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# Toxicokinetic Profiles of $\alpha$ -ketoglutarate Cyanohydrin, a Cyanide Detoxification Product, Following Exposure to Potassium Cyanide

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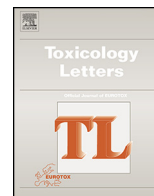
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# Toxicokinetic profiles of $\alpha$ -ketoglutarate cyanohydrin, a cyanide detoxification product, following exposure to potassium cyanide



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## HIGHLIGHTS

- The toxicokinetic behavior of  $\alpha$ -KgCN in swine was investigated.
- Measuring plasma  $\alpha$ -KgCN provides definitive confirmation of cyanide exposure.
- Treatment of cyanide poisoning with cobinamide renders  $\alpha$ -KgCN an ineffective diagnostic marker.

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## ABSTRACT

Poisoning by cyanide can be verified by analysis of the cyanide detoxification product,  $\alpha$ -ketoglutarate cyanohydrin ( $\alpha$ -KgCN), which is produced from the reaction of cyanide and endogenous  $\alpha$ -ketoglutarate. Although  $\alpha$ -KgCN can potentially be used to verify cyanide exposure, limited toxicokinetic data in cyanide-poisoned animals are available. We, therefore, studied the toxicokinetics of  $\alpha$ -KgCN and compared its behavior to other cyanide metabolites, thiocyanate and 2-amino-2-thiazoline-4-carboxylic acid (ATCA), in the plasma of 31 Yorkshire pigs that received KCN (4 mg/mL) intravenously (IV) (0.17 mg/kg/min).  $\alpha$ -KgCN concentrations rose rapidly during KCN administration until the onset of apnea, and then decreased over time in all groups with a half-life of 15 min. The maximum concentrations of  $\alpha$ -KgCN and cyanide were 2.35 and 30.18  $\mu$ M, respectively, suggesting that only a small fraction of the administered cyanide is converted to  $\alpha$ -KgCN. Although this is the case, the  $\alpha$ -KgCN concentration increased >100-fold over endogenous concentrations compared to only a three-fold increase for cyanide and ATCA. The plasma profile of  $\alpha$ -KgCN was similar to that of cyanide, ATCA, and thiocyanate. The results of this study suggest that the use of  $\alpha$ -KgCN as a biomarker for cyanide exposure is best suited immediately following exposure for instances of acute, high-dose cyanide poisoning.

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## 1. Introduction

Cyanide can be found in food (Vetter, 2000), smoke from fires (Becker, 1985; Brenner et al., 2010a; Purser et al., 1984), and cigarettes (Xu et al., 2011, 2012), and industrial facilities (Ma and Dasgupta, 2010; Smith et al., 2010; Zdrojewicz et al., 1996). It is easily procured and could be used as a weapon of mass destruction (Viswanath and Ghosh, 2010). Human exposure to cyanide

produces toxic effects by binding to the iron and copper in the active site of cytochrome c oxidase, thereby inhibiting the enzyme (Baskin et al., 2004). Depending on the dose, this can result in histotoxic anoxia (Baskin et al., 2004), cellular hypoxia (Conn, 1978), respiratory failure (Conn, 1978; Fasco et al., 2007; Way, 1984), and eventual death. Because cyanide is a rapidly acting poison, and cyanide exposure is relevant to both the military and public sectors, toxicokinetic information on cyanide and its detoxification products is important for understanding the behavior of cyanide following exposure. Cyanide can be metabolized and detoxified through a number of routes, including those outlined in Fig. 1. The two major routes of cyanide detoxification are conversion to thiocyanate in the presence of a sulfur donor (Ansell and Lewis, 1970; Baskin et al., 2004) and production of 2-amino-2-thiazoline-4-carboxylic acid (ATCA) from reaction with cystine (Ansell and

Abbreviations:  $\alpha$ -KgCN,  $\alpha$ -ketoglutarate cyanohydrin;  $\alpha$ -Kg,  $\alpha$ -ketoglutarate.

