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Preparation and biodistribution of ^{32}P-labelled hydroxocobalamin and comparison with ^{57}Co-labelled cobinamide and cyanocobalamin

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To study whether in biodistribution studies the phosphorus-containing side-chain of cobalamin and the central cobalt atom behave differently, ^{32}P- and ^{57}Co-labelled hydroxocobalamins were biosynthesized. A considerably higher specific activity of the ^{32}P-labelled compound was achieved than before. Both labelled hydroxocobalamins were administered in physiological quantities subcutaneously and orally to rats. The distributions of the two isotopes did not differ significantly. To explain previously observed slight deviations from this pattern, radiolabelled dicyano cobinamide and cyanocobalamin were prepared and studied in an analogous manner. Generally they behaved as hydroxocobalamin, but with greater accumulation in the liver, possibly reflecting the cyanide-detoxifying function of this organ.

Keywords: cobalamin; vitamin B_{12}; [^{57}Co]dicyano cobinamide; [^{57}Co]cobalamin; [^{32}P]cobalamin

Introduction

Following the isolation of vitamin B_{12}, cobalamin (Cbl), a number of radiolabels were introduced into the molecule,[1] but soon radiocobalt isotopes became almost exclusively used as tracers. Using radiocobalt-labelled Cbl, usually in cyano form (CN-Cbl), a large number of observations have been made regarding the absorption, transport, distribution, excretion, and nutritional requirement of this vitamin. From these observations the picture emerged early that in mammals the biological half-life of the vitamin is very long and that little breakdown of the molecule occurs in metabolism.[2] However, the cobalt label cannot unconditionally be assumed to reflect the behaviour of the entire Cbl molecule and especially not its side-chains. Our study was therefore undertaken to control the relevance of the numerous data obtained with the radiocobalt-labelled compound. For two reasons ^{32}P was chosen as the label. In metabolism, phosphorus is a very mobile atom. In Cbl-dependent enzyme reactions the side-chain-containing phosphorus is known to alternate between being bound or unbound to the central cobalt atom[3] and it may therefore tend to dissociate. One further reason for investigating the stability of the Cbl molecule is the possibility that breakdown could be a cause of Cbl-deficiency-like chronic conditions, common especially in the elderly.[4]

Previously, we managed by biosynthesis to achieve a specific activity of [^{32}P]hydroxocobalamin (OH-Cbl), permitting administration of the vitamin to rats in amounts corresponding to pharmacological doses used in treating Cbl-deficient patients.[5] Following improvements, our biosynthetic procedure now gave products that could be administered in physiological quantities and compared with [^{57}Co]OH-Cbl. Using our new preparations we found that the phosphorus apparently does not dissociate from the cobalt-containing part of the molecule and that the biodistribution of OH-Cbl is the same after parenteral or oral administration. In our previous study[5], we made some observations suggesting slightly different behaviour of the two labelled Cbls, possibly due to loss of phosphorus in metabolism. We therefore conducted similar studies with the phosphorus-free derivative dicyano cobinamide (dicyano Cbi). As CN-Cbl formation could easily contaminate OH-Cbl, we also compared the biodistributions of CN-Cbl and OH-Cbl.

Results and discussion

Preparation of labelled compounds

The production and use of ^{32}P-labelled Cbl in metabolic studies have been hindered by the short half-life of the isotope and low specific activity of the vitamin preparation resulting from large amounts of inactive phosphorus present in conventional microbiological media. Higher specific activity was now achieved by modifying the cultivation of *Propionibacterium*, and possibly because we purchased the strain from another

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supplier. We produced eight batches of [^{32}P]CN-Cbl and two of [^{57}Co]CN-Cbl. The specific activity of the ^{32}P-labelled compound varied between 3.8 and 2211.3 kBq μg^{-1} (0.1–59.8 $\mu Ci\,\mu g^{-1}$). The best specific activity achieved was now 1000 times higher than previously achieved by us and a million times than reported in the pioneering work of Smith *et al.*[1] The specific activities of some batches were still low, obviously related to the difficulty of the organism to thrive in low-phosphorus medium. The specific activities of two batches of [^{57}Co]CN-Cbl were 57 and 5.1 kBq μg^{-1} (1.54 and 0.14 $\mu Ci\,\mu g^{-1}$), respectively. Labelled [^{57}Co]dicyano Cbi and [^{57}Co]OH-Cbl were prepared from the former batch.

