New approach to the solution chemistry of bismuth citrate antiulcer complexes

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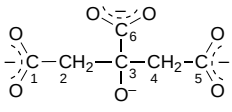
The use of ^{13}C-labelled citrate together with diffusion-ordered 2D [^{1}H,^{13}C] HSQC NMR spectroscopy has allowed the detection of cluster complexes formed by a bismuth(III) antiulcer complex in aqueous solution at pH 7.4.

Citrate is an important biological ligand for metal ions. It is present at high concentration (*ca.* 200 μM) in blood plasma and forms strong complexes not only with natural metal ions such as Ca^{II}, Mg^{II} and Fe^{III}, but also with ions of toxic (*e.g.* Al^{III}),[1] therapeutic (*e.g.* Bi^{III}) and diagnostic (*e.g.* $^{67}Ga^{III}$)[2] importance. Notable features of the chemistry of citrate are the low pK_a values of its three carboxyl groups (3.13, 4.76 and 5.40 at 25 °C, I = 0.1 M),[3] and, consequently, its existence as a trianion at biological pH (7.4), and the ability of the more highly charged metal ions to deprotonate the hydroxyl group (pK_a *ca.* 11.0)[4] giving rise to very strong metal–alkoxide bonds. Since citrate is a dendritic ligand with seven potential oxygen donors, it is perhaps not surprising that both solution equilibria and solid-state structures of metal citrate complexes are complicated, and often dominated by oligomer formation.



citrate

For example, X-ray crystallography has demonstrated the existence of an iron(III) citrate dimer,[5] an aluminium(III) citrate trimer[6] and a wide variety of structures (dimers, cubes, chains, sheets) for bismuth(III) citrate containing additional hydroxide and oxide ligands.[7] These form the basis of antiulcer drugs such as Colloidal Bismuth Subcitrate (CBS) and Ranitidine Bismuth Citrate (RBC).[8] Equilibria between different types of bismuth citrate complexes in aqueous solution are poorly understood and difficult to study. We show here that the combined use of ^{13}C-labelled citrate and a novel 2D diffusion-ordered [^{1}H,^{13}C] HSQC NMR method provides new insights into the structures and dynamics of bismuth(III) citrate complexes in aqueous solution and can readily be applied to studies of a wide range of other metal citrate complexes.

The normal 1D ^{1}H NMR spectrum of ranitidine bismuth citrate in the region δ 2.0–3.4 is characterized by a combination of sharp intense resonances from ranitidine (which is not bound directly to Bi^{III}),[9] some relatively intense AB quartets and some very broad peaks close to the baseline assignable to citrate. In order to investigate the types of Bi-bound citrate present in ranitidine bismuth citrate solutions at pH* 7 (pH meter reading for D_2O solution), we prepared the complex using citrate labelled at C2 and C4 with >95% ^{13}C.‡ As can be seen in Fig. 1, the peaks in the 2D [^{1}H,^{13}C] HSQC NMR spectrum cover a remarkably large chemical shift range in both the ^{1}H (δ 2.3–3.4) and ^{13}C (δ 47–57) dimensions. The appearance of the spectrum was the same at ranitidine bismuth citrate concentrations of 2 and 5 mM. At a given ^{13}C shift, the ^{1}H peaks appear as doublets of doublets due to the presence of $^{13}C_XH_AH_B$ spin systems, and the two CH_2 groups within one citrate ligand can be non-equivalent, as has been observed for $Na_2[Bi_2(cit)_2]$ by solid-state ^{13}C CP MAS NMR spectroscopy.[7e] The peaks in region 1 of the 2D [^{1}H,^{13}C] HSQC NMR spectrum (Fig. 1) have shifts close to those of free (unbound) citrate, whereas the marked high frequency ^{13}C shifts and pronounced separation of H_A and H_B peaks in region 3 suggest that these cross-peaks arise from rigidly-bound citrate ligands which place H_A and H_B in markedly different environments (*e.g.* bridging ligands in clusters). To investigate the dynamic properties of these bismuth(III) citrate species we measured their self-diffusion coefficients using 2D diffusion-edited heteronuclear single quantum coherence NMR spectroscopy (DOSY-HSQC).

