Pharmacokinetics of Intravenous Nitrosylcobalamin, an Antitumor Agent, in Healthy Beagle Dogs: A Pilot Study

ANNETTE M. SYSEL[1], WALTER I. HORNE[2], JÖRG M. STEINER[3], JAN S. SUCHODOLSKI[3] and JOSEPH A. BAUER[1]

[1]BNOAT Oncology, Inc., Akron Innovation Campus, Akron, OH, U.S.A.;
[2]Northeast Ohio Medical University, Rootstown, OH, U.S.A.;
[3]Gastrointestinal Laboratory, Department of Small Animal Clinical Sciences, Texas A&M University, College Station, TX, U.S.A.

Abstract. *Background/Aim: Nitrosylcobalamin (NO-Cbl) is a cobalamin-based anti-tumor agent. This study evaluated the pharmacokinetic parameters of NO-Cbl following intravenous administration in dogs. Materials and Methods: Four dogs received 10 mg/kg, 20 mg/kg and 40 mg/kg intravenous bolus doses of NO-Cbl, with a 14-day washout period between doses. Blood samples were collected at baseline and post-dosing, and noncompartmental pharmacokinetic parameters were determined. Results: Average peak serum concentrations of 2265, 5523 and 13,866 pg/mL were achieved following single-dose bolus intravenous administration of 10 mg/kg, 20 mg/kg and 40 mg/kg of NO-Cbl respectively. The average area under the curve was 12,697 h × pg/mL, 24,497 h × pg/mL and 44,976 h × pg/mL respectively, with an average elimination half-life of 16.2 h, 13.5 h and 13.1 h respectively. Conclusion: These results can be used to determine the dose and dosing intervals for clinical trials evaluating NO-Cbl in humans and companion animals.*

The term vitamin B_{12} refers to a class of substances comprising a corrin ring with four pyrroline elements linked to a central cobalt atom. These substances are collectively termed cobalamin, and each of the different 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 for vitamin B_{12} measure all of these forms following conversion to cyanocobalamin.

Nitrosylcobalamin (NO-Cbl) is a novel anticancer agent that is comprised of nitric oxide (NO) bound to the upper axial ligand position of vitamin B_{12} (cobalamin) (1). NO-Cbl is recognized by vitamin B_{12} binding proteins (2) and can be assayed using standard cobalamin immunoassays (3). NO-Cbl functions as a biological 'Trojan horse', utilizing the transcobalamin II (TCII) transport protein and transcobalamin II cell surface receptor (TCII-R) (4) to target NO-Cbl to cancer cells (5). Once NO-Cbl is internalized within cancer cells through TCII-R-mediated endocytosis, NO is liberated from vitamin B_{12}, resulting in decreased cellular metabolism, activation of apoptotic mechanisms and inhibition of survival pathways within the cancer cells (5-8). Uptake of NO-Cbl in cancer cells is increased over that in normal cells in two ways: (i) cancer cells produce TCII that can be used to scavenge, bind and transport NO-Cbl to the cells (9-11), and (ii) cancer cells express greater numbers of TCII receptors on their cell surface than do normal cells, enabling accumulation of NO-Cbl within the cells (12-15). Furthermore, the release of NO from NO-Cbl occurs only in an acidic intracellular environment, resulting in minimal systemic or local toxic effects (5). A major advantage of NO-Cbl administration is its tumor-specific accumulation.

The National Cancer Institute's Developmental Therapeutics Program independently tested NO-Cbl in a human tumor 60 cell line screening and in general, colon, ovarian and breast carcinomas as well as central nervous system tumors were most responsive to the anti-growth effects of NO-Cbl (5). More recently, a case study in dogs showed impressive anti-tumor efficacy of NO-Cbl in the treatment of spontaneous thyroid carcinoma, malignant peripheral nerve sheath tumor, anal gland adenocarcinoma and spinal meningioma (16). In the dogs of this study, NO-Cbl decreased tumor size by 43-100% over a twice daily course of treatment ranging from 10 weeks to 15 months' duration with no evidence of systemic or local toxicity.

This article is freely accessible online.

Correspondence to: Dr. J.A. Bauer, BNOAT Oncology, Inc., Akron Innovation Campus, 411 Wolf Ledges Parkway, Suite 105, Akron, OH 44311, U.S.A. E-mail: jbauer@bnoat.com

Key Words: Nitrosylcobalamin, vitamin B_{12}, cobalamin, nitric oxide, intravenous, pharmacokinetics, dogs.

