

Cobalt-vitamin B-12 interrelationships in liver of fetuses and infants

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The concentrations of cobalt and vitamin B-12 were measured in 90 autopsy samples of liver from fetuses and newborn infants from the city of Brasilia, Brazil. Gestational age varied from 19–42 weeks. Vitamin B-12 was assayed by L. leichmannii, and cobalt by electrothermal atomic absorption spectrophotometry. Concentrations on a wet weight basis of vitamin B-12 ranged from 83–758 ng/g of liver (293.15 ± 14.91 ; mean \pm SEM), and of cobalt ranged from 6–45 ng/g liver (17.68 ± 0.85 ; mean \pm SEM). The percentage of cobalt as vitamin B-12 ranged from 23–100 (72.4 ± 1.8 ; mean \pm SEM). The percentage of cobalt as vitamin B-12 was not affected by fetal development measured as liver weight ($r = 0.08$; $P < 0.4$). Liver weight was not significantly correlated with concentrations of either cobalt ($r = -0.16$; $P < 0.14$) or vitamin B-12 ($r = -0.15$; $P < 0.14$). However, the correlation between cobalt and vitamin B-12 concentrations was highly significant ($r = 0.83$; $P < 0.0001$). Concentration of vitamin B-12 can be derived from cobalt concentration by the equation [vitamin B-12] = $14.62x[Co] + 34.48$.

Keywords: human; liver; fetuses; Co; vitamin B-12

Introduction

Cobalt is an essential trace element considered to be present in the body only as a preformed component of the vitamin B-12 molecule.¹ There are more than 20 naturally occurring analogues of vitamin B-12 that contain cobalt, some of them found in mammals.² Not all cobalamins, including some which have been found in human tissues, possess vitamin B-12 activity.^{3,4} Liver is the principal storage organ for vitamin B-12.

The nutritional interrelationships between cobalt and vitamin B-12 have been studied extensively in domestic ruminant species.⁵ In cows and sheep fed adequate amounts of cobalt, most of the cobalt can be accounted for in the liver as vitamin B-12. However, with low dietary intake of cobalt, only one-third of it exists in the liver as the vitamin.⁵ It was suggested that in this situation elemental cobalt appears to be in forms unavailable for vitamin B-12 synthesis.⁵

The human infant is born with ample hepatic reserves of vitamin B-12 that seem to increase in adult life.^{6,7} While many studies have reported concentra-

tions of vitamin B-12 in human fetal liver,^{6,8–11} only a few have dealt with hepatic cobalt.^{12–14} It would appear that none have dealt with both nutrients.

Because of the presence of cobalt in the cobalamin molecule, several chemical methods for vitamin B-12 determination have been proposed based on detection of the metal. Atomic absorption spectrophotometry has been used successfully to determine vitamin B-12 from cobalt in pharmaceutical preparations^{15–17} with extremely low levels of inorganic cobalt, and in feeds¹⁸ after extraction of the vitamin.

With the development of the graphite furnace in the instrumentation for atomic absorption spectrometry, limit of detection has improved from the μg to the ng level.¹⁹ Therefore using graphite furnace techniques for the metal and the traditional microbiological assay for the vitamin, we studied the concentrations of cobalt and vitamin B-12 during human fetal development. Cobalt and vitamin B-12 concentrations did not vary according to sex or stage of fetal development. It was found that cobalt concentration may be a useful predictor of vitamin B-12 concentration in human tissue.

Materials and methods

Ninety liver samples of fetuses (19–42 weeks of gestation) and infants (less than 30 days) who died from various causes

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Table 1 Gestational age, liver weight, concentrations of cobalt, and vitamin B-12 in livers of fetuses and infants

	N	Mean	SEM	Range
Gestational age, ^a weeks	84	32.8	0.6	19-42
Cobalt, ng/g	90	17.68	0.8	6.0-45.0
Vitamin B-12, ng/g	90	293.2	14.9	83.0-758.0
% Cobalt as Vitamin B-12	90	72.4	1.8	23.0-100
Liver weight, g	90	104.06	5.5	23.0-290.0
Total Co in liver, µg	90	1.77	1.25	0.18-7.74
Total B-12 in liver, µg	90	29.37	2.17	3.65-130.37

^aThere were six fetuses without record for gestational age.

in the city of Brasilia, Brazil were collected during autopsies. For each sample, the cause of death, length of gestation, weight of liver, and sex were recorded. Gestation age was ascertained from the last menstrual period. Within hours of death, approximately 10 g were cut from the central portion of the right lobe of the liver, immediately frozen, and kept below -20° C until analysis. Before analysis each sample was divided into four portions. Two of these portions were further subdivided into smaller segments of approximately 2 g and 1 g for cobalt and vitamin B-12 determinations, respectively.