Animal experiments

Mixtures of the Cbl and Cbi preparations were administered by two routes, by subcutaneous injection (denoted by *s.c.*) or intragastric gavage (i.g.) to 54 male rats weighing 250–650 g. Owing to the greater specific activity, we were now able to administer physiological doses, i.e. a mass of maximally $\approx$20 ng, a dose corresponding to those given orally to humans in radiovitamin B_{12} absorption tests and known to require the intrinsic factor mechanism for efficient absorption[2]. The doses in the injection series varied depending on the specific activity achieved by the biosynthesis. Whenever the administered mass was large, the findings were controlled with smaller doses, and the results reported here were all obtained with physiological doses. The observations we occasionally made with somewhat larger doses did not differ from the results obtained with physiological doses and described below.

The experiments reported here were preceded by preliminary studies on 20 rats, whose doses and times of sacrifice varied. The results are available[6] and are in agreement with the ones presented here.

Biodistribution of labels after s.c. and oral administration of [^{57}Co] and [^{32}P] hydroxocobalamin

In the animals given [^{32}P]OH-Cbl and [^{57}Co]OH-Cbl simultaneously by injection, the radioactivity distributed itself among organs in the same manner as observed previously and corresponding to that observed by microbiological cobalamin assay.[5,7] The bulk of the radioactivity was found in the largest organs, the kidney and liver. There was no statistical difference between the distribution of the two labels in various organs expressed as percentage of administered dose per gram tissue (Figure 1(A) and (B)). Also, there was no statistical difference in their distribution among organs after intragastric and s.c. administration. This is somewhat surprising considering the complicated absorption mechanism of Cbl (Figure 2(A) and (B)).

Our conclusion is that in the metabolism of the rat, the phosphorus and cobalt atoms in the Cbl molecule accompany