Although the metabolism of vitamin B_{12} and its analogs has been extensively studied in humans, limited data on vitamin B_{12} metabolism are available for dogs. Studies have demonstrated that vitamin B_{12} (specifically hydroxocobalamin) metabolism in dogs is similar to that in humans (17-20), and the pharmacokinetics of intravenous hydroxocobalamin have been described in six dogs (21). To date, pharmacokinetic analysis of intravenously-administered NO-Cbl has not been conducted in any species. The specific aim of this study was to establish a preliminary pharmacokinetic profile of NO-Cbl following intravenous administration in healthy Beagle dogs.

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.

NO-Cbl at a concentration of 40 mg/mL was administered by a single intravenous bolus 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 h prior to each dosing event and throughout the 24-h 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 h 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 vitamin B_{12} 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 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 the assay was 150 to 1000 pg/mL. Any samples with a cobalamin concentration exceeding 1000 pg/mL were diluted as specified in the kit instructions and reanalyzed.

Noncompartmental pharmacokinetic calculations were conducted using the Kinetica version 5.0 software (Thermo-Fisher Scientific, Waltham, MA, USA). The area under the curve (AUC) was calculated by the trapezoidal method and extrapolated to infinity. For pharmacokinetic analysis, the samples were baseline-subtracted. Differences in mean cobalamin concentrations compared to baseline were calculated using an unpaired 2-tailed Student's *t*-test with a pooled estimator of variance to determine statistical significance. Calculations were made using the Sigma Plot version 10.0 software (SPSS, Chicago, IL, USA). Significance was determined to be $p \leq 0.05$.

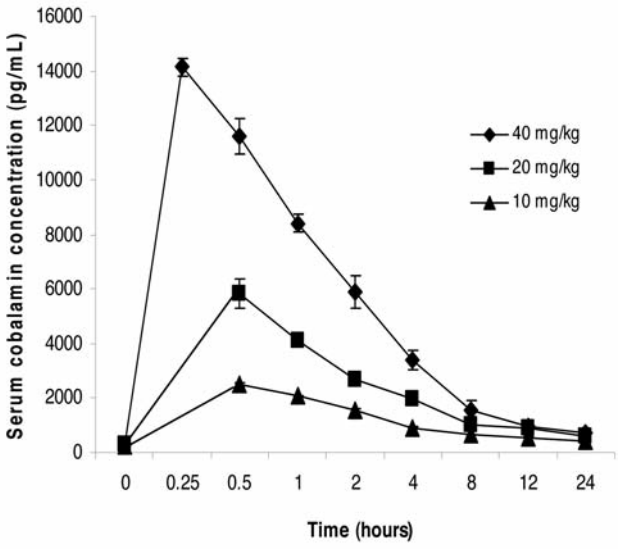

Figure 1. *Mean serum cobalamin (NO-Cbl) concentration-time profiles for 10, 20, and 40 mg/kg doses of nitrosylcobalamin administered intravenously to Beagle dogs. Data are expressed as mean±standard error of the mean (SEM).*

Results

Other than red/orange chromaturia, adverse effects were not observed in any of the dogs, either during or following intravenous administration of NO-Cbl. The weight of the dogs increased over the course of the study. Pre-dose (t=0 h) cobalamin concentrations were within the normal reference range for each dog at each dosing event. Serum cobalamin (NO-Cbl) levels increased following intravenous administration of NO-Cbl. For each dose at each time point following administration, serum NO-Cbl concentrations were significantly higher compared to pre-dose baseline concentrations ($p \leq 0.01$), and were still higher than baseline concentrations at the final sampling time (t=24 h). Average serum cobalamin concentration/time profiles for each NO-Cbl dose are illustrated in Figure 1.

Average peak serum concentrations (C_{max}) of 2265, 5523 and 13,866 pg/mL were achieved following single-dose bolus intravenous administration of 10 mg/kg, 20 mg/kg and 40 mg/kg of NO-Cbl, respectively. The average area under the curve (AUC_{last}) was 12,697 h × pg/mL (10 mg/kg dose), 24,497 h × pg/mL (20 mg/kg dose) and 44,976 h × pg/mL (40 mg/kg dose). The average/median elimination half lives (t1/2) were 16.2/14.3 h (10 mg/kg dose), 13.5/12.75 h (20 mg/kg dose) and 13.1/7.75 h (40 mg/kg dose) respectively. The pharmacokinetic parameters for each dog and each dose of NO-Cbl are summarized in Table I.

