Serum Folate: A Pharmacodynamic Biomarker of Intracellular Nitrosylcobalamin Activity Following Intravenous Administration in Dogs

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Abstract. *Background/Aim: Cobalamin and folate are interdependent co-factors of the methionine synthase pathway. This study evaluated the effect of intravenously-administered nitrosylcobalamin (NO-Cbl), a vitamin B12 analog, on serum folate concentrations in healthy dogs. Materials and Methods: Four dogs received a 10-mg/kg, 20-mg/kg and 40-mg/kg intravenous bolus dose of NO-Cbl, with a 14-day washout period between doses. Blood samples were collected at baseline and post-dosing, and serum cobalamin and folate concentrations were measured. Results: For each dose, serum cobalamin concentrations were inversely correlated with serum folate concentrations. Spearman rank correlation co-efficient values were –0.976 (10 mg/kg, p<0.0096), and –1.0 (20 mg/kg, p<0.008; 40 mg/kg, p<0.0046). Conclusion: Cellular uptake of NO-Cbl, following intravenous administration exerted a biological effect on folate, similar to that previously described for other vitamin B12 analogs. Serum folate concentration may serve as a pharmacodynamic biomarker of intracellular nitrosylcobalamin activity following intravenous administration.*

Cobalamin (vitamin B12) and folate (vitamin B9) are water-soluble molecules that serve as co-factors for specific enzymes that are crucial for cellular metabolism. Cobalamin refers to a class of substances comprising of a corrin ring with four pyrroline groups linked to a central cobalt atom. Each of the various cobalt-linked upper axial ligands denotes a different name: methyl (methylcobalamin; Me-Cbl), 5-deoxyadenosine (adenosylcobalamin; Ado-Cbl), hydroxyl (hydroxocobalamin; OH-Cbl), cyanide (cyanocobalamin; CN-Cbl), and more recently nitric oxide (nitrosylcobalamin, NO-Cbl). Routine immunoassays can be used to measure each of these forms following conversion to cyanocobalamin (1, 2).

Nitrosylcobalamin (NO-Cbl), a novel vitamin B12 analog, is comprised of nitric oxide (NO) bound to the upper axial ligand position of cobalamin (3). Developed as a potential anticancer agent, NO-Cbl functions as a biological 'Trojan horse', utilizing the vitamin B12 transcobalamin II (TCII) transport protein and transcobalamin II cell surface receptor (TCII-R) to specifically target NO-Cbl to cancer cells (4). Once NO-Cbl is internalized into cancer cells through TCII-R-mediated endocytosis, NO is liberated from cobalamin, resulting in decreased cellular metabolism, activation of apoptotic mechanisms and inhibition of survival pathways (4-7). Serum concentrations of NO-Cbl can be measured using standard cobalamin immunoassays (1).

Folate is a term that refers to a family of compounds containing a pteridine ring joined to both p-aminobenzoic acid and glutamic acid (2). Multiple single-carbon groups, including methyl (-CH_3), formyl (-CHO) and methylene (-CH_2-) are cross-linked between the amino group of the pteridine ring and the amino group of p-aminobenzoic acid, resulting in different forms of folate (2, 8). The primary function of each of these forms of folate is the transfer of single-carbon groups from the central folate atom to various enzymes involved in key biosynthetic reactions. Most routine immunoassays measure a composite blend of all forms of folate (2, 9).

Cobalamin and folate function as interdependent co-factors in the methionine synthase metabolic pathway, which serves as the final step in the conversion of homocysteine (a non-proteogenic amino acid) to methionine (an essential sulfur-containing amino acid). Methionine synthase, the enzyme

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responsible for catalyzing the final step of this conversion, contains the co-factor methylcobalamin (the major form of vitamin B12 in serum) and utilizes the folate substrate N^5-methyltetrahydrofolate (mTHF; the major form of folate in serum) (10). In the first step of this two-step conversion, methylcobalamin is formed by the transfer of a methyl (-CH_3) single-carbon group from N^5-mTHF, resulting in the production of methylcobalamin and tetrahydrofolate (THF), the reduced form of N^5-mTHF. In the second step, methylcobalamin transfers the -CH_3 group to homocysteine, thereby regenerating the cobalamin co-factor and producing methionine. Most of the methionine formed is converted to S-adenosylmethionine, which serves as a universal -CH_3 group donor for DNA, RNA, hormones, neurotransmitters, membrane lipids and proteins (11). Tetrahydrofolate produced by the methionine synthase pathway is predominantly used in the synthesis of DNA. During the course of the methionine synthase reaction, the cobalamin co-factor is regenerated, but the folate substrate N^5-mTHF is irreversibly reduced from its precursor, methylenetetrahydrofolate, and ultimately becomes 'trapped' in the reaction.

