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Quantification of α-ketoglutarate cyanohydrin in swine plasma by ultra-high performance liquid chromatography tandem mass spectrometry

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ABSTRACT

Determination of exposure to cyanide can be accomplished by direct cyanide analysis or indirectly by analysis of cyanide detoxification products, such as thiocyanate and 2-amino-2-thiazoline-4-carboxylic acid. A potentially important marker and detoxification product of cyanide exposure, α-ketoglutarate cyanohydrin (α-KgCN), is produced by the reaction of cyanide and α-ketoglutarate. Therefore, an ultra high-performance liquid chromatography tandem mass spectrometry method to determine α-KgCN in plasma was developed. Swine plasma was spiked with α-KgCN and α-KgCN-d_4 (internal standard) and proteins were precipitated with 1% formic acid in acetonitrile. After centrifugation, the supernatant was dried, reconstituted, separated by reversed phase high performance liquid chromatography and analyzed by tandem mass spectrometry. The method produced a dynamic range of 0.3–50 μM and a detection limit of 200 nM for α-KgCN. Furthermore, the method produced a %RSD of less than 13% for all intra- and inter-assay analyses. The stability of α-KgCN was poor for most storage conditions tested, except for −80 °C, which produced stable concentrations of α-KgCN for the 30 days tested. The validated method was tested by analysis of α-KgCN in the plasma of cyanide-exposed swine. α-KgCN was not detected pre-exposure, but was detected in all post-exposure plasma samples tested. To our knowledge, this method is the first reported analytical method for detecting α-KgCN in any matrix.

1. Introduction

Cyanide exposure can occur in a variety of ways, including accidental, suicidal, or homicidal. General population exposure can occur through smoke inhalation from cigarettes or fires, consuming cyanogenic glycosides found in foods, and working in industrial facilities that use cyanide [1]. Furthermore, cyanide exposure can also occur by the use of cyanide as a chemical warfare agent [2]. Once in the body, the toxicity of cyanide stems from its ability to inhibit cytochrome c oxidase, thereby disrupting oxygen transport to mitochondria [3]. Therefore, the confirmation of cyanide exposure is important to administer treatment in a timely fashion and monitor health conditions after exposure. The direct analysis of cyanide to confirm exposure has serious limitations, due to cyanide's volatility, reactivity, and short half-life in biological fluids [4–6]. Cyanide exists as both hydrogen cyanide (HCN) and the cyanide ion (CN^-) which are in rapid equilibrium with each other. Under normal biological conditions, cyanide exists mainly as HCN, which is extremely volatile and rapidly eliminated from biological matrices [4]. If cyanide exists as CN^-, it is nucleophilic and will rapidly react with various species in biological matrices, thereby eliminating free cyanide from the sample [7]. These limitations have led to the exploration of biomarker analysis for indirect determination of cyanide exposure.

Indirect analysis of cyanide has mainly focused around thiocyanate and 2-amino-2-thiazoline-4-carboxylic acid (ATCA), the major products of cyanide detoxification. Thiocyanate has shown promise as a marker of cyanide exposure and various methods exist for the analysis of thiocyanate in biological fluids [6]. However, disadvantages of thiocyanate analysis for cyanide exposure have been reported. Ballantyne reported that thiocyanate concentrations fluctuated during various sample storage conditions and recovery of thiocyanate from whole blood was low [8]. Furthermore, thiocyanate can be formed from metabolism of other compounds besides cyanide [9]. ATCA has also shown promise as an alternative marker of cyanide exposure and a few methods have been developed for the analysis of ATCA in biological fluids [7,10–12]. Although there is limited information on its relevance as a biomarker for cyanide exposure, Petrikovics et al. suggested that

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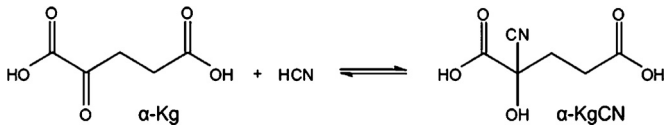

Fig. 1. Proposed reaction pathway for the conversion of α-Kg into α-KgCN.

