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A simple, convenient and direct method for assessing the purity of cobalamins

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Abstract

A straightforward and simple, but powerful and direct, method is presented for both the detection and quantitation of cobalamin impurities in either commercial cobalamins or in metastable cobalamins (Cbls), such as RSCbls. The method is, quite simply, the use of the aromatic region of the ^{1}H NMR of cobalamins; it is a method developed as an outgrowth of our work preparing metastable thiolatocobalamins (RSCbls) and is a method that proved necessary for characterizing those (and by inference other) cobalamins unstable to HPLC separation conditions (i.e., and, therefore, where the normally powerful HPLC method so commonly used in cobalamin chemistry fails). Despite considerable, prior, modern multidimensional NMR literature on cobalamins, the present method has not yet been indicated explicitly, nor has anyone reported previously the NMR data required to prove that the method works (i.e., the data for a series of cobalamins and their common impurities proving that they have different chemical shifts in the aromatic region of their ^{1}H NMR when examined under identical NMR solvent, pH and other conditions). The direct NMR method is easy to perform, readily quantitated and applicable to species unstable to the HPLC conditions required to separate cobalamin impurities. The results have allowed quantitation of the 5–11% impurities in, for example, commercial HOCbl·HX, results which document that some commercially available cobalamins are not as pure as the manufacturers' claims.

Keywords: Cobalamins; Purity

1. Introduction

Recently, we synthesized a series of isolable thiolatocobalamins (RSCbls): pentafluorophenylthiolatocobalamin (C_6F_5SCbl), cyclohexylthiolatocobalamin ($C_6H_{11}SCbl$), cysteinylcobalamin (CysCbl) and glutathionylcobalamin (GluSCbl) [1,2]. However, we experienced unusual difficulties in quantitating the purity of the first three RSCbls, as all three were unstable (decomposing to H_2OCbl^+) under the HPLC elutant conditions of H_2O/MeOH required to separate RSCbl from its possible H_2OCbl^+ contaminant (i.e., the starting material for the synthesis). HPLC is the normally powerful, traditional method used by most B_{12} scientists to determine the purity of cobalamins. However, this method can be used only if the cobalamin and its suspected contaminants are both stable and separable under some set of HPLC conditions. In addition, the impurities must be known and their HPLC behaviour previously examined, since conditions must be chosen where all cobalamin species separate from one another. In some cases, species differing only by a dynamic, prototopic equilibrium such as HOCbl and H_2OCbl^+ are common impurities that are difficult to separate reproducibly from one another, at least under the HPLC conditions that we and others commonly employ [1], and even with close attention to pH control in an attempt to keep the cobalamin all in one form or the other (i.e., HOCbl or H_2OCbl^+) on the HPLC column.

A search for alternative methods to identify and quantitate the purity of cobalamins turned up no other well-recognized, generally useful, and powerful method (UV–Vis spectroscopy generally suffers from the problem of overlapping bands). Further consideration of potentially applicable methods leads to NMR as an obvious possible choice, and a scrutiny of the available ^{1}H NMR data for cobalamins[1], espe-

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[1] Noteworthy aspects of the NMR work reported in the valuable paper provided elsewhere [4] include: (i) the statement in the Experimental section that "The purity of the B_{12} derivatives was established by ^{1}H NMR and ^{31}P NMR and by reversed-phase HPLC" (although no further details, nor mention of the use or significance of the aromatic region, were noted); (ii) a focus on the B2, B4, B7 and C10 protons (among others) and their correlations with the *trans* alkyl R group (the B7 signal correlating well