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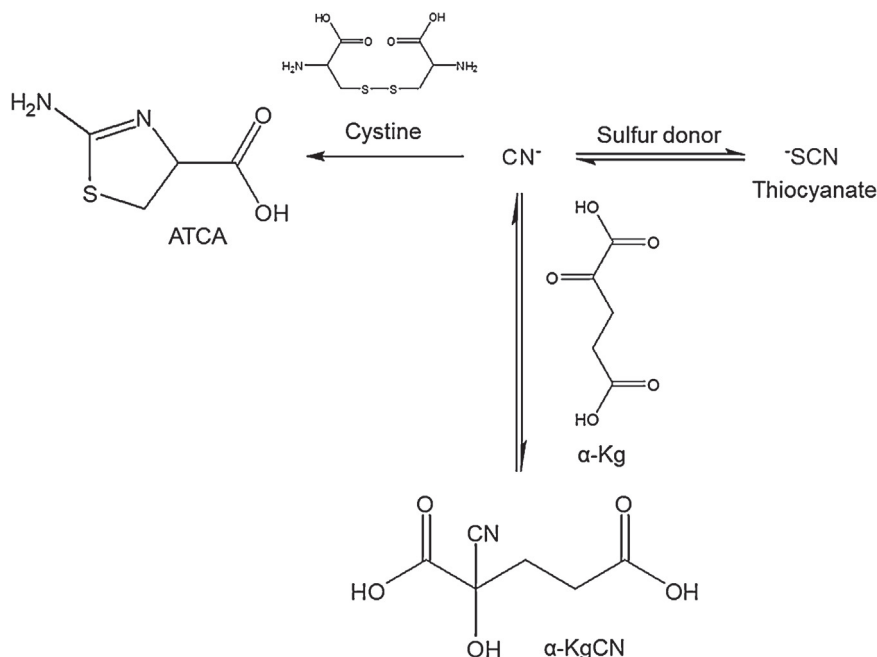


Fig. 1. Cyanide metabolism and detoxification pathways.

Lewis, 1970; Nagasawa et al., 2004). As an alternative detoxification pathway, cyanide can react with endogenous  $\alpha$ -ketoglutarate ( $\alpha$ -Kg) to form  $\alpha$ -ketoglutarate cyanohydrin ( $\alpha$ -KgCN) (Baskin et al., 2004; Baskin and Brewer, 1997) in animals. This detoxification pathway is likely important when the thiocyanate and ATCA pathways are overwhelmed, and will be investigated in this study.

Evaluation of the toxicokinetic behavior of cyanide and its breakdown products provides insight into the best marker for verification of cyanide exposure. Such studies have been conducted for cyanide (Dirikolu et al., 2003; Leuschner et al., 1991; Sousa et al., 2003) and its major detoxification products, thiocyanate (Leuschner et al., 1991; Sousa et al., 2003) and ATCA (Petrikovics et al., 2012), in various animal models. The results of these studies are presented in Table 1. Leuschner et al. (1991) investigated the toxicokinetics of cyanide in rats following acute potassium cyanide exposure by gavage at 1.0 mg KCN/kg body weight. The time of peak concentration ( $T_{max}$ ), 2 min, suggests that cyanide is rapidly distributed with this mode of exposure. Leuschner et al. (1991) also performed a chronic cyanide exposure study over a 13-week period. In that study, the blood cyanide concentrations ranged from 16.0 to 25.5  $\mu$ M and the thiocyanate plasma concentrations ranged from 341 to 877  $\mu$ M for rats given KCN at 160 mg/kg body weight per day in drinking water. The results of the 13-week study suggested that

chronic cyanide exposure at the dose used does not lead to saturation of cyanide detoxification pathways (Leuschner et al., 1991). Sousa et al. (2003) evaluated the toxicokinetics of blood cyanide and plasma thiocyanate in rats and pigs following oral potassium cyanide exposure at 3.0 mg KCN/kg body weight; over a 24 h period, blood cyanide concentrations ranged from 0.5 to 89.0  $\mu$ M and 1.0 to 57.5  $\mu$ M, and thiocyanate plasma concentrations ranged from 19.0 to 58.1  $\mu$ M and 18.0 to 42.8  $\mu$ M, in rats and pigs respectively. The results of this study suggest that about 65–75% of absorbed cyanide is converted to thiocyanate, which is in close agreement with the 80% predicted by Ansell and Lewis (1970). Petrikovics et al. (2012) studied the toxicokinetics of ATCA in rats following intravenous (IV) injection of ATCA at 100 mg/kg body weight. Although this study did not address the in vivo generation of ATCA from cyanide exposure, it is one of the first studies to address the distribution and elimination of ATCA. The plasma ATCA ranged from 0.96 to 18.5  $\mu$ M, and showed a consistent 5-fold increase over endogenous concentrations between 2.5 and 48 h post-exposure (Petrikovics et al., 2012). These findings suggest that the use of ATCA as a biomarker is promising, but further evaluation of the toxicokinetics of ATCA following cyanide exposure should be undertaken.