Vitamin B-12 was assayed by a microbiological method using ATCC 7830 *Lactobacillus leichmannii* as the test microorganism. The samples were homogenized in 0.1 N acetate buffer, pH 4.6, using a homogenizer (Thomas Scientific, Swedesboro, NJ, USA). The volume of the homogenate was made up to 20 mL with the same acetate buffer. Four aliquots of 2 mL were taken from the homogenate and digested with 5 mL of a freshly prepared solution of 0.6 g of papain (Biobras, Montes Carlos, Brazil) and 0.02 g of NaCN (Merck, Rio De Janeiro, Brazil) in 100 mL of 0.1 N acetate buffer, for 1 hour at 60° C, followed by 10 minutes of incubation at 100° C. After cooling, the volume was made up to 50 mL. The solution was then passed through filter paper and 5 mL of the filtrate were diluted to 100 mL with water. Five mL of this final solution was used in the microbiological assay according to the Association of Official Analytical Chemists.²⁰ A standard curve containing known amounts of the vitamin was run with each assay, and growth of the microorganisms was assessed by optical density at 550 nm in a Varian 634 spectrophotometer (Varian, São Paulo, Brazil). The average recovery of the method was 123.3% ± 9.5 ($n = 4$). The concentration of the vitamin B-12 standard was checked spectrophotometrically at 550, 361, and 278 nm according to manufacturer's instructions (Merck). Culture media were purchased from Difco Laboratories (Detroit, MI, USA).

For the cobalt determination, approximately 2 g of liver were digested in 3 mL of concentrated nitric acid (Suprapur, Merck, Darmstadt, Germany) under 1.0 kgf/cm² of pressure for 15 minutes, evaporated to dryness, and the volume made up to 2 mL with 0.5% (vol/vol) nitric acid (Suprapur).

The determination of cobalt was done by graphite furnace atomic absorption spectrometry (Varian Spectra AA, Varian Techtron Pty Ltd., Victoria, Australia) with atomization from the tube wall in pyro-coated graphite tubes. The sample volume used in atomization was 20 µL and the standard additions method was applied. The parameters for the graphite furnace operation were charring temperature 1700° C and atomization temperature 2500° C. The accuracy and precision of the method were evaluated by using a National Bureau of Standards (NBS) 1577a certified bovine liver.

The proportion of cobalt as vitamin B-12 was calculated as: % cobalt as vitamin B-12 = 4.34[vitamin B-12]/[Co]. The data were summarized as means ± SEM and ranges. Two-sample *t* test, Pearson correlations between variables, linear regression analysis, and data summarization were performed with SAS computer programs for PC (SAS Institute, Cary, NC, USA). A *P* value of less than 0.05 was considered significant.

Results

Summary data for concentrations, fetal age, and nutrients are shown in *Table 1*. The gestational age ranged from 19-42 weeks. Of the 90 samples, 70 came from stillborns or infants that died within 72 hr after birth, and 18 came from infants that lived from 3-12 days. Two infants were 1 month old. Most of the fetuses died of prematurity or perinatal causes. The mean concentration of vitamin B-12 and cobalt and their SEM are expressed on a wet weight basis. The calculated amount of cobalt as vitamin is expressed as mean and SEM of this percentage, assuming that cobalt is 4.34% of the vitamin B-12 molecule by weight.

Table 2 shows a summary of nutrient concentration data according to sex and liver weight as a proxy for stage of fetal development. There were no significant differences between sexes or stages of development for the concentration of vitamin B-12, cobalt, and cobalt as vitamin.

Figure 1 is a plot of the concentrations of cobalt versus vitamin B-12 ($r = 0.83$; $P < 0.0001$). *Figures 2 and 3* show that neither vitamin B-12 nor the per-

Table 2 Means (+/-SEM) of vitamin B12 and cobalt according to stage of development (liver weight) and sex of babies

	N	Vitamin B-12 ng/g	Cobalt ng/g	Co as B-12 %
Liver weight				
< 150 g	47	303.61 (19.01)	18.02 (1.14)	74.17 (2.52)
> 150 g	43	281.72 (23.42)	17.32 (1.27)	70.54 (2.53)
<i>P</i> *		0.278	0.682	0.785
Sex				
Male	45	312.13 (22.90)	18.71 (1.27)	72.58 (2.46)
Female	45	274.18 (18.95)	16.66 (1.11)	72.29 (2.62)
<i>P</i> *		0.214	0.362	0.677

*From two-sample *t* test.