Table I. *Pharmacokinetic parameters of 3 doses of nitrosylcobalamin following intravenous bolus administration in Beagle dogs.*

Dose		T_{max} (h)	C_{max} (pg/mL)	$t_{1/2}$ (h)	AUC_{last} (h × pg/ml)	C_{max} ratio	AUC ratio
10 mg/kg	Dog 1	0.5	2246	13.5	11919		
	Dog 2	0.5	2187	14.4	11666		
	Dog 3	0.5	2240	14.2	13131		
	Dog 4	0.5	2385	22.7	14070		
	Mean	0.5	2264.5	16.2	12696.5		
	Median			14.3			
	SD	0.00	84.60	4.35	1116.80		
	% CV	0.00%	3.74%	26.85%	8.80%		
20 mg/kg	Dog 1	0.5	6852	11.3	25910	3.05	2.17
	Dog 2	0.5	5905	21	24590	2.70	2.11
	Dog 3	0.5	4625	14.2	23222	2.06	1.77
	Dog 4	0.5	4708	7.5	24266	1.97	1.72
	Mean	0.5	5522.5	13.5	24497.0	2.4	1.9
	Median			12.75			
	SD	0.00	1061.88	5.70	1108.18	0.52	0.23
	% CV	0.00%	19.23%	42.25%	4.52%	21.08%	11.83%
40 mg/kg	Dog 1	0.25	14001	16.3	39617	6.23	3.32
	Dog 2	0.25	12942	26.9	36051	5.92	3.09
	Dog 3	0.25	14010	4.8	52748	6.25	4.02
	Dog 4	0.25	14510	4.5	51486	6.08	3.66
	Mean	0.25	13865.8	13.1	44975.5	6.1*	3.5*
	Median			7.75			
	SD	0.00	660.17	10.70	8389.65	0.16	0.40
	% CV	0.00%	4.76%	81.53%	18.65%	2.55%	11.47%

*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 the 10 and 20 mg/kg doses). T_{max}, time-to-maximum serum concentration following drug administration; C_{max}, maximum serum concentration; $t_{1/2}$, elimination half-life; AUClast, area under the serum concentration-time curve from time zero to time of last measurable concentration; C_{max} ratio, maximum serum concentration of a dose compared to that of the lowest dose and calculated by dividing the C_{max} dose by the C_{max} lowest dose; AUC ratio, area under the curve of a dose compared to that of the lowest dose and calculated by dividing the AUCdose by the AUClowest dose; SD, standard deviation; %CV, coefficient of variation calculated by dividing the standard deviation by the mean and expressed as a percentage.

Discussion

This pilot study was conducted to investigate the initial pharmacokinetic parameters of NO-Cbl, following intravenous administration in dogs.

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. Even then, serum cobalamin (NO-Cbl) concentrations were determined to be high at this earlier time point. As a result, it is likely that time-to-maximal concentration (T_{max}), maximum concentration (C_{max}), elimination half-life ($t_{1/2}$) and area under the curve (AUC) values were underestimated. Furthermore, addition of an earlier time point for the 40 mg/kg dose likely resulted in an overestimation of both the C_{max} ratio and AUC ratio for the 40 mg/kg dose. Based on these results, 1, 3, 5, 10, and 15 min sampling time points will be added to future pharmacokinetic analyses for intravenously-administered NO-Cbl.

Compared to pre-dose baseline concentrations, post-administration serum cobalamin (NO-Cbl) concentrations were significantly higher ($p \leq 0.01$) at each sampling time point for all doses, and were still higher than baseline concentrations at the final sampling time point (t=24 h). For this study, food was withheld from the dogs for 12 h prior to each dosing event and for 24 h throughout the sampling period, resulting in a cumulative 36-h fasting period. A 62-h fasting period would have been necessary to effectively extend the sampling period to 48-h post dosing, and this increase approached the limits of concern by the University's Institutional Animal Care and Use Committee. However, a longer sampling period will need to be justified for future pharmacokinetic analysis studies in order to accurately determine the elimination parameters for intravenously-administered NO-Cbl.

In this study with a small sample size, the median elimination half-life ($t_{1/2}$) values were less sensitive to outlying cobalamin (NO-Cbl) concentrations than the average $t_{1/2}$ values. Interestingly, the median elimination half-life decreased

with each increase in NO-Cbl dose: 14.3 h (10 mg/kg), 12.75 h (20 mg/kg) and 7.75 h (40 mg/kg). These results are consistent with those reported in a pharmacokinetic study of hydroxocobalamin (OH-Cbl) following intravenous administration in dogs (21). In that study, the elimination half-life was significantly lower at 140 mg/kg (6.0 h) than at 70 mg/kg (7.9 h). At this time, no clear explanation can be provided for this finding. However, this finding is clinically important because it suggests that use of lower doses of NO-Cbl may effectively increase the dosing interval.

This study has established initial pharmacokinetic data for intravenously-administered NO-Cbl in dogs. These preliminary results will be used as a starting point to design additional pharmacokinetic studies of NO-Cbl, and will be used to aid in selecting dose and dosing intervals in clinical trials evaluating NO-Cbl for treatment of tumors in both humans and companion animals.