plasma ATCA might not be a good biomarker for cyanide exposure based on a toxicokinetic study in rats [13]. Conversely, ATCA has been suggested as an advantageous marker of long-term low-level cyanide exposure [7,10–12]. Other markers of cyanide exposure include cyanide-protein adducts [3,14,15] and cyanocobalamin [16–18]. Cyanide-protein adducts may serve as excellent long-term markers of cyanide exposure, but the utility of this marker for rapid analysis has not been assessed, and the synthesis of the standards for this technique can be costly and demanding [19]. Although cyanocobalamin is a potential marker, hydroxocobalamin, which sequesters cyanide to form cyanocobalamin, is a treatment for cyanide exposure [20–22]. Therefore, the use of hydroxocobalamin as a treatment would convolute the use of cyanocobalamin as a biomarker. Furthermore, detection of cyanocobalamin for cyanide exposure is limited due to photodegradation [23,24]. Considering the limitations concerning current biomarkers of cyanide exposure, novel markers should be considered.

Cyanide is known to react with carbonyl compounds to form cyanohydrins [25]. In biological systems, cyanide is converted to α-ketoglutarate cyanohydrin (α-KgCN) through an equilibrium reaction with α-ketoglutarate (α-Kg) (Fig. 1) [6]. Because α-Kg resides in the plasma [26], the ability to determine concentrations of α-KgCN may allow for verification of cyanide exposure. Therefore, the objective of this project was to develop an analytical method to determine cyanide exposure by detection of α-KgCN in plasma. Because oral dosing of α-Kg has been shown to mitigate the toxicity of cyanide exposure [25,27–30], this method should also be beneficial in aiding studies of α-Kg as a therapeutic treatment for CN^- poisoning.

2. Experimental

2.1. Reagents and materials

All reagents were at least HPLC grade. Sodium cyanide (NaCN) and all solvents were purchased from Fisher Scientific (Fair Lawn, NJ, USA). α-Kg and α-ketoglutaric acid-d_6 (α-Kg-d_6) were purchased from Sigma–Aldrich (St. Louis, MO, USA). LC/MS grade formic acid was purchased from Thermo Scientific (Rockford, IL, USA). Swine (*Sus scrofa*) plasma (non-sterile with sodium citrate anti-coagulant) was acquired from the Veterinary Science Department at South Dakota State University. Cyanide-exposed swine plasma was received from Wilford Hall Medical Center (Lackland Air Force Base, TX). One animal (about 50 kg) was sedated, endotracheally intubated, and maintained under anesthesia with inhaled isoflurane. After acclimation, KCN (4 mg/mL) was infused intravenously (0.17 mg/kg/min) until apnea. Arterial blood was sampled at baseline, 5 min into cyanide infusion, at apnea, every 2 min after apnea for 10 min, and then every 10 min for 60 min. Whole blood (20 mL) was withdrawn from the animal at each time point. The blood (4 mL) was then placed into EDTA tubes and centrifuged to separate the plasma from the red blood cells, resulting in a final plasma volume of 1 mL for each sample. The plasma was shipped overnight on dry ice to South Dakota State University, where it was immediately frozen upon arrival and stored at −80 °C until needed.

2.2. Synthesis of α-KgCN and α-KgCN-d_4

α-KgCN was synthesized according to an adapted procedure of Green and Williamson [31], by first adding α-Kg and an equimolar amount of NaCN to water and stirring at room temperature for 30 min. The resulting solution was filtered and the solvent was removed by rotary evaporation to afford a white, sticky product. Characterization was achieved by ^{13}C NMR, along with ESI-MS operated in negative polarity mode. ^{13}C NMR (CD_3OD, 400 MHz): δ 178, 120, 70. ESI(−)-MS: m/z 172.0, 144.7, 101.0, 44.8. An isotopically-labeled internal standard, α-KgCN-d_4 was synthesized and characterized as described above for α-KgCN, with α-Kg-d_6 replacing α-Kg. ^{13}C NMR (CD_3OD, 400 MHz): δ 178, 172, 122, 73. ESI(−)-MS: m/z 176.1, 149.1, 105.1, 44.8. The characterization of α-KgCN and α-KgCN-d_4 by ^{13}C NMR did not show the presence of α-Kg. Furthermore, Green and Williamson [31] reported that no free acid (α-Kg) was evident in their final product.