Recently, Mitchell et al. (2013) established an analytical method to quantify the cyanide detoxification product,  $\alpha$ -KgCN, but a toxicokinetic profile of  $\alpha$ -KgCN following cyanide exposure has not been performed. Knowledge of  $\alpha$ -KgCN's toxicokinetic profile will provide a better understanding of cyanide's absorption and elimination by this alternative pathway and might show that  $\alpha$ -KgCN has advantages over other markers of cyanide exposure for verification of cyanide exposure. Therefore, we completed a toxicokinetic analysis of  $\alpha$ -KgCN in potassium cyanide-exposed swine and compared it with data for cyanide and its other detoxification products. We also studied the behavior of cyanide and its detoxification products during administration of cobinamide, a next-generation treatment for cyanide exposure (Brenner et al., 2010a,b; Broderick et al., 2006; Chan et al., 2010, 2011; Zou et al., 2012). Furthermore,  $\alpha$ -Kg has been suggested as a cyanide antidote (Bhattacharya et al., 2002; Bhattacharya and Vijayaraghavan, 1991, 2002; Hume et al., 1995; Mathangi et al., 2011; Norris et al., 1990; Tulsawani et al., 2005), and the results of this study may be important for  $\alpha$ -Kg therapeutic studies.

Table 1

Toxicokinetic parameters for cyanide, thiocyanate, and ATCA in rats and swine.  $C_{max}$ ,  $T_{max}$ , and  $t_{1/2}$  are designated as the peak blood or plasma concentration, peak time, and elimination half-life, respectively.

Species	Analyte <sup>a</sup>	$C_{max}$ ( $\mu$ M)	$T_{max}$ (min)	$t_{1/2}$ (min)
Rats	Cyanide	6.2 <sup>b</sup> , 89.0 <sup>c</sup>	2 <sup>b</sup> , 15 <sup>c</sup>	14 <sup>b</sup> , 38 <sup>c</sup>
	Thiocyanate	58.1 <sup>c</sup>	360 <sup>c</sup>	348 <sup>c</sup>
	ATCA	18.5 <sup>d</sup>	120 <sup>d</sup>	150 <sup>d</sup>
Swine	Cyanide	57.5 <sup>c</sup>	30 <sup>c</sup>	32 <sup>c</sup>
	Thiocyanate	42.8 <sup>c</sup>	360 <sup>c</sup>	297 <sup>c</sup>

<sup>a</sup> Cyanide was analyzed from whole blood, and thiocyanate and ATCA were analyzed from plasma.

<sup>b</sup> Leuschner et al. (1991).

<sup>c</sup> Sousa et al. (2003).

<sup>d</sup> Petrikovics et al. (2012).

## 2. Experimental

### 2.1. Reagents and materials

All reagents and materials were at least HPLC grade.  $\alpha$ -K<sub>g</sub>CN and  $\alpha$ -K<sub>g</sub>CN-d<sub>4</sub> were synthesized as previously reported (Mitchell et al., 2013). Labeled thiocyanate (Na<sup>13</sup>C<sup>15</sup>N) and cyanide (Na<sup>13</sup>C<sup>15</sup>N) were acquired from Isotech (Miamisburg, OH). Labeled ATCA-d<sub>2</sub> was synthesized in the lab of Dr. Nagasawa at the Department of Veterans Affairs Medical Center (Minneapolis, MN). Aquohydroxocobinamide was synthesized as described previously, and converted to a dinitro derivative by adding two molar equivalents of sodium nitrite (Chan et al., 2010, 2011). Sodium cyanide, sodium tetraborate decahydrate, sodium hydroxide, and Millex®-GV syringe filters (0.22  $\mu$ M) were purchased from Fisher Scientific (Fair Lawn, NJ). Sodium thiocyanate was obtained from Acros Organics (Morris Plains, NJ). Formic acid (LC/MS grade) and pentafluorobenzyl bromide (PFB-Br) were obtained from Thermo Scientific (Rockford, IL). Tetrabutylammonium sulfate (TBAS) was purchased from Sigma–Aldrich (St. Louis, MO). ATCA was obtained from Chem-Impex International (Wood Dale, IL). Oasis mixed-mode cationic exchange (MCX) columns were acquired from Waters Corporation (Milford, MA). *N*-methyl-*N*-trimethylsilyl-trifluoroacetamide (MSTFA) was acquired from Pierce Chemical Company (Rockford, IL).