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cially the aromatic region NMR data, suggested that this region of the NMR might be more useful as a criterion of cobalamin purity (and quantitatively so) than generally recognized [3–9]. The simple, but very useful, finding reported herein is that the aromatic region of the one-dimensional ^{1}H NMR spectrum of cobalamins, XCbl (X = RS, Me, Ado, CN, HO, H_2O), exhibits distinctive chemical shifts. While we at first thought that the present results might be too obvious for their own short report, reflection revealed that the present communication should prove of use to others as evidenced by: (i) the earlier "B_{12} prime" controversy[2] [10–17] that turned out to be due to a cobalamin monocarboxylic acid impurity [16] (i.e., and where the earliest studies [10–15] would have benefited from the use of aromatic region ^{1}H NMR studies of cobalamins such as those reported herein); and (ii) the detection and quantitation, via the present studies, of impurities in commercial cobalamins beyond the limits claimed by the manufacturers, results which provide the logical next step to improve the purity of several commercial cobalamins. Hence, we have taken time to report the present modest, but we believe useful, studies with the expectation that they may be interesting in particular to three groups: the suppliers of commercial cobalamins, newcomers[3] in the vitamin B_{12} field, and perhaps experienced B_{12} researchers who have struggled, as we did [1,2], with the impurities in commercial cobalamins and while determining the purity of RSCbl or other RXCbl cobalamins[4] that are not amenable to standard HPLC examination.

2. Experimental

The following were all purchased from Sigma: methylcobalamin (MeCbl, stated purity by manufacturer, ~99%), 5′-deoxyadenosylcobalamin (AdoCbl, stated purity by manufacturer, ~98%), cyanocobalamin (CNCbl, stated purity by manufacturer, ~99%), the hydrochloride salt of HOCbl (HOCbl·HCl, stated purity by manufacturer, ≥98%) and the acetate salt of HOCbl (HOCbl·HOAc, stated purity by manufacturer, ≥97%). C_6F_5SCbl, $C_6H_{11}SCbl$ and GluSCbl were synthesized as reported elsewhere [1,2].

^{1}H NMR spectra were recorded in D_2O on either a Varian Mercury-300 or Inova-300 spectrometer operating at room temperature and were referenced internally to 0 ppm with either TMS (CD_3OD) or TSP (D_2O). Anaerobic RSCbl solutions for NMR measurements were prepared in a Vacuum Atmospheres glove box (N_2, ≤2 ppm O_2).

3. Results and discussion

Fig. 1 gives the aromatic region of the ^{1}H NMR spectrum of $C_6H_{11}SCbl$ in anaerobic CD_3OD. Five well-separated signals are observable at $\delta = 7.17$, 6.99, 6.42, 6.21(d) and 6.03 ppm, arising from the B2, B4 and B7 signals of the α-dimethylbenzimidazole ligand, the C10 of the corrin and the R1 of the ribose (Fig. 2). Significantly, small impurity signals are also observable at $\delta = 7.25$, 6.88, 6.46 and 5.96 ppm, and integration of the signals at 7.25, 6.88 and 5.96 ppm, against those for the cobalamin, allows quantitation of the cobalamin as ~93 ± 2% pure. Hence, despite the complexity of the ^{1}H NMR spectrum of the cobalamin for $\delta < 5$ ppm, the aromatic region of the ^{1}H NMR spectrum of cobalamins is remarkably simple, affording a quick and easy method for determining the percentage of impurities in cobalamins.

Table 1 summarizes ^{1}H NMR aromatic chemical shifts in D_2O for a number of cobalamins. Significantly, no two cobal-

with the *trans* R group, and with the ^{31}P shifts, but the B2 and C10 chemical shifts being "erratic"); and (iii) especially their concluding statement in the NMR subsection of this paper, a statement fully fortified by the findings reported herein, that "In conclusion, some of the NMR resonances appear to be promising handles for evaluating changes in cobalamin structure, but further work is obviously needed". Refs. [30–56] in this paper [4] provide a valuable list of prior correlations of ^{1}H, ^{31}P and ^{13}C NMR data obtained for cobalamins.