2.3. Sample preparation

Swine plasma (100 μL) was spiked with internal standard (IS), α-KgCN-d_4 (20 μL of 100 μM), and 1% formic acid in acetonitrile (400 μL) to initiate protein precipitation. The resulting solution (pH of approximately 2), was then vortexed for 5 min and centrifuged for 15 min at 16,000×g (13,100 rpm, room temperature). After centrifugation, an aliquot of the supernatant was transferred to a 4 mL screw-top vial and dried under N_2(g) for 20 min at room temperature (Reacti-vap III, Pierce, Rockford, IL, USA). The volume of supernatant transferred was evaluated at 100, 300, and 400 μL, with 300 μL producing the optimum LC conditions in terms of peak symmetry and band broadening. After drying, the sample was reconstituted with formic acid (10 μL of 10 M) and water (300 μL) and was syringe-filtered (Teflon, 0.22 μm) to remove particulates. The volume of water for reconstitution was optimized at 300 μL, based on peak symmetry and band broadening. After filtration, an aliquot (100 μL) of the filtrate was transferred to a glass insert (150 μL) which was placed in a screw-top autosampler vial (2 mL) and analyzed by ultra-high performance liquid chromatography tandem mass spectrometry (UHPLC–MS–MS).

2.4. Analysis of α-KgCN

Analysis of α-KgCN was conducted by UHPLC–MS–MS on a Shimadzu UHPLC (LC-20AD, Shimadzu Corp., Kyoto, JPN) and an AB Sciex Q-trap 5500 MS (Applied Biosystems, Foster City, CA, USA). Samples were separated by reversed-phase (RP) chromatography using a Phenomenex Synergi 2.5 μ Fusion-RP 100 Å column (2.00 × 50 mm) (Phenomenex, Torrance, CA, USA). Mobile phases consisted of 1% formic acid in H_2O (mobile phase A, pH 2.1) and 1% formic acid in MeOH (mobile phase B). An aliquot (10 μL) of the sample was separated by gradient flow at 40 °C with a flow rate of 0.15 mL/min. The concentration of B was increased from 0% to 100% over 2 min, held at 100% for 1 min, and then ramped back down to 0% over 1 min and held constant for 2 min to re-equilibrate the column between samples. Detection of α-KgCN was achieved using electrospray ionization (ESI)–MS–MS operating in negative polarity. N_2 (50 psi) was used as the curtain gas. The ion source was operated at −4500 V and a temperature of 750 °C with a flow rate of 90.0 psi for both the nebulizer (GS1) and heater (GS2) gasses. The collision cell was operated with an entrance potential of −10.0 V and a collision potential of −11.0 V at a medium collision gas (N_2) flow rate. α-KgCN and α-KgCN-d_4 were analyzed in the MS by multiple reaction monitoring (MRM) with the parameters outlined in Table 1.

Table 1
Selected MRM transitions, optimized declustering potentials (DPs) and collision energies (CEs) for the detection of α-KgCN and α-KgCN-d_4 by MS–MS analysis.

Compounds	Q1 Mass (*m*/*z*)	Q3 Mass (*m*/*z*)	Time (ms)	DP (V)	CE (V)
α-KgCN (quantitation)	172.0	145.1	40.0	−26.47	−11.53
α-KgCN (identification)	172.0	57.0	40.0	−32.85	−24.82
α-KgCN-d_4 (quantitation)	176.1	149.0	40.0	−26.92	−13.28
α-KgCN-d_4 (identification)	176.1	61.2	40.0	−31.76	−29.00

2.5. Calibration and quantification

α-KgCN spiked swine plasma calibration standards (0.2, 0.3, 0.5, 1, 3, 5, 10, 30, 50, 100, and 300 μM) were prepared and analyzed to evaluate the linear range. Quality control (QC) standards at low, medium, and high concentrations (0.75, 4, and 20 μM) were used to determine the inter- and intra-assay accuracy and precision. The intra-assay accuracy and precision were determined over 1 day with quintuplicate analysis of each QC standard, and inter-assay accuracy and precision were evaluated over 3 days (within a 9 calendar day period) with quintuplicate analysis of the QC standards each day.