### 2.2. Animal studies

The animal studies were conducted at Wilford Hall Medical Center (Lackland Air Force Base, TX) in accordance with The Guide for the Care and Use of Laboratory Animals, and were approved by the Wilford Hall Clinical Research Division Institutional Animal Care and Use Committee; Wilford Hall is accredited by the American Association for Laboratory Animal Science. A total of 31 Yorkshire pigs (~50 kg) were sedated, intubated, and anesthetized with isoflurane. KCN was injected intravenously at 0.17 mg/kg/min until apnea occurred. At one minute post-apnea, the animals received either saline by IV injection (control group, *N* = 11) or 12.5 mg/kg cobinamide by IV (*N* = 10) or intraosseous (IO) (*N* = 10) injection. Arterial blood was sampled prior to cyanide exposure, 5 min after the start of cyanide infusion, at apnea, and at 2, 4, 6, 8, 10, 20, 30, 40, 50, and 60 min post-apnea. EDTA was added to an aliquot of blood, and the plasma was separated from the red blood cells by centrifugation and shipped on ice to South Dakota State University. Upon receipt, the EDTA-treated plasma was frozen and stored at -80 °C until used.

### 2.3. Preparation and analysis of swine plasma for $\alpha$ -K<sub>g</sub>CN

Plasma was prepared and analyzed for  $\alpha$ -K<sub>g</sub>CN according to a previously established method (Mitchell et al., 2013). Briefly, 1% formic acid in acetonitrile was added to the plasma, and the precipitate was removed by centrifugation. The resulting supernatant was concentrated by drying under N<sub>2</sub>(g) and then reconstituted in aqueous formic acid. The reconstituted sample was analyzed using ultrahigh-performance liquid chromatography tandem mass spectrometry, and  $\alpha$ -K<sub>g</sub>CN was quantified by monitoring the 172.0 to 145.1 *m/z* transition.

### 2.4. Preparation and analysis of swine plasma for cyanide and thiocyanate

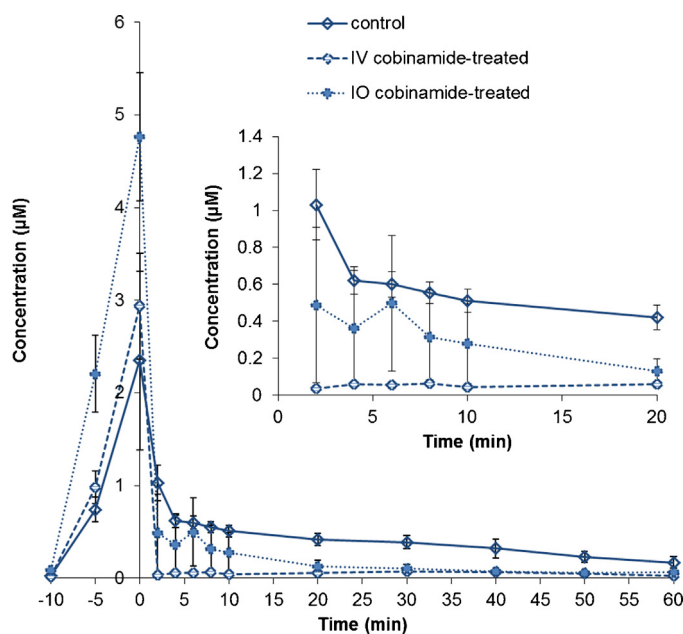
Cyanide and thiocyanate were measured simultaneously according to Bhandari et al. (2012). Briefly, tetrabutyl ammonium sulfate and pentafluorobenzyl bromide (PFB-Br) were added to plasma, followed by vortexing for 2 min, and heating at 70 °C for 1 h. Samples were then centrifuged at 9300  $\times$  g for 4 min, and the organic layer was analyzed by chemical ionization gas-chromatography mass-spectrometry (GC–MS) with ions 208 and 240 *m/z* selected for quantification of PFB-CN and PFB-SCN, respectively.

### 2.5. Preparation and analysis of swine plasma for ATCA

Plasma was analyzed for ATCA according to Logue et al. (2005). Briefly, proteins were precipitated from the plasma by addition of 1% HCl in acetone (v/v). The supernatant was diluted with 0.1 M HCl and applied to a mixed-mode cation exchange solid phase extraction column. After washing the column, ATCA was eluted using NH<sub>4</sub>OH:CH<sub>3</sub>OH:H<sub>2</sub>O (25:50:25) in 0.1 M HCl, and the samples were dried at 40 °C. MSTFA in hexane (30% v/v) was added to the dried samples, and they were heated at 50 °C for 1 h to chemically modify ATCA to ATCA-(TMS)<sub>3</sub> for GC–MS analysis with ion 362 *m/z* used for quantification.