[2] A putative "B_{12} prime" form ("B_{12}") of cyanocobalamin found at ~0.08% levels [10] in commercial cyanocobalamin (CNCbl) and initially thought to be an isomeric form of cyanocobalamin [10–15] was subsequently shown to be monocarboxylic acids of cyanocobalamin [16]. Hence, and since HOCbl·HX are most likely prepared commercially from CNCbl (Sigma was unable to supply us with this information), it is possible that the impurity is one of the monocarboxylic acids of CNCbl. However, the aromatic ^{1}H NMR chemical shifts of the impurities in D_2O ($\delta = 7.24$, 6.83, 6.67, 6.43, 6.19 and 6.07 ppm (present in both HOCbl·HCl and HOCbl·HOAc), pD = 6.1 ± 0.2, room temperature, internally referenced to TSP) are clearly different from those of CNCbl-b-monocarboxylic acid ($\delta = 7.28$ (B7), 7.10 (B2), 6.54 (B4), 6.37 (R1) and 6.10 (C10) [17]; in D_2O, pH 7.3, room temperature, referenced to TSP) or CNCbl-e-monocarboxylic acid ($\delta = 7.29$ (B7), 7.13 (B2), 6.52 (B4), 6.37 (R1) and 6.10 (C10) [17]; in D_2O, pH 7.3, room temperature, referenced to TSP). It has been claimed that a HOCbl′ form of HOCbl can constitute up to 15% of HOCbl stored in a refrigerator for a couple of years [10]. (When asked, Sigma advised us that their current batch of HOCbl·HOAc is about 10 years old.)

Since it is also likely that the "HOCbl′" form of HOCbl is a mixture of mono-carboxylic acids of HOCbl, the ^{1}H NMR chemical shifts of our impurity should be compared with these species to see whether the impurity is a mono-carboxylic acid of HOCbl. Unfortunately, the isolation and ^{1}H NMR of mono-carboxylic acids of HOCbl have not yet been reported in the literature. Sigma informed us that they are also concerned with the purity of their HOCbl·HOAc, and have recently investigated it for possible cyanocobalamin (1–3%) and dicyanocobinamide contaminants ($(NC)_2Cbi$ was not detected; i.e., it is presumably at levels ≤1%).

[3] We suspect that most experienced researchers in the cobalamin field are well aware, at least qualitatively, of the impurities in commercial cobalamins, although such impurities are little discussed in the open literature. However, we believe that in general most experts do not know: (i) the identity of the cobalamin impurities, (ii) their exact, quantitative percentages, and (iii) the fact that they are readily and quantitatively identified via the aromatic region of their ^{1}H NMR spectra.

[4] Specifically, RXCbl where X = other X (besides S), XH, or XR where X is a heteroatom with at least one unshared electron pair so that the RXCbl will therefore be generally much less stable under protic conditions than the better known RCbls.

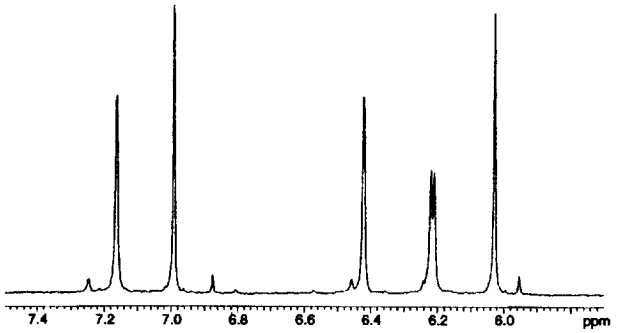

Fig. 1. ^{1}H NMR spectrum of the aromatic region of $C_6H_{11}SCbl$ ($\delta = 7.17$, 6.99, 6.42, 6.21(d) and 6.03 ppm) in anaerobic CD_3OD at room temperature. The results demonstrate the use of the aromatic region of ^{1}H NMR to access cobalamin purity (integration and comparison of the small impurity peaks at $\delta = 7.25$, 6.88 and 5.96 ppm with those for C_6F_5SCbl indicate a $7 \pm 2\%$ impurity in the product).