2.6. Stability and recovery

The stability of α-KgCN in swine plasma was assessed at each storage condition using low (0.75 μM) and high (20 μM) QC standards (each in triplicate). Short-term stability experiments were evaluated for the stability of α-KgCN in the autosampler (prepared samples), on the bench-top, and over multiple freeze-thaw cycles. For autosampler stability, QC standards were prepared, placed in an autosampler at 15 °C, and analyzed at 0, 2, 4, 8, 12 and 24 h. For bench-top stability, the QC standards were allowed to stand at room temperature for 0, 2, 4, 8, 12 and 24 h prior to analysis. Freeze-thaw stability was conducted over three cycles after the QC standards were initially analyzed (cycle 0). Each cycle consisted of storage at −80 °C for 24 h, thawing the standards unassisted at room temperature, preparing and analyzing the applicable QC standards, and refreezing the remaining (non-analyzed) standards. This protocol was continued until three cycles had elapsed. Long-term stability experiments were also conducted under various storage conditions (−80, −20 and 4 °C) for various times (0, 1, 2, 5, 12, 20, and 30 days). Low and high QC standards were prepared and stored at the desired temperature until analyzed.

Recovery experiments were conducted in order to determine the ability of the sample preparation protocol to extract α-KgCN from swine plasma. Swine plasma was spiked with α-KgCN at low, medium, and high QC concentrations and compared to aqueous α-KgCN samples at the same nominal concentrations. The recovery was calculated as a percentage by dividing the concentration of the low, medium, and high QC standards in plasma ($n = 5$ for each) against the same concentration of aqueous α-KgCN ($n = 5$ for each).

2.7. Data analysis

Calibration curves were developed by plotting the ratio of the MRM (172.0–145.1 *m*/*z*) peak area for the analyte (α-KgCN) and the MRM (176.1–149.0 *m*/*z*) peak area for the internal standard (α-KgCN-d_4) as a function of the α-KgCN concentration (μM) in plasma. Both weighted ($1/x$ and $1/x^2$) and unweighted calibration curves were prepared by least squares and a weighted ($1/x^2$) linear fit was found to best fit the calibration data as determined by the inspection of residual plots. The limit-of-detection (LOD) was determined at a signal-to-noise ratio of 3 over 3 separate days of analysis ($n = 5$ for each day) with baseline noise calculated as peak-to-peak noise directly adjacent to the α-KgCN peak. Precision was calculated as a percent relative standard deviation (%RSD) by dividing the standard deviation by the mean for each calibrator and QC standard. Accuracy (%) was determined by dividing the calculated concentration by the nominal concentration for each calibrator and QC standard. A %RSD of less than 15% and a percent accuracy of 100 ± 20% were used as criteria for inclusion of calibration standards and determination of the ULOQ and LLOQ. Stability was calculated as a percentage by dividing the concentration of the QC standard (low or high) from each time point (days or hours) by the concentration of the QC standard at time zero (the control). The α-KgCN was considered stable under a particular storage condition if this ratio was ≥85%.

3. Results and discussion

3.1. Analysis of α-KgCN from swine plasma

The mass spectra of α-KgCN and α-KgCN-d_4 produced by ESI(−)-MS are shown in Fig. 2. Fig. 2A shows the mass spectrum of α-KgCN with the ions important for the analysis of α-KgCN identified. The *m*/*z* ratio of 172.0 corresponds to the molecular ion of α-KgCN with the loss of a proton ($[M-H]^-$). The *m*/*z* ratio of 144.7 corresponds to the molecular ion of the precursor, α-Kg, minus a proton. The *m*/*z* ratio of 101.0 corresponds to the loss of a carboxyl group from α-Kg. The *m*/*z* ratio of 44.8 is a common fragment for a carboxyl group. ESI(−)-MS was also conducted on α-KgCN-d_4 and its mass spectrum is shown in Fig. 2B. α-KgCN-d_4 showed similar fragmentation compared with α-KgCN with the exception of a mass difference of +4 *m*/*z* for each major fragment, besides *m*/*z* 44.8, because of the replacement of 4 hydrogen atoms with deuterium atoms in the labeled compound.