### 2.6. Toxicokinetic and data analysis

Toxicokinetic parameters were determined according to methods described by the World Health Organization (1986) and Shargel et al. (2005). Analysis of  $\alpha$ -K<sub>g</sub>CN was completed with a one-compartment model, with *C*<sub>max</sub>, *T*<sub>max</sub>, *t*<sub>1/2</sub> and elimination constants (*K*<sub>e</sub>) obtained from the concentration-time curves. Area under the curve ([AUC]) after apnea was also determined from the concentration-time curve using the trapezoidal rule (Shargel et al., 2005). *C*<sub>max</sub>/*C*<sub>baseline</sub> was determined by dividing the maximum plasma concentration by the baseline concentration. The  $\alpha$ -K<sub>g</sub>CN data for the cobinamide and control animals were analyzed with a one-way analysis of variance and Bartlett's test for equal variances, which showed a



**Fig. 2.** Toxicokinetic profile of  $\alpha$ -K<sub>g</sub>CN in control, IV cobinamide-treated, and IO cobinamide-treated swine. Apnea, pre-exposure and 5 min infusion sample points are designated as “time 0, -10, and -5”, respectively. The plasma sampled at time zero was drawn prior to treatment, the -10 time point was obtained before infusion and the -5 time point was collected 5 min after exposure. Error bars denote standard error of the mean (SEM). Inset: “zoomed” representation of the plasma concentrations from 2 to 20 min post-apnea.

significance difference among the three groups. Therefore, two-tailed unpaired *t*-tests with Welch's correction were applied to each time point to evaluate statistical differences between the groups.

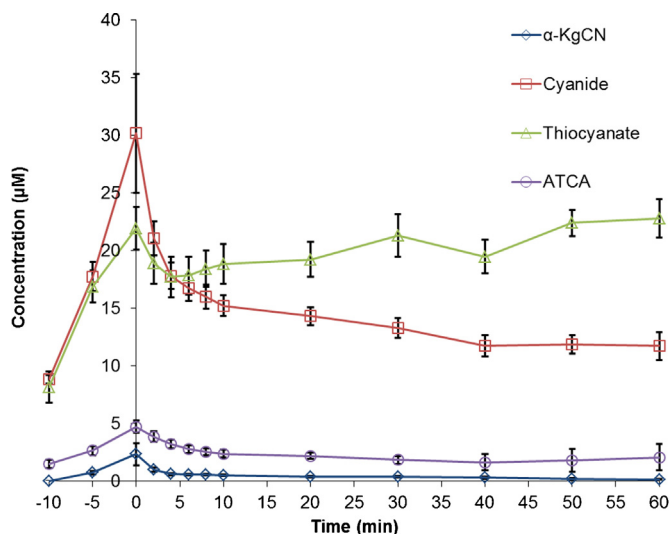
## 3. Results

### 3.1. Behavior of $\alpha$ -K<sub>g</sub>CN after cyanide exposure

The plasma  $\alpha$ -K<sub>g</sub>CN concentration similarly increased in all three experimental groups during cyanide infusion, but decreased with different kinetics after cyanide was stopped (at the onset of apnea) and cobinamide was injected (Fig. 2). In the control saline-treated group (solid line), the  $\alpha$ -K<sub>g</sub>CN concentration showed a typical exponential decrease for the duration of the experiment. In the group treated with IV cobinamide (dashed line),  $\alpha$ -K<sub>g</sub>CN concentrations showed a more rapid decrease compared to the control group. In the group treated with IO cobinamide (dotted line), the  $\alpha$ -K<sub>g</sub>CN concentrations fell at a similar rate to the IV-treated group, but the concentrations did not fall quite as low and were still well above baseline up to 10 min post-apnea. Significant differences between the control and IV cobinamide-treated groups were observed at all points except -5 and 0 min. Significant differences between control and IO cobinamide-treated groups were observed at -10, -5, 20, 30, and 50 min. In contrast, significant differences between the IV and IO cobinamide-treated groups were only found pre-apnea.

### 3.2. Comparison of the toxicokinetic profile of $\alpha$ -K<sub>g</sub>CN, cyanide, thiocyanate, and ATCA

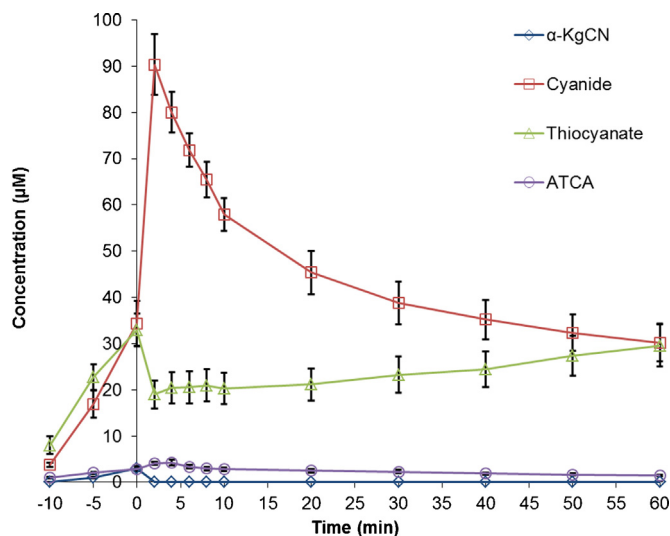
The toxicokinetic profile of  $\alpha$ -K<sub>g</sub>CN, ATCA, and cyanide in control animals were generally similar, with the exception that plasma cyanide concentrations were considerably higher compared to ATCA and  $\alpha$ -K<sub>g</sub>CN (Fig. 3). Also to be noted is that plasma ATCA did not decrease as rapidly as  $\alpha$ -K<sub>g</sub>CN, likely because ATCA