Acknowledgements

The Authors would like to thank Linda McCort (Northeast Ohio Medical University, Rootstown, OH) for her technical assistance and Sarah Read (Gastrointestinal Laboratory - Texas A&M University, College Station, TX) for performing the serum assays. The authors extend a special thanks to Carolyn Sterner of the Beagle Rescue League (Yardley, PA) for the successful adoption of all of the Beagles that were used in this study.

This project was funded with Federal funds from the National Cancer Institute, National Institutes of Health, Department of Health and Human Services, under SBIR Contract No. HHSN261201000107C to BNOAT Oncology, Inc.

References

1 Bauer JA: Synthesis, characterization and nitric oxide release profile of nitrosylcobalamin: a potential chemotherapeutic agent. Anti-Cancer Drug *9*: 239-244, 1998.

2 Bauer JA, Lindner DJ and Yadav S: Comparative kinetics and affinity analysis between vitamin B_{12} analogs and intrinsic factor. Biacore: Developments in Protein Interaction Analysis. Phoenix, Arizona; May 6-9, 2007.

3 Allen RH: Clinical role and current status of serum [cyano]cobalamin (vitamin B_{12}) assays. Ligand Q *4*: 37-44, 1981.

4 Seetharam B and Yammani RR: Cobalamin transport proteins and their cell-surface receptors. Expert Rev Mol Med *5*: 1-18, 2003.

5 Bauer JA, Morrison BH, Grane RW *et al*: Effects of interferon beta on transcobalamin II-receptor expression and antitumor activity of nitrosylcobalamin. J Natl Cancer Inst *94*: 1010-1019, 2002.

6 Bauer JA, Lupica JA, Schmidt H *et al*: Nitrosylcobalamin potentiates the anti-neoplastic effects of chemotherapeutic agents via suppression of survival signaling. PLoS ONE *2*: e1313, 2007.

7 Tang Z, Bauer JA, Morrison B and Lindner DJ: Nitrosylcobalamin promotes cell death via S nitrosylation of Apo2L/TRAIL receptor DR4. Mol Cell Biol *26*: 5588-5594, 2006.

8 Chawla-Sarkar M, Bauer JA, Lupica JA *et al*: Suppression of NF-kappa B survival signaling by nitrosylcobalamin sensitizes neoplasms to the anti-tumor effects of Apo2L/TRAIL. J Biol Chem *278*: 39461-39469, 2003.

9 Rabinowitz R, Rachmilewitz B, Rachmilewitz M and Schlesinger M: Production of transcobalamin II by various murine and human cells in culture. Isr J Med Sci *18*: 740-745, 1982.

10 Schohn H, Gueant JL, Girr M *et al*: Synthesis and secretion of a cobalamin-binding protein by HT 29 cell line. Biochem J *280*: 427-430, 1991.

11 Hall CA, Green-Colligan PD and Begley JA: Synthesis of transcobalamin II by cultured human hepatocytes. Biochim Biophys Acta *838*: 387-389, 1985.

12 Gupta Y, Kohli DV and Jain SK: Vitamin B_{12}-mediated transport: a potential tool for tumor targeting of antineoplastic drugs and imaging agents. Crit Rev Ther Drug Carrier Syst *25*: 347-379, 2008.

13 Seetharam B, Bose S and Li N: Cellular import of cobalamin (Vitamin B-12). J Nutr *129*: 1761-1764, 1999.

14 Seetharam B: Receptor-mediated endocytosis of cobalamin (vitamin B_{12}). Annu Rev Nutr *19*: 173-195, 1999.

15 Seetharam B and Li N: Transcobalamin II and its cell surface receptor. Vitam Horm *59*: 337-366, 2000.

16 Bauer JA, Frye G, Bahr A, Gieg J and Brofman P: Anti-tumor effects of nitrosylcobalamin against spontaneous tumors in dogs. Invest New Drugs *28*: 694-702, 2010.

17 Rappazzo ME and Hall CA: Cyanocobalamin transport proteins in canine plasma. Am J Physiol *222*: 202-206, 1972.

18 Rappazzo ME and Hall CA: Transport function of transcobalamin II. J Clin Invest *51*: 1915-1918, 1972.

19 Hall CA and Rappazzo ME: Uptake of protein bound vitamin B_{12} by canine organs. Proc Soc Exp Biol Med *146*: 898-900, 1974.

20 Hall CA and Rappazzo ME: Release of transcobalamin II by canine organs. Proc Soc Exp Biol Med 148: 1202-1205, 1975.

21 de La Coussaye JE, Houeto P, Sandouk P, Levillain P, Sassine A and Riou B: Pharmacokinetics of hydroxocobalamin in dogs. J Neurosurg Anesthesiol *6*: 111-115, 1994.

Received July 5, 2012
Accepted July 16, 2012