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# Simultaneous High-performance Liquid Chromatography-tandem Mass Spectrometry (HPLC-MS-MS) Analysis of Cyanide and Thiocyanate from Swine Plasma

Raj K. Bhandari
*South Dakota State University*

Erica Manandhar
*South Dakota State University*

Robert P. Oda
*South Dakota State University*

Gary A. Rockwood
*US Army Medical Research Institute of Chemical Defense*

Brian A. Logue
*South Dakota State University*, brian.logue@sdstate.edu

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RESEARCH PAPER

# Simultaneous high-performance liquid chromatography-tandem mass spectrometry (HPLC-MS-MS) analysis of cyanide and thiocyanate from swine plasma

**Raj K. Bhandari · Erica Manandhar · Robert P. Oda · Gary A. Rockwood · Brian A. Logue**

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**Abstract** An analytical procedure for the simultaneous determination of cyanide and thiocyanate in swine plasma was developed and validated. Cyanide and thiocyanate were simultaneously analyzed by high-performance liquid chromatography tandem mass spectrometry in negative ionization mode after rapid and simple sample preparation. Isotopically labeled internal standards, $Na^{13}C^{15}N$ and $NaS^{13}C^{15}N$, were mixed with swine plasma (spiked and nonspiked), proteins were precipitated with acetone, the samples were centrifuged, and the supernatant was removed and dried. The dried samples were reconstituted in 10 mM ammonium formate. Cyanide was reacted with naphthalene-2, 3-dicarboxaldehyde and taurine to form N-substituted 1-cyano[f]benzoisoindole, while thiocyanate was chemically modified with monobromobimane to form an SCN-bimane product. The method produced dynamic ranges of 0.1–50 and 0.2–50 μM for cyanide and thiocyanate, respectively, with limits of detection of 10 nM for cyanide and 50 nM for thiocyanate. For quality control standards, the precision, as measured by percent relative standard deviation, was below 8 %, and the accuracy was within ±10 % of the nominal concentration. Following validation, the analytical procedure successfully detected cyanide and thiocyanate simultaneously from the plasma of cyanide-exposed swine.

**Keywords** Bioanalysis · Method validation · Chemical warfare agent · Monobromobimane · Naphthalene-2,3-dicarboxaldehyde

R. K. Bhandari · E. Manandhar · R. P. Oda · B. A. Logue (✉)
Department of Chemistry and Biochemistry, South Dakota State University, Avera Health and Science Center 131, Box 2202, Brookings, SD 57007, USA
e-mail: brian.logue@sdstate.edu

G. A. Rockwood
Analytical Toxicology Division, US Army Medical Research Institute of Chemical Defense, 3100 Ricketts Point Road, Aberdeen Proving Ground, MD 21010, USA

## Introduction

The analysis of cyanide (as HCN or $CN^-$, inclusively represented as CN) in biological fluids is of forensic relevance because cyanide is a highly toxic chemical which blocks terminal electron transfer by binding to cytochrome c oxidase, resulting in cyanide-mediated histotoxic anoxia [1–3]. Cyanide is enzymatically metabolized in vivo to thiocyanate ($SCN^-$), in the presence of a sulfur donor (e.g., thiosulfate) [2, 3], as the major metabolic pathway.

Several analytical techniques have been successfully performed for the individual analysis of cyanide and thiocyanate from biological fluids, including spectrophotometry [4–6], gas chromatography–mass spectrometry (GC-MS) [7–9] and liquid chromatography [10–12]. While analysis of CN and $SCN^-$ can be performed separately, considering the large number of samples produced for therapeutic and other studies involving cyanide, there is a need for a rapid, accurate, and reliable method which can simultaneously determine cyanide and thiocyanate. Such an analytical method should simplify analysis and significantly reduce labor costs. Although many

**Table 1** Comparison of some important features of available methods for simultaneous cyanide and thiocyanate analysis from biological fluids

| Study | Analytical technique | LOD (μM) | | Time | | Biofluid(s) |
|---|---|---|---|---|---|---|
| | | CN | $SCN^-$ | Total[a] (h) | Analysis[b] (min) | |
| Imanari et al. [14] | HPLC-UV | 0.2 | 0.2 | 1.0 | 30 | Urine |
| Toida et al. [15] | HPLC-FLD | 0.02 | 0.02 | 7.0 | 24 | RBC/plasma |
| Chinaka et al. [16] | IC-UV-FLD | 0.0038 | 0.086 | 1.5 | 30 | Blood |
| Paul and Smith [17] | GC-MS | 1.0 | 5.0 | 0.9 | 6 | Saliva |
| Bhandari et al. [13] | GC-MS | 1.0 | 0.05 | 1.8 | 18 | Plasma |

