deprotonated at this pD when the same value can be observed in the case of free PPL only at pD $\simeq$ 10.

The pyridinium pk_A falls with respect to free PPL, as also established in the case of aldimines [21, 23]. The INH pyridinium function is also deprotonated at pD 7.4 since δ C-2,6 INH does not vary significantly from pD 7.4 to pD 10.3. Between these two pD we can notice clear differences for C-2 PPL and C-3 PPL chemical shifts. These differences are only assigned to the deprotonation of nitrogen involved in the hydrazone function.

TABLE II. Effect of Gallium on the δ ^{13}C-i of PPL-INH at pD = 10.3.

	Carbons	PPL-INH pD = 10.3	PPL-INH/Ga^{+++} pD = 10.3	Δ = (2) – (1)
PPL	C-2	152.6	156.1	+3.5
	C-3	159.6	160.3	+0.7
	C-4	121.6	122.4	+0.8
	C-5	132.1	133.2	+1.1
	C-6	136.1	136.9	+0.8
	C-2'	19.9	20.0	+0.1
	C-4'	149.6	156.8	+7.2
	C-5'	63.4	63.9	+0.5
INH	C-2,6	150.8	151.4	+0.6
	C-3,5	123.7	123.9	+0.2
	C-4	144.7	143.5	–1.2
	C-7	169.2	171.3	+2.1

Disappearance of hydrogen bonding (Fig. 1) and conformational modifications go with the deprotonation. Table II shows the effects of the introduction of gallium nitrate in a PPL-INH solution on δ ^{13}C values. The same results are obtained for the ratio L: Ga^{3+} = 1 and L: Ga^{3+} = 2. The presence of free ligand is only observed in spectra from a ratio L: $Ga^{3+} > 2$ (slow exchange) and up to a pD of 11. This last result corroborates the conclusion of the ^{71}Ga NMR study Fig. 3 (b)) indicating complex dissociation under strongly basic conditions.

The variations Δ = (2) – (1) registered for the δ ^{13}C-i values between free ligand and complexed ligand are compatible with a chelation of Ga^{3+} by

TABLE I. PPL-INH δ ^{13}C-i (ppm/TMS) *vs.* pD.

	Carbons	PPL pD = 7.4	PPL pD = 10.3	INH pD = 10.3	PPL-INH pD = 7.4	PPL-INH pD = 8.9	PPL-INH pD = 10.3
PPL	C-2	152.6	157.4	–	149.9	150.7	152.6
	C-3	165.3	168.1	–	153.0	154.9	159.6
	C-4	127.0	124.8	–	122.5	123.9	121.6
	C-5	137.0	133.3	–	131.6	132.5	132.1
	C-6	125.3	129.1	–	139.3	138.5	136.1
	C-2'	16.8	19.8	–	19.1	19.3	19.9
	C-4'	197.7	197.4	–	149.2	150.2	149.6
	C-5'	62.7	63.3	–	63.3	63.4	63.4
INH	C-2,5	–	–	151.2	151.1	151.3	150.8
	C-3,5	–	–	125.0	123.1	123.0	123.7
	C-4	–	–	143.7	139.3	141.5	144.7
	C-7	–	–	167.6	163.3	166.0	169.2

oxygen C-3 PPL-O^-, nitrogen C-4′ PPL-N and oxygen C-7 INH-O. Large effects are observed for C-4′ PPL (+7.2 ppm), C-7 INH (+2.1 ppm) and C-2 PPL (+3.5 ppm). The C-3 PPL value (Δ = +0.7 ppm) seems to be weak but can be explained by the disappearance of hydrogen binding on the phenolate anion occurring with the chelation step. Stabilization by complexation of a deprotonated form of the ligand will also be shown by ^{1}H NMR. The existence of three sites of complexation on the ligand is in good agreement with a 2:1 stoichiometry found for this complex, leading to a classical octahedral geometry around the metal ion.