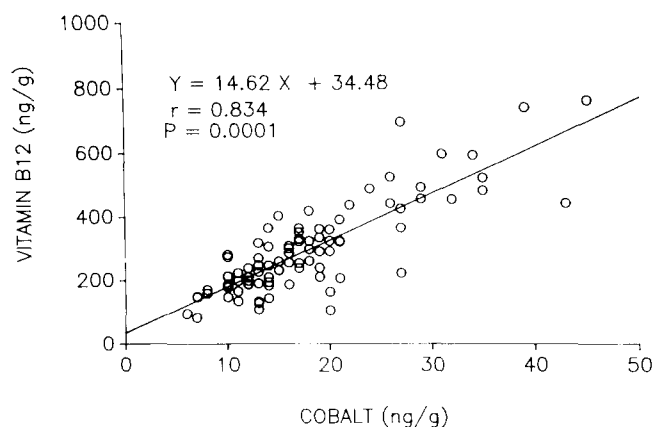


Figure 1 Concentrations of cobalt and vitamin B-12 in livers of fetuses and infants.

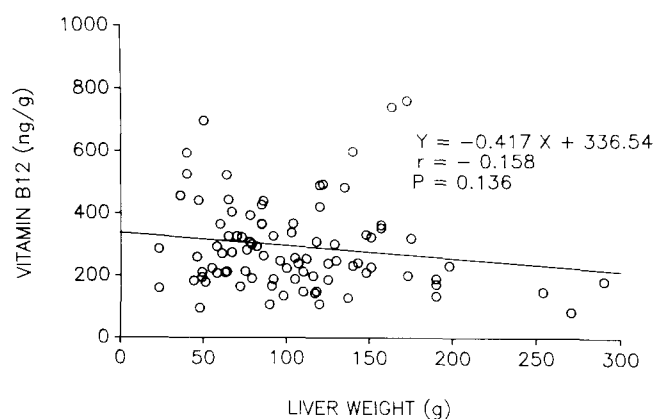


Figure 2 Plot of fetal development (liver weight) against vitamin B-12.

centage of cobalt as vitamin are affected by fetal development as indicated by liver weight. The coefficient of correlation between cobalt concentration and the percentage of cobalt as vitamin B-12 was also low (*Figure 4*) and not significant ($r = -0.177$; $P = 0.09$).

Discussion

The concentrations of vitamin B-12 reported here agree with those seen in studies done in Sweden,⁸ UK,⁶ India,⁹ Finland,⁷ and Mexico.^{10,11} The hepatic concentrations of vitamin B-12 in the present study were not affected by gestational age as indicated by size of fetus. This is in agreement with other reports but contrary to the findings of Vaz Pinto et al.¹⁰ Baker et al.⁹ reported an increase in total hepatic reserves of vitamin B-12 as a function of age. This is due to increases in liver weight but not in liver concentration of vitamin B-12. Liver weight did not affect vitamin B-12, and because there was a highly significant ($P < 0.0001$) correlation between concentrations of vitamin B-12 and cobalt, cobalt concentration in liver would not be expected to be affected by liver weight. Indeed this was the case.

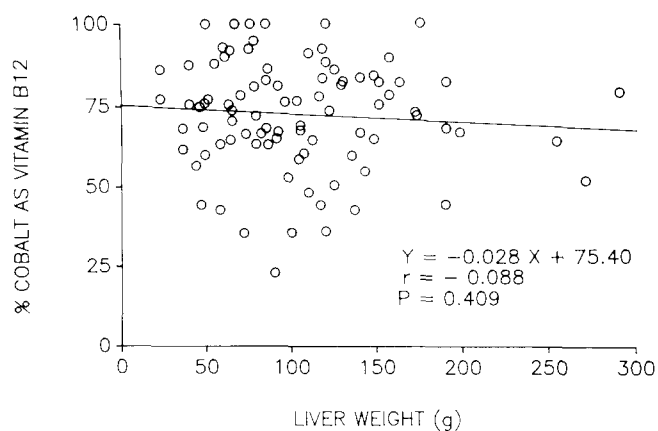


Figure 3 Plot of fetal development (liver weight) and percent of cobalt as vitamin B12 (assuming 4.34% of Co).

