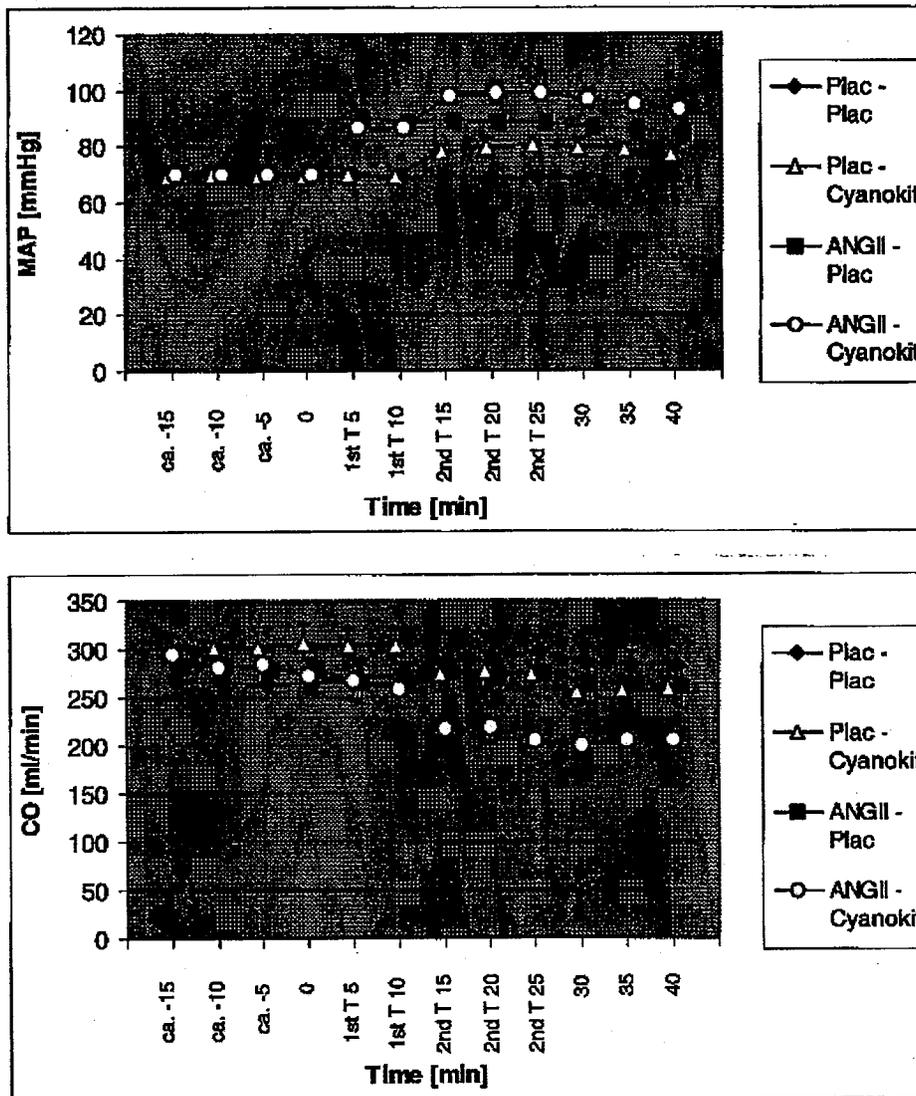


Pre-treatment (ca. -15 to 0 min), 1st treatment (1st T) (at time 0 min) with injection of L-NAME (30 mg/kg, i.v.) and placebo (solvent: 0.5 mg/kg, i.v.), respectively, 2nd treatment (2nd T) (10 to 25 min) by 15-min-infusion of Cyanokit® (75 mg/kg/15 min, i.v.) and placebo (solvent: 3 ml/kg/15 min, i.v.), respectively, and post-treatment (25 to 40 min); shown are mean arterial blood pressure (top: MAP), cardiac output (middle: CO) and total peripheral resistance (bottom: TPR); results are means ± SEM from 5 (Placebo-Placebo) and 7 animals (all other groups), respectively.

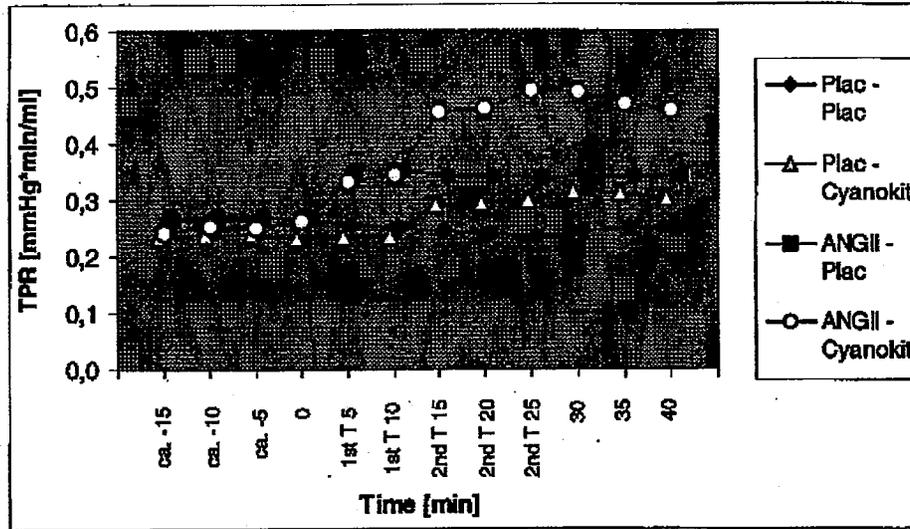
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Figure 2

Haemodynamics in Anaesthetized Placebo Treated Rabbit (Plac - Plac) or in Rabbits Treated with Angiotensin II (ANG - Plac), Cyanokit® (Plac - Cyanokit) or Angiotensin II and Cyanokit® in Combination (ANGII - Cyanokit)



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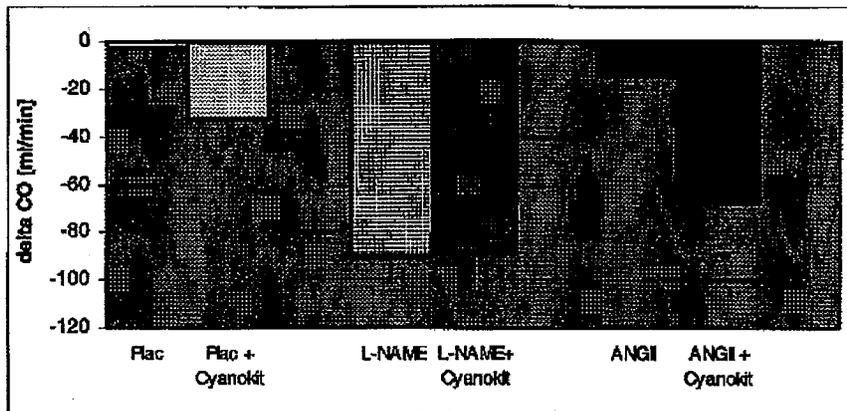
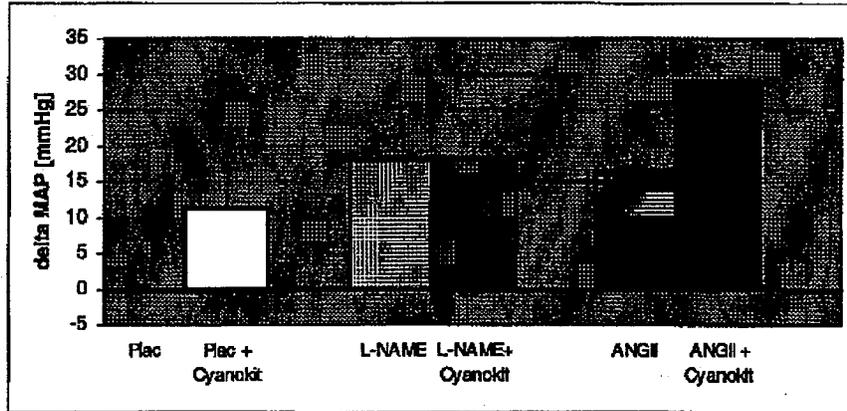


Pre-treatment (ca. -15 to 0 min), 1st treatment (1st T) (0 to 40 min) with continuous infusion of angiotensin II (0.05 μ g/kg/min, i.v.) or placebo application (solvent: 0.5 ml/kg, i.v.), respectively, 2nd treatment (2nd T) (10 to 25 min) by 15-min-infusion of Cyanokit[®] (75 mg/kg/15 min, i.v.) and placebo (solvent: 3 ml/kg/15 min, i.v.), respectively, and post-treatment (25 to 40 min); shown are mean arterial blood pressure (top: MAP), cardiac output (middle: CO) and total peripheral resistance (bottom: TPR); results are means \pm SEM from 5 (Placebo-Placebo) and 7 animals (all other groups), respectively.

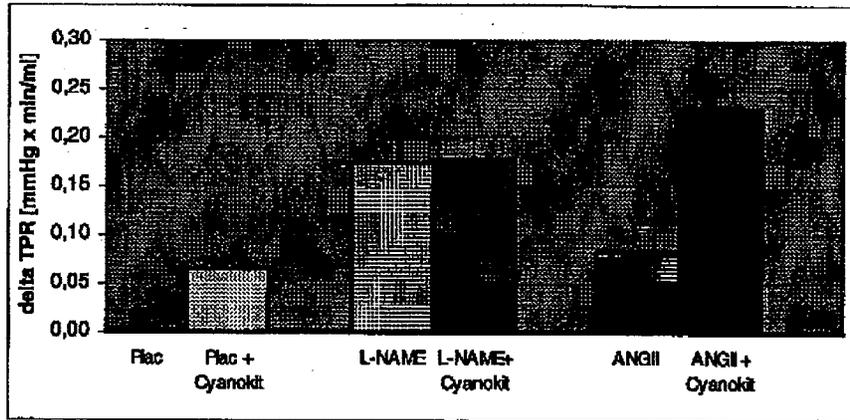
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Figure 3

Changes in Blood Pressure (MAP), Cardiac Output (CO) and Systemic Vascular Resistance (TPR) Induced by 1st and 2nd Treatments Taking pre-Treatment Values as References



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Reference values for the pre-treatment were taken at 0 min, for the 1st treatment at 10 min and for the 2nd treatment at 25 min; the 1st treatment (at time 0 min) consisted of the injection of L-NAME (30 mg/kg, i.v.), placebo (solvent: 0.5 mg/kg, i.v.) or infusion of angiotensin II (0.05 µg/kg/min, i.v. from 0 to 40 min), 2nd treatment (10 to 25 min) consisted of a 15-min-infusion of Cyanokit® (75 mg/kg/15 min, i.v.) and placebo (solvent: 3 ml/kg/15 min, i.v.), respectively; shown are changes in mean arterial blood pressure (top: delta MAP), in cardiac output (middle: delta CO) and in total peripheral resistance (bottom: delta TPR) related to the respective pre-treatment values; results are means ± SEM from 7 animals; significances of differences between 1st and 2nd treatments were evaluated using the two-tailed paired Student's t-test. * p<0.05, ** p<0.01, *** p<0.001

Discussion: The hemodynamic effects following hydroxocobalamin infusion into the anesthetized rabbit are comparable to those observed in healthy dogs (Riou, et al., 1991; Riou, et al., 1993), suggesting that the dog and the rabbit show similar hemodynamic effects following infusion of Cyanokit. The sponsor feels that the mechanism by which hydroxocobalamin leads to vasoconstriction is via inhibition of nitric oxide-mediated vasodilation.

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Study title: Cyanocobalamin: Characterization of Haemodynamic Effects in Anaesthetized Rabbits (EML 015722)

Key study findings: In contrast to hydroxocobalamin, cyanocobalamin did not result in hemodynamic changes that differed from those produced by vehicle alone. These results support the conclusion that hydroxocobalamin-induced hemodynamic effects are not related to the volume of infusate.

Study no.: PhD/0002,

Vol. 3, Tab 4.2.1.2.2, PhD/0002

Conducting laboratory and location: Cardiometabolic Research Darmstadt
Merck KGaA

Date of study initiation: August 31, 2005

GLP compliance: Unspecified

QA report: no

Drug, lot #, and % purity:

Cyanocobalamin, batch 9496 (date of release 02/09/05, retest dates 04/09/05 & 06/13/05), volume (9.375 mL/kg/15 min, IV)

Vehicle: physiological saline (9.375 mL/kg/15 min, IV)

Fentanyl: Janssen, Reg. No. 6762282.01.00

Pancuronium bromide: Organon, Reg. No. 6077468.00.00

Pentobarbital sodium: Narcoren, Reg. No. 208

Methods: Previous findings have suggested that high dose hydroxocobalamin infusion resulted in an increase in blood pressure in the dog model. To further characterize the potential for hydroxocobalamin to alter blood pressure the drug, cyanocobalamin (75 mg/kg/15 min, IV) was infused into anesthetized (fentanyl followed by pentobarbital sodium-Narcoren) male White New Zealand rabbits.

Results: Infusion of cyanocobalamin or vehicle did not alter MAP (mean arterial pressure), but decreased TPR (total peripheral resistance), and increased CO (cardiac output), and reduced HR (heart rate), and increased in SV (stroke volume). An equal volume of solvent alone produced similar changes.

Discussion: In contrast to hydroxocobalamin, cyanocobalamin did not result in hemodynamic changes that differed from those produced by vehicle alone. These results support the conclusion that hydroxocobalamin-induced hemodynamic effects are not related to the volume of infusate.

2.6.2.4 Safety pharmacology

Although classical safety pharmacology studies were not performed, the Sponsor cited results of a number of published studies in support of hydroxocobalamin safety. Since the Sponsor lacks ownership of these studies and the data which support the proposed product's safety, they can not be used for approval of this 505(b)(1) application.

NEUROLOGICAL EFFECTS

No studies were submitted.

CARDIOVASCULAR EFFECTS

The cardiovascular effects of the hydroxocobalamin have been examined in various animal models, including the dog and the rabbit.

Riou et al. (1991) characterized the hemodynamic effects of infusions of hydroxocobalamin (20, 70, and 140 mg/kg) in the conscious dog model. The results suggested that hydroxocobalamin infusion did not alter heart rate, mean arterial pressure, left ventricular end-diastolic pressure, PR interval, or QT interval. The highest dose tested, 140 mg/kg, resulted in a decrease in cardiac output (-19%), an increase in systemic resistance (+41%), and a decrease in stroke volume in non-anesthetized dogs in the absence of cyanide. Note that this dose was close to the dose (150 mg/kg) administered to cyanide treated dogs that resulted in 100% survival

Study Title: Study of the Cardio-vascular Effects in the Dog

Study no.: III.F

Vol. 12, Tab 4.2.3.7.7.3.1 III.F, p. 37

Conducting laboratory and location: _____

b(4)

Date of study initiation: study period in 1974

GLP compliance: no

QA report: no

Drug, lot #, and % purity:

Hydroxocobalamin, Batch RL 23918 (active substance), Purity: no information

Preparation for injection Batch 210 bis (HOCo 15000)

Hydroxocobalamin base 15 mg

Buffered aqueous solution QSP 2 mL for 1 vial

Sodium acetate 3.89 mg

Sodium chloride 8.20 mg

Acetic acid QSP pH 5.8 (about 2.68 mg)

Water for injection QSP 2 mL

Method

A dog (number of animals is not mentioned other than one), fasted for 24 hours, was administered 30 mg/kg, iv, of mebarbital sodium (0.5 mL/kg of Nembutal[®] 6%, then catheterized and infused with this anesthetic at a rate of 5 mg/kg/hour. Carotid artery pressure, respirations, ECG (D II lead), and heart rate were monitored. Test substances were administered through a catheter in the external saphenous vein. The responses to occlusion of the carotids, electrical stimulation of the distal end of the vagus nerve, intravenous injection of noradrenaline, 1 µg/kg, acetylcholine, 5 µg/kg or isoprenaline, 1 µg/kg.

Results

Injection of vehicle, 15, 30, 45, or 75 mg hydroxocobalamin no effect on any of the parameters recorded. There was no effect of hydroxocobalamin to perturbations of the cardiovascular system by mechanical, electrical, or chemical mediators.

PULMONARY EFFECTS

No studies were submitted.

RENAL EFFECTS

No studies were submitted.

GASTROINTESTINAL EFFECTS

A study was cited from the collection of studies comprising Study III.F (located in Vol. 12 Tab 4.2.3.7.7.3.1 concerning guinea pig stomach H ions, however this was not submitted.

ABUSE LIABILITY

No studies were submitted to characterize the abuse liability of hydroxocobalamin. The proposed product is a form of Vitamin B₁₂ and based on its known pharmacodynamic effects, such studies are not necessary. There is no abuse liability associated with this drug.

2.6.2.5 Pharmacodynamic drug interactions

There were no studies of drug interactions. Previous investigations were performed in order to determine the physical or chemical compatibility of Cyanokit® with different drugs and diluents for IV infusion available on the US market and/or in Europe:

Information from the European label is presented below:

- Cyanokit® is physically incompatible with Diazepam, Diprivan® 1 % injectable emulsion (propofol), Thiopental 0.5g, Nitroglycerin injection USP, Pentobarbital, Fentanyl, diluted Dopamine hydrochloride in Lactated Ringers Solution, diluted Dobutamine injection in 0.9% saline solution or in 5% dextrose solution or in Lactated Ringers Solution.
- Cyanokit® is chemically compatible with sterile saline (0.9% NaCl), Lactated Ringers Solution, or dextrose 5% (the most common diluents). Cyanokit® reconstituted with either sterile saline (0.9% NaCl), Lactated Ringers Solution, or dextrose 5% is stable for up to 6 hours between 5°C and 40°C.
- Cyanokit® is chemically incompatible with sodium thiosulfate injection and sodium nitrite injection.

Reviewer's Comment:

- 1) The wording about dobutamine may be confusing or misread to compatible solutions listed just below it.

"...diluted Dobutamine injection in 0.9% saline solution or in 5% dextrose solution or in Lactated Ringers Solution.

Cyanokit® is chemically compatible with sterile saline (0.9% NaCl), Lactated Ringers Solution, or dextrose 5% (the most common diluents)."

One might quickly read that dextrose solution and Lactated Ringers are in the incompatible paragraph, but in the compatible paragraph below and not realize at first it is dobutamine diluted in these solutions that is incompatible.

- 2) The third bullet refers to another currently available treatment for cyanide and wording to indicate this should be included.

In an emergency situation, the label would not be read but someone may be asked to quickly glance at it for any incompatibility/contraindication notes, therefore listing these items in 2 columns "Not compatible with" and "Compatible with" would seem more appropriate.

2.6.3 PHARMACOLOGY TABULATED SUMMARY

Not provided by sponsor.

2.6.4 PHARMACOKINETICS/TOXICOKINETICS

2.6.4.1 Brief summary

Hydroxocobalamin

Distribution of hydroxocobalamin is largely influenced by the coordinative binding to proteins. During the plasma protein binding, hydroxocobalamin reacts by replacing the coordinatively bound hydroxo ligand by accessible histidine- and thiol-groups of the proteins to form various cobalamin-(III) complexes. This ligand exchange is reported to be an equilibrium reaction such as the usual protein binding of drugs, exhibiting considerably slower rates of binding and release. Equilibrium concentrations were reached only within 1 to 2 hours after infusion.

Interestingly, this protein binding equilibrium exhibited a pronounced species-dependent difference, as evaluated from the average free fraction of cobalamins-(III). While humans showed the lowest free fraction of about 5%, the highest free fraction in the range of 24% was observed in rats.

