Forms of cobalamin and vitamin B_{12} analogs in maternal plasma, milk, and cord plasma

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Corrinoids are nutrients synthesized by microorganisms, both as biologically active compounds (cobalamins: hydroxo-, cyano-[vitamin B_{12}], and the two vitamin B_{12} coenzymatic forms: 5′ adenosyl- and methyl-cobalamin) and as biologically inactive molecules, the so-called analogs (cobinamides). Though the transport system in humans favors the biologically active forms of vitamin B_{12}, numerous studies have indicated the presence of analogs in human material.

The present study was undertaken to compare the number of analogs present in maternal plasma and milk and in cord plasma, and to determine if milk secretion is contaminated with potentially harmful vitamin B_{12} analogs. The concentrations of cobalamin and of cobinamides were measured in maternal milk, maternal plasma, and cord plasma. Cobalamin analogs were found in maternal plasma (34.6 ± 28.1 pmol/L) and in cord plasma (62.1 ± 32.0 pmol/L; $P < 0.005$), but not in maternal milk (0.6 ± 0.7 pmol/L). A combination of reverse phase high pressure liquid chromatography and radioisotopic dilution assay was used to identify the individual cobalamins. The major peak in cord plasma was identified as CH_3-cobalamin (39%), while the peaks of CH_3-cobalamin (29.75%) and ado-cobalamin (29.56%) were similar in the maternal plasma. The ado-cobalamin peak was higher in milk (41%) than in maternal or umbilical plasma.

We have confirmed the presence of analogs in plasma, but shown that milk is protected from vitamin B_{12} analogs, as none were found in the exocrine mammary secretion. Thus, biologically active vitamin B_{12} is selectively transported into milk.

We found cord plasma to contain significantly higher amounts of analogs than maternal plasma. Whether this represents conversion of active forms of the vitamin within the fetus or accumulation of analogs transferred during pregnancy remains to be elucidated. (J. Nutr. Biochem. 5:406–410, 1994.)

Keywords: corrinoids; vitamin B_{12}, analogs; milk; plasma; exocrine

Introduction

Vitamin B_{12} or cobalamin (cbl) is an essential nutrient for human and mammal cells because metazoa have no gene coding for its synthesis. Cobalamins are synthesised only by microorganisms (bacteria, yeast, and some algae).[1,2] Human plasma contains several microbiologically active forms of cobalamin, including hydroxo-cbl (OH-Cbl), cyano-cbl (CN-Cbl), 5′deoxyadenosyl-Cbl (Ado-Cbl), and methyl-cbl (CH_3-Cbl).[3] CN-cbl, the most stable form of cobalamin, was the first form to be isolated.[4] Ado-cbl and CH_3-cbl are the two physiologically active forms of cobalamin that are required as cofactors for two enzymes, L-methylmalonyl-coenzyme A mutase and methionine synthetase, in humans and other animals.[5–7]

Cobinamides are vitamin B_{12} analogs that have no vitamin activity. Cobalamin analogs have been found in human serum, bile, feces, and liver;[8–12] animal tissues,[13] foodstuffs; and vitamin supplements.[14] These analogs could be potentially harmful, but few data are available on their toxicity. Coates et al.[15] showed that some cobalamin analogs have no activity and may even inhibit the growth and development of chicks. Siddons et al.[16] noted that the Cbl analog, 2-methyl-2-aminopropanol-benzimidazole-cobalamin caused a severe demyelinisation of nerve fibers when given to baboons deficient in vitamin B_{12}. Kondo et al.[17] showed that

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the exposure of rats to nitrous oxide caused cobalamin inactivation and induced the conversion of cobalamin to analogs in the liver but did not lead to neurological disorders. Kanazawa et al.[18] have suggested that analogs are not as well absorbed as cobalamin, and that the amount absorbed might be insignificant. Kolhouse et al.[19] argued that the absorption and dissemination of analogs into animal tissues is prevented by several mechanisms. They are bound with low affinity to an intrinsic factor, and they are taken up by the ileum or absorbed into the body in very small amounts compared with vitamin B_{12}. All of the analogs bind to haptocorrin (transcobalamin I + III) with high affinity and are cleared from plasma by hepatocytes. This has given rise to the concept of the entero-hepatic circulation of corrinoids, by which cobalamin analogs are removed from the plasma and excreted in bile.[20] This has been confirmed in humans.[12]