Cobalamin supplementation has been associated with a significant decrease in serum folate concentration in both cats and humans (12-14). It is hypothesized that the increase in serum cobalamin concentration induces an increase in methionine synthase activity, resulting in increased intracellular demand for mTHF to drive the methionine synthase reaction (12). Since the serum folate concentration is typically only 1% to 2% of the intracellular concentration, the shift in mTHF distribution from extracellular to intracellular compartments is reflected, most noticeably, in the serum concentration (12). Thus, it is believed that increased folate consumption driven by cobalamin supplementation is observed as an overall decrease in serum folate concentration.

Pharmacodynamic (PD) biomarkers are objectively measured characteristics that provide evidence of a direct pharmacological effect of a targeted drug on a target (15, 16). Use of PD biomarkers is becoming increasingly important during the pre-clinical and early-clinical stages of targeted-anticancer drug development. As critical tools in this process, PD biomarkers provide proof-of-concept of biologic mechanisms, assist in determination of optimal dose and schedule, increase understanding of response and resistance mechanisms, permit design of rational combination therapies, facilitate interpretation of clinical data and support regulatory drug submission. While intracellular uptake of NO-Cbl has been demonstrated *in vitro* (4), an *in vivo* biological marker for the intracellular uptake of NO-Cbl has not yet been elucidated. The purpose of this study was to determine the effect of intravenous NO-Cbl administration on serum folate concentrations in healthy dogs, and to assess whether folate response can potentially be used as a pharmacodynamic biomarker of NO-Cbl uptake and intracellular activity.

Materials and Methods

Experimental animals consisted of 4 intact, 10-month-old male Beagle dogs, ranging in weight from 9.5 to 10.5 kg, purchased from Marshall BioResources (North Rose, NY, USA). Dogs were housed in the Comparative Medicine Unit at the Northeast Ohio Medical University (Rootstown, OH, USA) and were monitored daily. All study procedures were approved by the University's Institutional Animal Care and Use Committee. A 14-day acclimation period was provided prior to the start of the study. Throughout the study dogs were fed a commercial diet containing 170 mcg/kg vitamin B12 and 3.3 ppm folic acid (Advanced Protocol® High Density Canine Diet, Purina LabDiet®, St. Louis, MO, USA).

NO-Cbl at a concentration of 40 mg/mL was synthesized as previously described (3, 4). NO-Cbl was administered to all dogs by a single intravenous bolus injection through a 22-gauge butterfly catheter placed into the cephalic vein. Doses of 10, 20, and 40 mg/kg NO-Cbl were administered, with a 14-day washout period between each administered dose. Dogs were re-weighed prior to each dosing event. Food was withheld from the dogs for 12 hours prior to each dosing event and throughout the 24-hour sampling period. Blood samples were collected from the jugular vein at 0 (pre-dose), 0.25 (40 mg/kg dose only), 0.5, 1,2, 4, 8, 12 and 24 hours after dosing. Whole-blood samples were placed in rapid serum separating tubes (BD Vacutainer Rapid Serum Tube [RST], Becton, Dickinson & Co., Franklin Lakes, NJ, USA) containing a gel separator and a thrombin additive and approved for both vitamin B12 and folate analysis per the product insert. The samples were mixed immediately by gentle inversion and centrifuged within 15 min of collection according to the manufacturer's directions. Blood samples were spun in a refrigerated centrifuge (Kendo Sorvall RT 6000 Chilled Centrifuge, Block Scientific Inc., Bohemia, NY, USA) at 23˚C, for 10 min at 1278 ×g. Serum from each sample was transferred to a cryotube and stored at –20˚C. Frozen serum samples were shipped to the Gastrointestinal Laboratory at Texas A&M University (College Station, TX, USA) for automated determination of cobalamin and folate concentrations using a solid-phase, competitive chemiluminescent enzyme immunoassay, according to manufacturer's instructions (Immulite 2000, Siemens Healthcare Diagnostics, Deerfield, IL, USA). The reportable range for cobalamin was 150 to 1,000 pg/ml, and for folate was 1-24 ng/mL. Any samples with a cobalamin concentration exceeding 1,000 pg/mL or a folate concentration exceeding 24 ng/mL were diluted as specified in the kit instructions and re-analyzed.