[a] Total estimated time including sample preparation and final analysis

[b] Time necessary for completion of the analytical technique (not including sample preparation)

methods exist for the individual analysis of CN and $SCN^-$ [3], few methods have been developed for their simultaneous determination in biological fluids [13–17]. These methods are summarized in Table 1. Imanari, Toida and co-workers [14, 15] reported high-performance liquid chromatography (HPLC) methods based on the König reaction [18, 19] for analysis of CN and $SCN^-$ in urine with spectrophotometric detection [14] and blood with fluorometric detection [15]. For both methods, CN and $SCN^-$ were separated using a strong-base anion exchange column and subsequently reacted with chloramine-T, pyridine, and barbituric acid. Although the Imanari et al. [14] method only required 1 h to complete, a much longer sample preparation time, 7 h, was necessary for the modification of this method for blood samples [15]. In 1998, Chinaka et al. [16] reported an ion chromatographic method for the simultaneous determination of CN and $SCN^-$ in blood, where CN was derivatized with naphthalene-2,3-dicarboxaldehyde (NDA) and taurine for fluorometric detection, while unreacted $SCN^-$ was detected spectrophotometrically. While this method produced excellent limits of detection (LODs) for CN and $SCN^-$, the baseline found for $SCN^-$ was high, other anions common to blood were found to interfere with $SCN^-$ analysis, and the method took 1.5 h to complete. In 2006, Paul and Smith [17] reported a method for simultaneous analysis of CN and $SCN^-$ using GC-MS after reaction of both anions with pentafluorobenzyl bromide (PFB-Br). The method had a number of disadvantages, including relatively high LODs, the method was only applicable to human saliva, and the internal standard used did not correct for variations in the derivatization reaction. Recently, we developed a similar method for the simultaneous analysis of CN and $SCN^-$ in swine plasma using PFB-Br with GC-MS analysis [13]. The method featured excellent accuracy, precision, and LODs. However, the analysis time was long with an overall analysis time (sample preparation and GC-MS analysis) of approximately 2 h.

The goal of the work presented here was to develop a rapid and robust HPLC-MS-MS method for the simultaneous determination of CN and $SCN^-$ as a complementary method to those already established, with anticipated advantages including rapid analysis time, low LODs, and high selectivity. The developed method was applied to simultaneously determine CN and $SCN^-$ in the plasma of cyanide-exposed swine.

## Experimental

### Materials

#### *Reagents and standards*

Sodium cyanide, sodium hydroxide (NaOH), and all solvents (HPLC-grade or higher) were purchased from Fisher Scientific (Fair Lawn, NJ, USA). Sodium thiocyanate was purchased from Acros Organics (Morris Plains, NJ, USA). NDA was obtained from Tokyo Chemical Industry, America (Portland, OR, USA). Taurine was acquired from Alfa Aesar (Ward Hill, MA, USA). Monobomobimane (MBB) was purchased from Fluka Analytical through Sigma-Aldrich (St. Louis, MO, USA). Ellman's reagent (5,5′-dithiobis 2-nitrobenzoic acid) was obtained from Thermo Scientific (Hanover Park, IL, USA). Isotopically labeled internal standards, $NaS^{13}C^{15}N$ and $Na^{13}C^{15}N$, were acquired from Isotech (Miamisburg, OH, USA). Ammonium formate was purchased from Sigma-Aldrich (St. Louis, MO, USA).

Single cyanide and thiocyanate stock solutions (1 mM each) were prepared and diluted to the desired working concentrations for all experiments. Stock solutions of NDA (4 mM) and taurine (50 mM) were prepared in methanol and deionized water, respectively. Ellman's reagent (10 mM) was prepared in phosphate buffer (0.01 M, pH 7). A MBB solution (4 mM) was prepared in 0.1 M borate buffer (pH 8.0). The NDA, taurine, Ellman's reagent, and MBB solutions were stored at 4 °C in the dark. (*Note: Cyanide is released as HCN from solutions with pH values near or below the $pK_a$ of HCN ($pK_a$ = 9.2). Thus, all aqueous standards containing cyanide were prepared in 10 mM NaOH and handled in a well-ventilated hood).*

#### *Biological fluids*

Citrate anti-coagulated swine (*Sus scrofa*) plasma was obtained through the Veterinary Science Department at South

Dakota State University and plasma from cyanide-exposed swine was obtained from the laboratory of Dr. Vikhyat S. Bebarta at Wilford Hall Medical Center (Lackland Air Force Base, TX). For the cyanide-exposed swine, 11 swine (about 50 kg each) were intramuscularly injected with 1.7 mg/kg potassium cyanide. Blood samples were collected (4 mL), placed in EDTA tubes, and centrifuged to separate the plasma. The plasma samples (500 μL) were then frozen and shipped on ice to South Dakota State University. Upon receipt, all plasma samples were stored at -80 °C until analyzed. All animal procedures were conducted with the guidelines stated in "The Guide for the Care and Use of Laboratory Animals" (National Academic Press, 1996). The research facility where the plasma was gathered was AALAS (American Association for Laboratory Animal Science) accredited and all the animal protocols were approved by the appropriate institutional review board.