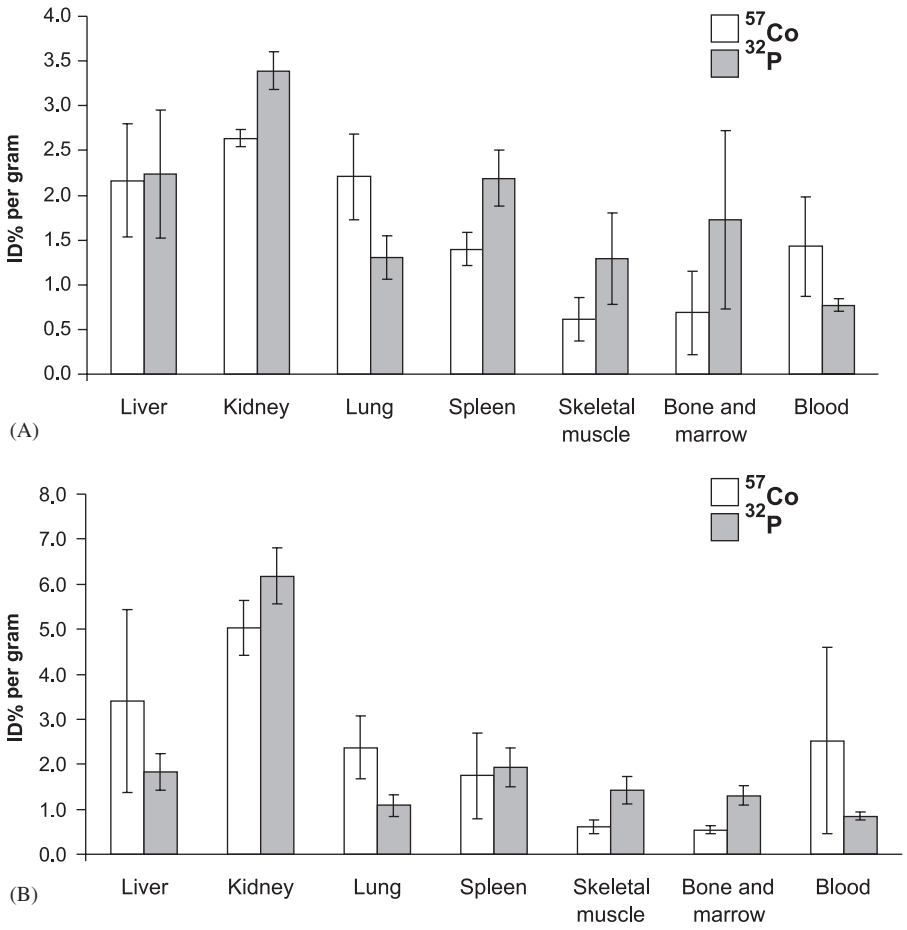

Figure 1. Distribution of the isotopes in various tissues expressed as mean $\pm$ standard deviation (s.d.) after intragastric (A) and subcutaneous (B) administration of [^{57}Co]CN-Cbl and [^{32}P]OH-Cbl. The animals (n = 4 in both series) were sacrificed 4 days after administration.

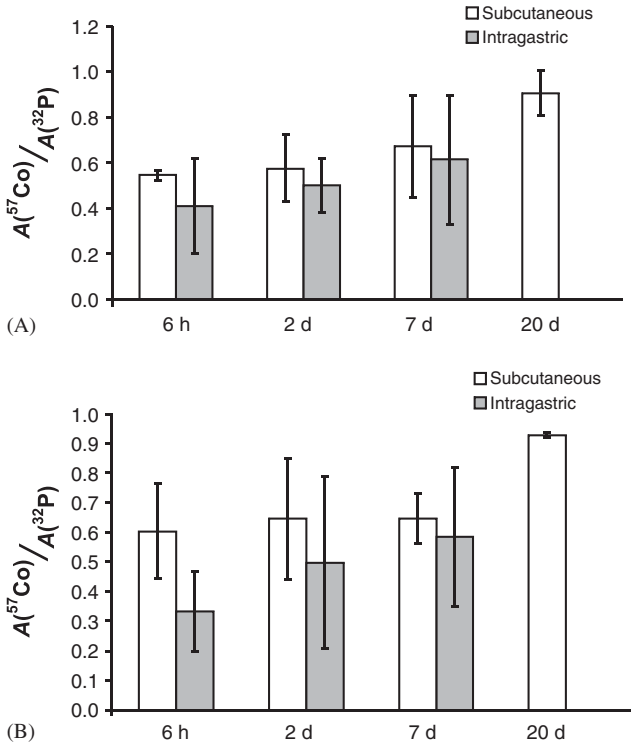

Figure 2. Ratio of the isotopes (mean ± s.d.) in the liver (A) and kidney (B) after intragastric and subcutaneous administration of [^{57}Co]OH-Cbl and [^{32}P]OH-Cbl. The number of animals in the i.g. series is 3 (sacrificed at 6 h), 3 (2 d), and 3 (7 d). The number of animals in the s.c. series is 4 (6 h), 4 (2 d), 5 (5 d), and 3 (20 d).

