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Optimisation of the Preparation and Purification of Three Monocarboxylic Acid Derivatives of Vitamin B_{12} and Their Characterisation by ^{13}C NMR

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Abstract

Conditions are established (1 M HCl, 50 °C, 42% yield) for optimum conversion of cyanocobalamin to the b-, d- and e-monocarboxylic acid derivatives. Their separation by a combination of anion exchange column chromatography and high performance liquid chromatography using a semi-preparative scale reversed phase column, and their unambiguous characterisation by 300 MHz ^{13}C NMR spectroscopy, are described.

Introduction**

Vitamin B_{12} derivatives are tightly bound by a wide variety of proteins (with association constants as high as 10^{11} [1]). The affinity of the protein for the cobalamins is generally independent of the nature of the β-ligand, but is critically dependent upon the integrity of the nucleotide loop [2–4]. We have recently isolated and purified to homogeneity by photodissociative affinity chromatography a haptocorrin from chicken serum [5]. Both the success of the affinity chromatography procedure in which the cobalamin moiety is immobilised by attachment to the β-ligand position, and the modest retarding effect which binding by the protein causes to the rate of replacement of H_2O by both HCN and CN^-, suggests that the cobalamin binding site of this protein leaves the β-ligand position relatively open to the solvent [5, 6].

The way proteins anchor the cobalamins to the binding site has not been clearly established and may involve, for example, hydrogen bonding between the protein and the propionamide side-chains which point to the α-face. We are currently investigating this possibility and have focussed attention on the b-, d- and e-propionamide side-chains of the corrin ring.

It is known [7, 8] that mild acid hydrolysis of cyanocobalamin produces a mixture of monocarboxylic acids (MCAs) derived from the propionamide side-chains b, d and e, which are apparently more readily susceptible to acid hydrolysis than the acetamide side-chains a, c and g [9]. Procedures which are available for the preparation of the MCAs [10, 11] involve incubation of cyanocobalamin in 0.5 to 1.0 M HCl at 37 °C for about 4 h, followed by their isolation from unreacted starting material and side products, and their separation, by anion exchange chromatography. Although the d-MCA is readily separated from the b- and e-MCA, repeated (and time consuming) passage through anion exchange columns is required for the resolution of the latter. We wish to report here a rational optimisation of the hydrolysis conditions for obtaining the MCAs from cyanocobalamin and an improved method for separation of b-MCA and e-MCA by HPLC. ^{13}C NMR spectroscopy is used to unambiguously distinguish between the b- and e-MCAs.

Experimental

HPLC work was done on a SpectraPhysics SP 8800 ternary gradient pump coupled to a Linear UVis 200 detector and a Varian 4290 computing integrator. Reversed phase HPLC work was done either on a 100 × 4.6 mm 5 micron RP-18 analytical column or a 250 × 10 mm D18-2510 20 micron Aquapore semi-preparative column (both Brownlee Laboratories); ion-exchange chromatography by HPLC was performed on a 250 × 4.6 mm Spherisorb S5 SAX column (PhaseSep). ^{13}C NMR spectra were obtained on a Brücker MSL 300 superconducting

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**Abbreviations used in this paper: Cbl, cobalamin; CNCbl, B_{12}, cyanocobalamin; MCA, monocarboxylic acid; b-MCA, Coα(α-5,6-dimethylbenzimidazolyl)-Coβ-cyanocobamic acid a,c,d,e,g-pentamide; d-MCA, Coα(α-5,6-dimethylbenzimidazolyl)-Coβ-cyanocobamic acid a,b,c,e,g-pentamide; e-MCA, Coα(α-5,6-dimethylbenzimidazolyl)-Coβ-cyanocobamic acid a,b,c,d,g-pentamide.

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NMR spectrometer operating at 75.470 MHz. Samples (*c.* 30 mg) were dissolved in water and the pH was adjusted to 7.0 with 0.1 M KOH. A deuterium lock signal and reference standard were provided by a solution of trimethylsilylpropionate (TSP) in D_2O contained in a concentric insert (Wilmad). Approximately 30000 scans were obtained on a 17000 Hz sweep width in 32 K data sets with a 30° pulse angle and a 2.7 s pulse repetition rate with continuous noise-modulated broad-band ^1H decoupling. Data sets were transformed without exponential multiplication and had a digital resolution of 1.0 Hz.

Column Chromatography

Dowex 1 X 2 anion exchange resin (chloride form) was converted into the acetate form by exhaustive washing with 1.0 M sodium acetate until the supernatant was free of chloride, and packed into a 570 X 30 mm column. Elution was by gravity at a flow rate of between 2.5 and 3 ml min^{-1}. XAD absorbent (Sigma), used for desalting all reaction mixtures, was exhaustively defined and packed into a 300 X 22.5 mm column. Salts were eluted from the column using deionised water as eluent. Subsequently, adsorbed B_{12} derivatives were eluted using 50% acetonitrile. The organic solvent was evaporated on a flash evaporation unit, and the aqueous phase routinely concentrated down to *c.* 10 ml.