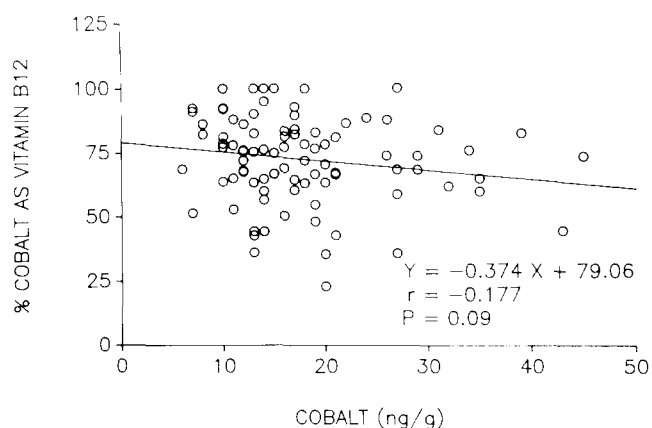


Figure 4 Plot of concentration of cobalt and percent of cobalt as vitamin B-12 (assuming 4.34% of Co).

There is indication that serum cobalamins in females may be higher than in males.²¹ However, no significant differences between sexes were found in the liver concentration of vitamin B-12 or cobalt.

The few published analyses of cobalt in infant livers were done by different methods, such as spark source mass spectrophotometry,¹³ flame absorption spectrometry,¹² and neutron activation analysis.¹⁴ The present results are in the range reported by Shand et al.¹³ Comparisons with other studies are difficult, because Widdowson et al.¹² reported a range of values below the recommendation for flame absorption spectrometry, while Alexiou et al.¹⁴ worked with only eight samples.

Contrary to the situation in ruminant species, there are no indications of benefits from cobalt ingestion in humans. The low coefficient of correlation between cobalt and the percentage of cobalt as vitamin B-12 ($r = -0.177$, $P = 0.09$) indicates that proportionality of cobalt as vitamin is not likely to change because of different concentrations of cobalt in the fetal liver or during fetal development.

There is no evidence of vitamin B-12 deficiency during pregnancy in Brasilia, possibly because of the

absence of strict vegetarians. Therefore the range of variation in the proportion of cobalt as vitamin B-12 could be due in part to the presence of different forms of cobalt. A calculated average of 27.6% of cobalt exists in forms not accounted for by the microorganism response. This fraction may include cobalamins that, although inactive in *L. leichmannii*, may be biologically active in humans. Kondo et al.² and Muir and Landon²² found analogues of vitamin B-12 in fetal circulation, and it is possible that these analogues may be present in liver. It is not clear in which forms cobalt other than cobalamins can be present in human liver. In fetuses and infants, direct contribution of cobalt from dietary sources other than the mother's milk were very small. The microorganism used in our assay is relatively specific for cobalamins but also responds to some non-cobalamin analogues. Therefore we do not know the exact proportion of elemental cobalt to cobalt in the vitamin B-12 that can be detected by *L. leichmannii*. It should be noted that the microbiological assay can incorporate an error of 10%.¹⁶

With the current improvements in analytical tools it is possible to predict concentrations of vitamin B-12 from concentrations of cobalt at the low levels of the vitamin found in animal fluids and tissues. Although prediction of the vitamin from cobalt has been reported for quality control of pharmaceutical preparations,^{15-17,19} its use in nutritional formulations has been limited because of contaminant cobalt.¹⁸ Within the detection limit achievable by graphite furnace techniques, it is now possible to predict vitamin B-12 concentrations and therefore extend its application to nutritional studies. This could reduce some difficulties reported with microbiological assays,^{16,23} but not those due to non-cobalamin cobalt-containing vitamin B-12 analogues found in human serum and tissue, which only radioassays can recognize.²⁴

References

- 1 Ellenbogen, L. (1984). Vitamin B-12. In *Handbook of Vitamins*, (L.J. Machlin, ed.) p. 497-547, Marcel Dekker, Inc., New York, NY, USA
- 2 Kondo, M., Kolhouse, J.F., and Allen, R.H. (1980). Presence of cobalamin analogues in animal tissue. *Proc. Natl. Acad. Sci. USA* **77**, 817-821
- 3 Kolhouse, J.F., Kondo, H., Allen, N.C., Poddell, E., and Allen, R.H. (1978). Cobalamin analogues are present in human plasma and can mask cobalamin deficiency because current radioisotope dilution assays are not specific for true cobalamin. *N. Engl. J. Med.* **299**, 785-792
- 4 Kanazawa, S. and Herbert, V. (1983). Noncobalamins vitamin B-12 analogues in human red cells, liver and brain. *Am. J. Clin. Nutr.* **37**, 774-777