Fig. 3 shows representative chromatograms of α-KgCN spiked swine plasma and cyanide-exposed swine plasma (pre- and post-exposure). The α-KgCN elutes at approximately 1.6 min with some degree of tailing, which is most likely caused by the interaction of exposed silica support with α-KgCN. The tailing did not interfere with quantification of α-KgCN. Furthermore, the method shows excellent selectivity for α-KgCN as shown by the absence of co-eluting peaks in the pre-exposure swine plasma chromatogram. In fact, no other peaks are seen in the pre-exposure swine plasma chromatogram.

3.2. Linear dynamic range

Standard curves were generated in the range of 0.2–300 μM α-KgCN in swine plasma. Calibration standards at 0.2, 100, and 300 μM were found to be outside the LLOQ or ULOQ, resulting in a linear dynamic range from 0.3 to 50 μM as described by a weighted ($1/x^2$) curve validated over 3 separate days of analysis (within 9 calendar days). Therefore, the dynamic range of α-KgCN in swine plasma is over 2 orders of magnitude. The LOD was found to be 200 nM α-KgCN in swine plasma validated over a 7-day period with 3 separate days of analysis ($n = 7$ for each day).

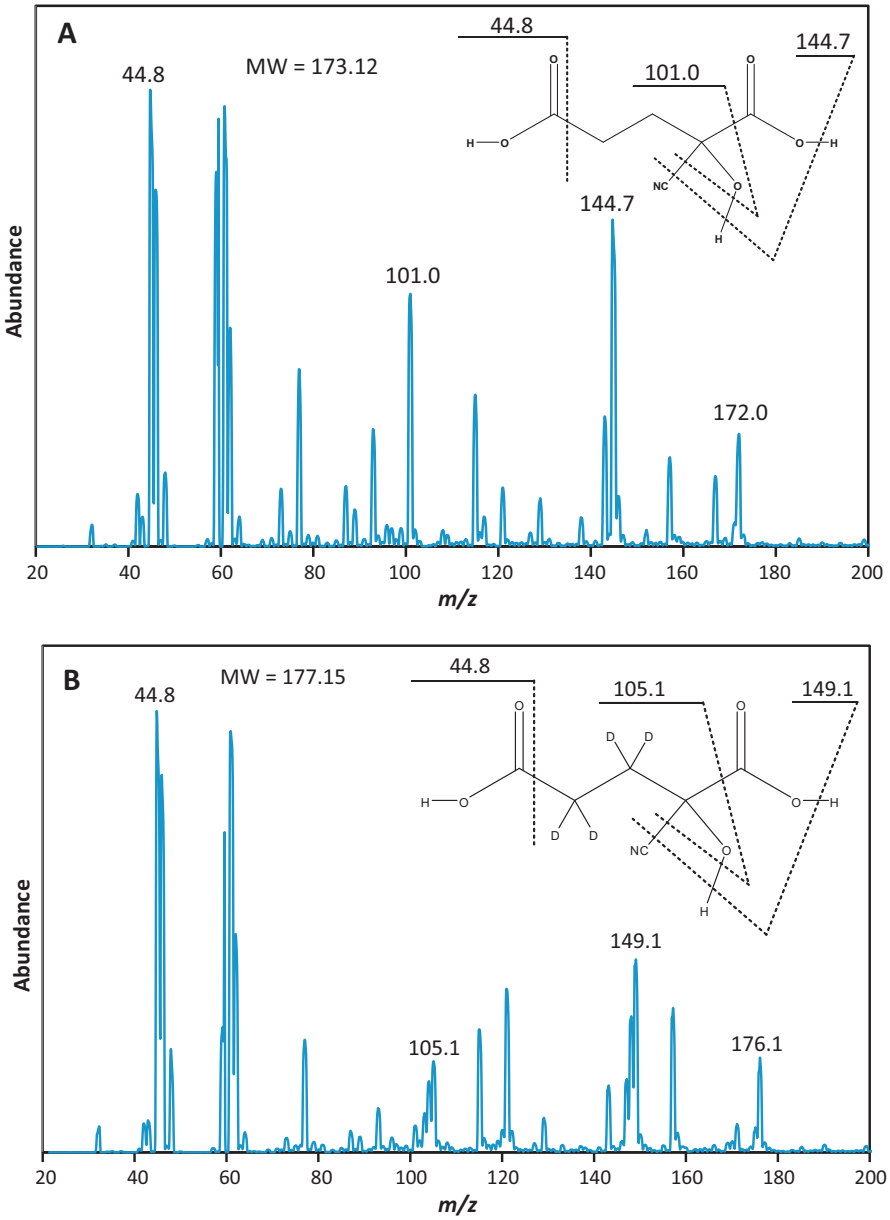

Fig. 2. ESI(−) mass spectra of α-KgCN (A) and α-KgCN-d_4 (B) with identification of the abundant ions. (A) The α-KgCN ion at 172.0 *m/z* corresponds to $[M-H]^-$ and the ion at 144.7 *m/z* corresponds to $[\alpha\text{-Kg-H}]^-$, the precursor to α-KgCN. (B) The α-KgCN-d_4 ion at 176.1 *m/z* corresponds to $[M-H]^-$ and the ion at 149.1 *m/z* corresponds to $[\alpha\text{-Kg-}d_4\text{-H}]^-$. Insets: Structures of α-KgCN (A) and α-KgCN-d_4 (B) with abundant fragments indicated.

3.3. Accuracy and precision