The Spearman rank correlation co-efficient was used as a non-parametric measure of the statistical dependence between serum cobalamin (NO-Cbl) and folate concentrations following intravenous NO-Cbl administration. The correlation co-efficient for each dose of NO-Cbl was calculated using an online computer software program (Free Statistics Software, Office for Research Development and Education, version 1.1.23-r7, URL http://www.wessa.net). A perfect Spearman rank correlation co-efficient of –1.0 was assigned when each of the variables was a perfect inverse correlation function of the other. Significance was set at $p \leq 0.01$.

Results

Average serum cobalamin (NO-Cbl) and folate concentrations at each sampling time point for each NO-Cbl dose are summarized in Table I.

Table I. *Average serum cobalamin (NO-Cbl) and folate concentrations following intravenous bolus administration of 3 doses of nitrosylcobalamin (NO-Cbl).*

	10 mg/kg NO-Cbl		20 mg/kg NO-Cbl		40 mg/kg NO-Cbl	
	Cobalamin (pg/mL)	Folate (ng/mL)	Cobalamin (pg/mL)	Folate (ng/mL)	Cobalamin (pg/mL)	Folate (ng/mL)
0 h (baseline)	229	20.3	285	18.6	282	16.9
0.25 h*	-	-	-	-	14,148	1.8
0.5 h	2,493	5.9	5,807	4.5	10,816	2.0
1 h	2,076	5.5	4,130	4.6	8,405	2.9
2 h	1,545	6.2	2,683	4.8	5,911	2.9
4 h	909	8.2	1,936	5.9	3,387	4.0
8 h	667	10.5	1,040	7.3	1,551	5.7
12 h	562	11.6	863	8.4	924	6.9
24 h	440	16.7	599	14.2	708	12.0

*An earlier sampling time point (t=0.25 h) was added for the 40-mg/kg dose (t=0.5 h was the first sampling time point for both the 10- and 20-mg/kg doses).

Pre-dose (t=0 h) serum cobalamin concentrations were slightly below the lower limit of the reference interval (251-908 pg/mL) for all 4 dogs at the 10 mg/kg dose (221, 224, 228, 242 pg/mL), but were within the normal reference interval for all dogs at both the 20 mg/kg and 40 mg/kg doses. High serum cobalamin (NO-Cbl) concentrations at the first post-administration sampling time point (t=0.5 h) following administration of both the 10 mg/kg and 20 mg/kg doses of NO-Cbl, prompted the addition of an earlier sampling time point (t=0.25 h) for the 40 mg/kg dose. Time to average maximum serum cobalamin (NO-Cbl) concentration (T_{max}), following NO-Cbl administration was 0.5 h (10 mg/kg and 20 mg/kg doses), and 0.25 h (40 mg/kg dose).

Pre-dose (t=0 h) serum folate concentrations were within the normal reference interval (7.7-24.4 ng/mL) for all dogs at all doses with the exception of one dog at the 10-mg/kg dose, whose baseline serum folate concentration was slightly increased (31.2 ng/mL). Time to lowest average serum folate concentration (T_{min}) was 1 h (10-mg/kg dose), 0.5 h (20-mg/kg dose), and 0.25 h (40-mg/kg dose). Average serum cobalamin and folate concentration/time profiles for each dose of NO-Cbl are shown in Figure 1.

A significant inverse correlation between average serum cobalamin (NO-Cbl) and folate concentrations was observed for all time points and for all doses following intravenous NO-Cbl administration. Spearman rank correlation co-efficient values were –0.976 (10-mg/kg dose; 6 degrees of freedom, $p<0.0096$), and –1.0 (20-mg/kg dose, 6 degrees of freedom, $p<0.008$; 40-mg/kg dose, 7 degrees of freedom, $p<0.0046$). There was no significant difference in correlation co-efficient values between NO-Cbl doses. Scatter plots of rank order for each dose of NO-Cbl are shown in Figure 1.

Discussion

Serum cobalamin concentrations at baseline (t=0) were slightly below the age-appropriate reference interval for all dogs at the 10-mg/kg dose, but were within the normal reference interval for all dogs at both the 20-mg/kg and 40-mg/kg doses of NO-Cbl. Low levels of dietary vitamin B12 may have contributed to low-serum cobalamin concentrations in the dogs at the start of the study. Tissue cobalamin concentrations may have sufficiently increased following the first intravenous bolus dose (10 mg/kg) of NO-Cbl, subsequently resulting in normal baseline (t=0) cobalamin concentrations for both the 20-mg/kg and 40-mg/kg doses.