Conversion of B_{12} to Monocarboxylic Acids

The conversion of CNCbl to the MCAs was investigated at various temperatures and acid concentrations by dissolving 0.1 g CNCbl (Roussel) in 10 ml aqueous acid solution at the appropriate temperature in a round bottom flask suspended in a thermostatted water bath. Periodically, a 100 μl sample was withdrawn and the reaction quenched by adding this to 5 ml of a solution of appropriate concentration of sodium acetate to give a final pH of *c.* 7. After filtration, 20 μl of this solution was analysed by HPLC using the SAX ion exchange column. For routine preparation of the MCAs, 1.0 g of CNCbl was dissolved in 100 ml 1.0 M HCl at 50 °C and incubated for 2 h (see below). The reaction mixture was then cooled in ice and the pH adjusted to 6 by addition of solid K_2HPO_4. After desalting on XAD, and concentration of the solution to *c.* 50 ml, the material was introduced onto an anion exchange column from which unreacted starting material, and neutral and cationic products were eluted with water. The eluent was then changed to 50 mM NaAc, pH 4.5, resulting in elution of one band from the column whilst a diffuse band migrated halfway down the column. The eluent was then changed to 50 mM NaAc, pH 5.3, and the rest of the material was eluted from the column.

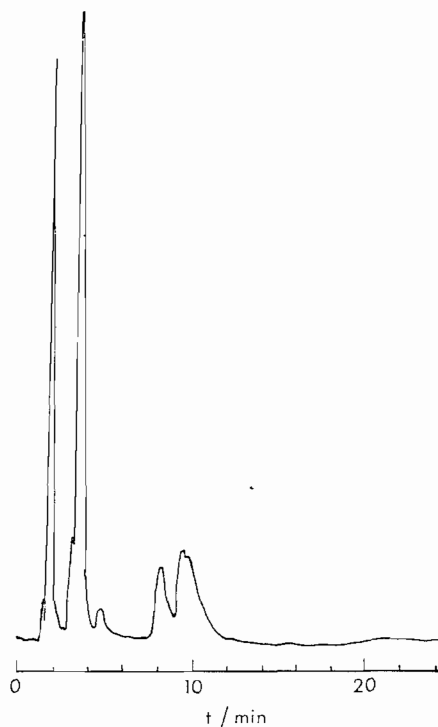

Fig. 1. HPLC chromatogram of conversion of B_{12} to MCAs. SAX column, detection at 360 nm. Solvent A = 50 mM NaAc, pH 4.5; solvent B = 50 mM NaAc, pH 5.3; flow rate = 2 ml min^{-1}. Elution program: from $t = 0$ to $t = 5$ min, 100% A; linear increase to 100% B between $t = 5$ and $t = 10$ min; 100% B between $t = 10$ and $t = 15$ min; linear return to 100% A between $t = 15$ and $t = 20$ min.

Results and Discussion

Conversion of B_{12} to Monocarboxylic Acids

The conversion of CNCbl to the three MCAs is readily monitored by HPLC using a strong anion exchange (SAX) column using the conditions given in the caption to Fig. 1. Unreacted CNCbl is eluted at 3.4 min, and the three MCAs between 8 and 11 min. Other reaction products are evident, with an especially prominent peak at $t = 1.8$ min. This peak co-elutes with aquocobalamin (B_{12a}) and with one of the isomers of aquocyanocobinamide (Factor B). It appears likely that some photolysis of B_{12} to B_{12a} occurred during the reaction [12], although this never amounted to more than *c.* 10% of the starting material, even under the most rigorous conditions used. However, we cannot exclude the possibility that acid-induced cleavage of the nucleotide occurred as well. A very broad peak is evident at around 21 min (Fig. 1). This peak increases in prominence with reaction time and is probably due to formation of di- and tricarboxylic acids. Since these peak(s) were in all cases very broad, the extent of their formation could not be quantified.