**Fig. 3.** Toxicokinetic profile of  $\alpha$ -KgCN, cyanide, thiocyanate, and ATCA in control swine. Apnea, pre-exposure and 5 min infusion sample points are designated as “time 0, –10, and –5”, respectively. The plasma sampled at time zero was drawn prior to treatment, the –10 time point was obtained before infusion and the –5 time point was collected 5 min after exposure. Error bars denote SEM.

formation is not an equilibrium reaction, as is production of  $\alpha$ -KgCN. Thiocyanate behaved quite differently compared to the other cyanide exposure markers, decreasing directly after apnea (2 and 4 min) and then rising gradually for the duration of the experiment (Fig. 3).

In the animals treated with IV cobinamide, cyanide, thiocyanate, ATCA and  $\alpha$ -KgCN showed the typical increase in concentration prior to apnea as cyanide was being absorbed and distributed (Fig. 4). However, cyanide concentrations increased sharply at 2 min post-apnea and then decreased. ATCA concentrations increased until 4 min post-apnea, before starting to decrease. Thiocyanate and  $\alpha$ -KgCN concentrations both decreased immediately following apnea, but thiocyanate then gradually increased starting at 2 min post-apnea.



**Fig. 4.** Toxicokinetic profile of  $\alpha$ -KgCN, cyanide, thiocyanate, and ATCA in IV cobinamide-treated swine. Apnea, pre-exposure and 5 min infusion sample points are designated as “time 0, –10, and –5”, respectively. The plasma sampled at time zero was drawn prior to treatment, the –10 time point was obtained before infusion and the –5 time point was collected 5 min after exposure. Error bars denote SEM.

**Table 2**

Toxicokinetic parameters for  $\alpha$ -KgCN, cyanide, and ATCA in control animals following IV-infusion of KCN (0.17 mg/kg/min) until apnea.

Analyte	$C_{\max}$ ( $\mu$ M)	$t_{1/2}$ (min)	$K_e$	[AUC] ( $\mu$ M min)	$C_{\max}/C_{\text{baseline}}$
$\alpha$ -KgCN	2.35	15	0.0462	25.6	102.2
Cyanide <sup>a</sup>	30.18	27	0.0258	474.4	3.1
ATCA <sup>a</sup>	4.73	14	0.0499	75.4	3.4

<sup>a</sup> The toxicokinetic data for cyanide and ATCA in swine plasma will be reported by Bhandari et al.

### 3.3. Toxicokinetics of $\alpha$ -KgCN, cyanide, and ATCA

Toxicokinetic parameters for  $\alpha$ -KgCN, cyanide and ATCA in control animals are presented in Table 2; values for thiocyanate could not be determined due to the increasing concentrations observed after apnea. A one-compartment model best represents the toxicokinetic behavior of  $\alpha$ -KgCN post-apnea, similar to Bhandari et al. (Publication pending) for cyanide and ATCA.  $\alpha$ -KgCN, cyanide, and ATCA all exhibited  $T_{\max}$  at apnea (0 min). Among all the markers, cyanide provided the highest  $t_{1/2}$  and  $C_{\max}$  values (although both could not be determined for thiocyanate).  $\alpha$ -KgCN and ATCA produced similar toxicokinetic values.

## 4. Discussion

The increase in plasma  $\alpha$ -KgCN concentrations before apnea, when cyanide is being infused, shows that a portion of the cyanide administered is quickly converted to  $\alpha$ -KgCN. After apnea, when the cyanide infusion is stopped, the metabolism and distribution of cyanide dominates and  $\alpha$ -KgCN concentrations rapidly decrease. Because  $\alpha$ -KgCN formation is an equilibrium reaction (Fig. 1), the rapid decrease in cyanide rapidly consumes  $\alpha$ -KgCN as the equilibrium favors the reactants. The sudden decrease in  $\alpha$ -KgCN levels in the IV and IO cobinamide-treated animals post-apnea was expected considering that cobinamide was administered just after apnea. Cobinamide has a high affinity for two cyanide ions (Brenner et al., 2010b), and, therefore, free cyanide in the plasma is rapidly sequestered after treatment, causing a decrease in free cyanide, which leads to the consumption of  $\alpha$ -KgCN as the equilibrium shifts toward the production of  $\alpha$ -Kg and cyanide (Fig. 1).