Metabolism of hydroxocobalamin is mainly characterized by exchange reactions of the hydroxo-ligand with other physiological ligands that exhibit high affinity to the cobalamin-(III). Thus, the binding of hydroxocobalamin to plasma proteins may be regarded as reversible metabolism. Besides, hydroxocobalamin forms low molecular derivatives with coordinating physiological compounds such as thiols, histidine, thiocyanate, and others. Accordingly, several high pressure liquid chromatography (HPLC) peaks have been detected in in vitro incubates of plasma with hydroxocobalamin during studies performed by the Sponsor.

Hydroxocobalamin is known to react with cyanide forming cyanocobalamin in vivo, even at the very low physiological concentrations of both reactants. Due to the extremely high complex stability ($K_{\text{diss}} \approx 10^{12} \text{ M}^{-1}$), cyanocobalamin is regarded as a physiological end product of hydroxocobalamin especially during cyanide intoxication. This reaction is known to proceed very rapidly with reaction rates of $660 \text{ M}^{-1}\text{s}^{-1}$ and $320 \text{ M}^{-1}\text{s}^{-1}$ for CN^- and HCN , respectively (in aqueous buffers, extrapolated for 37°C).

Excretion of hydroxocobalamin occurs mainly via the renal route. In the literature, it is reported that hydroxocobalamin is excreted in the urine in dogs (see 4.3.61). In human volunteers, the mean renal clearance of hydroxocobalamin (2.5 g to 10.0 g) amounted to

58% to 74% of total clearance.

Cyanocobalamin

Distribution: In contrast to hydroxocobalamin, cyanocobalamin does not exhibit the coordinative protein binding property due to the very high stability of the cyanide ligand bond, but was reported to have a non-covalent protein binding property of about 50% in humans. Accordingly, cyanocobalamin is more rapidly excreted via the kidneys than hydroxocobalamin.

Metabolism: Cyanocobalamin is the most stable cobalamin-(III) complex known, rendering significant in vivo metabolism by ligand exchange at high concentrations unlikely.

Excretion: Cyanocobalamin at high concentrations is completely excreted via the renal route as indicated in the literature. The predominant half-life was 0.3 to 0.5 hours in rats, 0.8 to 1.0 hours in dogs and 9.3 ± 3.2 hours in humans. Conclusively, cyanocobalamin excretion is 3 to 10 times faster (depending on the species) compared to hydroxocobalamin. TK data are available from a literature study in rats performed with repeated IV administration of 1, 5, 25, and 100 mg/kg/d cyanocobalamin for 6 months. TK in dogs was investigated during 2-week IV administration (40, 100, and 400 mg/kg). In both species, C_{max} and AUC values increased less than proportionally to the dose of cyanocobalamin, which was partly due to an increasing clearance at higher doses observed in both studies. No indication of any accumulation was found in either study, which is in agreement with the short predominant half-lives found in rats (0.3 to 0.5 hours) and in dogs (0.8 to 1.0 hours). The exposure to cyanocobalamin in lethally intoxicated dogs from the efficacy study was 13-fold lower than in the highest dose group of the dog cyanocobalamin toxicity study.

2.6.4.2 Methods of Analysis

Reactions of Hydroxocobalamin with Plasma Constituents and Bioanalytical / Pharmacokinetics Considerations

From the published literature it was known that hydroxocobalamin freely exchanges the hydroxo-ligand by coordinating amino acid residues of plasma proteins (histidine and cysteine) to form non-filterable protein complexes, and by low molecular weight ligands in plasma such as thiols, histidine, and thiocyanate. In order to determine the overall exposure to these cobalamins-(III) complexes and to determine the free concentration, these derivatives were quantified in sum and given as "total cobalamins-(III)" and "free cobalamins-(III)," respectively.

Hydroxocobalamin reacts with plasma constituents to form various cobalamin-(III) complexes by replacing the coordinatively bound hydroxo ligand. In plasma, endogenous compounds acting as cobalamin-(III) ligands appear to be mainly proteins (probably bound via accessible histidine- and possibly thiol-groups) but also low

molecular weight compounds such as thiols, histidine, glycine, thiocyanate and others. These high- and low-molecular weight cobalamin-(III) complexes formed with physiological ligands react with cyanide, as the latter is the strongest ligand known for cobalamin-(III). Thus, all derivatives of hydroxocobalamin formed in plasma may be regarded as active components against cyanide poisoning (although individual reaction rates with cyanide are not known). Therefore, the sum of high- and low-molecular weight cobalamin-(III) complexes can be considered the relevant parameter in evaluating efficacy, exposure, and distribution of hydroxocobalamin.

Safety margin calculations are usually based on the free fraction of a drug, which is calculated from the total plasma concentration and applying a protein binding factor. In contrast to most other drugs, which usually bind to proteins by rapidly equilibrating hydrophobic interactions, the specific binding of hydroxocobalamin to plasma proteins is a quite slow equilibrium reaction. Thus, its' free fraction changes significantly over a comparatively long time period and hence undergoes a rather complex in vivo kinetic since the elimination occurs in parallel via the kidney. Consequently, conversion of the total cobalamins-(III) concentration to the free cobalamins-(III) concentration applying a protein binding factor for safety margin calculations was considered inappropriate. Instead it was replaced by the ex-vivo measurement of the sum of so-called free cobalamins-(III) in plasma which reflects hydroxocobalamin, plus all cobalamins with the hydroxoligand replaced by low molecular weight plasma ligands.

Total Cobalamins

Hydroxocobalamin undergoes a rapid ligand exchange with coordinating amino acid residues such as histidine and cysteine to form high molecular non-filterable plasma protein complexes, and with low molecular ligands such as thiols, histidine, and thiocyanate forming so-called "free cobalamins-(III)." Such coordinative ligand exchanges are known to be equilibrium reactions which proceed much more slowly than the usual non-covalent protein binding of drugs. Thus, the free fraction of cobalamins-(III) changes significantly over a comparatively long time period and hence undergoes complex in vivo kinetics. Hydroxocobalamin added to plasma or serum reacts rapidly with protein histidine and -SH groups and small physiological compounds via ligand exchange, yielding a variety of high and low molecular complex derivatives. The sum of these complex derivatives is termed 'Total Cobalamins-(III)'. Therefore, bioanalytical methods were developed and validated to measure both, the exposure to the free fraction of cobalamins, termed "free cobalamins-(III)", as well as to the sum of cobalamins administered, termed "total cobalamins-(III)."

Total Cobalamins-(III) were quantitated by reacting all cobalamin complexes with excess cyanide to form cyanocobalamin. Excess cyanide was removed with glacial acetic acid, the samples were filtered by ultrafiltration to separate proteins and the ultrafiltrates were analyzed by HPLC-UV.

Table 2.6.4-1 Overview of Bioanalytical Methods Used in Pharmacokinetic / Toxicokinetic Studies

Analyte	Species	Matrix	Method	Validation Study No	Reference of Validation	Conc. Unit Used in Validation	Method Used in Study No.*
Hydroxocobalamin	Dog	Native plasma	HPLC-UV	MPK/ Hydroxo. 03.01	4.2.2.1.4.2	µg/mL	T8348
Hydroxocobalamin	Dog	Acidified plasma	LC-MS/MS	DMPK 133-03	4.2.2.1.4.1	µg/mL	T8355
Total cobalamins-(III)	Rat	Native plasma	HPLC-UV	DMPK 03-05	4.2.2.1.1.4	µg eq/mL	T15098
Total cobalamins-(III)	Dog	Native plasma	HPLC-UV	DMPK 98-04	4.2.2.1.1.6	µg/mL	T8374 N106342
Total cobalamins-(III)	Human	Native plasma	HPLC-UV	DMPK 70-04	4.3.35	µg/mL	EML 015722-H101
Total cobalamins-(III)	Human	Acidified urine	HPLC-UV	DMPK 123-04	4.3.37	µg/mL	EML 015722-H101
Free cobalamins-(III)	Rat	Plasma ultrafiltrate	HPLC-UV	DMPK 04-05	4.2.2.1.1.5	µg eq/mL	T15096
Free cobalamins-(III)	Dog	Plasma ultrafiltrate	HPLC-UV	DMPK 122-04	4.2.2.1.1.7	µg/mL	T8374
Free cobalamins-(III)	Human	Plasma ultrafiltrate	HPLC-UV	DMPK 114-04	4.3.38	µg/mL	EML 015722-H101
Cyanocobalamin	Dog	Native plasma	HPLC-UV	DMPK 135-04	4.2.2.1.3.1	µg/mL	T8380
Cyanocobalamin	Human	Native plasma	HPLC-UV	DMPK 216-05	4.3.38	µg/mL	DMPK 205-05

* The concentration unit used during the bioanalytical studies was µg eq/mL throughout. The use of µg/mL during most validations is due to historical reasons (see 2.6.4.2.1.3).

Bioanalytics of Total and Free Cobalamins-(III)

The bioanalytical method employed for the determination of total cobalamins-(III) is based on the general reaction of all cobalamin complexes to form cyanocobalamin quantitatively in the presence of excess cyanide. Following this derivatization performed in native plasma samples, the samples are ultrafiltrated to separate the proteins and analyzed by HPLC-UV using cyanocobalamin calibrators prepared in plasma. The non-protein-bound cobalamin-(III) complexes, i.e., free cobalamins-(III) are measured in protein free plasma ultrafiltrates prepared immediately after sample collection at 4°C to prevent ongoing ex vivo equilibration. All free cobalamin-(III) derivatives are quantitatively transformed into cyanocobalamin by incubation with excess cyanide and analyzed by HPLC-UV using cyanocobalamin calibrators prepared in ultrafiltrate. Both methods have been validated in rat, dog, and human plasma within the calibration range of 0.1 to 500 µg eq/mL or µg/mL, respectively. Both analytes have been shown to be sufficiently stable in plasma of all species for at least 12 weeks at -20°C.

The concentration unit, µg/mL, was used during the dog and human validations referring to the amount of hydroxocobalamin spiked to the plasma samples. Due to different molecular weights of all derivatives summed up as total cobalamins-(III), however, the analyte concentration can not be given as µg/mL strictly speaking, and therefore µg eq/mL was finally used during the rat validation and the bioanalytical studies in all species reflecting the molecular weight of the common cobalamin entity without any specific ligand. The difference between hydroxocobalamin given as µg/mL and total cobalamin-(III) derivatives given as µg eq/mL is only 1.3%.

Physiological plasma concentrations of cobalamin derivatives are clearly <LLOQ (about 0.001 µg eq/mL), and therefore did not contribute at all to the high cobalamin concentrations that had been administered during the Merck KGaA studies.

The HPLC-UV method initially developed for assaying "hydroxocobalamin" in native plasma samples revealed the instability of the analyte for the first time rendering this assay inappropriate. This instability, which was certainly due to the binding of hydroxocobalamin to plasma proteins, could be stopped by acidic methanol precipitation of the plasma samples, enabling accurate and precise analysis of calibration samples and quality controls if precipitated immediately after the preparation.

The LC-MS/MS method used acidification of the plasma to stabilize the analyte. Acidification of plasma is known to prevent the complexation reaction of hydroxocobalamin (probably by protonating the ligand histidine), thereby ensuring stability of hydroxocobalamin in calibration and quality control samples which are prepared in pre-acidified plasma. However, the conditions for in vivo samples are different: Binding to plasma proteins has already occurred in vivo and is stopped upon addition of acid at the time point of sample collection. Despite acidification re-liberating a part of the bound cobalamins-(III), it is not cleaved quantitatively from the proteins. In conclusion, this method underestimates the real concentrations and should be considered semi-quantitative as well.

Cyanocobalamin

Stability of Cyanocobalamin in Biological Matrices: Cyanocobalamin is the most stable cobalamin-(III) complex known ($K_{diss}: 10^{-12}/M$). Therefore, no reactivity with plasma constituents, as observed for hydroxocobalamin was expected. This was corroborated by all method validations using cyanocobalamin as reference in quality control plasma samples.

Bioanalytics of Cyanocobalamin: Cyanocobalamin was analyzed in plasma samples using the same chromatographic method as used for total and free cobalamins-(III), which separated cyanocobalamin from other free cobalamin derivatives. Sample preparation required an initial separation of the plasma proteins by ultrafiltration. While such facile sample preparation is sufficient for all PK/TK studies where cyanocobalamin itself has been administered to animals, specific care has to be taken during pharmacological studies where cyanide is reacting in vivo with hydroxocobalamin to form cyanocobalamin. At early time points after hydroxocobalamin infusion, unreacted hydroxocobalamin may still react ex vivo in the blood samples with cyanide for instance liberated from the MetHb-CN complex thus elevating the cyanocobalamin concentration above its actual in vivo level.

2.6.4.3 Absorption and Kinetics

In the rat studies, hydroxocobalamin was injected intraperitoneal, and in the dog studies it was infused intravenously.

Hydroxocobalamin: In the dog study, a dose proportional increase of AUC was demonstrated for both free cobalamins-(III) and total cobalamins-(III). The predominant half-lives were 3 and 5 hours in the rat and 6 and 8 hours in the dog for free and total cobalamins-(III), respectively. Total body clearance of free cobalamins-(III) was 0.38 to 0.50 L/kg and was approximately 6 to 7 times higher compared to total cobalamins-(III).

Cyanocobalamin: Measurements of cyanocobalamin were performed with repeated IV administration of 1, 5, 25, and 100 mg/kg/d for 6 months TK in dogs and during 2 week IV administration (40, 100, and 400 mg/kg). In both species, C_{max} and AUC values increased less than proportionally to the dose of cyanocobalamin, which was partly due to the increasing clearance at higher doses observed in both studies. No indication of any accumulation was found during both studies, which is in agreement with the short predominant half-lives found in rats (0.3 to 0.5 h) and in dogs (0.8 to 1.0 h). The summary table below was reproduced from the sponsor's submission:

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Table 2.4-6 Species Comparison of C_{max} and AUC of Total and Free Cobalamins-(III)

Dose (mg/kg)	Gender	Rat Toxicity Study (IP) T15096		Dog Toxicity Study (IV) T8374		Human Safety Study (IV) EML 015722-H101	
		C _{max} ¹	AUC ²	C _{max} ¹	AUC ²	C _{max} ¹	AUC ²
Free Cobalamins-(III)⁶							
75	M+F	-	-	-	-	112.7 ±20.8 ³	394.6 ±36.9 ³
150	M	-	-	410 ±54	308 ±32	197.2 ±40.3 ⁴	813.8 ±153.3 ⁴
150	F	-	-	429 ±31	304 ±22		
300 ⁵	M	140	611	511 ±61	608 ±68	-	-
300 ⁵	F	181	705	676 ±218	785 ±327	-	-
1200	M	-	-	1480 ±160	3520 ±1520	-	-
1200	F	-	-	1650 ±320	2730 ±130	-	-
Total Cobalamins-(III)⁶							
75	M+F	-	-	-	-	579.0 ±112.6 ³	9422.9 ±2991.6 ³
150	M	-	-	773 ±66	2350 ±180	995.3 ±149.1 ⁴	15681.1 ±2571.5 ⁴
150	F	-	-	922 ±73	2160 ±120		
300 ⁵	M	292	2660	1260 ±180	4110 ±490	-	-
300 ⁵	F	373	2810	1540 ±390	4850 ±1120	-	-
1200	M	-	-	3370 ±460	18800 ±5300	-	-
1200	F	-	-	3320 ±370	14500 ±428	-	-

1 (µg eq/mL)

2 (µg eq/mL×h)

3 5.0 g dose

4 10.0 g dose

5 NOAEL in T15096 and T8374

6 all means rounded to 3 significant digits, SD with same precision as the mean

2.6.4.4 Distribution

The distribution of hydroxocobalamin is largely influenced by the coordinative binding to proteins. During the plasma protein binding, hydroxocobalamin reacts by replacing the coordinatively bound hydroxo ligand by accessible histidine- and thiol-groups of the proteins to form various cobalamin-(III) complexes. This ligand exchange is reported to be an equilibrium reaction such as the usual protein binding of drugs, exhibiting considerably slower rates of binding and release. Equilibrium concentrations were reached only within 1 to 2 hours after infusion. This protein binding equilibrium exhibited a pronounced species-dependent difference, as evaluated from the average free fraction of cobalamins-(III) (see Table 2.4-5). While humans showed the lowest free fraction of about 5%, the highest free fraction in the range of 24% was observed in rats.

Table 2.4-5 Species Differences of Average Free Fraction of Cobalamins-(III)

Dose (mg/kg)	Gender	Average Free Fraction of Cobalamins-(III) in Plasma (%) [*]		
		Rat (T15096) IP	Dog (T8374) IV	Human (EML 015722-H101) IV
75	F			4.2 ¹
	M			
150	F		14.1	5.2 ²
	M		13.1	
300	F	25	16.2	
	M	23	14.8	
1200	F		18.8	
	M		18.7	
mean	±SD	24	16.0±2.4	4.7

* calculated as mean AUC_{free} / mean AUC_{total}

1 5 g dose

2 10 g dose

The reason for this species difference is unknown.

Hydroxocobalamin: Hydroxocobalamin binds in vivo to plasma proteins coordinative at the cobalt site and to a high extent reaching about equilibrium concentrations 1 to 2 hours after infusion. Average free fractions calculated as AUC ratios of free to total cobalamins were found to be 24% in rats, 16% in dogs, and 5% in humans, respectively.

Cyanocobalamin: In contrast, cyanocobalamin does not exhibit this coordinative protein binding property due to the very high stability of the cyanide-ligand bond. In humans, it has a non-covalent protein binding property of about 50%. Thus, cyanocobalamin is more rapidly distributed in the body and undergoes more rapid renal excretion than hydroxocobalamin.

2.6.4.5 Metabolism

Hydroxocobalamin:

Hydroxocobalamin forms low molecular derivatives by ligand exchange with physiological compounds such as proteins by interaction of thiols, histidine, thiocyanate, and others at the coordinating cobalt site. In a cyanide-intoxicated individual, cyanocobalamin formation predominates due to its high formation rate.

Due to the high molecular weight of hydroxocobalamin, interaction with drug metabolizing enzymes such as CYP P450 is unlikely to occur and therefore PK interaction studies were judged not necessary. In addition, since hydroxocobalamin is

intended for single antidote administration, induction studies were also determined to be unnecessary.

Cyanocobalamin:

Due to the extremely high complex stability ($K_{\text{Diss}} 10^{-12}/\text{M}$), cyanocobalamin is regarded as a physiological end product of hydroxocobalamin especially during cyanide intoxication. This reaction is known to proceed very rapidly with reaction rates of $660 \text{ M}^{-1}\text{s}^{-1}$ and $320 \text{ M}^{-1}\text{s}^{-1}$ for CN^- and HCN , respectively. Thus, cyanocobalamin is the most stable cobalamin-(III) complex known, rendering a significant in vivo metabolism at least via ligand exchange highly improbable.

2.6.4.6 Excretion

Hydroxocobalamin and cyanocobalamin are mainly excreted via urine. Cyanocobalamin excretion is 3 to 10 times faster (depending on the species) compared to hydroxocobalamin due to the extensive protein binding of the latter. Excretion of hydroxocobalamin occurs mainly via the renal route. It is reported that hydroxocobalamin is excreted in the urine in dogs. In human volunteers, the mean renal clearance of hydroxocobalamin (2.5 g to 10.0 g) amounted to 58% to 74% of total clearance.

The mean predominant half-lives were 3 and 5 hours in the rat, 6 and 8 hours in the dogs, and 28 and 31 hours in humans for free and total cobalamins-(III), respectively. Total body clearance of free cobalamins-(III) in dogs was 0.38 to 0.50 L/h/kg and was approximately 6- to 7-fold higher compared to total cobalamins-(III).

2.6.4.7 Pharmacokinetic drug interactions

There were no studies submitted.

2.6.4.8 Other Pharmacokinetic Studies

There were no studies submitted.