The intra- and inter-assay accuracy and precision were evaluated for low, medium, and high QC standards over 3 days of analysis (Table 2). The method produced good accuracy and precision for the concentrations tested, with intra-assay precision ≤11% and inter-assay precision ≤13% for all QC standards, and accuracy within ±10% of the nominal QC concentration. The accuracy and precision of analytical methods are typically considered acceptable if the %RSD (precision) is less than 15% and the percent accuracy of back-calculated concentrations is between 80% and 120% as compared to the nominal concentration.

3.4. Stability and recovery

The short-term stability of α-KgCN in swine plasma was evaluated on the bench-top and in the autosampler over 24 h. Prepared samples of α-KgCN exhibited excellent stability in the autosampler, with no more than 15% deviation from the control. Furthermore, freeze-thaw experiments showed that α-KgCN was stable in swine plasma at −80 °C over the 3 cycles tested. Conversely, the bench-top stability of α-KgCN was poor with α-KgCN concentrations falling significantly below 85% of the control within 2 h, showing that α-KgCN is quickly eliminated from swine plasma at room temperature.

The long-term stability of α-KgCN was evaluated for 30 days at −80, −20 and 4 °C. The α-KgCN was stable for 30 days at −80 °C, for 1 day at −20 °C, and was quickly eliminated at 4 °C, for both low and high QC concentrations. The results of the long-term study suggest that α-KgCN spiked swine plasma samples should be stored at −80 °C when possible. If samples need to be stored at −20 °C, they should be analyzed as soon as possible. Storage at >−20 °C is not recommended for plasma samples.

Table 2
The accuracy and precision of α-KgCN in swine plasma by UHPLC–MS–MS.

Nominal concentration (μM)	Intra-assay accuracy (%)[a]	Inter-assay accuracy (%)[b]	Intra-assay precision (%RSD)[a]	Inter-assay precision (%RSD)[b]
0.75	100	97	11	8.9
4	110	104	11	13
20	107	104	5.0	8.4

[a] Mean for 1 day of validation ($n = 5$).
[b] Mean for 3 days of validation ($n = 15$).