It has recently been shown that cobalamin analogs may stimulate cobalamin-dependent holo-methionine synthetase in vitro. Human apo-methionine synthetase can be converted to active holo-methionine synthetase by several cobalamin analogs. The activation involves reduction of the oxidized cob(III)alamine form to the reduced cob(II)alamin form.[21] The potent inhibition of mammalian methionine synthase activity by various cbl analogs in vivo could be due to inhibition of intracellular cbl transport or to inhibition of the enzymatic formation of cob(II)alamin, rather than to direct inhibition of the enzyme. Cob(II)alamin is the only form that can bind to the apo-enzyme.[21]

Relatively little is known about the way in which vitamin B_{12} is exchanged between the mother and the developing fetus. Vitamin B_{12} deficiency in the mother is frequently associated with congenital abnormalities.[22,23] A maternal vegan diet can cause nutritional vitamin B_{12} deficiency resulting in hematological[24] and serious neurological disorders.[25] Rappazzo et al.[26] found that the total vitamin B_{12} in several fetal tissues was significantly higher than in identical adult tissues.

This study was carried out to measure vitamin B_{12} analogs in exocrine secretions such as milk. The results were used to determine if the maternal organism protects this exocrine pathway from contamination with potentially harmful vitamin B_{12} analogs that are present due to their ileal absorption or the catabolism of vitamin B_{12}. Milk was analyzed because it is of interest to know if the milk corrinoid profile is different from that of the plasma, and thereby, if a newborn might receive a contamination by analogs from its mother.

The analog profiles of maternal and cord plasma were compared to analyze the exchange of vitamin B_{12} and analogs between the mother and the fetus. Radioisotopic dilution assays (RIDA) were used to evaluate true vitamin B_{12} and cobalamin analogs in maternal plasma, cord plasma, and maternal milk. The forms of cobalamin present in each fluid were then identified by reverse phase high pressure liquid chromatography (HPLC).

Methods and materials

Patients

Samples of maternal plasma and cord plasma were taken from 18 women immediately after they had given birth, and samples of milk were obtained from six of them. Cord blood was collected just after cutting the umbilical cord. Maternal blood samples were anticoagulated with EDTA, and cord blood samples with sodium citrate. The mean age of the mothers was 31.2 ± 6.5 years. Samples of milk (5 mL) were obtained by manual milking 5 to 7 days after birth and collected in glass tubes. None of the mothers were on any treatment affecting the vitamin B_{12} metabolism or on a supplement of the vitamin.

Experimental procedure

Chemicals. Crystalline hydroxo-, cyano, methyl-, 5′ deoxyadenosyl-cobalamin, cyano-cobinamide, used as standards for HPLC, and highly purified binding proteins (hog intrinsic factor and haptocorrin) were obtained from Sigma Chemicals Company (St Louis, MO USA), ^{57}Co-labeled CN-cobalamin (Amersham, France, specific activity 220 Ci/μg) was from Amersham, the LiChrospher RP 18 end capped column (5 μm silica, 250 mm *5 mm I.D.) was from Merck (Darmstadt, Germany).

Assay of vitamin B_{12} and analogs. The RIDA method described by Djalali et al.[9] was adapted from Kolhouse et al.[10] Okuda et al.[27] have shown that ado-cbl and CH_3-cbl are partially protected from light when bound to internal proteins or lyophilized. However, plasma and milk samples were extracted and assayed in the dark or dim red light. Because of difficulties of milk extraction, all samples (plasma and milk) were extracted with ethanol, as previously described, before incubation at 100° C[28] to ensure that corrinoids were separated from their binding proteins. To prevent the nonspecific binding of OH-Cbl to histidine residues of proteins, the samples (0.5 mL) were incubated for 2 hours at room temperature with excess cadmium acetate (0.2 M), added to four volumes of absolute ethanol preheated to 78° C, and mixed vigorously for 20 min at this temperature. The samples were cooled in an ice bath, centrifuged at 2000 *g* for 10 min, and the supernatant removed. The precipitate was mixed with two volumes of cold 80% ethanol (vol/vol), centrifuged as above, and the two supernatants were pooled and evaporated to dryness in a rotary evaporator at 40° C and taken up in 0.5 mL distilled water. Aliquots (0.1 mL) were mixed with 24 fmol ^{57}Co-labeled CN-cobalamin (in 1 mL of 0.082 M sodium tetraborate (pH 9.2) containing 0.02 M NaN_3, 0.02 M bovine serum albumin, 0.1 M urea, 0.3% thioglycerol, 0.05% dithiothreitol (wt/vol), and 0.3 mM KCN) and heated at 100° C for 15 min. This treatment converted all the corrinoids to their cyano-forms in line with the calibration standards.[29,30] Hog haptocorrin and hog intrinsic factor (9.1 fmol per tube) were used as binders. A hemoglobin-coated charcoal suspension was used to adsorb the free corrinoids. They were then removed by centrifugation at 2000*g* for 15 minutes. As intrinsic factor only binds cobalamin, while haptocorrin binds all corrinoids (cobalamin and inactive analogs), the assay distinguishes total corrinoids from true vitamin B_{12}. The difference was considered to be the contents of analogs. All samples were assayed in duplicate three times on 3 different days.