2.6.4.9 Discussion and Conclusions

The Sponsor relied on previously published animal data for a substantial aspect of the pharmacokinetic information. Hydroxocobalamin is administered intravenously, but they did not provide tissue distribution studies. From the toxicological findings of prolonged retention in some of the organs and tissues examined, this could become a safety issue upon recovery from cyanide poisoning. They did not submit original studies to determine metabolism or elimination, but provided published reports. It is recommended that tissue

distribution studies be conducted to adequately address hydroxocobalamin and cyanocobalamin distribution and the time course of elimination from tissues. In addition, it is recommended that blood sample be obtained such that it can be determined when hydroxocobalamin and cyanocobalamin concentrations become sufficiently low that reliable blood biochemistry analysis can be performed for those tests that rely on colorimetric analysis.

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2.6.4.10 Tables and figures to include comparative TK summary

Table 2.4-7 Cyanocobalamin C_{max} and AUCs Values in TK and PK Studies

Study	Nava-Ocampo AA et al. ^{1,2}	14-Day Toxicity Study T8380 ¹	Dog Efficacy Study N106342 ²	
Location in CTD	(see 4.3.34)	(see 4.2.3.2.2.1)	(see 4.2.1.1.2)	
Species	Rat	Dog	Dog	
Gender	M+F	M+F	M+F	
Cyanocobalamin Dose	100 mg/kg/d (NOAEL)	400 mg/kg/d (NOAEL)	-	
Hydroxocobalamin Dose	-	-	75 mg/kg	150 mg/kg
Cyanide Dose	-	-	0.92 mg/kg	
n	12	6	18	18
Mean C _{max} (µg eq/mL)	431 ±59.0	954 ±81	104 ±14.0	132 ±20.5
Mean AUC (µg eq/mL×h)	191 ±27.9	853 ±117	60.5 ±8.93	69.8 ±9.82

1 data of Day 1

2 original concentration data transformed to µg eq/mL using the factors of Table 2.6.4-2

Table 2.4-7 Cyanocobalamin C_{max} and AUCs Values in TK and PK Studies

Study	Nava-Ocampo AA et al. ^{1,2}	14-Day Toxicity Study T8380 ¹	Dog Efficacy Study N106342 ²	
Location in CTD	(see 4.3.34)	(see 4.2.3.2.2.1)	(see 4.2.1.1.2)	
Species	Rat	Dog	Dog	
Gender	M+F	M+F	M+F	
Cyanocobalamin Dose	100 mg/kg/d (NOAEL)	400 mg/kg/d (NOAEL)	-	
Hydroxocobalamin Dose	-	-	75 mg/kg	150 mg/kg
Cyanide Dose	-	-	0.92 mg/kg	
n	12	6	18	18
Mean C _{max} (µg eq/mL)	431 ±59.0	954 ±81	104 ±14.0	132 ±20.5
Mean AUC (µg eq/mL×h)	191 ±27.9	853 ±117	60.5 ±8.93	69.8 ±9.82

1 data of Day 1

2 original concentration data transformed to µg eq/mL using the factors of Table 2.6.4-2

Dog Study T8355*: Hydroxocobalamin Toxicokinetics

Dose (mg/kg/day)	Day	C _{0.083h} (µg/mL)	AUC(0-24h) (µg-h/mL)	C _{0.083h} (µg/mL)/dose	AUC(0-24h) (µg-h/mL)/dose
300	1	722	1147.4	2.41	3.83
	3	609	1206.5	2.03	4.02
600	1	978	2664.7	1.63	4.44
	3	1377	2469.7	2.30	4.12
1200	1	2352	8103.9	1.96	6.75
	3	1996	5986.3	1.66	4.99

* Doses were administered for 3 days to n=2/sex/dose beagle dogs.

Dog Study T8377*: Cyanocobalamin Toxicokinetics for a 400 mg/kg/day dose

Day	C (at 1 hour) (µg eq/mL)		AUC(1-inf) (µg eq/mL*h)		t _{1/2} (6-24hr) (h)		T _{1/2} (1-6 hr) (h)	
	M	F	M	F	M	F	M	F
3 (first day at this dose)	409	274	445	236	3.3	3.7	0.83	0.77
11 (day 9 of this dose)	446	273	534	260	3.7	4.2	0.84	0.80

* Doses were administered to n=1/sex. Dosing consisted of 40 mg/kg on day 1, 100 mg/kg on day 2, and 400 mg/kg on days 3-14.

Dog Study T8380*: Cyanocobalamin Toxicokinetics

Dose (mg/kg)	Day	Sex	C _{max} (µg eq/mL)	T _{max} (h)	C _{max} /D ¹	AUC _{tot} (µg eq/mL*h)	AUC _{tot} /D ²	Clearance (L/h/kg)	V _{ss} (L/kg)	t _{half} (1-6h) (h)
40	2	f	211	0.0992	5.37	149	3.79	0.266	0.204	0.799
		m	204	0.147	5.21	159	4.06	0.249	0.202	0.829
	12	f	236	0.0621	6.02	128	3.25	0.308	0.243	0.761
		m	219	0.0317	5.58	144	3.66	0.277	0.242	0.842
100	1	f	477	0.0629	4.87	309	3.15	0.319	0.251	0.739
		m	426	0.0696	4.34	357	3.64	0.279	0.241	0.825
	11	f	371	0.0982	3.78	291	2.97	0.338	0.263	0.727
		m	419	0.0701	4.27	331	3.37	0.305	0.255	0.823
400	1	f	924	0.0601	2.36	796	2.03	0.498	0.441	0.768
		m	984	0.143	2.51	911	2.32	0.436	0.404	0.815
	11	f	926	0.134	2.36	819	2.09	0.48	0.499	0.749
		m	855	0.143	2.43	845	2.15	0.468	0.516	0.812

1: C (µg eq/mL); D[mg eq/kg]

2: AUC [µg eq/mL x h]; D [mg eq/kg]

* Doses were administered to n=3/sex/dose. Dosing consisted of 40 mg/kg on day 1, 100 mg/kg on day 2, and 400 mg/kg on days 3-14.

2.6.5 PHARMACOKINETICS TABULATED SUMMARY

Table 2.4-6 Species Comparison of C_{max} and AUC of Total and Free Cobalamins-(III)

Dose (mg/kg)	Gender	Rat Toxicity Study (IP) T15096		Dog Toxicity Study (IV) T8374		Human Safety Study (IV) EML 015722-H101	
		C_{max} ¹	AUC ²	C_{max} ¹	AUC ²	C_{max} ¹	AUC ²
Free Cobalamins-(III)⁶							
75	M+F	-	-	-	-	112.7 ±20.8 ³	394.6 ±36.9 ³
150	M	-	-	410 ±54	308 ±32	197.2 ±40.3 ⁴	813.8 ±153.3 ⁴
150	F	-	-	429 ±31	304 ±22		
300 ⁵	M	140	611	511 ±61	608 ±68	-	-
300 ⁵	F	181	705	676 ±218	785 ±327	-	-
1200	M	-	-	1480 ±160	3520 ±1520	-	-
1200	F	-	-	1650 ±320	2730 ±130	-	-
Total Cobalamins-(III)⁵							
75	M+F	-	-	-	-	579.0 ±112.6 ³	9422.9 ±2991.6 ³
150	M	-	-	773 ±66	2350 ±180	995.3 ±149.1 ⁴	15681.1 ±2571.5 ⁴
150	F	-	-	922 ±73	2160 ±120		
300 ⁵	M	292	2660	1260 ±180	4110 ±490	-	-
300 ⁵	F	373	2810	1540 ±390	4850 ±1120	-	-
1200	M	-	-	3370 ±460	18800 ±5300	-	-
1200	F	-	-	3320 ±370	14500 ±428	-	-

1 (µg eq/mL)

2 (µg eq/mL·h)

3 5.0 g dose

4 10.0 g dose

5 NOAEL in T15096 and T8374

6 all means rounded to 3 significant digits, SD with same precision as the mean

2.6.6 TOXICOLOGY

2.6.6.1 Overall toxicology summary

General toxicology

The sponsor submitted both single-dose and repeat-dose toxicology studies in support of this NDA application. The key toxicology studies included a single dose and 28-day repeat-dose toxicology studies in the dog model for hydroxocobalamin and a 14-day repeat-dose toxicology study in the dog model for cyanocobalamin. Many of the studies, especially the rat studies, did not satisfy our recommended Guidances in terms of number of animals, gender of animals, length of treatment, and parameters measured. However, in total, the major toxicities could be identified.

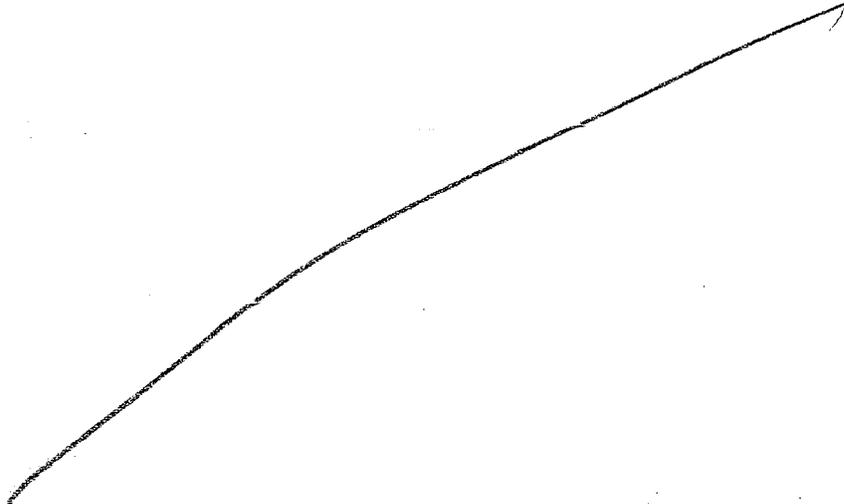
In the single dose rat studies, hydroxocobalamin was administered intraperitoneal at doses of 75 to 1000 mg/kg. The major clinical signs in rats included dyspnea, locomotor disturbance, piloerection, incomplete eyelid closure, sunken flank and reddish urine and reddish skin. The major organ toxicities were accumulation of fluid in the lungs at 1000 mg/kg (highest dose) and death in some animals (1000 mg/kg, and 1 female at 600 mg/kg), discolored and congested liver, discolored organs and tissues at ≥ 300 mg/kg including skin, abdominal fat, testes, and epididymides.

In the dog, all doses of hydroxocobalamin were administered intravenously. Single doses were 150, 300, and 1200 mg/kg. Repeated doses ranged from 75 to 1200 mg/kg/day. There were no deaths at any dose. The major clinical signs included reddish discoloration of the skin and mucous membranes and reddish urine at all doses. Wrinkles and or wheals about the head, swollen ears, and head edema developed, possibly signs of hypersensitivity or osmotic fluid shifts with drug distribution. At the higher doses of 300 and 1200 mg/kg, emesis and tremors were observed. During a recovery period, all these signs resolved. Consistent changes in liver enzymes were reported, but it was also noted that the reddish hydroxocobalamin may interfere with colorimetric analyzers. Validation of these measurements was not reported. Other changes in chemistry and hematology were not of toxicological significance. There were no changes in body weight or food consumption. There were no changes in EKG parameters, heart rate or blood pressure, but the times of measurement were only at 2 hours after administration. There were no ophthalmologic examinations. The target organs were kidney, liver, bone marrow and skin. The liver of high dose dogs was characterized by edema of intrahepatic sinuses with activation of Kupffer cells, multifocal small acute necrosis, and microgranulomas. The kidney findings included multifocal tubule eosinophilic casts, focal papilla hemorrhage, multifocal tubular dilatation, and crystalline intracytoplasmic deposits in the distal tubule. In the bone marrow, there was minimal to moderate single cell necrosis that appeared to be dose-dependent in incidence and severity. These were thought to be macrophages. The ratios of hematopoietic cells were not altered. In the gall bladder, adrenals and fat tissue, hemorrhages were present. With time post-treatment the

occurrence and severity of these findings decreased, but resolution was not complete by 2 weeks post-treatment.

Cyanocobalamin, the product of cyanide and hydroxocobalamin reaction, was tested in a 2-week repeat-dose study in dogs at 400 mg/kg and did not produce evidence of effects different from those described for hydroxocobalamin.

Immunities of the drug product that have been identified consisted of



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Genetic toxicology

Studies were conducted for both mutagenic and clastogenic potential of hydroxocobalamin. In reverse mutation bacteria assays, hydroxocobalamin, at doses up to 5000 µg/plate, was not mutagenic to strains of *S. typhimurium* and *E. coli* in the absence or presence of S9 mix. Hydroxocobalamin was not mutagenic, at the TK locus (5-trifluorothymidine resistance) in mouse lymphoma cells using a fluctuation protocol at doses of 158 to 5000 µg/mL, with or without S9.

In the in vivo clastogenic assay, hydroxocobalamin was administered to male rats at doses up to 140 mg/kg, sufficient to induce clinical signs of toxicity, but did not induce micronuclei in rat bone marrow polychromatic erythrocytes and did not alter the ratio of normochromic to polychromic erythrocytes. Therefore, hydroxocobalamin was not clastogenic in this assay.

Carcinogenicity

There were no carcinogenicity studies submitted in support of this NDA application, nor would such studies be required for an acute indication.

Reproductive toxicology

Embryofetal toxicity studies were conducted with rats and rabbits in 1974. These studies did not meet current GLP and ICH guidelines and therefore could not be used to support the reproductive safety of the proposed product.

Local Tolerance

There were no separate studies on local tolerance. The Sponsor did not indicate abnormal clinical or histopathological findings for injection sites in the dog toxicology studies. In the rat studies following intraperitoneal injection, some abdominal pathological findings were attributed to the injection.

Hypersensitivity-like reactions occurred in the dog studies, evidenced by swelling of regions of the head and ears and the presence of wrinkles or wheals soon after intravenous administration of hydroxocobalamin. A similar reaction was noted in one male treated with a dose of 400 mg/kg cyanocobalamin. These reactions resolved within a few hours or by the following day, somewhat dependent on dose administered. In study T8348, the 300 mg/kg (high dose) hydroxocobalamin infusion resulted in these clinical signs on subsequent dosing days, suggesting this was not a hypersensitivity reaction, but may reflect fluid shifts within the body from an undefined cause, since the injected compounds are supposedly iso-osmotic.

Special toxicology

Hydroxocobalamin was not phototoxic to *in vitro* cultures of mouse fibroblasts at doses up to 1000 µg/mL with or without exposure to UVA light wavelengths. Toxicity measures of this assay reflect only the most serious degree, that of cell death. Phototoxicity may also cause less severe cellular reactions in skin resulting in erythema, swelling, and eschar formation, altered DNA (thymidine dimmer, adduct formation) which may lead to mutagenesis and tumors, not detectable by this assay. This assay did not test UVB wavelengths, nor does it consider other types of cells in the skin.

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Study title: EMD 415 722 - Acute Toxicity Study in Rats after Intraperitoneal Administration

Key study findings: Male and female rats were administered a single dose of 300, 600, 800, or 1000 mg/kg hydroxocobalamin, intraperitoneal. The following key findings were obtained:

1. Clinical signs were noted at all doses tested, including reddish discoloration of the skin.
2. The maximum non-lethal dose for females was 300 mg/kg, which corresponds to a human equivalent dose of 2903 mg/60 kg person based on a body surface area comparison. The maximum non-lethal dose for males was 1000 mg/kg, which corresponds to a 9677 mg/60 kg person. In this study, females appear to be somewhat more susceptible to toxicity.
3. A NOAEL of 300 mg/kg, IP, for the rat could be established, which corresponds to a human equivalent dose of 2903 mg/60 kg person based on a body surface area comparison.

Study no.: T15741

Vol. 6, Tab 4.2.3.1.1, T15741

Conducting laboratory and location: Merck KGaA, Institute of Toxicology
64271 Darmstadt, Germany

Date of study initiation: Nov 18, 2003

GLP compliance: yes

QA report: yes

Drug, lot #, and % purity:

EMD 415722, Hydroxocobalamin (Cyanokit), Batch 2056, Purity 94%

Vehicle: Physiological sodium solution

Methods

Male and female rats (— : WU, 7 to 9 weeks of age, n=3/sex/dose) were administered a single IP injection of 300, 600, 800 or 1000 mg/kg hydroxocobalamin in a volume of 20 mL/kg. The 300 mg/kg dose was only administered to females, and the 800 mg/kg dose was only administered to males. They were observed for the first 6 hours, then daily for the following 14 days. Body weights were obtained on days 2, 4, 6, 8, 11, 13 and 15. All were subjected to pathological exam at early death or sacrificed on day 15. A potency ratio was calculated from a modified logit analysis of the dose response curve (Unkelbach and Wolf, 1985). This modification used the "1/(2n) rule" of Cobb and Church (1983).

Results

Mortality: Deaths occurred in the 600 and 1000 mg/kg dose groups as follows: One female at 600 mg/kg, 3 females at 1000 mg/kg, and 1 male at 1000 mg/kg. Deaths were generally noted after 5 hours after drug administration up to day 2.

Clinical Signs: Signs of toxicity were seen immediately after intraperitoneal administration and up to day 6 post-treatment (see table below). These consisted of

piloerection, incomplete eyelid closure, sunken flanks, retention of feces, reddish urine, abdominal position, locomotor disturbance, skin reddened, and dyspnea.

Body Weight: At the 800 and 1000 mg/kg dose, weight gain during the first few days posttreatment was reduced.

Pathology: Specific organs and tissue used for histopathology were not described, but from the results presented, these included liver, lung, testes and epididymis, and visible lesions. Animals that died showed an accumulation of reddish fluid in the thoracic and abdominal cavity, red discoloration of the peritoneum and the skin (anogenital region, most likely due to excretion of discolored urine). Some animals showed blood congestion in the liver and the lung and one animal showed a dilation of the small intestine with fluid content. Animals that were sacrificed showed focal fat necroses in the abdominal cavity. One animal dosed with 600 mg/kg showed reddish fluid in the abdominal cavity. One animal dosed with 800 mg/kg showed in the testes unilateral necroses of seminiferous tubules with concurrent calcification. The latter is of questionable toxicological relevance because of its unilateral appearance and lack of apparently dose-dependency.

Pathology of Animals that Died

Macroscopic findings:

Organ/Tissue with diagnosis	300 mg/kg 3 m	600 mg/kg 3 m/3 f	800 mg/kg 3 m	1000 mg/kg 3 m/3 f
Thorax, fluid reddish	-	328	-	316, 317, 318, 311
Abdomen, fluid reddish	-	328	-	316, 317, 318, 311
Skin (ano-genital region), discoloration reddish	-	328 (with subcutis and penis)	-	311
Peritoneum, discoloration reddish	-	-	-	316, 317, 318, 331
Liver, mottled	-	-	-	311
Liver, congestion blood	-	-	-	316, 317, 318
Lung, discoloration red	-	-	-	311
Small intestine, dilation with fluid content	-	328	-	-

Microscopic findings:

Organ/Tissue with diagnosis	300 mg/kg 3 m	600 mg/kg 3 m/3 f	800 mg/kg 3 m	1000 mg/kg 3 m/3 f
Liver, hyperemia	-	-	-	311
Lung, hyperemia and atelectasis	-	-	-	311

Pathology of Animals Sacrificed

Macrosscopic Findings:

Organ/Tissue with diagnosis	300 mg/kg 3 m	600 mg/kg 3 m/3 f	800 mg/kg 3 m	1000 mg/kg 3 m/3 f
Abdomen, fat tissue, discoloration white	-	-	331, 332, 333	313
Abdomen, fluid reddish	-	321	-	-
Testes, discoloration white	-	322 unilateral (no histology exam)	331 unilateral	312 unilateral (no histology exam)
Testes, small	-	322 (no histology exam)	331	-
Epididymides, discoloration white	-	322 unilateral (no histology exam)	333 unilateral	-
Epididymides, small	-	-	331	-

Histologic Findings:

Organ/Tissue with diagnosis	300 mg/kg 3 m	600 mg/kg 3 m/3 f	800 mg/kg 3 m	1000 mg/kg 3 m/3 f
Abdomen, fat necrosis	-	-	331, 332, (333 tissue missing)	313
Testes, atrophy bilateral and necrosis of tubules with calcification unilateral l	-	-	331	-
Epididymides, lack of spermia	-	-	331	-
Epididymides, spermatogenic germinal cells, unilateral	-	-	333	-

The estimated median lethal doses (LD₅₀) after an observation period of 14 days, were 937 (721 - 1218) mg/kg for combined males and females, 1383 (604 - 3166) mg/kg for males, and 642 (379 - 1087) mg/kg for females, but there was no statistical difference between the sexes.