^{1}H NMR allows us to confirm complex formation and also to derive the stability range in relation with pD. Under pD 6.6, gallium hydroxide precipitates, revealing dissociation of the complex; similarly, above pD 11 free ligand appears in the solution. Between these two limits, the complex exists in aqueous solution, with no detectable dissociation.

Figure 4 shows the variations of chemical shifts for the uncoupled protons of PPL-INH (H-C-4′PPL, H-C-6PPL and H_3 C-2 PPL) with respect to pD. This figure also shows the charges with pH for the 2:1 gallium complex.

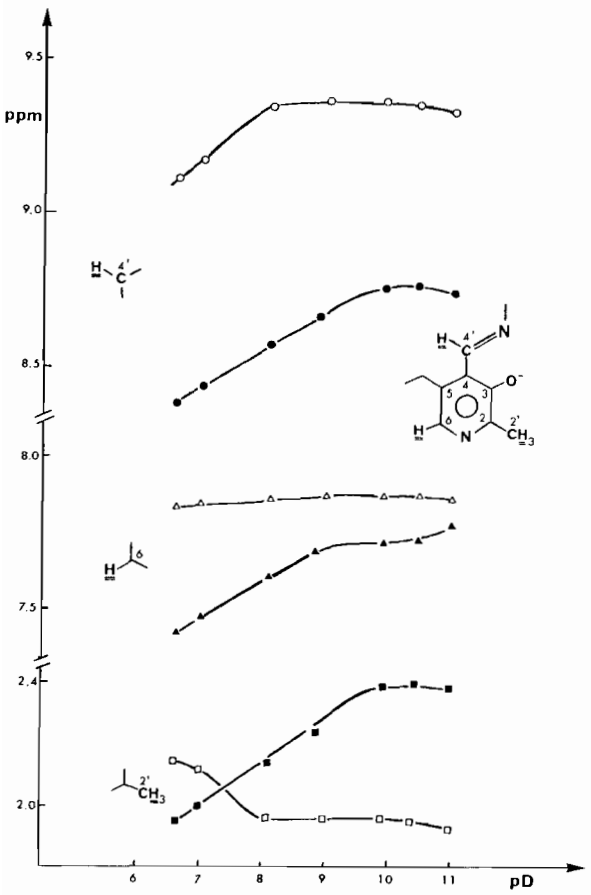

Fig. 4. The effects of 1.0 *M* $Ga(NO_3)_3$ on the variation of the ^{1}H chemical shifts of PPL-INH with pD (open symbols indicate the presence of Gallium).

The chemical shifts of the three protons do not change significantly above pD 10 when only free ligand is in solution. In the presence of gallium we observe the same kind of variation starting at pD 8. This implies that an unprotonated form of the ligand is stabilized by complexation with gallium. In this case, lowering the pD can affect only those functions which are not involved in complexation, such as PPL pyridinic nitrogen under pD 8.

Slow exchange between free and complexed ligand has allowed us to verify the stoichiometry of the complex. ^{1}H NMR spectra of a 4 L: 1 Ga^{3+} solution shows two peaks of identical intensity, one for the free ligand and one, shifted, for the bound ligand.

Conclusion

Complex formation of gallium 3+ in aqueous solution with new ligands incorporating the hydrazone function can be studied by NMR. This ligand appears to be an excellent chelating agent for Ga^{3+}. The complex formed has a 2 L: 1 Ga^{3+} stoichiometry and is stable in a broad pH range (from 6 to 11). This property allows identification of the species present in aqueous solution and encourages us to extend our work to other hydrazones derived from pyridoxal phosphate. Studies on other families of ligands are already in progress, and represent a necessary prerequisite for further investigations on the therapeutic properties of this class of complexes. In this respect, the established hydrosolubility of $(PPL\text{-}INH)_2Ga^{3+}$ complex at neutral pH enables us to realise biological and pharmacological tests.

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