The within-assay and between-assay coefficients of variation were, respectively, 7.6% and 12.3% for plasma, and 9.4% and 14.3% for milk. As milk is a fluid notorious for behaving anomalously in analytical assays, the recovery of corrinoids after milk extraction were checked. The recoveries (89.3 ± 3.1%) were not different from that of corrinoids extracted from plasma.[11]

Reverse phase HPLC

Reverse phase HPLC was carried out as previously described.[9] The cobalamins were identified by their retention times. Hydroxo-, cyano, methyl-, 5′ deoxyadenosyl-cobalamin, and cyano-cobinamide were used as standards. Three pools, one for the 18 maternal plasmas, a second for the 18 cord plasmas, and the third for the

six milk samples, were desalted on Sep Pak cartridges (Waters, Millipore, France). Corrinoids were separated by HPLC on a RP 18 Lichrosorb (Merck) column using a 10 to 50% acetonitrile gradient in 0.085 M phosphoric acid adjusted to pH 3.0 with triethanolamine and detected at 365 nm. One-half minute fractions were collected. HPLC separated identifiable peaks and provided fractions of purified corrinoids that were then quantified by RIDA. Each 0.5 min fraction was assayed by RIDA for vitamin B_{12} and analogs (three injections for each pool). Individual cobalamins in the three pools (maternal milk, maternal plasma, and cord plasma) were identified by a combination of reverse phase HPLC and RIDA. The RIDA detected nanogram quantities of corrinoids.

Statistical analysis. The nonparametric Wilcoxon signed-rank test was used to compare groups, and the Spearman rank test was used to determine correlation coefficients. All values are means plus or minus standard deviations.

Results

Total corrinoids and cobalamin

The amounts of true vitamin B_{12} and analogs in cord plasma, maternal plasma, and maternal milk are given in *Figure 1*. The maternal plasma contained 297.6 ± 196.8 pmol/L true vitamin B_{12}, and 34.6 ± 28.1 pmol/L analogs. The analogs accounted for 9.7 ± 9.4% of the total corrinoids in maternal plasma. The cord plasma contained 298.0 ± 106.0 pmol/L true vitamin B_{12}, and 62.1 ± 32.0 pmol/L analogs, with analogs accounting for 14.9 ± 10.0% of the total corrinoid content. The maternal milk contained 270.2 ± 39.8 pmol/L true vitamin B_{12}, but only 0.7 ± 0.8% analogs. The concentration of analogs in the cord plasma was significantly higher than that of maternal plasma ($P < 0.005$), while that of milk was significantly lower than the analog contents of either maternal ($P < 0.025$) or cord plasma ($P < 0.001$).

There was a correlation between the true vitamin B_{12} concentration in maternal plasma and milk (rho = 0.943, $P < 0.05$). The vitamin B_{12} concentrations in maternal, cord plasma, and milk were similar.

Forms of corrinoids

Individual cobalamins in samples were identified by their retention times compared with those of corrinoid standards

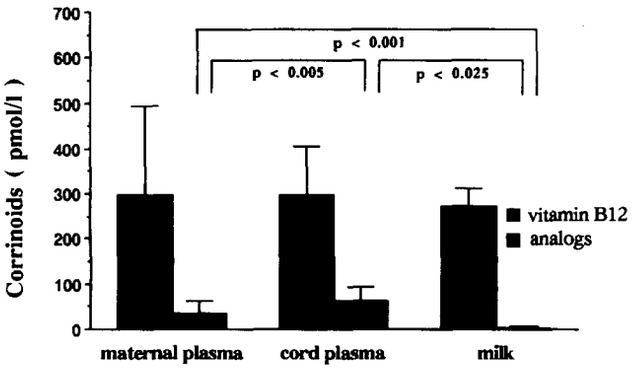

Figure 1 Vitamin B_{12} and analogs in maternal plasma, milk, and cord plasma. The nonparametric Wilcoxon signed-rank test was used to compare groups.