Comparing the cobinamide-treated groups to the control animals, the main difference occurs immediately following apnea, when plasma cyanide sharply increases and  $\alpha$ -KgCN sharply decreases. The increase in cyanide and decrease of  $\alpha$ -KgCN in the treated animals is likely the result of rapid cyanide extraction from the red blood cells into the plasma through cobinamide sequestration of cyanide (Nath et al., 2013). This phenomenon would result in less free cyanide in the plasma even though the total (free and sequestered) cyanide concentration increases. The sequestration of cyanide causes a sudden decrease in  $\alpha$ -KgCN concentrations. ATCA also showed an increase in concentration until about 4 min post-apnea, which could be explained by conversion of small amounts of free cyanide released by dicyano cobinamide or aquocyanocobinamide (Blackledge et al., 2010).

Thiocyanate also showed interesting behavior in the control and cobinamide-treated animals. The increase in thiocyanate concentrations, as cyanide is infused into the animal, is expected because of the large fraction of cyanide converted to thiocyanate as the major detoxification pathway of cyanide (Ansell and Lewis, 1970; Baskin et al., 2004; Sousa et al., 2003). After the infusion is stopped, a sudden decrease in the thiocyanate concentration occurs, because less free cyanide is available and the combination of thiocyanate distribution and elimination is more rapid than the conversion of cyanide to thiocyanate. Over time, the rate of conversion of cyanide to thiocyanate increases as rhodanese's activity increases

(rhodanese is the enzyme mainly responsible for enzymatic conversion of cyanide to thiocyanate) (Wrobel and Frendo, 1992; Wrobel et al., 2004). After 2–4 min, thiocyanate elimination is not fast enough to match the rate of conversion of cyanide to thiocyanate, causing a buildup of thiocyanate in the plasma (Wrobel and Frendo, 1992; Wrobel et al., 2004). Chan et al. (2010) also observed an increase in plasma thiocyanate concentrations as cyanide was released from red blood cells and converted to thiocyanate.

We found a statistical difference between the  $\alpha$ -K<sub>g</sub>CN concentrations of the control and cobinamide-treated animals, suggesting that  $\alpha$ -K<sub>g</sub>CN is eliminated from the plasma at a faster rate when cobinamide is administered. Animals receiving IV cobinamide showed the fastest elimination of  $\alpha$ -K<sub>g</sub>CN from the plasma, but it was certainly comparable to that in animals receiving IO cobinamide, suggesting the two routes of administration allowed similar distribution profiles for cobinamide. Previous studies conducted in Gottingen minipigs have shown that IO- and IV-administration of the cyanide antidote, hydroxocobalamin, to non-cyanide-poisoned animals produce similar distribution profiles (Murray et al., 2012). Significant differences were also seen pre-apnea in all groups, which can be explained due to interanimal variability.

Comparison of the toxicokinetic parameters of  $\alpha$ -K<sub>g</sub>CN to those of cyanide and ATCA (Table 2), shows that  $\alpha$ -K<sub>g</sub>CN behaves similarly to ATCA, although ATCA had the largest  $K_e$  value, suggesting it is eliminated faster from the plasma than cyanide or  $\alpha$ -K<sub>g</sub>CN. Comparison of the [AUC] values, establishes that  $\alpha$ -K<sub>g</sub>CN had the lowest overall plasma concentrations throughout the study, supported by its fast rate of elimination, low  $C_{max}$  concentrations, and low baseline concentrations. We will present a more detailed description of the toxicokinetic behavior of cyanide, thiocyanate and ATCA in a future publication.