Study T15741 Results, Single dose Hydroxocobalamin, Batch 2056

Dose (mg/kg)	300	600	800	1000
Females, n=	3	3	-	3
Males, n=	-	3	3	3
Mortality	0	1 F: day 2	0	3 F: within 6 hours 1 M: within 23 hours
Estimated Lethal Dose (LD ₅₀) (mg/kg) Median (95% confidence interval)	937 (721 - 1218) combined sexes 1383 (604 - 3166) males 642 (379 - 1087) females			

Dose (mg/kg)	300		600		800		1000	
Clinical Signs								
dyspnea	Within 1 h to 24 h		M, F: Within 15 m to 24 h, F: to d 2		M: within 15 m to d 2		M: Within 15 m to d 2 F: within 15 m to 6 h	
locomotor disturbance	Within 1 h to 24 h		M, F: Within 15 m to 24 h F: to d 2		M: within 15 m to d 2		M: Within 15 m to d 2 F: within 15 m to 6 h	
piloerection	F: -		M, F: Within 4 h to 24 h		M: within 3 h to d 2		M: Within 4 h to 24 h F: within 4 h to 6 h	
incomplete eyelid closure	F: -		M, F: Within 4 h		M: within 6 h to 24 h		M: Within 2 h to 24 h F: within 2 h to 6 h	
sunken flank	Within 2 h to 4 h		M: - F: d 2 to d 3		M: d 2 to d 6		M: d 2 F: -	
reddish urine	F: Within 4 h to d 2		M, F: Within 2 h to 24 h F: d 3		M: within 1 h to d 4		M: Within 2 h to d 2 F within 2 h to 6 h	
Discolored urine	-		-		-		M: Within 6 h to 24 h F: -	
retention of feces	-		-		-		M: d 2 F: -	
abdominal position	-		M: - F: d 2		-		M: Within 2 h to 6 h F: -	
skin reddened	-		M, F: Within 6 h to 24 h		M: within 4 h to 24 h		M: Within 2 h to d 2 F: within 2 h to 6 h	
Body weight	M	F	M	F	M	F	M	F
gain to day 2	-	7%	10%	6%	1%	-	4	-
gain to day 8	-	14%	27%	11%	14%	-	24	-
gain to day 15	-	21%	47%	17%	34%	-	49	-

Abbreviations: d = day, h = hour, m=minutes

Study title: EMD 415722 -Acute Toxicity Study in Rats after Fractionated Intraperitoneal Administration of 1000 mg/kg

In the previous study, T15741, all female rats dosed with 1000 mg/kg hydroxocobalamin (administered as a single dose) died within 6 hours. This study was undertaken to dose 1000 mg/kg as 3 fractionated doses, 333 mg/kg IP, at 6 hour intervals.

Key study findings: Under the given experimental conditions of this acute intraperitoneal toxicity study, after fractionated administration of 1000 mg/kg test material formulation in female rats, the test material EMD 415722 revealed no lethal toxicity. Signs of intoxication appeared already after the first administration and were more pronounced after the second and third injections but no mortality was seen.

Study no.: T15765

Vol. 6, Tab 4.2.3.1.2, T15765

Conducting laboratory and location: Merck KGaA, Pharma Ethicals, Global Preclinical R&D, 64271 Darmstadt, Germany

Date of study initiation: Jan 16, 2004

GLP compliance: yes

QA report: yes

Drug, lot #, and % purity:

EMD 415722, (Cyanokit, Hydroxocobalamin), Batch 2056. According to the sponsor, this batch of drug substance has a total impurity content of ~%.

Vehicle: physiological sodium chloride solution

Methods

Females rats (WU, 9 weeks of age, n=3) were administered a total of 1000 mg/kg hydroxocobalamin over 12 hours as 3 fractionated doses of 333 mg/kg in a volume of 20 mL/kg, every 6 hours. Animals were monitored for the first 7 hours, then daily for 4 days. Body weights were obtained before treatment and on days 2 and 4. All rats were subjected to a pathology exam on day 5.

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Results

Mortality: There were no deaths.

Clinical Signs: After the first administration, clinical signs included reddish urine, locomotor disturbance, and dyspnea within the first hour and observed during the first day. With the second administration piloerection and edema around the snout were observed within the first hour and during the first day. With the third administration, incomplete eyelid closure and temporary abdominal positioning were observed and during the first hour and during the first day. Some of these signs lasted up to 24 hours.

Body Weight: Body weight gain increased in all 3 animals, but was much lower than animal treated at lower doses in previous studies (6% gain on day 2 and 8% gain on day 4 relative to day 1 body weights).

Pathology: There were no histopathological findings that could be attributed to hydroxocobalamin administration. One animal showed a focal hemorrhage in the abdominal fat tissue that was considered to be related to the application procedure and not hydroxocobalamin. (Note: The reasoning for this conclusion was not provided).

Study title: EMD 415722 (Hydroxocobalamin) –Single Intraperitoneal Injection in Rats

Key study findings: A single dose of hydroxocobalamin was administered to male and female rats at 300 mg/kg, IP, to determine the toxicokinetics of free and total cobalamins. There were no pronounced gender differences in terms of exposure.

Study no.: T15096

Vol. 6, Tab 4.2.3.1.3, 15096

Conducting laboratory and location: Merck KGaA, Institute of Toxicology, 64271 Darmstadt, Germany

Toxicokinetics: Institute of Drug Metabolism and Pharmacokinetics, Merck KGaA, D-85567 Grafing, Germany

Date of study initiation: July 8, 2005

GLP compliance: yes

QA report: yes

Drug, lot #, and % purity:

EMD 415722 (Cyanokit, Hydroxocobalamin), Batch 2059, Purity 94%

Vehicle: 0.9% saline

Methods

EMD 415722 was administered intraperitoneal, 3 mL/kg, at a dose of 300 mg/kg to Wistar rats (WU, n=6/sex, 10 weeks of age, males 314 g, females 210 g). The control group consisted of 3 males and 3 females. Appearance, behavior and mortality were checked. Body weight was recorded once before treatment for calculation of the administration volume. Blood for determination of free and total cobalamins was sampled from the retroorbital plexus at 15 and 30 minutes, 1, 3, 6, and 24 hours after administration of 300 mg/kg EMD 415722. Blood from the control rats was sampled 30 minutes, 3, and 24 hours after administration of the vehicle. After the last blood sampling for toxicokinetics, the rats were killed by a pain-free method, but they were not further examined.

Results

Mortality: All rats survived to their scheduled death.

Clinical Signs: Reddish urine occurred in 3 of 6 males and 3/6 females treated with hydroxocobalamin.

Toxicokinetics: Male rat number 4 showed anomalous low levels of both "free" and "total" cobalamins-(III), which were not observed in the other similarly treated rats so it was excluded from the analysis (value at 15 min =0.7 µg eq/mL, compared to >136.0 µg eq/mL for other rats at this time. The reviewer concurs with the decision to exclude this animal from the toxicokinetic evaluation.

For "total" cobalamins-(III), the C_{max} was 373 and 292 µg eq/mL for female and male rats, respectively, and was reached 1 h after administration. Interindividual variability

expressed as CV% was low and varied between 1.70 to 15.6%. The terminal half-lives were 4.7 and 5.3 h and the calculated AUC_{last} was 2810 and 2660 $\mu\text{g eq}^*\text{h/mL}$ for female and male rats, respectively. For "free" cobalamins-(III) the maximum observed plasma concentrations C_{max} were 181 (female) and 140 $\mu\text{g eq/mL}$ (male), which were reached within 1 h after administration. The measured concentrations declined rapidly, which was also reflected by the terminal half-life of 2.9 h. The calculated AUC_{last} values were 705 for female and 611 $\mu\text{g eq}^*\text{h/mL}$ for the male rats. Inter-individual variability expressed as CV% was low and varied between 4.68 to 19.2%. There were no gender specific differences in the pharmacokinetic parameters of "free" and "total" cobalamins-(III).

Toxicokinetic analysis of Study T15096

Analyte	Sex	C_{max} ($\mu\text{g eq/mL}$)	T_{max} (h)	T_{half} (h)	AUC_{last} $\mu\text{g eq}^*\text{h/mL}$
Free cobalamins - (III)	m	140	0.5	2.9	611
	f	181	1	2.9	705
Total cobalamins - (III)	m	282	1	5.3	2660
	f	373	1	4.7	2810

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Figure 3 Mean plasma concentration time curves for free and total Cobalamins (III) in female rats

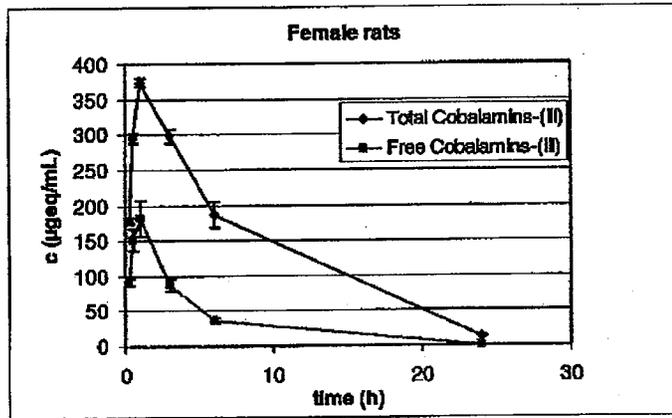
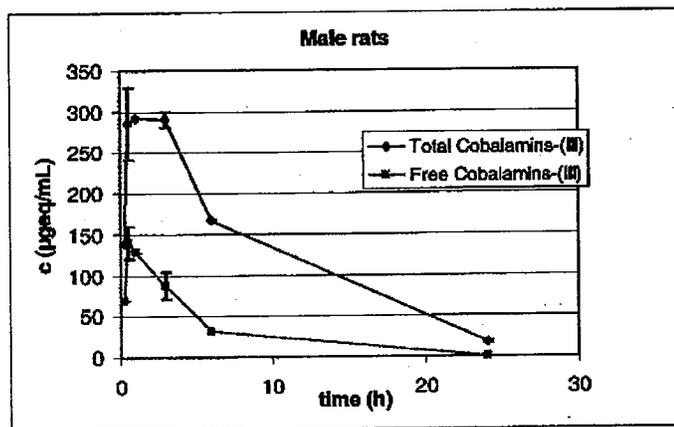


Figure 4 Mean plasma concentration time curve for free and total Cobalamins in male rats



IMPURITIES**Study title: EMD 415722 (Cyanokit® 2.5 g Batch 9337) - Acute Toxicity Study in Rats after Intraperitoneal Administration**

Key study findings: Female rats were treated with 300, 600, or 1000 mg/kg hydroxocobalamin via intraperitoneal injection. The maximum nonlethal dose was 600 mg/kg for females, but similar clinical signs were observed in all treatment groups.

Reviewer's Comments: This study cannot be used to support the safety of the drug product, specifically due to the use of only female animals, lack of hematology and clinical chemistry data, and incomplete histopathological analysis. There was no control group. However, based on the information in the study, the maximum nonlethal dose was 600 mg/kg for females, which corresponds to a human equivalent dose of 5806 mg per 60 kg person, based on a body surface area comparison.

Study no.: T15948

Vol. 11, Tab 4.2.3.7.6.1 T15948

Conducting laboratory and location: Merck KGaA, Pharma Ethicals, Global Preclinical R & D, 64293 Darmstadt Germany

Date of study initiation: Nov 12, 2004

GLP compliance: yes

QA report: yes

Drug, lot #, and % purity:

EMD 415722 (Hydroxocobalamin), Batch 9337, Purity 92.4%

Vehicle: physiological saline

Methods

Female rats (Wistar-Kyoto, WU, 9 weeks of age, 162-172 g body weight) were administered 300, 600, or 1000 mg/kg EMD 415722 intraperitoneal (n=3/dose) in a volume of 20 mL/kg. All animals were checked for at least 6 hours following injection, then checked daily for two weeks. Body weights were obtained before treatment and on days 2, 4, 6, 8, 11, 13, and 15. All animals which died prior to scheduled sacrifice or were sacrificed at the end of the study, on day 15, were necropsied. Histopathology was performed on heart, lung, liver, kidneys and small intestine. There was no control (vehicle-treated) group in this study. A potency ratio was calculated from a modified logit analysis of the dose response curve (Unkelbach and Wolf, 1985). This modification used the "1/(2n) rule" of Cobb and Church (1983).

b(4)**Results**

Mortality: Two deaths occurred in the 1000 mg/kg group, at 6 hours and 23 hours after intraperitoneal administration

Clinical Signs: Signs of toxicity were seen immediately after intraperitoneal administration and lasted up to day 7. At the 300 mg/kg dose, these consisted of dyspnea

and locomotor disturbance within the first 15 minutes, followed by piloerection and incomplete eyelid closure within 60 minutes, sunken flank within 3 hours and reddish urine within 4 hours.

Body Weight: During the first study days, there was a dose-related reduction in body weight gain.

Pathology: Histopathology was performed on the heart, lung, liver, kidney and small intestine and gross lesions. The two rats that died following drug administration had red discoloration of the body and an increased amount of red fluid in the thoracic cavity. One of these rats had brown fat atrophy. Of the rats that were sacrificed at the end of the study, two rats (one at the 600 and one at the 1000 mg/kg dose) showed focal or multi focal white nodules, 1-2 m in diameter in fat tissue of the abdominal cavity. One also had adhesions between the ovary or urinary bladder with the abdominal wall. Histologically, these lesions were diagnosed granulomas.

For female rats, the estimated lethal IP dose (LD₅₀) after an observation period of 14 days, was 859 (574 -1285) mg/kg, (median, 95% confidence interval).

Study T15948 Results, Single dose Hydroxocobalamin, Batch 9337

Dose (mg/kg)	300	600	1000
Females, n=	3	3	3
Mortality	0	0	2 (66.7%)
Estimated Lethal Dose (LD ₅₀) (mg/kg) Median (95% confidence interval)	859 (574-1285)		
Clinical Signs			
dyspnea	Within 15 m to 24 h	Within 15 m to d 2	Within 15 m to d 2
locomotor disturbance	Within 15 m to 24 h	Within 15 m to d 2	Within 15 m to d 2
piloerection	Within 1h to 4 h	Within 1 h to 24 h	Within 1 h to d 2
incomplete eyelid closure	Within 1h to 4 h	Within 1 h to 24 h	Within 15 m to 24 h
sunken flank	Within 2 h to 4 h	Within 2 h to 24 h, d 6 to d 7	Within 2 h to d 7
reddish urine	Within 4 h to 24 h	Within 2 h to d 3	Within 2 h to d 3
retention of feces	-	d 6	d 2, d 4-6
abdominal position	-	-	Within 15 m to 24 h
skin reddened	-	-	Within 2 h to 24 h
Body weight			
gain to day 2	7%	4%	2%
gain to day 8	14%	7%	10%
gain to day 15	22%	16%	24%

Abbreviations: d = day, h = hour, m=minutes

Study title: EMD 415722 (Cyanokit® 2.5 g, Batch 2080) - Acute Toxicity Study in Rats after Intraperitoneal Administration

Key study findings: Female rats were treated with 300, 600, or 1000 mg/kg hydroxocobalamin via intraperitoneal injection. The maximum nonlethal dose was 600 mg/kg for females, but similar clinical signs were observed in all treatment groups.

Reviewer's Comments: This study cannot be used to support the safety of the drug product, specifically due to the use of only female animals, lack of hematology and clinical chemistry data, and incomplete histopathological analysis. There was no control group. However, based on the information in the study, the maximum nonlethal dose was 600 mg/kg for females, which corresponds to a human equivalent dose of 5806 mg per 60 kg person, based on a body surface area comparison.

Study no.: T16400

Vol. 12, Tab 4.2.3.7.6.2 T16400

Conducting laboratory and location: Merck KGaA, Institute of Toxicology, 64271 Darmstadt, Germany

Date of study initiation: Oct 17, 2005

GLP compliance: yes

QA report: yes

Drug, lot #, and % purity:

EMD 415722 (Hydroxocobalamin), Batch 2080, Purity 89.8%

Vehicle: physiological saline

Methods

Female rats (Wistar-Kyoto WU, 9 weeks of age, 160-180 g body weight) were administered 300, 600, or 1000 mg/kg EMD 415722 intraperitoneally (n=3/dose) in a volume of 20 mL/kg. All animals were checked for at least 6 hours following injection, and then checked daily for two weeks. Body weights were obtained before treatment and on days 2, 4, 6, 8, 11, 13, and 15. All animals which died prior to scheduled sacrifice or were sacrificed at the end of the study, on day 15, were necropsied. Histopathology was performed on heart, lung, liver, kidneys and small intestine. There was no control (vehicle-treated group) in this study. A potency ratio was calculated from a modified logit analysis of the dose response curve (Unkelbach and Wolf, 1985). This modification used the "1/(2n) rule" of Cobb and Church (1983).

Results

Mortality: Deaths occurred in two rats, dosed with 1000 mg/kg, within 6 hours after administration.

Clinical Signs: Signs of toxicity were seen immediately after intraperitoneal administration for the first 24 hours at the 300 and 600 mg/kg dose and up to day 7 for the 1000 mg/kg dose. These consisted of piloerection, incomplete eyelid closure, sunken

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flanks, retention of feces, reddish urine, abdominal position, locomotor disturbance, skin reddened, and dyspnea.

Body weight: Dose-related reduction in body weight gain occurred in all three group during the first week. The surviving animal in the 1000 mg/kg dose lost weight during this time.

Pathology: Histopathology was performed on the heart, lung, liver, kidney and small intestine and gross lesions. Animals that died had reddish discoloration of all organs and tissues. One animal showed firm content in the cecum without further macroscopic organ changes. Histology of the cecum revealed no abnormal findings. One rat in the 600 and one in the 1000 mg/kg dose groups that were sacrificed at 2 weeks had multifocal adhesions between organs and tissues in combination with white particles in the abdominal cavity. They also had mild focal fat necroses with mixed inflammation in the abdominal cavity.

The median estimated lethal doses (LD₅₀) for female rats after 14 days was stated as 859 (574 -1285, 95% confidence interval) mg/kg in the results section 4.1.3, Table 9, and summary results, but in the summary conclusion stated as 642 (379 – 1087) mg/kg.