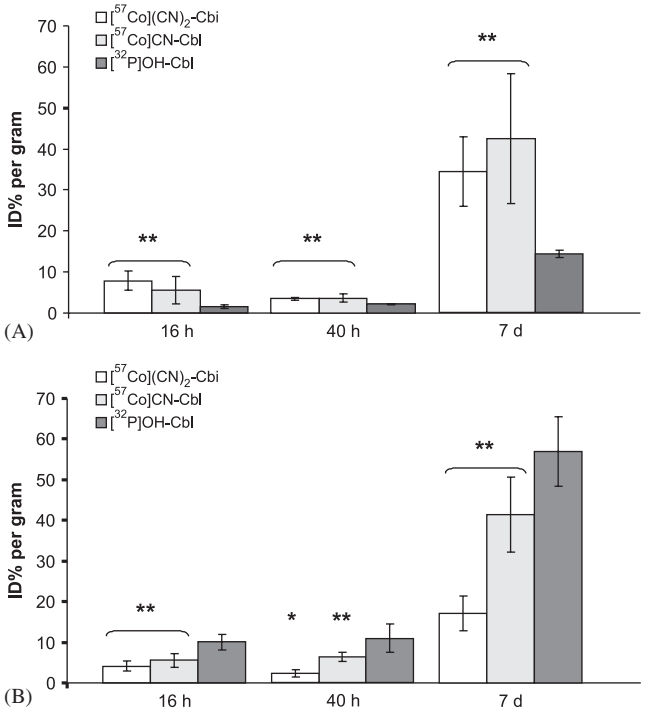

Figure 3. Accumulation of radioactivity (mean ± s.d.) to the liver (A) and kidney (B) after subcutaneous administration of the radiolabelled corrins. ^{57}Co radioactivity from either CN-Cbl or $(CN)_2$-Cbi is compared with ^{32}P radioactivity at each time point; ** denotes a highly significant difference with $p \leqslant 0.01$ and * denotes a barely significant difference with $p \leqslant 0.05$. The number of animals was 3, 3, and 4 at 16 h, 4 d, and 7 d, respectively, for the series receiving [^{57}Co]dicyano cobinamide and [^{32}P]OH-Cbl. *N* was 4, 3, and 4 at 16 h, 4 d, and 7 d, respectively, for the series receiving [^{57}Co]CN-Cbl and [^{32}P]OH-Cbl.

each other, and apparently, the entire side-chain-containing 5,6-dimethylbenzimidazole-ribofuranose-phosphate-aminopropanol does not dissociate. Our results tally with a recent study using CN-Cbl labelled with ^{14}C or ^{13}C in the dimethylbenzimidazole moiety.[8,9] In experiments on one person, the carbon-labelled compound was found to behave in a way consistent with the behaviour of the pure vitamin[8] but in urine possible breakdown products non-detectable with cobalt-labelled Cbl were found. The occurrence of such phenomena demonstrates the importance of studies similar to our present one.

Experiments with labelled cobinamide, cyanocobalamin, and hydroxocobalamin

These experiments were performed to explain our previous[5] findings where a change in the ^{57}Co/^{32}P ratio was obtained suggesting slow breakdown of the Cbl molecule into a phosphorus-lacking compound such as Cbi. As OH-Cbl has great affinity for cyanide, another explanation of our previous findings could be the spontaneous formation of CN-Cbl, known to be less well retained after injection than OH-Cbl and to have weaker affinity for protein.[2,10]

[^{57}Co]Cbi and [^{32}P]OH-Cbl were injected s.c. in symmetric sites and the distribution observed up to 7 d. In another series, [^{57}Co]CN-Cbl and [^{32}P]OH-Cbl were injected s.c. in different sites. Typical results are shown in Figure 3(A) and (B). The hepatic uptake of both Cbi and CN-Cbl was greater than that of OH-Cbl. In the kidney more OH-Cbl accumulated than CN-Cbl and the uptake of Cbi was relatively low.

The liver is known to remove non-cobalamin corrins.[11] The presence of Cbi could thus explain our previous results. CN-Cbl also accumulated more in the liver than OH-Cbl and we administered Cbi in its dicyano form. A common denominator is possibly cyanide, known to be detoxified in the liver.[12] Close scrutiny of our previous findings[5] suggests that spontaneous formation of CN-Cbl is the more likely of the two explanations.

CN-Cbl was long considered to be *the* vitamin. However, later this form was suspected to be an artifact or even toxic[10] and OH-Cbl is currently regarded as the more physiological form. Mellman *et al.*[13] observed differences between the cyano and hydroxo forms in their liver uptake and incorporation into coenzymes, the latter being quickly incorporated as coenzymes to Cbl-dependent apoenzymes.

Conclusions

In our biodistribution studies, ^{32}P- and ^{57}Co-labelled OH-Cbl behaved in the same manner. Consequently, cobalt-labelled Cbl is a good tracer for metabolic purposes. Also, Cbl labelled elsewhere in the side-chain containing the phosphorus and dimethylbenzimidazole will most likely behave in the same manner.