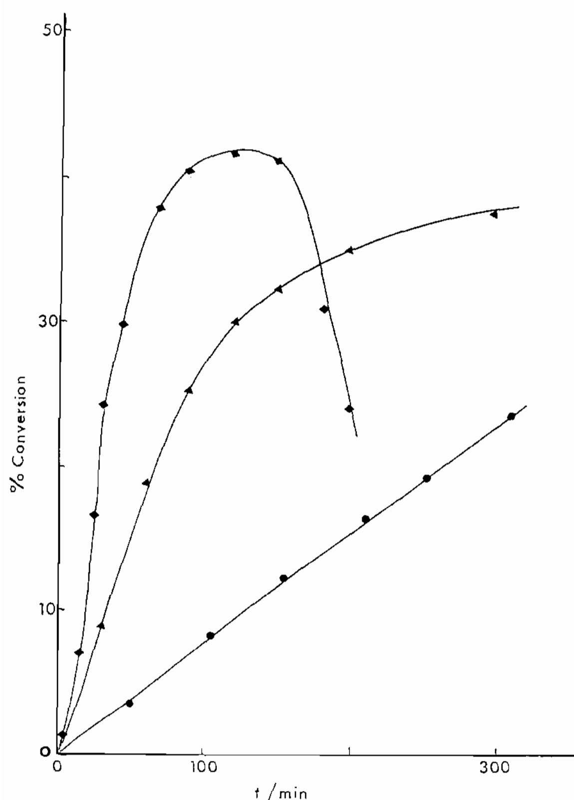

Fig. 2. Conversion of B_{12} to the three monocarboxylic acids by hydrolysis with 1.0 M HCl at (●) 30 °C; (▲) 40 °C; (◆) 50 °C.

Peaks which have approximately the same retention time as CNCbl itself are probably due to (unidentified) acid-induced modifications of the molecule which do not result in an overall change in its charge. The identification of these producs was not attempted, but, since the corrin ring is known to be very stable in acid solution [13], it is unlikely to involve changes in the ring itself.

The dependence of the conversion of CNCbl to MCAs as a function of (i) temperature at constant HCl concentration and (ii) HCl concentration at constant temperature, was investigated; the results are shown in Figs. 2 and 3. Optimum conversion (42%) is obtained after 2 h hydrolysis with 1.0 M HCl at 50 °C after which the amount of MCAs decreases as further hydrolysis to di- and tricarboxylic acids occurs. With an HCl concentration of 2.0 M, conversion to di- and tricarboxylic acids becomes too competitive with formation of MCAs, with a maximum yield of less than 30%, albeit in a relatively short reaction time.

From these studies, we conclude that the optimum conditions for preparation of the MCAs of B_{12} are by hydrolysis of CNCbl with 1.0 M HCl at 50 °C for 2 h.

Isolation of Monocarboxylic Acids

The three MCAs are readily separated by HPLC on a reversed phase column (see Fig. 4). The first band to be eluted from the anion exchange column

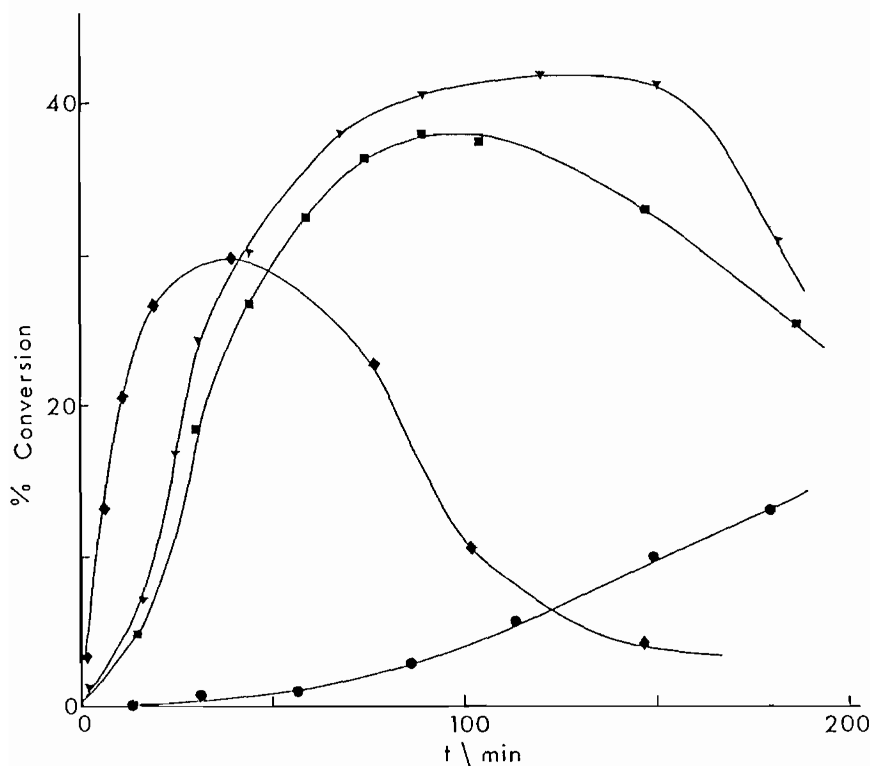

Fig. 3. Conversion of B_{12} to the three monocarboxylic acids at 50 °C in (●) 0.1 M HCl; (■) 0.5 M HCl; (▲) 1.0 M HCl; (◆) 2.0 M HCl.