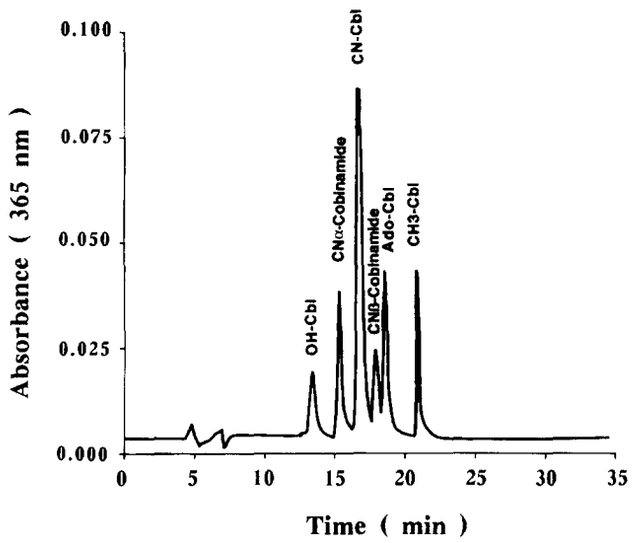

Figure 2 Identification by HPLC of corrinoids standards: OH-cbl, CN-cbl, Ado-cbl, CH_3-cbl, and CN-cobinamide.

(Figure 2). Figure 3 shows the individual cobalamins in the three pooled samples after reverse phase HPLC and RIDA on the collected fractions. The profiles of maternal and cord plasma were similar. The major peak in cord plasma was identified as CH_3-Cbl, while the peaks of CH_3-Cbl and Ado-Cbl were similar in the maternal plasma. The AdoCbl peak in milk was larger than in maternal or umbilical plasma. The relatively high substance concentration of cobalamins other than CH_3-Cbl and adoCbl is most likely caused by a limited protection toward lights during sampling and handling of the samples prior to the analytical work.

The two physiologically active forms of vitamin B_{12}, AdoCbl and CH_3-Cbl, accounted for 29.7% and 29.5% of maternal plasma, 29.6% and 39% of cord plasma, and 41% and 15% of maternal milk. One HPLC peak was shown by RIDA to be a cobalamin analog. The retention time of this peak was that of dicyano-cobinamide. The very small peak obtained for analogs in milk confirms the analog value obtained from entire milk sample.

Discussion

The concentrations of cobalamin and cobalamin analogs were determined in maternal plasma, cord plasma, and maternal milk. The most interesting observations of the present study were the findings that, while there are cobalamin analogs in maternal plasma, there are more in cord plasma but none in maternal milk. Relatively little is known about the way in which vitamin B_{12} and its analogs are transferred from the mother to the developing fetus, although the total vitamin B_{12} in fetal tissues is significantly higher than that in identical adult tissues.[26] Vitamin B_{12} deficiency in the mother is frequently associated with congenital abnormalities.[22–25]

Some authors[31] question whether vitamin B_{12} analogs exist and consider them to be artefacts of analysis when an RIDA is used. The fact that there were no analogs in milk, while there were in serum argues against this. Moreover,

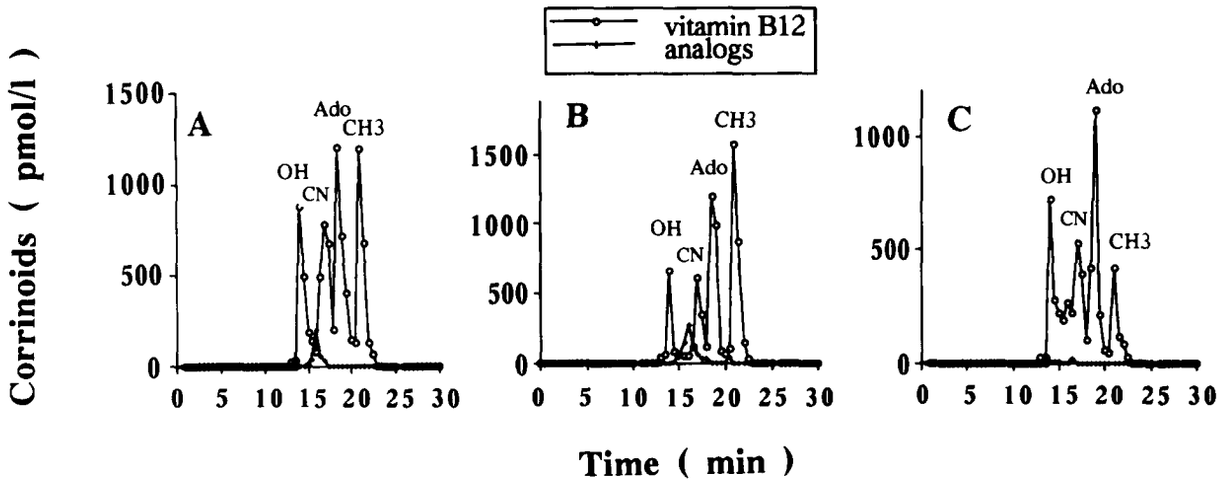

Figure 3 Identification of corrinoids present in maternal plasma (A), cord plasma (B), and milk (C). Fractions obtained by HPLC were assayed by RIDA method for vitamin B_{12} (intrinsic factor as binding protein) and total corrinoids (haptocorrine as binding protein). Analogs correspond to the difference between the two assays.