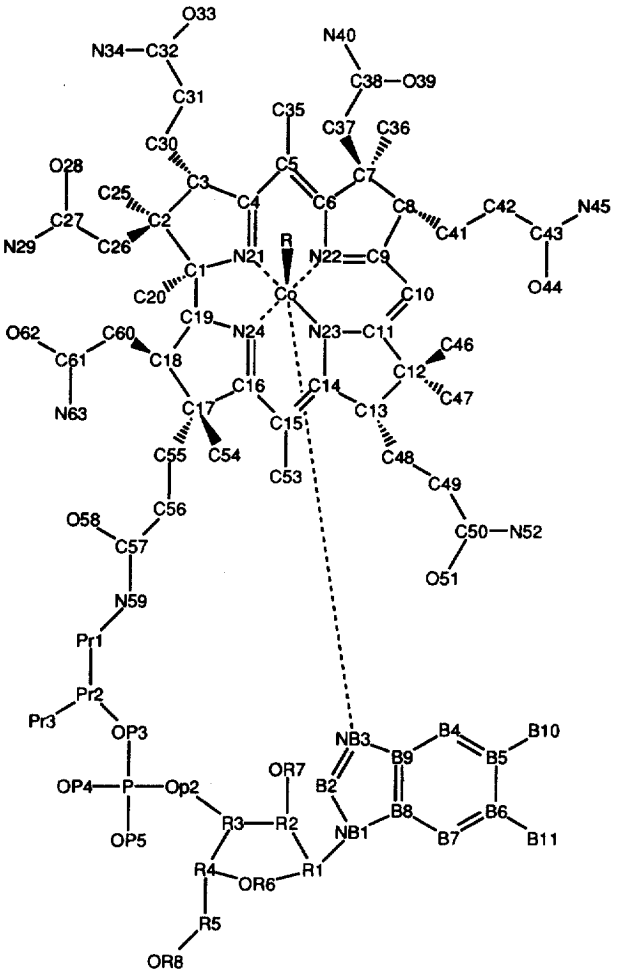

Fig. 2. Atom designations for cobalamins.

amins examined have exactly the same set of aromatic chemical shifts; hence, the aromatic region of the ^{1}H NMR of cobalamins is a quick and easy method by which one can identify and quantitate a given cobalamin's purity. The same conclusion can also be drawn from Table 2, data summarizing the ^{1}H NMR aromatic chemical shifts in CD_3OD for a number of thiolatocobalamins.

Table 1
Aromatic ^{1}H NMR chemical shifts for cobalamins in D_2O at 25°C; referenced to TSP

Cobalamin	δ (ppm) (assignment)
MeCbl [a]	7.18 (B7), 6.97(B2), 6.28 (B4), 6.27 (R1), 5.91 (C10)
AdoCbl [b,c]	8.19 (A2), 8.00 (A8) 7.16 (B7), 6.95 (B2), 6.26 (R1), 6.24 (B4), 5.93 (C10)
CNCbl [d]	7.28 (B7), 7.10 (B2), 6.51 (B4), 6.36 (R1), 6.09 (C10)
N_3Cbl [d]	7.19 (B7), 6.84 (B2), 6.45 (B4), 6.27 (R1), 6.19 (C10)
H_2OCbl$^+$ [d]	7.16 (B7), 6.51 (B2), 6.44 (B4), 6.26 (C10), 6.22 (R1)
HOCbl [d]	7.17 (B7), 6.74 (B2), 6.50 (B4), 6.25 (R1), 6.07 (C10)
GluSCbl[e]	7.25 (B7), 6.97 (B2), 6.42 (B4), 6.33 (R1), 6.12 (C10)

[a] Ref. [19].
[b] Ref. [20].
[c] A2 and A8 signals from the β-5′-deoxyadenosyl ligand of AdoCbl.
[d] Ref. [21].
[e] Ref. [22].