When OH-Cbl was used, the biodistribution was the same after oral or parenteral administration. Soon after administration, CN-Cbl accumulated more in the liver than OH-Cbl. Thus, in experiments of the present kind attention should be paid to the form of Cbl administered (CN-Cbl or OH-Cbl) and the possibility of spontaneous formation of CN-Cbl. The uptake of radioactive dicyano Cbi resembled that of CN-Cbl. This could reflect the role of the liver in detoxification to remove cyanide and to excrete non-vitamin corrins into the bile.

The biodistribution of orally and parenterally administered OH-Cbl was the same.

Experimental

Reagents and equipment

[^{32}P]5′-γ-ATP was purchased either from GE Healthcare (PT15068-5MCI, Amersham Radiochemicals, Little Chalfont, England) or PerkinElmer (NEG035C005MC, NEN Radiochemicals, PerkinElmer, Waltham, USA). [^{57}Co]$CoCl_2$ was purchased from GE Healthcare (CTS1-1MCI). OH-Cbl, CN-Cbl, and dicyano Cbi for standards were from Sigma-Aldrich (product numbers H7126, V2876, and C3021, respectively) as was cerium(III)nitrate hexahydrate (238538). All solvents were also purchased from there and were *pro analysi* or high-peformance liquid chromatography (HPLC) gradient grade (methanol). Preparative HPLC was performed on a Waters HPLC system. TLC was run as an ascending run on 20 cm × 20 cm aluminum-backed Merck F_{254} silica gel plates. The solvent was 30:45:25:2 (v/v/v/v) 2-butanol–2-propanol–H_2O–NH_3 (28%).

Biosynthesis and isolation of radiolabelled cobalamins

The methods for biosynthesis and isolation of radiolabelled Cbls have been described.[5] [^{32}P]CN-Cbl was converted to [^{32}P]OH-Cbl and the concentration of Cbl was assayed microbiologically[14] with *Lactobacillus delbrueckii* subsp. *lactis*. ^{57}Co- and ^{32}P-labelled Cbls were biosynthesized in cultures of *Propionibacterium freudenreichii* subsp. *freudenreichii* strain NBRC 12424 (NITE Biological Resource Center, Chiba, Japan). [^{57}Co]Cbl was synthesized in a cobalt-free medium modified from that described by Ye *et al.*[15]. The culture was supplemented with 7.2 MBq [^{57}Co]$CoCl_2$ per litre. Biosynthesis of [^{32}P]Cbl was carried out in low-phosphorus medium prepared as described[6]. A total of 185 MBq [^{32}P]5′-γ-ATP per litre was added in three batches during anaerobic growth. In both biosyntheses *P. freudenreichii* was first cultured anaerobically in +30°C for 72 h, and then aerobically 72 h with 250 rpm shaking at the same temperature. Accumulated propionic acid was neutralized with 8 M NaOH between the anaerobic and aerobic incubations. Radiolabelled Cbls were extracted from bacterial cells as CN-Cbl as described.[16] CN-Cbl was converted to OH-Cbl by overnight photolysis in dilute hydrochloric acid[17].

Preparation of [^{57}Co]Dicyanocobinamide

[^{57}Co]dicyano Cbi was prepared from [^{57}Co]CN-Cbl by hydrolysis with cerium(III)hydroxide by the modification of a method for the preparation of OH-Cbl[18]. A total of 50 μl of [^{57}Co]CN-Cbl with specific activity of 0.057 MBq μg^{-1} was mixed in a 50 ml conical centrifuge tube with 5 ml 1 M sodium hydroxide and 6 ml 0.33 M cerous nitrate. A total of 5 μg of non-radioactive CN-Cbl was added to the reaction as a carrier. The mixture was incubated in +95°C water bath for 2.5 h, cooled, and filtered first through Whatman 3MM filter paper (Whatman, Bretford, England) and then with a 0.22 μm PVDF syringe filter (Millipore, Billerica, USA). [^{57}Co]dicyano Cbi was extracted from the filtrate with 30:70 (v/v) 88% phenol–benzene and from the organic phase with a 1:2 (v/v) mixture of 99.5% 1-butanol and distilled water. The final aqueous phase was evaporated to dryness at +45°C overnight. The residue was dissolved in 100 μl distilled water and converted to dicyano form by adding 10 μl of 5% (w/v) potassium cyanide. The product was characterized with TLC.