## Methods

### *Sample preparation*

Plasma (spiked or non-spiked, 200 μL) was added to a 2 mL micro-centrifuge vial along with 50 μL each of 100 μM $NaS^{13}C^{15}N$ and $Na^{13}C^{15}N$. Acetone (400 μL) was added to the sample to precipitate plasma proteins and the vial was vortexed for 2 min and then centrifuged for 5 min at 13,200 rpm (16,200×g; Thermo Scientific Legend Micro 21R Centrifuge, Waltham, MA, USA). An aliquot (500 μL) of the supernatant was then transferred to a 4-mL glass screw-top vial and dried under $N_2$ (g) for 15 min at room temperature (RT) (Reacti-vap III, Pierce, Rockford, IL, USA). After drying, the sample was reconstituted with 200 μL of 10 mM aqueous ammonium formate. NDA and taurine (50 μL each) were added and mixed thoroughly to produce an N-substituted 1-cyano[f]benzoisoindole (CBI) (Fig. 1). An aliquot (100 μL) of Ellman's reagent was added to react with free thiols in solution and vortex-mixed (1 min). MBB (100 μL) was then added to produce the SCN-bimane complex shown in Fig. 2. The sample was heated on a block heater (VWR International, Radnor, PA, USA) at 70 °C for 15 min. After filtration with a 0.22 μm tetrafluoropolyethylene membrane syringe filter, an aliquot of the prepared sample (100 μL) was transferred into a screw-top autosampler vial (2 mL) with a 150-μL glass insert for subsequent HPLC-MS-MS analysis. The analysis of cyanide through reaction with NDA to form CBI was originally suggested by Sano et al. [20]. To our knowledge, the analysis of $SCN^-$ using MBB to produce an SCN-bimane product is first suggested here. In previous studies, it was thought that MBB reacts with free thiols only [21, 22].

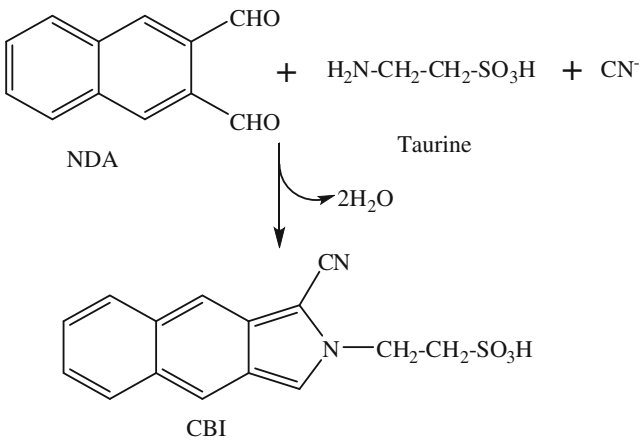


**Fig. 1** Schematic representation of the reaction of NDA and taurine in the presence of cyanide to form an N-substituted 1-cyano[f]benzoisoindole (*CBI*) complex

### *HPLC-MS-MS analysis*

Prepared samples were simultaneously analyzed for CBI and SCN-bimane (Figs. 1 and 2) using a Shimadzu HPLC (LC-20AD, Shimadzu Corp., Kyotu, Japan) with a Phenomenex Kinetex XB-C18 RP column (50×2.10 mm, 2.6 μ 100 Å) protected by a Synergi 2.5 μ Fusion-RP 100 Å C18 (both Phenomenex, Torrance, CA, USA) guard cartridge (10× 2.00 mm, i.d.). Each chromatographic analysis was carried out with mobile phase components of aqueous 10 mM ammonium formate (mobile phase A) and 10 mM ammonium formate in methanol (mobile phase B). An aliquot (10 μL) of the prepared sample was separated by gradient flow at 0.25 mL/min and 40 °C. The concentration of B, initially 50 %, was increased linearly to 100 % over 3 min, held at 100 % for 1 min, decreased linearly to 50 % over 1 min, and held constant for 2 min to re-equilibrate the column between samples. An AB Sciex Q-trap 5500 MS-MS (Applied Biosystems, Foster City, CA, USA) with multiple reaction monitoring (MRM) was used to detect CBI and SCN-bimane using electronspray ionization (ESI)-MS-MS operated in negative polarity. Nitrogen gas (30 psi) was used as the curtain and nebulization gas. The dwell time was 100 ms for all MRM transitions. The ion source was operated at −4,500 V and 500 °C with neubilizer (GS1) and heater (GS2) gas pressures at 40.0 and 60.0 psi, respectively. The collision cell was operated with an entrance potential of −5.0 V and a cell potential of −7.4 V, with a medium collision gas pressure.