Table 2
Aromatic ^{1}H NMR chemical shifts for thiolatocobalamins in CD_3OD at room temperature; referenced to TMS

Cobalamin	δ (ppm)
C_6F_5SCbl [a]	7.17, 6.78, 6.42, 6.19(d), 6.04
$C_6H_{11}SCbl$ [b]	7.17, 6.99, 6.42, 6.21(d), 6.03
GluSCbl [b]	7.17, 7.01, 6.46, 6.20(d), 6.06

[a] See Ref. [1].
[b] See Ref. [2].

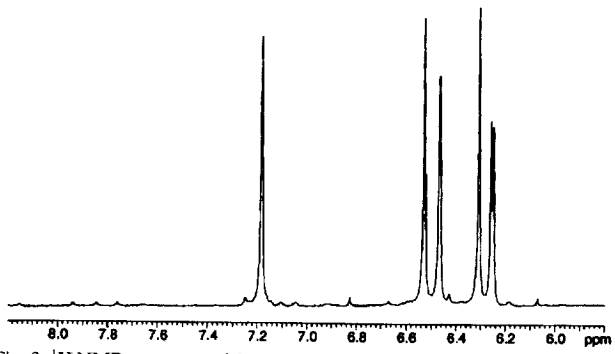

Fig. 3. ^{1}H NMR spectrum of the aromatic region of commercial HOCbl · HCl at room temperature in D_2O, pD 6.0 ± 0.1. Signals arising from H_2OCbl$^+$ are observable at $\delta = 7.18$, 6.53, 6.47, 6.31 and 6.25(d). Impurities at the $5 \pm 2\%$ level can be seen at $\delta = 7.94$, 7.85, 7.76, 7.25, 7.10, 7.04, 6.83, 6.67, 6.43, 6.19 and 6.07 ppm.

Fig. 3 gives the ^{1}H NMR aromatic region of Sigma's HOCbl · HCl in D_2O, pD 6.0 ± 0.1. Note that D_2O rather than CD_3OD is the preferred solvent, since the simplified spectrum in D_2O, compared to that in CD_3OD[5], allows ready detection

[5] We suspect, as one source of the more complicated spectra in methanol, precedented ion-pairing effects (i.e., in methanol: see the Cl^--dependent ion-pairing effects in methanol presented in Figs. 2 and 3 elsewhere [18] for a RCo[macrocycle]$^+Cl^-$ B_{12} model complex); there is also the possibility of solvate isomerism such as ClCoCbl versus Cl^--[(CD_3OD)CoCbl]$^+$. The exact speciation of HOCbl · HCl in methanol has not been investigated further, except to show that the peaks seen in CD_3OD are influenced by added LiCl. Practically speaking, the key result here is that D_2O is the preferred solvent.

of the impurities [18]. Signals attributable to H_2OCbl^+ are observed at $\delta = 7.18$, 6.53, 6.47, 6.31 and 6.25(d) ppm, and are in reasonable agreement with literature values (see Table 1, 5th entry). Signals arising from the impurities are also seen at $\delta = 7.94$, 7.85, 7.76, 7.25, 7.10, 7.04, 6.83, 6.67, 6.43, 6.19 and 6.07 ppm, and the individual ^1H signals integrate as $5 \pm 2\%$. Hence HOCbl·HCl from Sigma is, at best, 93–97% pure. The impurity chemical shifts were unchanged by the addition of 0.50 M LiCl (see Section 4), although the chemical shifts of the H_2OCbl^+ peaks were broadened considerably, indicating that a $H_2OCbl^+ + Cl^- \rightleftharpoons ClCbl + H_2O$ equilibrium probably occurs (somewhat faster than the NMR timescale). This experiment with added LiCl nevertheless demonstrates that none of the impurity peaks are attributable to ClCbl, and also rules out H_2OCbl^+ to Cl^- ion-pairing of any type as responsible for the smaller impurity peaks.