Although DOSY-HSQC has been alluded to in the literature,[10] the present use appears to be the first direct application. One-dimensional homonuclear and heteronuclear diffusion-ordered NMR methods have been used recently to correlate chemical shifts and molecular self-diffusion coefficients. In view of the limitation of peak overlap in 1D ^{1}H NMR spectra, techniques have been developed which combine diffusion methods with standard 2D homonuclear techniques *e.g.* DOSY-COSY,[11] DOSY-NOESY[12] and diffusion-ordered TOCSY.[13] These depend on the existence of either scalar or dipolar coupling between spins and are useful only if cross-peaks from different species are not degenerate. The heteronuclear DOSY-HSQC method in combination with specific ^{13}C labelling can overcome the problem of overlap of ^{1}H resonances, and also provides greatly increased sensitivity in the ^{13}C dimension compared to direct heteronuclear observation.

Self-diffusion coefficients (D) were evaluated from measurements of volume integrals [$A(g_d)$] of peaks in 2D [^{1}H,^{13}C] DOSY-HSQC NMR spectra acquired using the pulse sequence

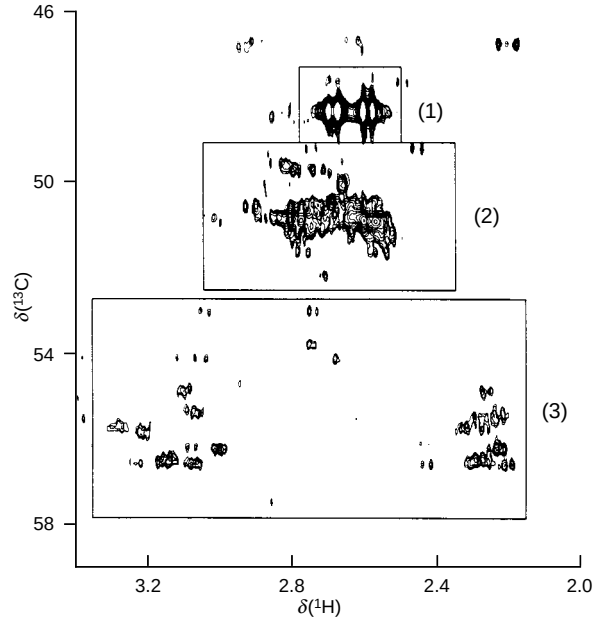


Fig. 1 2D [^{1}H,^{13}C] HSQC NMR spectrum of 5 mM ranitidine bismuth citrate (pH* 7.4) with diffusion weighting at a bipolar gradient strength of 25.05 mT m^{-1} acquired using the scheme in Fig. 2. The volume integrals of peaks in areas labelled 1–3 were used to measure self-diffusion coefficients. The total areas of each region were determined but the behaviour of the individual peaks in each region appear to be similar.

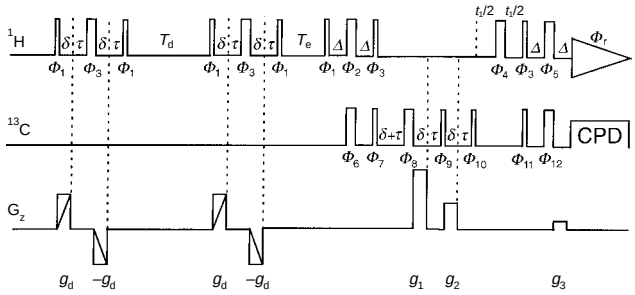


Fig. 2 Pulse sequence used for 2D [^{1}H,^{13}C] DOSY-HSQC experiments. Along the ^{1}H and ^{13}C schemes, narrow and wide vertical bars represent 90° and 180° pulses, respectively. Phase cycling: $\phi_1 = \phi_2 = \phi_5 = \phi_6 = \phi_8 = \phi_{10} = x$; $\phi_3 = y$; $\phi_4 = x, x, -x, -x$; $\phi_7 = 4(y), -4(y)$; $\phi_{11} = x, -x$; $\phi_r = x, -x, x, -x, -x, x, -x, x$. An XY32 composite pulse (CPD) decoupling scheme was applied during acquisition. Diffusion period T_d = 120 ms; settling period prior to the start of the HSQC section of the experiment T_e = 30 ms; gradient duration δ = 2 ms; gradient recovery period t = 100 μs; $\Delta = 1/4J_{HC}$ = 1.84 ms; gradients g_1, g_2 and g_3 were set in the ratio 80 : 30 : 20 with gradient strengths of 60.7, 24.1 and 15.17 mT m^{-1}, respectively. Rectangular gradients were used throughout.

shown in Fig. 2. This uses gradients to select for the correct quantum transitions. The peak volumes vary with the applied gradient strength (g_d) according to eqn. (1):[13c]

$$A(g_d) = \exp[-D(2\gamma g_d \delta)^2(T_d + \tau/2 + 4\delta/3)] \quad (1)$$

where γ is the gyromagnetic ratio of the observed nucleus, T_d the diffusion time, τ the time interval between bipolar gradients, and δ the gradient duration.