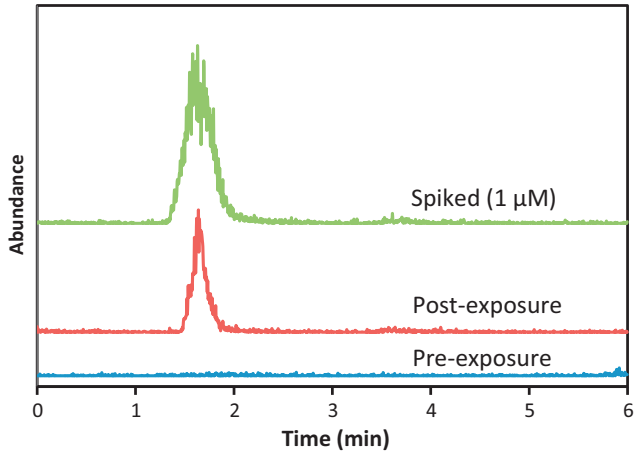

Fig. 3. Chromatograms of α-KgCN spiked swine plasma (upper trace) and the plasma of cyanide-exposed swine, pre-exposure (lower trace), and post-exposure (middle trace). The chromatograms represent the signal response of the MRM transition 172.0→145.1 *m/z*.

The recovery of α-KgCN from swine plasma at low, medium, and high QC concentrations was 14%, 22%, and 27%, respectively. Acidification of the swine plasma before spiking in α-KgCN, did not significantly increase the recovery (25%, 23%, and 30% for low, medium and high QC standards, respectively). Heating the swine plasma to precipitate proteins and cooling back to room temperature before spiking in α-KgCN actually decreased the recovery (7%, 4%, and 4% for low, medium, and high QC standards, respectively). Because enzyme activity should at least be reduced when heating and acidifying the plasma, the consistently low recovery is most likely due to facile equilibrium between α-Kg and α-KgCN. Consequently, cyanide may undergo various side reactions that remove it from the swine plasma such as protein binding [3,14], ATCA formation [7,32], or evaporation of HCN [4,33]. Further experiments will be considered to address these concerns. A potential avenue for future research in this area would be to compare α-KgCN spiked aqueous and plasma samples by mass spectral analysis to elucidate reactions involving α-KgCN.

3.5. *The analysis of cyanide-exposed swine plasma*

The described method was applied to the analysis of α-KgCN in plasma samples obtained from cyanide-exposed swine. Fig. 3 shows representative chromatograms of plasma collected from swine before and after cyanide exposure. The peak for α-KgCN observed around 1.6 min and the absence of co-eluting peaks in the pre-exposed swine sample indicate that the analysis is selective for α-KgCN. Overall, the results indicate that the analytical method presented can be used to quickly and easily analyze α-KgCN in the plasma of cyanide-exposed swine.

3.6. *α-KgCN as a marker of cyanide exposure*

The suggested use of α-KgCN as a cyanide biomarker was confirmed with the observation of α-KgCN in the plasma of cyanide-exposed swine after exposure (Fig. 3). The major advantage of using α-KgCN as a biomarker is that it was not detected endogenously in the plasma. The disadvantages of using α-KgCN as a cyanide marker are poor recovery and limited stability in plasma. Although the stability of α-KgCN was poor under most conditions, it was shown to be stable for at least 30 days at −80 °C, which is significantly better than cyanide and thiocyanate in plasma (found to be stable for 2 and 5 days, respectively [34]) but worse than ATCA (found to be stable for at least 3 months under a variety of storage conditions [7]). Further studies on the toxicokinetic behavior of α-KgCN were undertaken in order to further evaluate its use as a marker of cyanide poisoning [35].

4. Conclusions

An analytical method for the determination of α-KgCN, a potential alternative marker of cyanide exposure, was created and validated. This method shows the ability to detect α-KgCN in swine plasma at low concentrations, as indicated by an LOD of 200 nM. Furthermore, α-KgCN can be quantified accurately and precisely in swine plasma, at sub-μM concentrations. The method allows α-KgCN to serve as a biological marker for cyanide exposure and should aid in studies of therapeutic treatment of cyanide exposure with α-Kg. Future work will include the application of the method to analyze α-KgCN from the plasma of cyanide-exposed swine and investigations pertaining to the low recovery of α-KgCN from swine plasma. To our knowledge, the method developed here is the first reported analytical method for detecting the cyanide detoxification product, α-KgCN, in any matrix.

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contained herein are the private views of the authors and are not to be construed as official or as reflecting the views of the Department of the Army, the National Institutes of Health, the National Science Foundation or the Department of Defense.

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