Plasma concentrations of  $\alpha$ -K<sub>g</sub>CN were relatively low in all animals compared to cyanide and thiocyanate because a relatively low amount of cyanide was detoxified by the  $\alpha$ -K<sub>g</sub>CN pathway. It has been suggested that about 80% of cyanide is converted to thiocyanate in the presence of a sulfur donor (Ansell and Lewis, 1970; Baskin et al., 2004; Sousa et al., 2003) and another 15–20% of cyanide is metabolized by L-cystine to produce ATCA (Ansell and Lewis, 1970). This would suggest that only a small percentage of cyanide is converted to other detoxification products in non-treated (control) animals, including cyanocobalamin (Astier and Baud, 1995; Butte et al., 1982; Chatzimichalakis et al., 2004) and cyanide-protein adducts (Fasco et al., 2007; Youso et al., 2010, 2012), which is consistent with the low plasma concentrations of  $\alpha$ -K<sub>g</sub>CN. Based on the measured  $\alpha$ -K<sub>g</sub>CN concentrations and detoxification of cyanide by the thiocyanate and ATCA pathways, we estimate that about 0.1–1.7% of the cyanide dose was converted to  $\alpha$ -K<sub>g</sub>CN. This estimation was done by dividing the maximum concentrations of  $\alpha$ -K<sub>g</sub>CN by the total maximum concentrations of cyanide, thiocyanate, ATCA and  $\alpha$ -K<sub>g</sub>CN of cobinamide-treated and control animals after factoring in the distribution of cyanide between red blood cells and plasma (70–96% of blood cyanide resides in the red blood cells (Baar, 1966; Lundquist et al., 1985). The percentage of cyanide in plasma increases as the cyanide dose increases, because the red blood cells become saturated with cyanide (Lundquist et al., 1985). Further studies (i.e., radioisotope experiments) would have to be undertaken to accurately calculate how much cyanide participates in the  $\alpha$ -K<sub>g</sub>CN pathway.

This study suggests that use of  $\alpha$ -K<sub>g</sub>CN as a biomarker for cyanide exposure would be most applicable in instances of acute, high-dose cyanide poisoning soon after exposure. The major advantage of using  $\alpha$ -K<sub>g</sub>CN as a marker for cyanide exposure is the low, if not undetectable, levels of endogenous  $\alpha$ -K<sub>g</sub>CN in the plasma, making cyanide exposure easy to detect from elevated

$\alpha$ -K<sub>g</sub>CN concentrations. Comparing the maximum cyanide, thiocyanate, ATCA and  $\alpha$ -K<sub>g</sub>CN plasma concentrations to their endogenous (baseline) concentrations, shows that  $\alpha$ -K<sub>g</sub>CN has a much higher  $C_{max}/C_{baseline}$ , suggesting that measuring plasma  $\alpha$ -K<sub>g</sub>CN can provide a definitive confirmation of cyanide exposure. Although there are several potential advantages of  $\alpha$ -K<sub>g</sub>CN as a cyanide exposure marker, its rapid elimination, especially in the presence of cobinamide, may limit its use.

To our knowledge, this work provides the first reported toxicokinetic profile of  $\alpha$ -K<sub>g</sub>CN in any animal. The ability to measure  $\alpha$ -K<sub>g</sub>CN in plasma would be beneficial in studies using  $\alpha$ -K<sub>g</sub> as a cyanide antidote (Bhattacharya et al., 2002; Bhattacharya and Vijayaraghavan, 1991, 2002; Hume et al., 1995; Mathangi et al., 2011; Tulsawani et al., 2005). The equilibrium constant for the reaction of  $\alpha$ -K<sub>g</sub>CN ( $K_{f,\alpha-KgCN}$ ) was estimated by assuming the reaction was at equilibrium at apnea. The  $\alpha$ -K<sub>g</sub>CN concentration (2.35  $\mu$ M) was divided by the remaining cyanide concentration (i.e., 30.18  $\mu$ M – 2.35  $\mu$ M = 27.83  $\mu$ M) and the remaining endogenous  $\alpha$ -K<sub>g</sub> concentration (i.e., 23.95  $\mu$ M – 2.35  $\mu$ M = 21.60  $\mu$ M (Dabek et al., 2005)). Based on the calculated equilibrium constant ( $K_{f,\alpha-KgCN} = 3.9 \times 10^{-3}$ ), the conversion of  $\alpha$ -K<sub>g</sub> into  $\alpha$ -K<sub>g</sub>CN is not favorable. Therefore, the use of  $\alpha$ -K<sub>g</sub> as a therapeutic may not be very effective, but further studies would have to be undertaken to determine  $\alpha$ -K<sub>g</sub>CN's efficacy in minimizing the lethality of cyanide following exposure.

Future work should address the absorption, distribution, and elimination of  $\alpha$ -K<sub>g</sub>CN in other animals to determine the most appropriate animal model for evaluating the behavior of  $\alpha$ -K<sub>g</sub>CN in humans following cyanide exposure. Rigorously determining the  $K_{f,\alpha-KgCN}$ , and the amount of cyanide that participates in the  $\alpha$ -K<sub>g</sub>CN pathway, would produce a clear picture of the role of  $\alpha$ -K<sub>g</sub> in cyanide detoxification, both naturally and as a therapeutic.

### Conflict of interest statement

The authors declare no conflict of interest.

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