As can be seen from Fig. 3, the diffusion coefficients associated with peaks in regions 2 and 3 are less than half that associated with region 1, which in turn is less than half of the value measured for citrate itself. If we make the assumption that the molecules concerned are approximately spherical (with radius, r) then, according to the Stokes–Einstein equation, the diffusion coefficient is proportional to r^{-1}. If free citrate is assumed to have a diameter of *ca*. 4 Å then the bismuth(III) citrate complexes giving rise to the peaks in regions 2 and 3 have diameters of *ca*. 20 Å. This is close to the size of the dodecanuclear cluster $[Bi_{12}O_8(cit)_8]^{12-}$ in the crystalline state.[7d] In this structure some of the citrate ligands form bridges between three Bi^{III} ions and such a coordination mode might

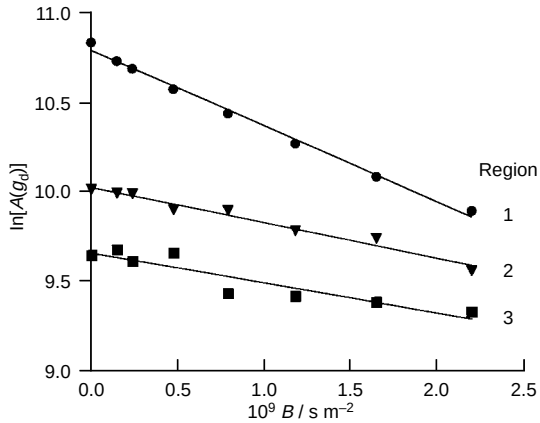


Fig. 3 Plots of ln[$A(g_d)$] *vs.* $B = q^2(T_d + \tau/2 + 4\delta/3)$ where $A(g_d)$ is the (arbitrary) volume integral at a diffusion gradient strength g_d, and $q = (2\gamma g_d \delta)$, and g_d varies from 8.35 to 125.25 mT m^{-1} in increments of 16.7 mT m^{-1}, for peaks in region 1 (●), region 2 (▼) and region 3 (■) of Fig. 1. The slope is proportional to D, the self-diffusion coefficient, giving the values: 4.3, 1.9, 1.7 and 10.2 × 10^{-10} m^2 s^{-1} for regions 1, 2, 3 and free citrate, respectively.

give rise to the unusual shifts we observe, although several types of cluster species appear to be present in solution.

In conclusion we have shown that the use of diffusion-ordered 2D [^{1}H,^{13}C] HSQC NMR spectroscopy, together with ^{13}C-enriched citrate, allows the sensitive detection of a wide range of types of bound citrate in aqueous solutions of the antiulcer drug ranitidine bismuth citrate at pH* 7.4. The unusual ^{1}H,^{13}C shifts and slow diffusional behaviour of some of these species provide a new method of characterising citrate cluster complexes. The identification of cluster species in solution is particularly important since they may be taken up by cells by a different mechanism compared to low molecular mass complexes. We are applying similar methods to the study of a range of biologically important citrate complexes.

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Notes and References

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‡ Bismuth citrate was prepared by mixing 16 mg [^{13}C2,^{13}C4]citric acid (0.08 mmol, supplied by GlaxoWellcome) with 35.6 mg bismuth nitrate (0.076 mmol, Sigma) in 2 ml water and heating at 80 °C for 1 h, followed by filtering and washing three times with water, and drying in a desiccator. Ranitidine bismuth [^{13}C]citrate was prepared by dissolving 8 mg of bismuth [^{13}C]citrate and 1.6 mol equiv. of ranitidine in 1 ml D_2O and adjusting the pH* to 7.4 by addition of ranitidine. NMR experiments were performed on a Varian ^{Unity}INOVA 600 NMR spectrometer operating at a ^{1}H resonance frequency of 599.613 MHz using a triple resonance [^{1}H,^{13}C,^{15}N] probe equipped with a *z*-field gradient capability. The *z*-field gradients were accurately calibrated with a sample of doped water at 25 °C using a 1D diffusion experiment. 2D [^{1}H,^{13}C] DOSY-HSQC data were typically acquired with 16 transients into 512 complex data points over a ^{1}H frequency width of 1 kHz for each of 2 × 64 t_1 increments over a ^{13}C frequency width of 2.5 kHz using an XY32 decoupling scheme during t_2.

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