Study T16400 Results, Single dose Hydroxocobalamin, Batch 2080

Dose (mg/kg)	300	600	1000
Females, n=	3	3	3
Mortality	0	0	2 (66.7%)
Estimated Lethal Dose (LD ₅₀) (mg/kg) Median (95% confidence interval)	859 (574 - 1285)		
Clinical Signs			
dyspnea	Within 15 m to 4 h	Within 15 m to 24 h	Within 15 m to d 3
locomotor disturbance	Within 15 m to 24 h	Within 15 m to 24 h	Within 15 m to d 3
piloerection	Within 1h to 4 h	Within 1 h to 6 h	Within 1 h to d 3
incomplete eyelid closure	Within 1h to 4 h	Within 1 h to 6 h	Within 15 m to 24 h
sunken flank	Within 2 h to 4 h	Within 2 h to d 2	Within 2 h to d 7
reddish urine	Within 4 h to 24 h	Within 3 h to 24 h	Within 2 h to 6 h, d 2 to d 4
Discolored urine	-	-	Within 6 h to 24 h
retention of feces	-	-	d 2-d3
abdominal position	-	-	Within 2 h to 6 h
skin reddened	-	Within 3h to 24 h	Within 2 h to d 3
Body weight			
gain to day 2	7%	5%	-4%
gain to day 8	12%	8%	-1%
gain to day 15	19%	18%	9%

Abbreviations: d = day, h = hour, m=minutes

DOG**HYDROXOCOBALAMIN****Study title: EMD 415722 - Intravenous Tolerance Study in Beagle Dogs**

Key study findings: Dogs were administered a single intravenous dose of hydroxocobalamin (150, 300, or 1200 mg/kg). The following key findings were made:

1. A NOAEL was not identified.
2. The lowest dose tested, 150 mg/kg, was associated with reddening of the skin, wrinkled and/or wheals of the region of the head, mild elevations in liver enzymes with evidence of microgranulomas, and minimal intracellular deposition of crystalline material in the kidney.
3. The lowest dose tested corresponds to a human equivalent dose of 5,000 mg/60 kg person based on a body surface area comparison. The 300 mg/kg dose corresponds to 10,000 mg/kg human equivalent dose (body surface area) and the 1200 mg/kg dose corresponds to 40,000 mg/60 kg person.
4. The target organs of toxicity include the skin, liver, kidney, and bone marrow.

Study no.: T8374

Vol. 7, Tab 4.2.3.1.4, T8374

Conducting laboratory and location: Merck KGaA, Institute of Technology, 64271 Darmstadt, Germany

Toxicokinetics: Institute of Drug Metabolism and Pharmacokinetics, Merck KGaA, Am Feld 32, D-85567, Grafing, Germany

Date of study initiation: Oct 22, 2004

GLP compliance: yes

QA report: yes

Drug, lot #, and % purity:

EMD 415722 (hydroxocobalamin), Batch 2066, Purity 94.6%.

Vehicle: physiological saline

Methods

A single dose of hydroxocobalamin (0, 150, 300 or 1200 mg/kg, iv., 10 mL/min of 25 mg/mL) was administered to beagle dogs (n=4/sex/dose; 14-14.5 months of age; males: 7.6-10 kg; females 6.4-8.2 kg). Two dogs of each sex/dose combination were observed for 2 week following treatment (recovery dogs). Clinical signs and food consumption were monitored daily. Body weight was measured weekly. Hematology and electrolytes were performed from samples collected before infusion, at the half time of infusion, 5, 20, 45 minutes, and 1, 2.5, 4, 6, and 24 hours after treatment and at the end of the 14 day recovery period. Enzymes ALAT, ASAT and AP were determined at 24 and 72 hours (recovery dogs only) after treatment and at day 14. Blood samples were also obtained at these timepoints for toxicokinetic analysis. Gross and histopathology was performed on all dogs. Non-recovery dogs were killed and necropsied at 24 hours after infusion.

Results

Mortality: There were no premature deaths.

Clinical Signs: Clinical signs of drug reaction such as red colored urine, skin and mucous membranes were seen in all dose groups, Wrinkles and/or wheals in the region of the head transiently seen in one male (458788) dosed with 150 mg/kg and in some dogs dosed with 300 or 1200 mg/kg, Swollen ears were evident in dogs dosed with 300 or 1200 mg/kg, In addition, vomiting, and tremor were observed in some dogs dosed with 300 or 1200 mg/kg, Furthermore, one male (466314) showed a low pulse and three dogs (466314m, 460138m, 465725f) defecated during the treatment with 1200 mg/kg. It is considered that these symptoms, i.e., vomiting, tremor, low pulse and defecation were caused rather by the infusion per se than by the treatment with EMD 415722. All these symptoms proved to be reversible in the course or at the end of the recovery period.

Food consumption and body weight: There were no differences from control animals. One female 466349 dosed with 1200 mg/kg had reduced body weight and reduced food consumption during 4 days of the recovery period.

Hematology: There was a decrease (64-99%) in platelets in the 1200 mg/kg dose group. In addition, 1 control dog showed also a slight decrease in platelets at 45 minutes after treatment. In addition, there was a transient increase in red blood cell count (10-20%), hematocrit (11-20%) and hemoglobin (8-21%) in dogs at all hydroxocobalamin doses for up to 1 hour after treatment. These corresponded with subcutaneous edema.

Clinical Chemistry: A slight increase in ALAT (1.5-fold) was seen in the male 462025 dosed with 150 mg/kg. At 1200 mg/kg, a slight to moderate increase in ALAT (1.6-9 -fold) was evident in some dogs (see table below). ASAT values were slightly increased (1.6-1.9 -fold) in two males (458788, 463013) dosed with 150 or 300 mg/kg, respectively. At 1200 mg/kg, a slight to moderate increase in ASAT was evident in some dogs (see table below). A minor increase in alkaline phosphatase (1.7-2.4 -fold) was observed in one female (459911) dosed with 300 mg/kg and in some dogs dosed with 1200 mg/kg (see table below). However, one control female (455720) and 2 females (462360, 455258) dosed with 150 mg/kg showed already a minor elevation in alkaline phosphatase in week -1. All these changes proved to be reversible at the end of the 14-day recovery period.

Reviewer's Comment: The reddish coloration from hydroxocobalamin may interfere with laboratory analysis using colorimetric techniques. Usually these include standard analysis of ALAT, ASAT, alkaline phosphates, and hemoglobin, all of which had relatively variable increases after dosing. It was not mentioned if the procedure were validated for these measurements in the presence of hydroxocobalamins.

ALAT values in selected dogs dosed with 150 or 1200 mg/kg:

Dose (mg/kg)	Animal No./Sex	ALAT (U/L)			
		Before dosing	24 hours after dosing	3 days after dosing	14 days after dosing
150	462025/m	47	63	57	71
1200	460138/m	45	74	78	44
	465725/f	28	271	-	-
	466349/f	29	90	64	32

- = not measured

ASAT values in selected dogs dosed with 150 or 1200 mg/kg:

Dose (mg/kg)	Animal No./Sex	ASAT (U/L)			
		Before dosing	24 hours after dosing	3 days after dosing	14 days after dosing
150	458788/m	40	76	78	33
300	463013/m	44	73	-	-
1200	466578/m	44	92	-	-
	460138/m	52	123	122	49
	459547/m	35	112	67	38
	466870/f	39	70	-	-
	465725/f	29	117	-	-
	466349/f	25	240	39	33

- = not measured

Alkaline phosphatase values (AP) in selected dogs dosed with 150, 300 or 1200 mg/kg:

Dose (mg/kg)	Animal No./Sex	AP (U/L)			
		Before dosing	24 hours after dosing	3 days after dosing	14 days after dosing
0	455720/f	220	241	-	-
150	462360/f	295	329	-	-
	455258/f	264	138	129	198
300	459911/f	165	294	-	-
1200	466578/m	105	221	-	-
	459547/m	96	230	176	89
	466870/f	138	237	-	-
	466349/f	154	318	233	73

- = not measured

Pathology:

At main kill, the necropsy revealed treatment-related reddish discolorations of multiple organs and tissues mainly those rich in collagen and elastic fibers as follows:

Red discoloration MAIN KILL	Group 1 0 mg/kg 2m/2f	Group 2 150 mg/kg 2m/2f	Group 3 300 mg/kg 2m/2f	Group 4 1200 mg/kg 2m/2f
Skin mild moderate		2/2	2/2	2/2
Abdominal cavity, peritoneum minimal mild moderate			1/0 0/2	2/2
Aorta, intima mild				1/1
Bone mild moderate			1/1	1/1

Red discoloration MAIN KILL	Group 1 0 mg/kg 2m/2f	Group 2 150 mg/kg 2m/2f	Group 3 300 mg/kg 2m/2f	Group 4 1200 mg/kg 2m/2f
Kidney, papilla mild moderate			1/1	1/2
Nerve, sciatic mild				1/2
Stomach, mucosa mild				1/0
Trachea mild moderate			2/2	2/2
Urinary bladder, mucosa minimal mild moderate			1/1	1/0 0/1 1/1
Esophagus, mucosa mild				1/1

In addition, a treatment-related reddish discoloration of the urine was present in animals of all dose groups at main kill. Only EMD 415722 treated dogs showed unilateral or bilateral hematomas at the injection sites. There was no dose dependency. After recovery, one dog of the high dose group (466349, f) showed a mild red discoloration of the renal papilla, the subcutaneous tissue of the skin, and the urine.

Organ Weights: The following organ weights were determined: terminal body weight (after exsanguination), heart, liver, left and right kidneys separately, spleen, thymus,

testes or ovaries (together), prostate or uterus, adrenals (together), thyroids with parathyroids (together), pituitary, and brain (cerebrum, cerebellum, medulla)

The liver weights of high dose females were slightly decreased in comparison to the control. Also, one control dog of the recovery showed a comparable low relative liver weight, and the weight of a low dose female (compare dog 462742f, group 2) was even lower than that of the high dose female liver weight. The liver weights of the mid dose were comparable to the control. Therefore, this change is evaluated to be of no toxicological relevance. After recovery, the liver weights of both high dose males were relatively high, but still in the normal range seen also in controls (compare dog 455916m, control, main kill).

Histopathology: The following tables were reproduced from the sponsor's submission.

The liver showed the following main findings at main kill:

Findings MAIN KILL	Group 1 0 mg/kg 2m/2f	Group 2 150 mg/kg 2m/2f	Group 3 300 mg/kg 2m/2f	Group 4 1200 mg/kg 2m/2f
Edema, sinus minimal mild			1/-	1/1 1/1
Activation, Kupffer cell minimal mild				1/2 1/-
Microgranuloma mild moderate massive	-/1	-/2	1/2	-/1 1/- -/1
Necrosis, small, acute, multifocal mild			-/1	1/-

All high dose dogs showed an edema of the intrahepatic sinuses accompanied by minimal to mild activation of Kupffer cells. In one mid dose male a minimal edema of the sinuses was seen without activated Kupffer cells. Microgranulomas were seen in all groups including control but the degree and incidence was slightly more pronounced in the mid and high dose group. Multifocal small acute necroses were seen in one mid dose female (459911) and one high dose male (466578) and were considered to be related to treatment.

After recovery, the following main findings were seen:

Findings RECOVERY	Group 1 0 mg/kg 2m/2f	Group 2 150 mg/kg 2m/2f	Group 3 300 mg/kg 2m/2f	Group 4 1200 mg/kg 2m/2f
Deposition, eosinophilic, Kupffer cell, focal minimal		1/-	-/1	
Deposition, eosinophilic, Kupffer cell, diffuse minimal mild			2/- -/1	-/1 2/1
Necrosis, small, focal mild		1/-		1/-

Focal and diffuse deposition of eosinophilic material in Kupffer cells (most likely test substance related) was seen in all dose groups. The degree and incidence increased with dose. The incidence of microgranulomas was slightly more pronounced in the high dose group, Focal small necroses were seen in one low and one high dose male. This finding is focal, seen only in one dog per group without dose dependency and known to occur spontaneously. It is, therefore, considered to be of no treatment-related relevance.

Kidney

At main kill the following main findings were seen in the kidneys:

Findings MAIN KILL	Group 1 0 mg/kg 2m/2f	Group 2 150 mg/kg 2m/2f	Group 3 300 mg/kg 2m/2f	Group 4 1200 mg/kg 2m/2f
Cast, eosinophilic, tubule, multifocal minimal moderate				1/- -/1
Cast, Bowman's space, eosinophilic, focal mild				-/1
Dilatation, tubular, multifocal minimal moderate				1/- -/1
Hemorrhage, medulla, interstitial, focal, unilateral mild				-/1
Hemorrhage, papilla, submucosal, focal mild				-/1
Deposition, crystalline, intracytoplasmic, tubule, distal minimal mild		1/-		1/- 1/-

In one high dose female (465725) moderate multifocal eosinophilic casts mainly in tubules but focally also in the Bowman's space of some glomeruli with moderate tubular

dilatation were detected. This finding is evaluated to be due to the administration of EMD 415722, In the other female (466870), focal acute mild hemorrhages in the interstitial tissue of the medulla or in the submucosa of the papilla were seen. Whether these hemorrhages are treatment related or not is questionable. Crystalline intracytoplasmic depositions were seen in the two high dose males and in one low dose male. One high dose male (566578) showed minimal multifocal eosinophilic casts and dilated tubules in addition. The crystalline deposition in the low dose male (and also in one of the high dose males) was not accompanied by other changes attributable to treatment and was not seen after recovery, therefore their occurrence is not evaluated as an adverse effect.

After recovery the following main findings were detected:

Findings RECOVERY	Group 1 0 mg/kg 2m/2f	Group 2 150 mg/kg 2m/2f	Group 3 300 mg/kg 2m/2f	Group 4 1200 mg/kg 2m/2f
Dilatation, tubular, cystic, multifocal massive				-/1
Dilatation, tubular, multifocal mild				2/-
Cast, eosinophilic, tubule, multifocal mild moderate				1/- -/1
Basophilia, tubular, cortex, multifocal mild moderate				1/- 1/1

Findings RECOVERY	Group 1 0 mg/kg 2m/2f	Group 2 150 mg/kg 2m/2f	Group 3 300 mg/kg 2m/2f	Group 4 1200 mg/kg 2m/2f
Infiltrates, mononuclear, interstitial, multifocal mild				2/1
Degeneration, vacuolar, tubular, focal, unilateral minimal				2/-
Degeneration, vacuolar, tubular, multifocal moderate				-/1
Hydropic change, tubular, multifocal moderate				-/1
Deposition, eosinophilic, tubular, proximal mild moderate				1/- -/1
Microgranuloma, focal mild		1/-		-/1

The findings seen after recovery were more pronounced than those detected at main kill. One high dose female (466349) showed the most pronounced findings of moderate to massive degree: cystic dilatation of tubules, eosinophilic casts in the proximal tubules, vacuolar degeneration and hydropic cytoplasmic change, tubular basophilia, eosinophilic deposition in tubular cytoplasm, and interstitial mononuclear infiltrates. In the two high dose males, minimal to moderate treatment related findings were seen: dilatation of tubules, eosinophilic casts in the tubules, vacuolar degeneration, tubular basophilia, eosinophilic deposition in tubular cytoplasm, and interstitial mononuclear infiltrates. In the second female, only focal unilateral changes (focal tubular cytoplasmic basophilia and focal micro granuloma, mild) were seen which also occur spontaneously and in controls and are, therefore, evaluated as spontaneous findings. Also one low dose male (462025) showed focal tubular cytoplasmic basophilia and focal micro granulomas, which are evaluated to be of spontaneous origin. In quality, the following findings are comparable to some findings above evaluated to be treatment related. Due to their focal appearance and low degree and their spontaneous occurrence also seen in controls they are evaluated to be of spontaneous origin. Overall, the changes do not appear to be dose-related, are minimal to mild in nature, and can not easily be attributed to the treatment.

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Findings MAIN KILL	Group 1 0 mg/kg 2m/2f	Group 2 150 mg/kg 2m/2f	Group 3 300 mg/kg 2m/2f	Group 4 1200 mg/kg 2m/2f
Basophilia, tubular, cortex, focal, unilateral minimal mild		1/-	1/1	
Cast, eosinophilic, papilla, focal, unilateral minimal		2/-		
Cast, eosinophilic, tubule, focal, unilateral minimal				1/-
Cast, tubule, hemorrhagic, focal minimal			-/1	
Infiltrates, mixed, interstitial, focal, unilateral minimal			1/-	
Infiltrates, mononuclear, interstitial, focal minimal			-/1	
Necrosis, small, medulla, focal minimal				-/1
Basophilia, tubular, cortex, focal, unilateral minimal mild	1/1	-/1		-/1
Cast, eosinophilic, tubule, focal, unilateral minimal	1/-			
Cast, tubule, hemorrhagic, focal, unilateral minimal	-/1			

Findings MAIN KILL	Group 1 0 mg/kg 2m/2f	Group 2 150 mg/kg 2m/2f	Group 3 300 mg/kg 2m/2f	Group 4 1200 mg/kg 2m/2f
Basophilia, tubular, cortex, focal, unilateral minimal mild		1/-	1/1	
Cast, eosinophilic, papilla, focal, unilateral minimal		2/-		
Cast, eosinophilic, tubule, focal, unilateral minimal				1/-
Cast, tubule, hemorrhagic, focal minimal			-/1	
Infiltrates, mixed, interstitial, focal, unilateral minimal			1/-	
Infiltrates, mononuclear, interstitial, focal minimal			-/1	
Necrosis, small, medulla, focal minimal				-/1

Findings RECOVERY	Group 1 0 mg/kg 2m/2f	Group 2 150 mg/kg 2m/2f	Group 3 300 mg/kg 2m/2f	Group 4 1200 mg/kg 2m/2f
Basophilia, tubular, cortex, focal, unilateral minimal mild	1/1	-/1		-/1
Cast, eosinophilic, tubule, focal, unilateral minimal	1/-			
Cast, tubule, hemorrhagic, focal, unilateral minimal	-/1			

Hydroxocobalamin treated dogs had reddish discoloration of the subcutis and urine in all dose groups, and of multiple other organs and tissues (mainly those in collagen and elastic fibers) in 300 and 1200 mg/kg dose groups. Hematomas were present at injection site in hydroxocobalamin treated dogs, but not control dogs. The discoloration appeared to be reversible since at 2 weeks following treatment, only one dog in the 1200 mg/kg group had mild red discoloration of renal papilla, subcutis and urine.

2.6.6.3 Repeat-dose toxicity

RAT

HYDROXOCOBALAMIN

Study title: 3-Month Toxicity Study in the Rat

Key study findings: Subcutaneous administration of 5 or 50 mg/kg hydroxocobalamin to rats resulted in no detectable toxicity.

Reviewers Comments: This study was completed in 1974 and was submitted to support the application to the French authorities. This study lacks data concerning the purity of the hydroxocobalamin and identification of the composition of the solvent control. Individual animal data are not provided. In many cases, the variations associated with mean values are not provided. There was no toxicokinetic information. Therefore this study does not meet current guidance recommendations, and cannot be used to support the safety of the proposed drug product.

Study no.: III.A.2.1

Vol. 12, Tab 4.2.3.7.7.5.2, III.A.2.1

Conducting laboratory and location:

Date of study initiation: report dated 1974

GLP compliance: yes

QA report: yes

Drug, lot #, and % purity:

Hydroxocobalamin, unknown batch and purity

Vehicle: solvent (but not identified)

Methods

Doses: 5 and 50 mg/kg, saline and solvent controls

Species/strain: Rat

Number/sex/group or time point (main study): 10/sex/dose

Route, formulation, volume, and infusion rate: subcutaneous at 0.2 mL/100 g BW

Satellite groups used for recovery: 5/sex/dose were kept an additional 1 month without treatment

Weight: Mean weight: males, 215 g; females, 175 g

Results

Mortality: There were no deaths.

Clinical signs: There were no clinical sign abnormalities noted.

Body weights: There was no affect on bodyweight gain.

Food consumption: This was not determined.

Hematology: Measurements were obtained for red cell count and differential white cell count. There was no affect on absolute or relative blood cell counts.

Clinical chemistry: Measurements were obtained for total lipids, SGOT, SGPT, total cholesterol, proteins, urea, glucose, and hemoglobin. There were no changes in transaminases, cholesterol, urea or hemoglobin. The changes noted by the Sponsor are indicated in the table below, with statistically significant differences from saline control in bold.

Reviewer's Comment: These differences are not consider toxicologically significant. The solvent is undefined and may account for the changes in lipids and proteins rather than hydroxocobalamin.