we have recently proved that analogs are not a measurement artefact, but that they are present in plasma, bile, and feces,[11] but not in exocrine secretions like milk (this report) and seminal fluid (unpublished data).

Though this paper does not define whether analogs are actively transported from the mother to the fetus, we find it most likely that the analogs are created within the fetus. Vitamin B_{12} is transported both to the mammary glands and to the fetus via the same protein, transcobalamin. Transcobalamin binds mainly vitamin B_{12}, and after saturation with the vitamin, the complex is internalized via a specific transcobamin receptor present in most if not all cells of the body. Vitamin B_{12} internalized into the mammary glands is excreted directly into the milk. Vitamin B_{12} entering the fetus may well be metabolized to create the analogs observed in the present study. Another possibility is that minute amounts of analogs are transferred to the fetus and accumulated during pregnancy. The observation that little if any analogs are present in milk fits well with the selective absorption mediated by transcobalamin, but the result is somewhat surprising because the major cobalamin binding protein in milk is haptocorrin, a vitamin-binding protein known to bind both vitamin B_{12} and its analogs.

Vitamin B_{12} analogs differ from vitamin B_{12} in that they do not have Co 5,6-dimethyl benzimidazole, or they possess a modified benzimidazole group.[19] There are also other corrinoids that differ in the tetrapyrrole area or have a central atom other than cobalt, such as nickel, copper, or zinc. The analogs in human plasma may have two origins: synthesis by gut flora microorganisms, or cobalamin catabolism. The organism seems to be partially protected from absorption of analogs[19] because most of them do not bind to the vitamin B_{12} binding protein involved in absorption, the intrinsic factor, and thereby not to the ileal receptor, and most of them that do are retained on the membrane. In plasma, analogs have a greater affinity for haptocorrin than for transcobalamin II, which delivers corrinoids to tissues. The enhanced ability of haptocorrin to bind analogs enables them to be transported to the liver, where they are cleared by the asialo receptor. Analogs are then excreted via the bile and feces.[9,11]

The few analogs that are bound to transcobalamin II may enter a variety of tissues, although this has only been shown with synthetic analogs.[19] The complex transcobalamin II-cbl analogs could be taken up by pinocytosis, as is the transcobalamin II-cbl complex.[32] In the present study, no analogs were found in the exocrine mammary secretion, milk. Thus, milk is protected from analogs. The reason is unknown. Perhaps analogs do not enter human exocrine human cells, or mammary cells work like other cells and export analogs via a mechanism different from that of exocrine secretion.

The main forms of cobalamin present in maternal plasma, cord plasma, and milk are OH-cbl, CN-cbl, Ado-cbl, and CH_3-cbl, as determined by a combination of HPLC-RIDA. CH_3-cbl and Ado-cbl are the major forms of cobalamin in maternal plasma and cord plasma. Ado-cbl is the major form of cobalamin in maternal milk. Our results are in agreement with previous studies in human plasma[4,9] and in human milk, although Ado-cbl was not routinely separated from OH-cbl in this latter study.[33] One important observation is the increase in the CH_3-Cbl concentration in the cord plasma, in agreement with Linnell et al.,[34] who established a relationship between the increase in CH_3-cbl in the plasma of children and the need for active production of the methionine to ensure tissue growth in fetal and infant development. For this reason, the high CH_3-cbl concentration in umbilical plasma could be due to the fact that vitamin B_{12} is an essential nutrient for all cells, especially during development, as CH_3-cbl is a cofactor for the methylation of homocysteine to methionine.[3,6] Ado-cbl is the cofactor for conversion of methylmalonyl-CoA to succinyl-CoA. The reasons for the high CH_3-cbl concentration in infancy remains unknown. There could be an active transport process. Some authors have suggested that cobalamin is transferred to the fetus via the cord blood as CH_3-cbl[35].

In conclusion, the present paper indicates that milk, in contrast to plasma, contains little if any vitamin B_{12} analogs. Surprisingly, fetal plasma does contain analogs. Further studies are needed to clarify whether analogs are transported from the mother to the fetus or whether these substances are produced within the fetus.

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