- 5 Smith, R.M. (1977). Cobalt. In *Trace Elements in Human and Animal Nutrition*. Volume I, p. 143-179, Academic Press, Inc., New York, NY, USA
- 6 Hsu, J.M., Kawin, B., Minor, P., and Mitchell, J.A. (1966). Vitamin B12 concentrations in human tissues. *Nature* **210**, 1264-1265
- 7 Rappazzo, M.E., Salmi, H.A., and Hall, C.A. (1970). The content of vitamin B12 in adult and foetal tissue: A comparative study. *Br. J. Haematol.* **18**, 425-433
- 8 Killander, J. (1958). The vitamin B12 content of human fetal tissues. *Acta Chim. Scand.* **12**, 1356-1359
- 9 Baker, S.J., Jacob, E., Ajan, K.T., and Swaminathan, S.P. (1962). Vitamin B12 deficiency in pregnancy and puerperium. *Br. Med. J.* **1**, 1658-1661
- 10 Vaz Pinto, A., Torras, V., Figueroa Sandoval, J.F., Dillman, E., Ramirez Mateos, C., and Cordova, M.S. (1975). Folic acid and vitamin B12 determination in fetal liver. *Am. J. Clin. Nutr.* **28**, 1085-1086
- 11 Loria, A., Vaz-Pinto, A., Arroyo, P., Ramirez-Mateos, C., and Sanchez-Medal, L. (1977). Nutritional anemia. VI. Fetal hepatic storage of metabolites in the second half of pregnancy. *J. Ped.* **91**, 569-573
- 12 Widdowson, E.M., Chan, H., Harrison, G.E., and Milner, R.D.G. (1972). Accumulation of Cu, Zn, Mn, Cr, and Co in the human liver before birth. *Biol. Neonate* **20**, 360-367
- 13 Shand, C.A., Agget, P.J., and Ure, A.M. (1985). The spark-source mass spectrometric determination of the trace element composition of human foetal livers. In *Trace Elements in Man and Animal*. TEMA 5. p. 642-645, Commonwealth Agricultural Bureau, London
- 14 Alexiou, D., Grimanis, A.P., Grimani, M., Papaevangelou, G., Koumantakis, E., and Papadatos, G. (1977). Trace elements (zinc, cobalt selenium, bromine, gold) in human placenta and newborn liver at birth. *Ped. Res.* **11**, 646-648
- 15 Diaz, F.J. (1972). Determination of cyanocobalamin by atomic absorption spectrophotometry with a pre-mix air-acetylene flame. *Anal. Chim. Acta* **58**, 455-458
- 16 Peck, E. (1978). The application of flameless atomic absorption to the analysis of vitamin B12. *Anal. Letters* **B11**, 103-117
- 17 Berge, D.G., Plaum, R.T., Lehman, D.A., and Franck, C.W. (1986). Indirect determination of organic compounds by atomic absorption spectrometry: the study of vitamin B12 in pharmaceutical dosage forms. *Anal. Letters* **1**, 613-618
- 18 Whitlock, L.L., Melton, J.P., and Billings, T.J. (1976). Determination of vitamin B12 in dry feeds by atomic absorption spectrophotometry. *J. Assoc. Off. Anal. Chem.* **59**, 580-581
- 19 Medina-Escriche, J., Hernandez-Llorens, M.L., Llobat-Stelles, M., and Sevillano-Cabezas, A. (1987). Determination of vitamin B12 as cobalt by use of a catalytic-spectrophotometric method. *Analyst* **112**, 309-311
- 20 A.O.A.C. 1980. *Official Methods of Analysis*, 13th ed. Association of Official Analytical Chemists, Washington, DC, USA
- 21 Fernandes-Costa, F., Van Tonder, S., and Metz, J. (1985). A sex difference in serum cobalamin and transcobalamin levels. *Am. J. Clin. Nutr.* **41**, 784-790
- 22 Muir, M. and Landon, M. (1985). Endogenous origin of microbiologically inactive cobalamins (cobalamins analogues) in the human foetus. *Br. J. Haematol.* **61**, 303-306
- 23 Herbert, V. and Drivas G. (1982). Spirulina and vitamin B12. *J. Am. Med. Assoc.* **248**, 3096-3097
- 24 Das, K.C., Manusselis, C., and Herbert, V. (1991). Determination of vitamin B12 (cobalamin) in serum and erythrocytes by radioassay, and holotranscobalamin II (holo-TC II) and holo-haptocorrin (holo-TC I and III) in serum by adsorbing holo-TC II on microfine silica. *J. Nutr. Biochem.* **2**, 455-464