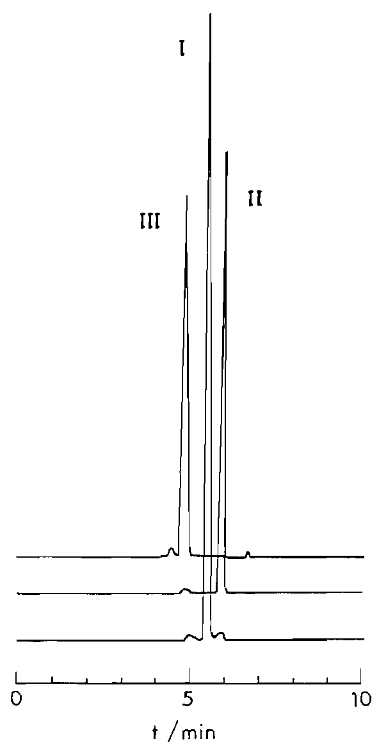

Fig. 4. HPLC chromatogram of the three MCAs on a reversed phase C-18 column. Detection at 360 nm. Solvent A = 50 mM phosphate, pH 6.5; solvent B = MeCN; flow rate = 1.5 ml min^{-1}. Elution program: at t = 0, 95% A and 5% B. Between t = 0 and t = 5, changed linearly to 85% A and 15% B, and held until t = 10 min. Between t = 10 and t = 15 min, changed linearly to 70% A and 30% B, and then linear return to starting conditions between t = 15 and t = 20 min. I: d-MCA; II: b-MCA; III: e-MCA (see text for assignments).

is a single species (*c.* 96%) (I in Fig. 4) with trace contamination by two other species which are probably the other two MCAs. By analogy with the results of Anton *et al.* [10], the eluted species is d-MCA. The second band eluted from the anion exchange column contained a mixture of the other two MCAs (II and III in Fig. 4), with a trace of the d-MCA present as well. The leading edge of this second fraction contained more of the MCA labelled as II in Fig. 4, whilst the tail had a greater amount of III. Based on the known behaviour of the unresolved MCAs on an anion exchange column [10] it is likely that II corresponds to b-MCA and III to e-MCA; the possible ambiguity in their assignments was resolved by ^{13}C NMR (see below).

The significant difference in retention times of the e- and b-MCAs was exploited in their resolution using a semi-preparative reversed phase HPLC column at a flow rate of 5.0 ml min^{-1} and the following elution program, where solvents A and B are as defined in the caption to Fig. 4. At t = 0, 95% A and 5% B, increased linearly from t = 0 to t = 20 min to 85% A and 15% B, held at this composition until t = 30 min, and then returned by linear gradient to starting conditions after 35 min. Sample volumes were 0.5 ml. The e-MCA eluted after 13.4 min, and the b-MCA after 17.4 min; they were collected manually as they eluted from the column.

After desalting, the resolved MCAs were precipitated from solution by addition of acetone. The d-MCA gave fine needle crystals, but the other two only an amorphous, fine precipitate which was collected on a 0.45 μm filter. The final yields obtained were 7.8% of d-MCA, 14.3% of b-MCA and 9.4% of e-MCA, in reasonable agreement with the yields previously reported [10]. HPLC analysis (Fig. 4) showed that the e-MCA was obtained with a purity of 95% and the b-MCA with a purity of 96%.

The ^{13}C NMR spectra of the e-MCA and b-MCA were assigned based on the previous work of Hogenkamp and co-workers [14, 15] and Marzilli and co-workers [16, 17]. The b-MCA (i.e. II of Fig. 4) shows significant differences from CNCbl ($\Delta\delta = \delta_{MCA} - \delta_{CNCbl}$ ppm) at the b-side chain carbon atoms C-30 (0.904), C-31 (2.792) and C-32 (3.224), with the differences increasing the closer the C atom is to the carboxylate functionality, and at C-3 (0.445), C-4 (0.549), C-5 (0.321) and C-6 (−0.411) of the A/B rings. Strictly analogous differences are seen in the e-MCA (III of Fig. 4), viz. the e-side chain C atoms C-48 (0.615), C-49 (2.435) and C-50 (3.458), and the C-ring C atoms C-11 (0.284), C-13 (0.441) and C-14 (0.554); the C-ring Me resonances appear not to be significantly affected.

We have succeeded in rationalising optimum conditions for the preparation of the MCAs of vitamin B_{12} by mild acid hydrolysis, and have described conditions for the improved separation of the b- and e-MCAs by HPLC. The ^{13}C NMR spectra leave no doubt as to their correct assignment and serve as further evidence that these species are true monocarboxylic acids of B_{12}, and not conformational isomers. The interaction of these MCAs with the haptocorrin from chicken serum will be reported on elsewhere.

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