Chemistry values after 3 months of treatment

Doses		saline	solvent	5 mg/kg	50 mg/kg
glucose	M	111 ± 15	104 ± 11	106 ± 27	87 ± 9
	F	136 ± 21	153 ± 21	126 ± 16	110 ± 15
lipids	M	563 ± 76	434 ± 73	447 ± 77	446 ± 62
Proteins	M	6.37 ± 0.31	6.73 ± 0.26	6.7 ± 0.27	7.0 ± 0.24

Urinalysis: Urine tests included pH, glucose, proteins, and blood. There were no abnormalities detected.

Gross pathology: The organs examined were heart, lung, spleen, liver, stomach, kidneys, brain, adrenals, testes and epididymis, ovaries, vagina, duodenum and ileum.

Organ weights: No changes were detected in the organs examined (heart, lung, spleen, liver, stomach, kidney, brain, adrenals, thyroid, testes, and ovaries)

Histopathology: Adequate Battery: yes

Peer review: no

The Sponsor attributed abnormal findings to intercurrent disorders of an infectious nature, since they were found in all animals, in the lungs (probably chronic viral pneumopathy), and with occasional renal and pancreatic changes. Hepatic islets of lymphocytes were attributable to the persistence of lymphopoiesis, which is normal in young rats. The frequency of these observations was the same in all groups of treated or control rats.

Images of cerebral anoxia, observed in the animals of which the brain had been fixed by immersion were attributed to delayed fixation, which is commonly observed under these conditions. Some brains were fixed by perfusion and in all of these cases the organs appeared to be histologically normal. Staining with Oil Red 0 for hepatic lipids after cryosectioning was negative in all groups.

DOG**HYDROXOCOBALAMIN****Study title: EMD 415722 -Intravenous Tolerance Study in Beagle Dogs**

Key study findings: The Sponsor interpreted the few clinical chemistry findings together with the pathological results as signs of tissue overload, rather than specific toxicological effects. They suggest a dose level of lower than 600 mg/kg should be selected as the top dose in a repeat-dose intravenous toxicity study.

Reviewer's Comment: There was only 1 female and 1 male used in this preliminary trial, therefore no conclusions would be very definitive. This study is also the only study in which interference by hydroxocobalamin during clinical chemistry testing was mentioned. However, neither the method nor the results of this investigation of potential interference were described.

Study no.: T8347

Vol. 9, Tab 4.2.3.2.1.3, T8347

Conducting laboratory and location: Merck KGaA, Institute of Technology, 64271 Darmstadt, Germany

Toxicokinetics: _____

Date of study initiation: Jan 23, 2003

GLP compliance: yes

QA report: yes

Drug, lot #, and % purity:

EMD 415722 (Cyanokit, hydroxocobalamin), Batch 2056, Purity 97.7%

Vehicle: pyrogen-free 0.9% sodium chloride

Methods

Doses: 150, 300, 600 mg/kg/day administered on days 1, 2, and 3-14, respectively

Species/strain: beagle dogs

Number/sex/group or time point (main study): 1/sex/dose

Route, formulation, volume, and infusion rate: intravenous infusion once daily for 2 weeks at 10 mL/min of 25 mg/mL hydroxocobalamin or vehicle

Age: males and females 12 months of age

Weight: males, 7.8 kg; females, 7.9 kg

Unique study design or methodology: the dogs were dosed at 150 mg/kg on day 1, 300 mg/kg on day 2 and 600 mg/kg on days 3 to 14.

Results

Mortality: checked daily

There were no deaths.

b(4)

Clinical signs: checked daily

Clinical signs included red urine and skin after all doses. Soft stools occurred on day 2 with the 300 mg/kg dose. On days 3-14, 600 mg/kg dosing resulted in swollen ears accompanied with head shaking observed on days 3 and 4 for up to 1 hour after treatment, and red skin and reddish skin secretions on days 5 to 14.

Body weights: measured on day -2, 4, and 5

There were no effects on body weight.

Food consumption: Diet was offered for 2 hour/day and the daily food intake was recorded

There were no effects on food consumption.

Ophthalmoscopy: examination at week 2, using a hand lamp, ophthalmoscope, fundus camera, slit-lamp biomicroscope

No ophthalmic effects were noted.

EKG: Lead II was evaluated although standard leads I, II, and III and Goldberger leads aVR, aVL, and aVF were recorded. EKG was monitored at week -1 and before and 2 hours after treatment on days 1, 2, 3, and 11. Dogs were conscious.

There were no effects on EKG parameters and there were no changes in heart rate with repeated dosing (see table below).

Heart Rate

Day	-3	1		2		3		11	
Time		0	+2	0	+2	0	+2	0	+2
M, n=1	147	132	129	126	114	117	102	122	105
F, n=1	132	138	123	120	114	105	117	135	111

Blood Pressure: Systolic, diastolic and mean arterial blood pressure were measured indirectly using a tail cuff system (oscillometric system) at the same times in the same dogs as EKG recordings. Dogs were conscious

There were no blood pressure changes.

Hematology: blood was obtained by jugular vein puncture, after fasted for 22 hours, during week -1 and at week 2 after treatment started.

There was an increase in erythrocyte sedimentation rate at 1 and 2 hours after administration in the male dog.

Hematology				
Parameter	600			
	M (n=1)		F (n=1)	
	Week -1	Week 2	Week -1	Week 2
Erythrocyte sedimentation rate				
After hour 1 (mm/h)	1	26		
After hour 2 (mm/2h)	3	55		

Clinical chemistry: blood was obtained by jugular vein puncture, after fasted for 22 hours, during week -1 and at week 2 after treatment started. It was mentioned that an interference test was conducted to determine if effects noted were treatment related or due to an interference of hydroxocobalamin with the method used.

Reviewer's Comment: The result of this determination was not mentioned.

There was a small increase in serum liver enzymes in the male and females as indicated in the table below.

Clinical Chemistry				
Parameter	600			
	M (n=1)		F (n=1)	
	Week -1	Week 2	Week -1	Week 2
ALAT (U/L)	20	63	26	64
ASAT (U/L)	29	98	32	54

Gross pathology:

At necropsy the connective tissue of the subcutis and other tissues rich in elastic and collagen fibers exhibited a reddish discoloration, especially the aorta, trachea, kidney, urinary bladder. The urine was also reddish.

Organ weights:

Organ weights were not measured.

Histopathology: Adequate Battery: yes

Peer review: in house discussion was mentioned

The liver was the major affected organ in both dogs. Massive deposition of eosinophilic material was observed in the sinusoid lining cells (Kupffer cells) and to a lesser degree in hepatocytes. The material was diffusely distributed in the cytoplasm of the Kupffer cells and was stored in large inclusion bodies in the liver cells. Single cell necrosis and small necroses of hepatocytes were found throughout the liver parenchyma, often associated with reactive inflammatory infiltrates.

The female exhibited a bile duct hyperplasia in addition to the liver findings mentioned above. There were also foam cell foci in the lung of the male and very few proteinaceous casts with concomitant chronic inflammation in the kidneys. It could not be determined if these were related to hydroxocobalamin treatment.

Study title: EMD 415 722 – 4 week intravenous toxicity study with an 8 week recovery period in beagle dogs

Key study findings: Beagle dogs administered daily intravenous doses of 0, 75, 150 or 300 mg/kg hydroxocobalamin for 4 weeks resulted in reversible toxicities, with the exception of degenerative and regenerative changes in the liver at the 300 mg/kg/day dose. If one considers the reversibility of the changes, the NOAEL dose was 150 mg/kg.

Reviewer's Comments: The pathologist report concludes that a NOEL cannot be established, but given the apparent reversibility of findings, a NOAEL of 150 mg/kg is reasonable. However, lingering presence of material in tissues and organs even 8 weeks after terminating treatment at the high dose, (only twice the expected high dose in people) reveals its extremely slow tissue elimination, possibly due to recycling and use as vitamin B₁₂ within the cells.

Study no.: T8348

Vol. 9, Tab 4.2.3.2.1.2, T8348

Conducting laboratory and location: Merck KGaA, Institute of Toxicology, 64271 Darmstadt Germany

Toxicokinetics: _____

Date of study initiation: April 17, 2003 (this study was performed before the 2-week study above)

GLP compliance: yes

QA report: yes

Drug, lot #, and % purity:

EMD 415722 (Cyanokit, hydroxocobalamin), Batch 2059, Purity 97.8%

Vehicle: pyrogen-free 0.9% sodium chloride

b(4)

Methods

Doses: 0, 75, 150, 300 mg/kg/day

There was no indication the drug concentration of the stock solutions used for dosing were verified by assay in this signed GLP study.

Species/strain: beagle dogs

Number/sex/group or time point (main study): 3/sex/dose

Route, formulation, volume, and infusion rate: intravenous infusion once daily for 4 weeks at 10 mL/min of 25 mg/mL hydroxocobalamin or vehicle

Satellite groups used for toxicokinetics or recovery: 2/sex/dose for 0 and 300 mg/kg/day dose groups, observed for 8 weeks following the termination of treatments.

Age: males and females 8-9 months of age

Weight: males, 7.2-10.9 kg; females, 6.4-8.5 kg

Results

Mortality: checked daily

There was no mortality.

Clinical signs: checked daily

Clinical signs included red urine, skin, mucous membranes, were all reversible during recovery.

Some dogs developed swollen ears, wrinkles and/or wheals on the head soon after infusion and lasting up to 1 hour. This response appeared dose related in frequency of occurrence. At the 300 mg/kg/day dose, some dogs exhibited this response for the entire 4 weeks of dosing.

At the 300 mg/kg/day dose one male developed a weak and low pulse and defecated and one female vomited soon after administration of the first dose (day 1). In weeks 1 and 2, one female exhibited salivation, associated with dosing.

Body weights: weekly from week -1

There was no effect on body weight.

Food consumption: Diet was offered for 2 hours each day and daily food intake was recorded.

There was no effect on food consumption.

Ophthalmoscopy: Examinations were completed at week -2, week 4 and week 12 (recovery animals) using a hand lamp, ophthalmoscope, fundus camera, slit-lamp biomicroscope.

There were no ophthalmic effects.

EKG: Lead II was evaluated although standard leads I, II, and III and Goldberger leads aVR, aVL, and aVF were recorded. EKG was monitored at week -1 and before and 2 hours after treatment in week 3 and week 12. Dogs were conscious.

There were no effects on EKG parameters or heart rate.

Blood Pressure: Systolic, diastolic and mean arterial blood pressures were measured indirectly using a tail cuff system (oscillometric system) at the same times in the same dogs as EKG recordings. Dogs were conscious.

There were no effects on blood pressure.

Hematology: Blood was obtained by jugular vein puncture, after fasted for 22.5 hours, during week -2 and at weeks 4 and 12 after treatment started.

The low dose female 7881 and the high dose male 4190 showed a distinct decrease in platelet count in week 4. This effect was not seen in any recovery group dogs. In

addition, erythrocyte sedimentation rates at 1 and 2 hours (ESR 1 and 2) were increased in one mid dose female (7881) in week 4. As the control male 1230 also showed an increase in ESR 2 in week 4 (see table below), the increase is not considered treatment-related.

Hematological Changes

Parameter	Dose (mg/kg)									
	0			75		150		300		
Week	-2	4	12 recovery	-2	4	-2	4	-2	4	12 recovery
Platelets (/nL)										
F 7881				221	40					reversible
M 4190								308	71	
Erythrocyte sedimentation rate										reversible
After hour 1 (mm/hr1) F 7881				1	20					
After hour 2 (mm/hr2) F 7881				1	30					
After hour 2 (mm/hr2) M 1230	4	16								

Clinical chemistry: Blood was obtained by jugular vein puncture, after fasted for 22.5 hours, during week -2 and at weeks 4 and 12 after treatment started.

A dose-dependent increase in ALT was seen in male and female dogs, however, the effect was more pronounced in the male dogs.

In addition, an increase in AP values was observed in two high dose males (7432, 1493) in week 4. Some treated females (1226, 5472, 7717, and 2257) showed an increase in AP already before treatment. In one control female (4705), an elevated AP-value was seen in week 4. Therefore, this effect was considered not treatment-related. These changes were reversible by the end of the recovery period.

Clinical Chemistry Changes

Parameter	Dose (mg/kg)									
	0			75		150		300		
Week	-2	4	12 recovery	-2	4	-2	4	-2	4	12 recovery
ALAT (U/L)										
Males										reversible
3517				37	178					
6219						30	273			
8013						52	110			
8697						55	113			
7432								22	380	
4190								34	484	
1072								33	291	
1493								30	381	
Females										
7881				33	65					
3623				32	70					
7717						37	84			
5472						32	76			

	3096								25	140		
ASAT (U/L)											reversible	
Males	3517			37	82							
	6219					29	85					
	8013					28	45					
	7432							29	128			
	4190							31	163			
	1072							28	117			
	1493							29	103			
Females	3096							30	78			
AP (U/L)											reversible	
Males	7432							279	832			
	1493							182	391			
Females	4705	280	400									
	1226			405	273							
	5472					303	213					
	7717					294	200					
	2257							322	259			
Urine:				increased incidence of oxalate crystals in urine week 4 also reversible								reversible

Urinalysis: The red color of the urine interfered with the analytical methodology and only sediment could be examined. The Sponsor stated an interference test (concentrations: 100 mg/L and 500 mg/L) was performed in order to check if the test material would interfere with the analytical methods used.

In comparison with the control dogs, a higher incidence of oxalate crystals was observed in the urine of the treated dogs in week 4. This effect was reversible by the end of the recovery period.

Gross pathology:

The necropsy revealed a treatment-related reddish discoloration of the skin in 3/6 low dose, 6/6 mid dose and 6/6 high dose animals. The severity of the finding increased dose dependently from minimal/mild in the low dose group to moderate/massive in the high dose group. A treatment-related reddish discoloration of the urine was present in 2/6 mid dose and 3/6 high dose animals. Withdrawal of treatment for 8 weeks resulted in resolution of the changes in the skin and the urine.

Organ weights:

The determination of body and organ weights indicated the following treatment-related changes: slightly increased liver, kidney and spleen weights in low, mid and high dose animals. No clear-cut histopathological correlates to these weight changes were found.

After a recovery period of 8 weeks, the weight changes were reversible with exception of the increased liver weights in high dose females.

Histopathology: Adequate Battery: yes

Peer review: yes (in house discussion with other pathologists)

The histopathological examination of the dogs at the end of 4 weeks of treatment indicated treatment-related findings in the liver, kidney, bone marrow, lymph nodes and spleen. The 75 mg/kg dose group showed minimal to mild macroscopic and histopathological visible treatment-related findings consisting of a discoloration of organs and histological changes in the liver, the kidney and the bone marrow. The 150 mg/kg dose group showed similar findings as the 75 mg/kg dose group but to a higher degree. The treatment-related findings in the low and mid dose group were judged by the Sponsor to be the consequence of an overload phenomenon with hydroxocobalamin.

In the 300 mg/kg dose group the overload phenomenon-related findings exceeded those of the low and mid dose group. In addition, adverse reactive and degenerative changes in the liver and the kidney were seen. After withdrawal of treatment for 8 weeks, only minor histopathological changes were seen in the liver and the bone marrow, therefore Sponsor considered this indicating at least partial reversibility of earlier findings.

Liver: In the liver two categories of treatment-related changes were seen consisting of overload-related findings only and of overload-related findings with additional reactive and degenerative changes. Signs of an overload phenomenon in low, mid and high dose animals (severity and incidence increased with dose):

- minimal to mild intracytoplasmic deposition of an eosinophilic material (most likely test substance related) in the sinusoidal Kupffer cells
- intracytoplasmic deposition of an eosinophilic material in the hepatocytes
- minimal to moderate peri vascular mixed cellular infiltrates
- minimal to moderate perivascular mononuclear cell infiltrates
- Signs of an overload phenomenon with additional adverse reactive and degenerative changes in the high dose group:
 - minimal to moderate degeneration of hepatocytes
 - minimal to mild single cell necroses of hepatocytes
 - minimal to mild bile duct proliferation
 - minimal to mild fibrosis

Kidney: In the kidney, as in the liver, overload-related findings only and overload-related findings with additional reactive and degenerative changes were observed. These increased in severity and incidence with increased dose:

- minimal to moderate intracytoplasmic deposition of an eosinophilic material (most likely test substance related) in the proximal tubules and focal minimal deposition of crystals in the urothelium of the renal pelvis in one low dose animal
- Signs of an overload phenomenon with additional adverse reactive and degenerative
- changes in the high dose group included single cell necroses of tubular cells

Bone Marrow: In the bone marrow, minimal to moderate single cell necroses were detected in treated dogs of all dose groups with a mild dose-dependent increase in incidence and degree. This finding was evaluated to be more a consequence of an overload phenomenon than a toxic effect on the haematopoietic cells.

Reviewer's Comment: This reason for judging this as not being a toxic effect on hematopoietic cells was not provided. Therefore, the reviewer is not convinced of this conclusion.

Spleen and Lymph Nodes: In the spleen, an activation of the lymph follicles was found in one mid dose and some high dose animals. In the lymph nodes a minimal intracytoplasmic deposition of an eosinophilic material in sinus macrophages was diagnosed in some high dose animals.

Heart: In the heart focal minimal mononuclear infiltrates were found in some low, mid and high dose animals. This finding was considered by the Sponsor to be of questionable toxicological relevance.

Recovery animals: The histopathological examination of the recovery animals revealed treatment-related findings in the liver and the bone marrow. In the liver minimal perivascular degeneration of hepatocytes, intracytoplasmic deposition of an eosinophilic material in sinusoidal Kupffer cells, minimal fibrosis and minimal bile duct proliferation was diagnosed in some high dose animals. Withdrawal of treatment for 8 weeks did not result in complete resolution of these findings but a tendency towards recovery was present. In the bone marrow single cell necroses were still present but at a lower incidence and severity also indicating some reversibility of earlier findings. In the kidney, spleen and lymph nodes all treatment-related findings recovered after withdrawal of treatment for 8 weeks with exception of a focal minimal deposition of crystals in the urothelium of the renal pelvis in one high dose animal. In the heart focal minimal mononuclear infiltrates were still found in high dose animals.

Toxicokinetics: Blood was obtained from the jugular vein on day 1 and in week 4 from all animals of the 75 and 150 mg/kg/day dose groups and from the 2nd, 3rd, and 4th animals/sex of the 0 and 300 mg/kg/day groups. Sampling times were before and at 1, 2, 3, 4, 6, and 24 hours after treatment. Blood was also obtained at week 4 before necropsy and 2 g of liver, heart and kidney were taken from all necropsied animals at this time for drug quantification.

Analysis of the plasma samples resulted in unreliable data since it was found that hydroxocobalamin was highly unstable in plasma samples unless the samples are spiked with 2% glacial acetic acid and the whole process of sampling and sample preparation is done under cooled conditions. Values were reported but not included in this review. The Sponsor stated that tissue samples have not been analyzed.

Study title: EMD 415722 -Intravenous Tolerance Study in Beagle Dogs

Key study findings: Administration of 300, 600 and 1200 mg/kg/day for 3 days resulted in reddish urine, skin, mucous membranes. Some animals also exhibited wrinkles and/or wheals on the head and/or swollen ears. The histopathological examination of dosing indicated treatment-related findings in the liver, kidney, bone marrow, heart, spleen, lymph nodes, blood vessels of the gall bladder, the fat tissue around the oviduct and the ciliary body of the eye.