For all doses of NO-Cbl, serum cobalamin (NO-Cbl) concentration rapidly increased following intravenous administration, and time to average maximum serum cobalamin (NO-Cbl) concentration (T_{max}) was evident at the first sampling time point post-administration. It is probable that use of earlier sampling time points would have established an even earlier serum cobalamin (NO-Cbl) T_{max}. The rapid effect of intravenous NO-Cbl administration on serum cobalamin (NO-Cbl) concentration was similar to the effect that has been observed following intravenous injection of other cobalamin analogs (17-20).

Conversely, for all doses, serum folate concentration rapidly decreased following intravenous NO-Cbl administration. Time to average minimum serum folate concentration (T_{min}) was evident at the first sampling time point post administration for both the 20 mg/kg and 40 mg/kg doses of NO-Cbl, and corresponded to the observed cobalamin (NO-Cbl) T_{max} for these doses. While not significant, T_{min} for the 10 mg/kg dose of NO-Cbl occurred slightly later than the observed cobalamin T_{max} for this dose. Low baseline (t=0) serum cobalamin

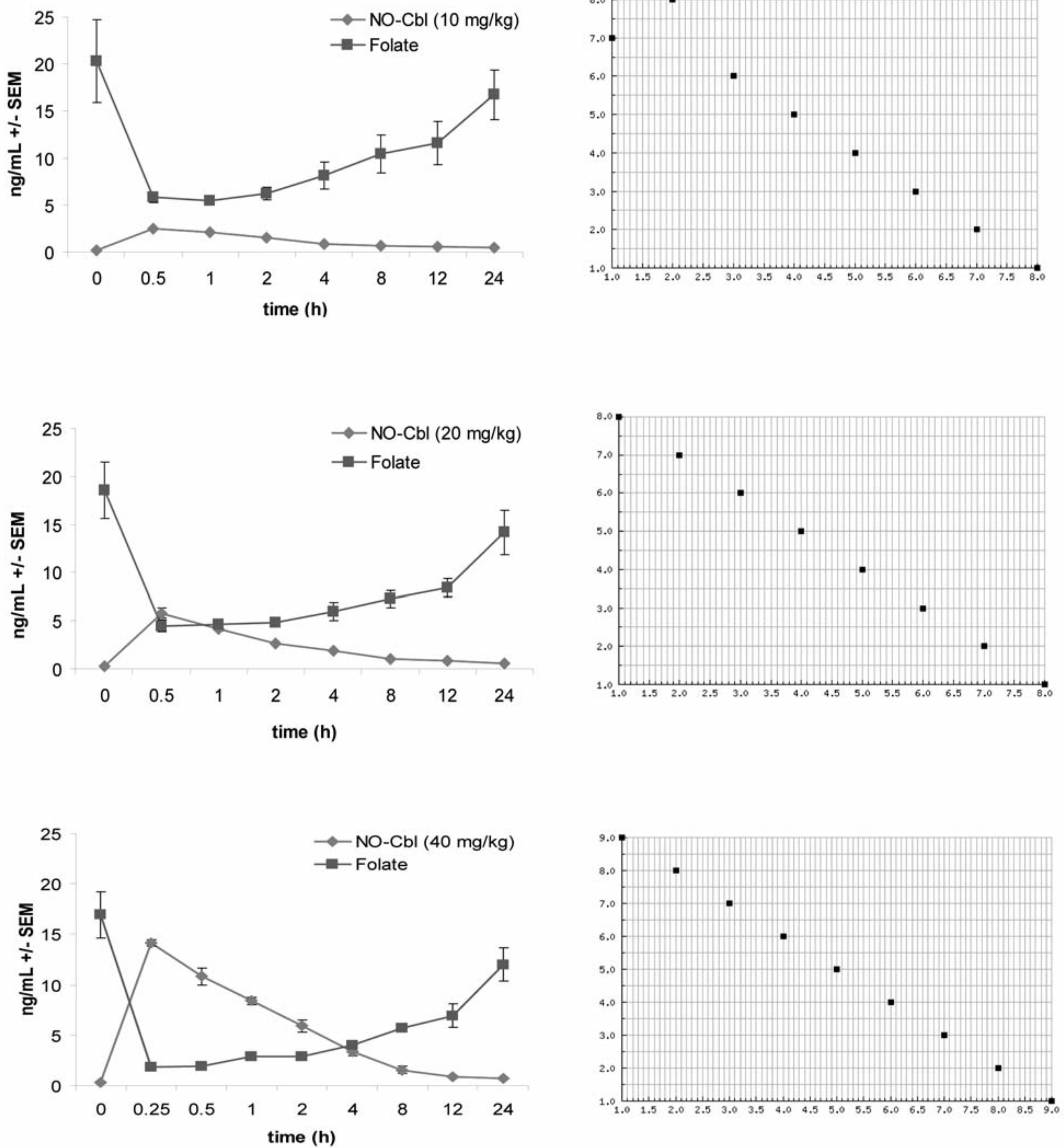

Figure 1. *Average serum cobalamin (NO-Cbl) and folate concentration/ time profiles (left) and scatter plots of rank order (right) in healthy Beagle dogs following an intravenous bolus dose of 10 mg/kg (top), 20 mg/kg (middle) or 40 mg/kg (bottom) nitrosylcobalamin (NO-Cbl). For the concentration/time profiles, data are expressed as mean±standard error of the mean (SEM). For the scatter plots, x axis represents folate concentration (ng/mL) and y axis represents cobalamin (NO-Cbl) concentration (ng/mL).*

concentration in all dogs at the start of the study may have contributed to this effect. Studies have shown that cell surface TCII-R levels are regulated by intracellular levels of cobalamin (21, 22); decreased serum cobalamin concentrations in dogs at the start of the study may have corresponded to expression of fewer cell surface TCII receptors, resulting in a longer lag time to endocytosis of NO-Cbl and to delayed induction of an intracellular effect on folate.

A highly significant inverse correlation was identified between serum cobalamin (NO-Cbl) and folate concentrations, following intravenous NO-Cbl administration. A similar effect on serum folate concentration has been described following cobalamin injection in both cats and humans (12, 13). Results of this study confirm that NO-Cbl was recognized by the cobalamin transport/delivery system and was capable of inducing a folate response similar to that observed with other cobalamin analogs. The consistent response of folate to intravenous NO-Cbl administration demonstrates a direct pharmacological effect of NO-Cbl on cellular function, and supports the use of folate as a reliable PD biomarker of intracellular NO-Cbl activity. Combined with pharmacokinetic and toxicity data, this folate biomarker will be useful in the evaluation of NO-Cbl dose-response relationships and in selection of a rational NO-Cbl dose and