Purification and characterization of the radioactive compounds

The tracers were purified with preparative HPLC using the gradient run described earlier by Chassaigne and Lobinski[19]. Solvent A was 50% (v/v) 25 mmol l^{-1} sodium acetate–50% methanol at pH 4.0. Solvent B was 25 mmol l^{-1} sodium acetate buffer at pH 4.0. A linear gradient of A was applied from 20 to 80% over a period of 30 min. The solvent was collected in 1 min fractions (500 μl) and those containing the desired tracer pooled and evaporated to dryness at +45°C overnight. All tracers were purified once more with HPLC immediately prior to administration to rats in order to remove impurities formed during storage.

Radiolabelled cobalamin preparations

For the biodistribution study radiolabelled Cbl tracers were dissolved in physiological saline and passed through a 0.22 μm sterile syringe filter. The radioactivity of each dose was adjusted so that ^{32}P- and ^{57}Co- labelled tracers would give roughly the same count rate in organ preparations.

Animals

Male Wistar Han™ rats (strain HsdRccHan™:WIST, Harlan Nederland, Horst, the Netherlands) weighing 250–650 g were utilized in the biodistribution studies. The rats were either pair housed or group housed in polycarbonate cages with aspen bedding in a horizontal airflow unit (Ehret, Emmendingen, Germany). The animals were provided with enrichment, such as aspen blocks and shavings. Pelleted rodent maintenance diet (Global Diet, Harlan Teklad, Oxon, England) and tap water were available *ad libitum*. A 12:12 lighting rhythm was maintained, with lights on from 6 a.m. to 6 p.m.

Biodistribution experiments

The experimental design was approved by the National Committee for Animal Experimentation in Finland (State Provincial Office of Southern Finland, Hämeenlinna, Finland). Selected Cbl preparations were administered to rats either via i.g. in a volume of 2 ml or s.c. in a volume of 0.5 ml. The vehicle was physiological saline in both cases. Animals (a total number of 54) were allocated to the following groups: [^{57}Co]OH-Cbl+[^{32}P]OH-Cbl, both i.g., [^{57}Co]OH-Cbl+[^{32}P]OH-Cbl, both s.c., [^{32}P]OH-Cbl+[^{57}Co]CN-Cbl, both i.g., [^{32}P]OH-Cbl+[^{57}Co]CN-Cbl, both s.c., and [^{32}P]OH-Cbl+[^{57}Co]dicyano Cbi, both s.c. Within each group the animals were sacrificed at specified times after the administration, ranging from 6 h to 20 d, but not all time points are represented in all groups.

The doses of [^{32}P]-Cbl given s.c. varied between 0.21 and 300 ng, but only one animal received more than 20.7 ng. The oral doses of [^{32}P]OH-Cbl varied between 0.21 and 300 ng, but only one animal received more than 20.7 ng. In no series did the animals receive more than 2.9 ng of the ^{57}Co-labelled compound. The total mass of Cbl given (i.e. the two-label mixture) was below 25 ng in all animals apart from the ones receiving an unphysiological dose of [^{32}P]OH-Cbl. The amount of [^{57}Co]dicyano Cbi administered could not be assessed microbiologically because of the insensitivity of the available test organism, but it

was determined spectrophotometrically[20] to be in the nanogram range.

Extraction and counting

These procedures have been described.[5] The method to extract cobalamin from tissues was the same as that used for bacterial cultures after biosynthesis of radioactive cobalamins. For experiments with co-administration of OH-Cbl and either CN-Cbl or dicyano Cbi, a weighed sample of homogenized tissue was dissolved in Soluene-350 tissue solubilizer (PerkinElmer, Waltham, USA) according to the manufacturer's instructions. Scintillation cocktail (OptiPhase HiSafe® 3, PerkinElmer) was added and the samples were light adapted 2 h prior to counting with a scintillation counter (RackBeta, Wallac, Finland). All counts were corrected for background and for decay from the time of administration until the time of radioactivity measurement.

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