Study no.: T8355

Vol. 8, Tab 4.2.3.2.1.1, T8355

Conducting laboratory and location: Merck KGaA, Institute of Technology, 64271 Darmstadt, Germany

Toxicokinetics: _____

Date of study initiation: Nov 21, 2003

GLP compliance: yes

QA report: yes

Drug, lot #, and % purity:

EMD 415722 (hydroxocobalamin), Batch 2066, Purity 95.5%

Vehicle: pyrogen-free 0.9% sodium chloride

Methods

Doses: 0, 300, 600, 1200 mg/kg/day

Species/strain: beagle dogs

Number/sex/group or time point (main study): 2/sex/dose

Route, formulation, volume, and infusion rate: intravenous infusion once daily for 3 days at 10 mL/min of 25 mg/mL hydroxocobalamin or vehicle

Satellite groups used for toxicokinetics or recovery:

Age: males and females 15-22 months of age

Weight: males, 7.9-10.4 kg; females, 5.9-9.5 kg

Results

Mortality: checked daily

There were no deaths.

Clinical signs: checked daily

All doses resulted in reddish urine, skin, and mucous membranes. Some animals also exhibited wrinkles and/or wheals on the head and/or swollen ears. Some symptoms last longer than 6 hours. At 600 mg/kg/day, one animal salivated after dosing and at 1200 mg/kg 3 animals vomited.

Body weights: measured on day -2, 4, and 5

There were no effects on body weight.

Food consumption: diet was offered for 2 hour/day and the daily food intake was recorded

There were no effects on food consumption.

EKG: Lead II was evaluated although standard leads I, II, and III and Goldberger leads aVR, aVL, and aVF were recorded. EKG was monitored at week -1 for all dogs and before and 2 hours after treatment only in the 1200 mg/kg dose dogs. Dogs were conscious. The effect of heart on QT interval was dissociated by Van de Water's equation.

There were no effects on EKG parameters or heart rate.

Blood Pressure: Systolic, diastolic and mean arterial blood pressures were measured indirectly using a tail cuff system (oscillometric system) at the same times in the same dogs as EKG recordings.

There were no effects on blood pressure.

Hematology: Blood was obtained by jugular vein puncture, after fasted for 22.5 hours, during week -1 and at week 1 after treatment started.

There were few animals per dose, so individual animals were considered rather than mean values. The percentage lymphocytes and number of platelets were reduced in some animals in the 1200 mg/kg dose group. The erythrocyte sedimentation rate was also greatly increased in some animals at 1 and 2 hours. Blood clotting was prolonged as determined by PT and PTT tests in one animal in the 300 mg/kg group after treatment.

Hematology Summary

Parameter	Day	0		300		600		1200	
		-6	4	-6	4	-6	4	-6	4
Lymphocytes (%)									
	F 9028			18.3	4.9				
	F 4126					21.6	9.7		
	F 7890							18.0	2.1
	M 2392							29.4	7.2
	M 4450							26.1	9.4
Platelets (/nL)									
	F 4916							486	67
	F 7890							306	65
	F 2392							348	68
Erythrocyte sedimentation rate									
After hour 1 (mm/hr1)	F 4916							1	22
After hour 2 (mm/hr2)	F 4916							2	105
After hour 1 (mm/hr1)	F 7890							1	22

After hour 2 (mm/hr2) F 7890							1	43
PT (sec)	F 9028		6.7	12.7				
PTT (sec)	F 9028		10.4	17.7				

Clinical chemistry: Blood was obtained by jugular vein puncture, after fasted for 22.5 hours, during week -1 and at week 1 after treatment started.

(Note: methods indicate Clinical Chemistry samples (ALAT, ASAT, AP, K+, and Iron) collected during week -1, tables indicate day -2, but legend to tables indicate day-6)

There were few animals per dose, so individual animals were considered rather than mean values. For serum liver enzymes ALT and AST, increased concentrations occurred in all dose groups in some animals. Alkaline phosphatase also increased in some animals in the 300 and 1200 mg/kg dose groups. Declines occurred in potassium and iron concentrations.

Reviewer's comment: In this study, it was mentioned that an interference test was conducted to determine if the increased enzyme levels were a real treatment effect or were an artifact from hydroxocobalamin interference with the method used to assay ALT and AST. However, the results of this analysis were not presented and it was not mentioned if the values reported were obtained by a validated method.

Summary of Clinical Chemistry

Parameter	0		300		600		1200	
	Day -6	Day 4						
ALAT (U/L) (=ALT)								
Males								
2499					224	191		
2392							35	83
4450							43	683
Females								
9028			34	5997				
5726			21	69				
4126					28	356		
4916							31	122
7890							32	1286
ASAT (U/L) (=AST)								
Males								
5432			36	74				
2279			34	60				
2499					44	110		
4100					40	78		
2392							43	161
4450							28	401
Females								
9028			26	3337				
5726			23	66				
4126					24	187		

1307					42	119		
4916							30	157
7890							28	1307
AP (U/L) (=ALP) Females								
9028			53	370				
4916							129	320
7890							212	478
Potassium (mmol/L) Males								
2499					4.65	3.75		
4100					4.81	3.78		
2392							4.51	3.39
4450							4.57	3.68
Females								
9028			4.49	3.41				
4126					4.79	3.27		
1307					4.91	3.54		
4916							4.60	3.54
7890							4.30	3.54
Iron (mmol/L) Males								
4450							27	12.1
Females								
9028			44	11				
7890							46.3	10.1

Gross pathology:

Gross pathology revealed treatment-related reddish discoloration of the skin and urine in all dose groups.

Organ weights (specify organs weighed if not in histopath table):

There were no treatment-related organ weight changes.

Histopathology: Adequate Battery: yes

Peer review: no

The histopathological examination after 3 days of dosing indicated treatment-related findings in the liver, kidney, bone marrow, heart, spleen, lymph nodes, blood vessels of the gall bladder, the fat tissue around the oviduct and the ciliary body of the eye.

Liver: Hepatocellular necroses were found in 3/4, 4/4 and 4/4 dogs of the 300, 600 and 1200 mg/kg/day dose groups, respectively. One dog in the low dose group exhibited massive necroses. Mixed infiltrates were present in treated dogs and controls, but the severity of the finding increased with dose. In the Kupffer cells, there was deposition of an eosinophilic material in 1/4, 3/4 and 4/4 dogs of the low, mid and high dose group, respectively. Also, deposition of a brown pigment was noted in 3/4 dogs of the low, mid and high dose group, respectively.

Kidney: In the kidneys dilation of tubules with proteinaceous casts was found in 2/4 and 4/4 dogs of the mid and high dose groups. The dilated tubules were lined by flattened epithelial cells with evidence of mitotic figures. Necrosis of single tubular cells was present in 2/4 mid dose and 4/4 high dose dogs. In the glomeruli a focal, segmental formation of thrombi was noted in all high dose dogs. Other findings e.g. focal plasma leakage, edema of tubular cells and hemorrhages were found in high dose dogs.

Bone Marrow: In the bone marrow, a few single cell necroses were found in 1/4, 3/4 and 4/4 dogs of the low, mid and high dose group, respectively. Minimal to mild deposition of a brown pigment was noted in 2/4 of the high dose dogs. The overall picture of the hematopoietic cells was however not altered.

Reviewer's Comment: In view of this cellular necrosis, quantitative analysis of the erythroid: myeloid ration would have been useful to confirm the subjective overall picture.

Heart: In the heart, minimal to mild focal degeneration of myofibers was diagnosed in the left ventricle of 2/4 high dose dogs.

Toxicokinetics: Blood was obtained from the jugular vein of all animals (or saphenous vein if hematomas prevented blood collection) on days 1 and 3, at times before and at 5, 1, 2, 3, 4, 6, and 24 hours after treatment.

There was no dose accumulation between day 1 and 3 of treatment.

Hydroxocobalamin Toxicokinetics

Dose (mg/kg/day)	Day	C _{0.083h} (µg/mL)	AUC _(0-24h) (µg-h/mL)	C _{0.083h} (µg/mL)/dose	AUC _(0-24h) (µg-h/mL)/dose
300	1	722	1147.4	2.41	3.83
	3	609	1206.5	2.03	4.02
600	1	978	2664.7	1.63	4.44
	3	1377	2469.7	2.30	4.12
1200	1	2352	8103.9	1.96	6.75
	3	1996	5986.3	1.66	4.99

Study title: 4 Week Toxicity Study in the Dog (Anphar Rolland)

Key study findings: Intramuscular administration 5 mg of hydroxocobalamin, 6 days out of 7, for 4 weeks, did not induce any clear signs of toxicity.

Study no.: III.A.2.2

Vol. 12, Tab 4.2.3.7.7.5.1, III.A.2.2

Conducting laboratory and location: _____

Date of study initiation: 1974

GLP compliance: no, performed prior to GLP existence

QA report: no

Drug, lot #, and % purity:

hydroxocobalamin, Batch and Purity not provided
vehicle: physiological saline

b(4)

Methods

Doses: 0 or 5 mg/kg/day

Species/strain: dogs

Number/sex/group or time point (main study): 3 males and 1 female per treatment group

Route, formulation, volume, and infusion rate: intramuscular administration in lumbar region for 6 of 7 days/week for 4 weeks; saline as 0.5 mL/kg/day; hydroxocobalamin as 0.33 mL/kg/day of 15 g/L solution

Age: 8 to 14 months of age

Weight: Not specified

Unique study design or methodology (if any):

ResultsMortality:

There were no deaths.

Clinical signs: observed daily

Injection seemed to cause pain, thought to be attributed to the concentration of hydroxocobalamin. Otherwise, there were no noted reactions to the injection.

Body weights: measured weekly

Only 1 dog (#145) in hydroxocobalamin group had weight loss (2.7 kg over 4 weeks), others had normal weight gains.

Reviewer's comment: dog 147 in this group lost weight also (1.4 kg in the first 2 weeks, then regained 0.6 kg the last 2 weeks.

Food consumption: weighed daily, weekly consumption calculated

No differences between groups in food consumption. No information about the dog with weight loss.

Ophthalmoscopy: not performed

EKG: not performed

Hematology: at 0, 2 and 4 weeks of treatment (blood cell counts, hematocrit and hemoglobin) at 0 and 4 weeks (differential white cell count), blood obtained from the anterior subcutaneous vein; (mentioned that hemoglobin assayed by colorimetric method)

In the 5 mg/kg/day treated animals, the mean red cell count rose from 5,700,000/mm³ to 7,180,000/mm³ in week 2 and to 7,750,000/mm³ in week 4. Corresponding increases occurred in mean hemoglobin from 15.35g to 17.90g, then to 18.10 g and hematocrit from 40.80% to 47.75 %, then to 51 %. The 2,000,000 red cell count increase was still within the normal range, but greater than the control group which increased by 800,000 cells.

Clinical chemistry: Blood was obtained from the anterior subcutaneous vein at 0, 2, and 4 weeks and assayed for glucose, urea, total protein and electrophoretic separation, alkaline phosphatases, transaminases (SGOT and SGPT), Na, K, Cl and Ca.

There was an increase in alkaline phosphates in one control male dog (#142) from 113 mU/mL to 280 mU/mL at 4 weeks.

There was a slight increase in SGPT in a male dog treated with 5mg/kg/day (#145) from 57 mU/mL to 76 mU/mL. at 4weeks

Urinalysis: at 0 and 4 weeks (pH and qualitative tests for glucose, bilirubin, blood proteins and ketone bodies)

Only a control dog (#140) showed any change, the presence of biliary pigments (++) at week 4.

Gross pathology: at 1 month of treatment

There were no treatment related pathologies.

Organ weights (liver, spleen, kidney, adrenal, gonads, thyroid, and heart):

There were no effects on organ weights.

Histopathology: Adequate Battery: no; few tissues examined: the above weighed organs plus stomach, jejunum, striated muscle (temporal) and lumbar muscle (injection site)

Peer review: no

There were no treatment related changes in histopathology

CYANOCOBALAMIN

Study title: Art. 524950 (Cyanocobalamin) – Intravenous Tolerance Study in Beagle Dogs**Key study findings:**

Reviewer's Comment: This is a preliminary dose range-finding study that included only a single animal per sex, the lack of data regarding the purity of the drug substance, and verification of the actual dose administered. Therefore, this study can not be used to definitively characterize the potential toxicity of high dose cyanocobalamin in the dog model.

Study no.: T8377

Vol. 11, Tab 4.2.3.2.2.2, T8377

Conducting laboratory and location: Merck KGaA, Institute of Toxicology, 64271 Darmstadt, Germany

Bioanalytics: _____

Toxicokinetics: Institute of Drug Metabolism and Pharmacokinetics, Merck KGaA, D-85567 Grafing

Histopathology: TPC Toxicologic Pathology Consultancy, D-24116 Kiel

Date of study initiation: Nov 25, 2004

GLP compliance: yes

QA report: yes

Drug, lot #, and % purity:

Cyanocobalamin (Solution of Art. 524950), Batch 04R0091B, Purity 97.6%

Vehicle: water for injection

b(4)

Methods

Doses: 40, 100, 400 mg/kg administered on day 1, day 2, and days 3-14, respectively

Species/strain: beagle dogs

Number/sex/group or time point (main study): 1/sex

Route, formulation, volume, and infusion rate: intravenous infusion, once daily, 10 mL/min of 8 mg/mL, for 2 weeks

Age: male: 47 months; female: 47 months

Weight: male: 9.8 kg; female: 8.4 kg

Unique study design or methodology: Increasing doses were administered over the first three days, starting with 40 mg/kg on day 1, then 100 mg/kg on day 2, followed by 400 mg/kg on days 3 through 14.

Results

Mortality: checked daily

Clinical signs: checked daily

At 40 mg/kg, red colored urine was seen in both dogs. Red colored urine, skin and mucous membranes were evident in both dogs after treatment with 100 and 400 mg/kg.

Body weights: recorded weekly

There were mild decreases in body weight (M, 6%; F, 5%)

Food consumption: recorded daily, food was offered for a 2 hour period each day

There was a small decrease in food consumption (M, 17%; F 31%)

EKG: Lead II was evaluated although standard leads I, II, and III and Goldberger leads aVR, aVL, and aVF were recorded at pre-dose (week -1), and before and 2 hours after treatment at week 2. Dogs were conscious.

There were no effects on EKG parameters.

There were no effects on heart rate.

Blood Pressure: Systolic, diastolic and mean arterial blood pressures were measured indirectly using a tail cuff system (oscillometric system) at the same times in the same dogs as EKG recordings. Dogs were conscious.

There were no effects on blood pressure.

Hematology: blood was obtained by jugular vein puncture, during weeks -2 and week 2.

There were no effects on hematology.

Clinical chemistry: blood was obtained by jugular vein puncture during weeks -2 and 2

There were no effects on clinical chemistry.

Gross pathology:

Necropsy revealed treatment-related reddish discolorations of the kidneys and the urine in both dogs, the male showed red discoloration of the subcutaneous tissue of the skin in addition.

Organ weights (specify organs weighed if not in histopath table):

No organs were weighed.

Histopathology: Adequate Battery: yes

Peer review: no

Kidneys: The male dog showed focal minimal eosinophilic depositions in the cytoplasm of the proximal tubules, which are evaluated as a minimal treatment related effect.

Bone marrow: Both dogs showed minimal single cell necroses in the bone marrow. In the female this finding was seen only focally. The single cell necroses were mainly characterized by basophilic apoptotic like nuclear detritus or, but more seldom, large light cells with subtle eosinophilic cytoplasmic threads and pyknotic nuclei. The affected cells are most likely macrophages. The number and the maturation of the hematopoietic cells seemed not to be altered. The single cell necroses are evaluated as a treatment related finding though comparable findings were seen in control dogs with a low incidence.

Reviewer's Comments: It would have been useful to verify these impression with quantitative data of the erythroid:myeloid ratio and to use special staining techniques (immunocytochemical to surface antigens) to verify that the cells were macrophages.

Injection site: At the injection sites mild to moderate hemorrhages were seen perivascular and in the vascular wall. In addition minimal to mild granulation tissue, focal minimal to mild necroses and mild intimal activation were detected. These findings were attributed to the administration technique rather than the test substance Art,524950.

Toxicokinetics: blood was obtained from the jugular vein in week 2 "after reaching the MTD." Sampling times were before and 1, 2, 4, 6, and 24 hours after treatment.

Cyanocobalamin Toxicokinetics (dose of 400 mg/kg/day)

Day	C (at 1 hour) (µg eq/mL)		AUC(1-inf) (µg eq/mL* <i>h</i>)		t _{1/2} (6-24hr) (h)		T _{1/2} (1-6 hr) (h)	
	M	F	M	F	M	F	M	F
3 (first day at this dose)	409	274	445	236	3.3	3.7	0.83	0.77
11 (day 9 of this dose)	446	273	534	260	3.7	4.2	0.84	0.80

Study title: Art. 524950 (Cyanocobalamine) – 2 week intravenous toxicity study in beagle dogs

Key study findings: Clinical findings such as red colored urine, skin and mucous membranes were completely reversible within the first week of the 2-week recovery period. Gross pathology revealed that red discoloration was still present in the epididymides of the high dose males killed 2 weeks after treatment was terminated. Histopathology revealed a slight eosinophilic deposition in the proximal renal tubular epithelium of both recovery group males and an increased number of macrophages in the sternal bone marrow of all recovery animals. Based on these results the NOAEL of this study can be considered to be 400 mg/kg.

Reviewer's Comment: This is the definitive study that was completed by the sponsor to characterize the potential toxicity of cyanocobalamin, which would be formed at high levels when hydroxocobalamin is administered to treat cyanide poisoning. The pathologist report indicated that a NOEL could not be determined due to discoloration of tissues. However, due to the partial resolution of findings during the 2 week non-treatment period, the reviewer concurs that a NOAEL of 400 mg/kg is reasonable.

Study no.: T8380

Vol. 10, Tab 4.2.3.2.2.1, T8380

Conducting laboratory and location: Merck KGaA, Institute of Toxicology, 64271 Darmstadt, Germany

Bioanalytics: _____

Toxicokinetics: Institute of Drug Metabolism and Pharmacokinetics, Merck KGaA, D-85567 Grafing

Histopathology: _____

Date of study initiation: Feb 4, 2005

GLP compliance: yes

QA report: yes

Drug, lot #, and % purity:

Cyanocobalamin (Solution of Art. 524950), Batch 9496, Purity no information
The cyanocobalamin solution was produced by Merck Santee s.a.s., 45400 Semoy France. HPLC assay of cyanocobalamin indicated 8.3 mg/mL, but the purity of the cyanocobalamin was not provided.

Vehicle: no information provided

Reviewer's Comment: Since this study was performed about 2 months after the preliminary study, the purity of cyanocobalamin and the vehicle used may be the same as the in preliminary study, but it was not stated.

Methods

Doses: 0, 40, 100, 400 mg/kg administered on days 1, 2, and 3-14, respectively

Species/strain: beagle dogs

Number/sex/group or time point (main study): 3/sex/dose

Route, formulation, volume, and infusion rate: intravenous infusion, once daily, 10 mL/min of 8 mg/mL, for 2 weeks

Satellite groups used for recovery: 2/sex, only the 400 mg/kg/day dose

Age: males, 8-8.5 months; females, 8-8.5 months

Weight: males, 6.3-8.9 kg; females 5.3-6.9 kg

Results

red urine, skin, mucous membranes in all dose groups,
reversible in high dose dogs on day 4 of recovery

400: wrinkles and/or wheals in head region and swollen ears on day 1 (n=1 M)

Clin Chem

Decreased platelets on day 1 and 11 in 100 mg/kg (n=1 F) and 400 mg/kg (n=2 F)

Mortality: checked daily

There were no deaths.

Clinical signs: checked daily

Clinical signs of red colored urine, skin, and mucous membranes occurred in all dose groups. In the high-dose recovery animals, these symptoms resolved by day 4, indicating reversibility. One high-dose male (9325) showed wrinkles and/or wheals in the region of the head and swollen ears on the first day of treatment. In addition, male (8841) in the high-dose group urinated during treatment on 2 days.

Body weights: recorded weekly from day -2

There was no effect on body weight.

Food consumption: Diet was offered for 3 hours each day and the daily food intake was recorded.