Fig. 4 gives the aromatic ^1H NMR region of Sigma's "acetate salt of HOCbl"[6]. The purity of this latter material was of special interest to us, as it was the starting material for our syntheses of C_6F_5SCbl and $C_6H_{11}SCbl$ [1,2]. Once again the major signals at $\delta = 7.17$, 6.52, 6.46, 6.30 and 6.24(d) ppm correspond to H_2OCbl^+. Prominent impurity signals are seen at $\delta = 7.24$, 6.82, 6.42, 6.38, 6.18 and 6.06 ppm, and individual ^1H NMR signals integrate as $11 \pm 2\%$ impurity. The spectrum was unaltered by the addition of 0.50 M LiOAc to the solution (see Section 4); hence, the impurity signals cannot be attributed to AcOCbl. The ^1H NMR spectrum of HOCbl·HOAc in CD_3OD has also been measured (spectrum not given), and contaminant signals are now observed at $\delta = 7.25$, 6.88, 6.46, 6.24 and 5.96 ppm in this solvent. Hence, the nearly 7% $\delta = 7.25$, 6.88, 6.46 and 5.96 ppm impurity seen in $C_6H_{11}SCbl$ [2] is carried through from the starting material, which contains the same impurity, but at levels of about 11%.

Our initial attempts to isolate and identify the impurity (which is also present in HOCbl·HCl) have so far been unsuccessful; such an identification is necessary before a rational attempt to remove the impurity from the starting material can be made. As noted earlier our guess here is that the impurities may be monocarboxylic acid derivatives of HOCbl·HX, analogous to the monocarboxylic acid derivatives of cyanocobalamin identified by Marzilli et al. [16]. Unfortunately, the monocarboxylic acids of HOCbl·HX have never been isolated and characterized; the present work identifies that task as a goal for future research.

Finally, a referee noted that the aromatic region of cobalamin spectra "may also be a useful reporter of cobalamin interactions with enzymes"; we agree. We further note that the the corrin C10 hydrogen aromatic region of the ^1H NMR is likely to be similarly useful for accessing the cobamide impurities in (dimethylbenzimidazole-free) cobinamides, although the key issue of whether the corrin C10 hydrogen chemical shift will always be detectably different for different cobinamides remains to be investigated.

[6] Note that, although Sigma calls this compound the "acetate salt of HOCbl", rigorously this cannot be so, as a charge balance means it must be the acetate salt of H_2OCbl^+ (i.e., HOCbl·HOAc).

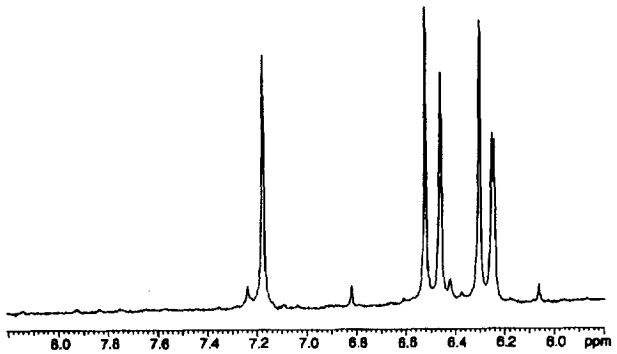

Fig. 4. ^1H NMR spectrum of the aromatic region of commercial HOCbl·HOAc at room temperature in D_2O, pD 6.0 ± 0.1. H_2OCbl^+ signals are observable at $\delta = 7.17$, 6.52, 6.46, 6.30 and 6.24(d). Impurities at the $11 \pm 2\%$ level can be seen at $\delta = 7.24$, 6.82, 6.42, 6.38, 6.18 and 6.06 ppm.

4. Supplementary material

Data on impurity chemical shifts by addition of 0.50 M LiCl (Fig. A) and 0.50 M LiOAc (Fig. B) are available from the authors.

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