schedule of administration. Additional applications may include further understanding of tissue responses and possible mechanisms of resistance to NO-Cbl therapy, evidence-based evaluation of NO-Cbl in combination therapy, and the possible development of an earlier clinical trial-endpoint able to demonstrate treatment effect and correlate with response and/or survival. Additional studies of the effect of NO-Cbl administration on folate concentrations in tumor-bearing patients will be necessary in the pursuit of these applications.

An interesting observation resulting from this study was the profound and prolonged effect exerted on serum folate concentrations by intravenous bolus doses of NO-Cbl. The observed folate response may warrant further investigation of the use of NO-Cbl as a potential anti-neoplastic cytotoxic folate antagonist. Classic folate antagonists used for the treatment of various cancers work by inhibiting dihydrofolate reductase (DHFR), the enzyme responsible for the reduction of dihydrofolate to THF, essential to single-carbon group transfer pathways (23). The ensuing folate deficiency results in cellular apoptosis, increased mutagenesis and inhibition of DNA synthesis in both normal and tumor cells (23, 24). Anti-tumor efficacy of the folate antagonists, currently in use, is limited by either inherent resistance or resistance acquired during the course of treatment (23). As a result, new folate antagonists, able to readily enter cells, target other folate-dependent enzymes, inhibit more than one biosynthetic pathways or affect multiple steps within a single pathway, are being developed to overcome the problem of resistance. A novel folate antagonist such as NO-Cbl that is capable of increased intracellular uptake and that can conceivably decrease tissue folate concentrations by means of increased methionine synthase activity would be less likely to generate significant resistance. Additional studies are needed to evaluate the effect of long-term NO-Cbl administration on serum folate concentrations, and to assess whether this effect demonstrates anti-tumor potential. Furthermore, studies will be necessary to assess possible adverse effects associated with long-term NO-Cbl administration on folate function and serum/tissue folate concentrations. Measurement of red blood cell folate concentrations may provide a more accurate measure of chronic folate status in future studies.

This study has established folate as a pharmacodynamic biomarker of intracellular NO-Cbl uptake and cellular response, following intravenous NO-Cbl administration. This study has also provided initial data to justify evaluation of NO-Cbl as a potential folate antagonist. Results from this study will be used to advance the regulatory submission and pre-clinical and early-clinical development of NO-Cbl as a targeted anticancer drug for use in both humans and companion animals, and to support further evaluation of additional mechanisms of action by which NO-Cbl may prove effective as an anti-tumor agent.

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