There was no effect on food consumption.

EKG: Lead II was evaluated although standard leads I, II, and III and Goldberger leads aVR, aVL, and aVF were recorded at pre-dose (week -2), at and before and 2 hours after treatment at week 2, and at the end of the recovery period in recovery dogs (day 25). Dogs were conscious.

There was no effect on EKG parameters or heart rate. Second degree AV block was seen in the females 4337 (control group) and 4698 (low dose group) in week -2. Additionally, the female 4698 showed a second degree AV block at 2 hours after dosing in week 2.

Reviewer's Comment: As acknowledged by the Sponsor, this effect is spontaneously seen in Beagle dogs and was not indicative of a treatment-related effect. However, its detection two weeks prior to cyanocobalamin administration, in two dogs, should have resulted in replacement of these dogs prior to initiation of the treatment phase. There were few dogs per treatment group, and for the characterization of toxicological effects, maximizing the number of "normal" dogs would be advantageous. Although not ideal, this did not impact the adequacy of the study.

Blood Pressure: Systolic, diastolic and mean arterial blood pressures were measured indirectly using a tail cuff system (oscillometric system) at the same times in the same dogs as EKG recordings. Dogs were conscious.

There was no effect on blood pressure.

Hematology: Blood was obtained by jugular vein puncture, after fasted for 21 hours, during week -2 and at days 8, 9 and 23 after treatment started.

Elevated erythrocyte sedimentation rates were seen in one control female at week 2 and one low dose male at weeks -2, -1, and 2 (see table below). The mid dose female 3521 and two high dose dogs (female 4949 and male 1847) showed transiently decreased platelet values on days 1 and 11. The tables below were reproduced from the sponsor's submission:

Erythrocyte sedimentation rates (mm/1 or 2 hours) in the control female 4655 and the male 3373 dosed with 400 mg/kg:

Dose (mg/kg)	Animal No./Sex	ESR	Week -2	Week -1	Week 2
0	4655/f	After 1 hour	1	-	10
		After 2 hours	1	-	20
400	3373/m	After 1 hour	27	22*	22
		After 2 hours	65	73*	55

* Due to elevated ESR values in week -2, additional blood sampling in week -1.

Transiently decreased platelet values of the mid dose female 3521 and two high dose dogs (female 4949 and male 1847):

Dose (mg/kg)	Animal No./Sex	Day	Platelets (/nL)
100	3521/f	1 (half of infusion)	53
		11 (before treatment)	68
400	1847/m	11 (5 min after treatment)	74
	4949/f	1 (4 h after treatment)	11
		11 (5 min after treatment)	22

Clinical chemistry: blood was obtained by jugular vein puncture, after fasted for 21 hours, during week -2 and at days 8, 9 and 23 after treatment started.

Elevated alkaline phosphatase values were seen in 1 control female (4337), 1 mid dose male (8159) and 1 high dose female (1197).

Alkaline phosphatase values (U/L) in the control female 4337, the mid dose male 8159 and the high dose female 1197:

Dose (mg/kg)	Animal No./Sex	Week -2	Week 2
0	4337/f	245	214
100	8159/m	122	205
400	1197/f	180	228

Urinalysis: only urine sediment was determined

There were no effects on urinalysis.

Gross pathology:

At necropsy, a minimal red discoloration was noted in the epididymides, skin, urinary bladder or kidneys in individual animals of treated groups. It was still present in the epididymidis of animals sacrificed at the end of the treatment-free recovery period, No microscopic correlation of this alteration was found at histopathologic examination, and thus, the toxicologic relevance of this finding remains unclear from this study.

Organ weights (specify organs weighed if not in histopath table):

There were no effects on organ weights.

Histopathology: Adequate Battery: yes
Peer review: no

Kidney: In the kidneys, a minimal eosinophilic deposition was noted in the tubular epithelium in three males and one female of the high dose group (400 mg/kg body weight) main study, A similar but more eosinophilic deposition was seen, slight in degree, in both recovery males of this group, An increased number of large macrophages was noted in the (sternal) bone marrow of the animals of the high dose group when compared with the control animals and is suspected to be treatment-related. The toxicologic relevance of this finding, however, remains unclear from this study.

A small number of microscopic lesions such as hemorrhage, inflammatory cell infiltration, pigment accumulation, fibrosis and/or endothelial proliferation in the affected veins was observed at the injection sites in both, control and treated animals. These incidental findings were considered to be due to mechanical irritation during injection procedures rather than to any irritant effects of the vehicle or test substance. The minimal to slight eosinophilic depositions in the proximal tubular epithelium observed in animals of the high dose group (main and recovery sacrifice) was suspected to be related to the treatment with cyanocobalamin.

Bone Marrow: An increased number of large macrophages were noted in the (sternal) bone marrow of the animals of the high dose group when compared with the control animals. The findings were suspected to be treatment-related, but its toxicologic relevance, however, remains unclear from this study. In the animals of the mid and low

dose group, the number of such cells was within the normal limits as noted in the control group.

Reviewer's Comments: There was no indication that the bone marrow macrophages were quantitated. Also the erythroid:myeloid ratio was not determined.

Toxicokinetics: blood was obtained from the jugular vein on day 1 and in week 2 from all animals of the 100 mg/kg/day dose groups and from the 2nd, 3rd, and 4th animals/sex/ of the 400 mg/kg/day groups. On treatment day 2 and week 2, samples were obtained from dogs in the 0 and 40 mg/kg/day groups. Sampling times were before and at the half time of infusion, 5 minutes, and 1, 2, 4, 6, and 24 hours after treatment.

There were no sex specific differences in the plasma concentrations of cyanocobalamin. In general, plasma concentrations and AUC values were similar on day 1 and in week 2. Dose-normalized C_{max} and AUC values increased from doses of 40 to 400 mg/kg, but were less than proportional to the dose. The Sponsor attributed this to a non-proportional increase to an increased total body clearance with increasing dose. The volume of distribution at steady state (V_{ss}) was higher in the highest dose group than in the intermediate and low dose animals.

Reviewer's Comment: Tissue uptake and sequestration may also explain these findings

Cyanocobalamin Toxicokinetics

Dose (mg/kg)	Day	Sex	C _{max} (µg eq/mL)	T _{max} (h)	C _{max} /D ¹	AUC _{tot} (µg eq/mL·h)	AUC _{tot} /D ²	Clearance (L/h/kg)	V _{ss} (L/kg)	t _{1/2} (1-6h) (h)
40	2	f	211	0.0992	5.37	149	3.79	0.266	0.204	0.799
		m	204	0.147	5.21	159	4.06	0.249	0.202	0.829
	12	f	236	0.0621	6.02	128	3.25	0.308	0.243	0.761
		m	219	0.0317	5.58	144	3.66	0.277	0.242	0.842
100	1	f	477	0.0629	4.87	309	3.15	0.319	0.251	0.739
		m	426	0.0696	4.34	357	3.64	0.279	0.241	0.825
	11	f	371	0.0992	3.78	291	2.97	0.338	0.263	0.727
		m	419	0.0701	4.27	331	3.37	0.305	0.255	0.823
400	1	f	924	0.0601	2.36	796	2.03	0.498	0.441	0.768
		m	984	0.143	2.51	911	2.32	0.436	0.404	0.815
	11	f	926	0.134	2.36	819	2.09	0.48	0.499	0.749
		m	955	0.143	2.43	845	2.15	0.468	0.516	0.812

1: C (µg eq/mL); D[mg eq/kg]

2: AUC [µg eq/mL x h]; D [mg eq/kg]

Study	T8374	T8348	T8355	T8380
Species	Dog Single dose	Dog Repeat dose	Dog Repeat dose	Dog cyanoc obala min
Adrenals	*X	*X	*X	*X
Aorta	X	X	X	X
Bone Marrow smear	X	X	X	X
Bone (femur)				
Bone sternum	X	X	X	X
Brain	*X	*X	*X	*X
Cecum	X	X	X	X
Cervix				
Colon	X	X	X	X
Duodenum	X	X	X	X
Epididymis	X	X	X	X
Esophagus	X	X	X	X
Eye	X	X	X	X
Fallopian tube	X	X	X	X
Gall bladder	X	X	X	X
Gross lesions				
Harderian gland				
Heart	*X	*X	*X	*X
Ileum	X	X	X	X
Injection site	X	X	X	X
Jejunum	X	X	X	X
Kidneys	*X	*X	*X	*X
Lachrymal gland				
Larynx	X	X	X	X
Liver	*X	*X	*X	*X
Lungs	X	X	X	X
Lymph nodes, cervical				
Lymph nodes mandibular	X	X	X	X
Lymph nodes, mesenteric	X	X	X	X
Mammary Gland	X	X	X	X
Nasal cavity				
Optic nerves	X	X	X	X
Ovaries	X	X	X	X
Pancreas	X	X	X	X
Parathyroid	X	X	X	X
Peripheral nerve				
Pharynx				
Pituitary	*X	*X	*X	*X
Prostate	*X	*X	*X	*X
Rectum	X	X	X	X
Salivary gland	X	X	X	X

Sciatic nerve	X	X	X	X
Seminal vesicles				
Skeletal muscle	X	X	X	X
Skin	X	X	X	X
Spinal cord	X	X	X	X
Spleen	*X	*X	*X	*X
Sternum				
Stomach	X	X	X	X
Testes	*X	*X	*X	*X
Thymus	*X	*X	*X	*X
Thyroid	*X	*X	*X	*X
Tongue				
Trachea	X	X	X	X
Urinary bladder	X	X	X	X
Uterus	X	X	X	X
Vagina	X	X	X	X
Zymbal gland				

X, histopathology performed

*, organ weight obtained

2.6.6.4 Genetic toxicology

Summary of Genetic Toxicology Studies

Study / Initiation Date	Cells	Hydroxocobalamin Dose / Batch	Result
MUTAGENICITY ASSAYS			
Bacterial reverse mutation assay			
T15570 (Feb 19, 2003)	<i>Salmonella typhimurium</i> : TA-98 TA-100 TA-1535	50 - 5000 µg/plate Batch 2056, 93.9% purity	-S9: negative +S9: negative
T15950 (Dec 1, 2004)		50 - 5000 µg/plate Batch 2070, 96.1% purity	-S9: negative +S9: negative
T15917 (Nov 4, 2004)	<i>Escherichia coli</i> : WP2uvrA	50 - 5000 µg/plate Batch 9337, 92.4% purity (stability testing batch)	-S9: negative +S9: negative
T16401 (Oct 11, 2005)		50 - 5000 µg/plate Batch 2080, 89.8% purity (stability testing batch)	-S9: negative +S9: negative
Mammalian Cells, <i>in vitro</i>			
T15575 (Apr 22, 2003)	Mouse lymphoma (L5178Y trK+/-)	158, 500, 1580 and 5000 µg Batch 2056, 93.9%	-S9: negative +S9: negative
CLASTOGENICITY ASSAYS			
Rat, <i>in vivo</i> Micronucleus Test			
T15574 (April 3, 2003)	rat, bone marrow, examined 24 or 48 hr after treatment	0, 14, 44.3 and 140 mg/kg, iv., single administration, Batch 2056, 93.9% purity	negative

Study title: EMD 415 722 (Cyanokit®) –Bacterial Mutagenicity Assay

Key findings: EMD 415 722 (hydroxocobalamin) at concentrations of up to 5000 µg/plate was not mutagenic to strains of *S. typhimurium* and *E. coli* in the absence or presence of S9 mix.

Study no.: T15570

Vol. 11, Tab 4.2.3.3.1.1, T15570

Conducting laboratory and location: Institute of Toxicology, Merck KGaA, 64271 Darmstadt, Germany

Date of study initiation: Feb 19, 2003

GLP compliance: yes

QA reports: yes

Drug, lot #, and % purity:

EMD 415722 (hydroxocobalamin), Batch 2056, Purity 93.9%

Vehicle: physiological saline

Methods

The following methods were used for studies T15570, T15950, T15917 and T16401.

Strains/species/cell line:

Salmonella typhimurium TA98, TA100, TA102, TA1535, TA1537
Escherichia coli WP2 *uvrA*

Doses used in definitive study: 50.0, 158, 500, 1580 and 5000 µg/plate

Basis of dose selection:

EMD 415722 (hydroxocobalamin) was initially tested at seven concentrations, separated by half-log intervals, 5, 15.8, 50.0, 158, 500, 1580 and 5000 µg/plate. This was followed by a second experimental series, usually 5 concentrations including at least 4 nontoxic concentrations. Since there was no precipitation of EMD 415722 on the agar plates and no visible toxicity of the bacterial lawn, the second series consisted of the highest 5 doses from the initial test.

Negative controls: physiological saline

Positive controls:

Strain	-S9		+S9	
	Compound	Dose (µg/plate)	Compound	Dose (µg/plate)
TA98	Daunomycin	4	2-aminoanthracene	2
TA100	N-ethyl-N'-nitro-N-nitrosoguanidine	5	2-aminoanthracene	2
TA102	cumene hydroperoxide	200	benzo[a]pyrene	10
TA1535	N-ethyl-N'-nitro-N-nitrosoguanidine	10	2-aminoanthracene	2
TA1537	9-aminoacridine	50	2-aminoanthracene	10
WP2 <i>uvrA</i>	N-ethyl-N'-nitro-N-nitrosoguanidine	5	2-aminoanthracene	10

Incubation and sampling times:

The plate incorporation method with and without addition of liver S9 mix from Aroclor 1254-pretreated rats was used. Plates were incubated for 2 to 3 days at 37°C. The metabolic activation solution (S9) was prepared from the livers of male Wistar rats aged 6-8 weeks, that received a single intraperitoneal injection of Aroclor 1254 (500 mg/kg body weight) dissolved in Miglyol 812 oil. On day 5 to 7 post-injection, animals were sacrificed, the livers were removed and processed sterilely to create S9 using standard techniques. The S9 was then frozen and stored in liquid nitrogen at -196°C. The mixture of S9 plus the added cofactors is termed S9 mix. Its composition is listed below. The first test series was conducted with 10 % S9, the second series conducted with 30 % S9 in the S9 mix.

Components	Quantity per mL S9 mix	
	1st Series	2nd Series
Liver homogenate (S9)	0.10 mL	0.30 mL
MgCl ₂ /KCl aqueous solution (0.4 M/1.64 M)	0.02 mL	0.02 mL
Glucose-6-phosphate, disodium salt	5 µmol	5 µmol
NADP, disodium salt	4 µmol	4 µmol
Sodium phosphate buffer (0.2 M, pH 7.4)	0.50 mL	0.50 mL
Distilled water	0.38 mL	0.18 mL

Every S9-batch is tested for its metabolic activity by the use of specific substrates, requiring different enzymes of the P450-isoenzyme family. The mutagenicity of 2-aminoanthracene, benzo(a)pyrene, and 3-methylcholanthrene is thus characterized once for every S9-batch. Revertant colonies were scored using either an Arek MiniCount colony counter or manually. The presence of precipitate and the background lawn of non-revertant cells were checked for each plate.

Results

Study validity:

Triplicate plates were used for each concentration of the test material and the positive controls. Twice as many solvent control plates were used for each bacterial strain.

Signs of toxicity included a reduction in the number of spontaneous revertants or a clearing of background lawn. Precipitation was checked so that the test material would not interfere with scoring of the colonies.

The phenotypic characteristics (amino-acid requirement for growth, presence of R-factor etc.) of the strains are periodically checked. The verification of the phenotype together with the number of revertants yielded for the negative and positive control groups in the current study contribute to the validity of the test system.

Clear increases in the number of revertants for *S. typhimurium* TA98, TA100, and TA1537 with all positive controls and for TA1535 with 2-aminoanthracene are used as an acceptance criterion for each S9-batch.

According to the publications of Levin et al. (1982) and Kier et al. (1986) and the historical controls of the laboratory, usually the following mean numbers of revertants are acceptable as negative (or solvent) controls for the bacterial strains used:

Strain	mean number of revertants
TA98	15 - 60
TA100	75 - 200
TA102	200 - 450
TA1535	3 - 37
TA1537	4 - 31
WP2 <i>uvrA</i>	10 - 70

The positive control test materials should induce a "clear increase" in the number of revertants as indicated in the table below.

Mean Number of Colonies (Solvent Control)	Maximal Mean Number of Colonies over the Actual Solvent Control (Test Material)	
≤ 10	≤ 9	≥ 30
≤ 30	≤ 19	≥ 40
≤ 80	≤ 29	≥ 80
≤ 200	≤ 49	≥ 120
≤ 500	≤ 79	≥ 200
Assessment:	"No Increase"	"Clear Increase"

A test material was defined as *non-mutagenic* in this assay if "no" or "weak increases" occur in the first and second series of the main experiment. ("Weak increases" randomly occur due to experimental variation.)

A test material is defined as *mutagenic* in this assay if there is a dose-related (over at least two test material concentrations) increase in the number of revertants is induced, the maximal effect is a "clear increase", and the effects are reproduced at similar concentration levels in the same test system. "Clear increases" occur at least at one test material concentration, higher concentrations show strong precipitation or cytotoxicity, and the effects are reproduced at the same concentration level in the same test system.

In all other cases, a third test series with the bacterial strain in question is performed. If the criteria for a positive test result are not fulfilled in at least two out of the three series, the test material is defined as being non-mutagenic in this test system.

Study outcome:

There was no precipitation of EMD 415722 on the agar plates. There was no clear toxicity to the bacteria. Each treatment with the positive controls led to a clear increase in revertant colonies, thus, showing the expected reversion properties of all strains and good metabolic activity of the S9 mix used. Negative and positive controls were within their expected ranges.

EMD 415722 showed no increase in the number of revertants of any bacterial strain with and without the addition of rat liver S9 mix (series 2 Table below, reproduced from the sponsor's submission). Thus, EMD 415722 was not mutagenic in this assay.

TABLE 3 / Series No.: 2

EMD 415 722: Summary of the Mean Number of Revertant Colonies T15570

Test Material	Concentration [µg/plate]	+/- S9- Mix	Mean revertant colonies / plate		
			TA 98	TA 100	TA 102
Solvent control		-	22	118	293
EMD 415 722	50	-	22	148	303
	158	-	20	128	301
	500	-	22	131	307
	1580	-	20	125	274
	5000	-	37	158	314
Solvent control		+	32	178	310
EMD 415 722	50	+	28	182	275
	158	+	36	185	338
	500	+	31	181	317
	1580	+	35	168	330
	5000	+	35	189	307
Positive controls	Name		DAON	ENNG	CUM
	Conc. [µg/plate]	-	4	5	200
	Revert./plate		108	755	828
Positive controls	Name		2-AA	2-AA	B(a)p
	Conc. [µg/plate]	+	2	2	10
	Revert./plate		79	380	813

Test Material	Concentration [µg/plate]	+/- S9- Mix	Mean revertant colonies / plate		
			TA 1535	TA 1537	WP2 uvrA
Solvent control		-	20	11	43
EMD 415 722	50	-	22	16	52
	158	-	21	15	57
	500	-	26	12	51
	1580	-	25	17	53
	5000	-	21	14	53
Solvent control		+	25	17	53
EMD 415 722	50	+	23	14	60
	158	+	31	21	50
	500	+	27	20	55
	1580	+	23	19	65
	5000	+	28	25	52
Positive controls	Name		ENNG	9-AA	ENNG
	Conc. [µg/plate]	-	10	50	5
	Revert./plate		970	314	1893
Positive controls	Name		2-AA	2-AA	2-AA
	Conc. [µg/plate]	+	2	10	10
	Revert./plate		102	82	110

DAON	Daunomycin	ENNG	N-Ethyl-N'-nitro-N-nitroso-guanidine
CUM	Cumene hydroperoxide	2-AA	2-Aminoanthracene
B(a)p	Benzo(a)pyrene	9-AA	9-Aminoacridine