### *Calibration, quantification, and LOD*

The calibration and quality control (QC) standards were prepared from aqueous cyanide and thiocyanate stock solutions (200 μM each). All the calibration standards for CN (0.01, 0.02, 0.05, 0.1, 0.2, 0.5, 1, 2, 5, 10, 20, 50, and 100 μM) and $SCN^-$ (0.01, 0.02, 0.05, 0.1, 0.2, 0.5, 1, 2, 5, 10, 20, 50 and 100 μM) were prepared in swine plasma. The peak area signal

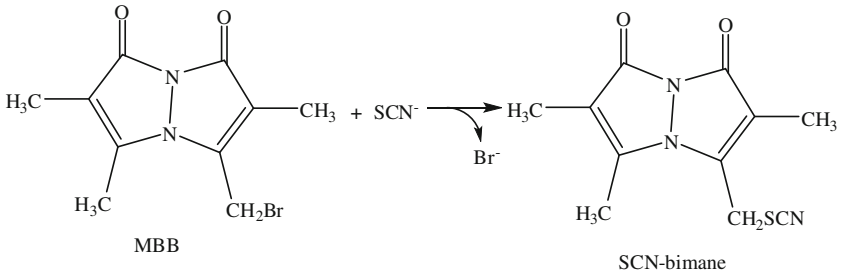


**Fig. 2** Schematic representation of the MBB thiocyanate reaction to form a SCN-bimane product

ratios (i.e., the peak area of the analyte transition divided by the peak area of the internal standard transition) were plotted as a function of calibrator concentration. Both nonweighted and weighted ($1/x$ and $1/x^2$) linear calibration curves were prepared by least squares and a nonweighted linear fit was found to best describe the calibration data for cyanide, with a $1/x^2$ weighted linear fit used for thiocyanate. A computer workstation running Analyst™ software 1.4.1. (Farmingham, MA, USA) was used for data acquisition and peak integration.

The upper limit of quantification (ULOQ) and the lower limit of quantification (LLOQ) were defined by investigation of calibrators which satisfied the following inclusion criteria: (1) a percent relative standard deviation of <10 % (as a measure of precision) and (2) a percent deviation within ±20 % back-calculated from the nominal concentration of each calibration standard (as a measure of accuracy). Three QC standard concentrations were prepared in swine plasma for CN (0.3, 3 and 15 μM as low, medium, and high, respectively) and $SCN^-$ (0.7, 4, and 15 μM as low, medium, and high, respectively) and were analyzed in quintuplicate ($N=5$) each day for 3 days. These QC standards were analyzed in parallel with the calibration standards. Intra-assay precision and accuracy of the method was assessed by analyzing replicates of the QC standards from each day's analysis. Inter-assay precision and accuracy of the method were calculated by comparing the QC standards from three separate days. The intra- and inter-assay investigations were performed within seven calendar days.

The LODs were estimated by analysis of multiple concentrations of CN and $SCN^-$ below their respective LLOQ. The LOD was defined as the lowest analyte concentration reproducibly producing a signal-to-noise ratio of 3 which contained both MRM transitions. Noise was calculated as the peak-to-peak noise directly adjacent to the analyte peak.

*Selectivity, stability, and recovery*

The ability to differentiate and quantify CBI and SCN-bimane in the presence of other plasma components (assay selectivity) was determined by comparing blank swine plasma (triplicate) with spiked swine plasma (15 μM, triplicate) by the procedure described earlier. Matrix effects were also investigated by creating a calibration curve in aqueous solution and one in plasma and evaluating the similarity of the curves. There was no significant difference between the two curves, indicating that matrix effects were not important. Symmetry of the chromatographic peaks, as measured by peak asymmetry ($A_s$), was evaluated by dividing the front-width by the back-width at 10 % peak height [23].

The short- and long- term storage stability of cyanide and thiocyanate was evaluated using swine plasma spiked with high and low QC concentrations of each analyte. For short-term stability, both the low and high QC samples were evaluated in the autosampler, on the bench-top, and under multiple freeze–thaw (FT) conditions. The autosampler stability of CBI and SCN-bimane was evaluated for prepared cyanide and thiocyanate QC standards (both high and low) after placing the QC standards in the LC autosampler at 15 °C and analyzing at approximately 0, 1, 2, 4, 8, 12, and 24 h. The bench-top stability of CBI and SCN-bimane was evaluated using QC standards which were allowed to stand at room temperature (RT) for 0, 1, 2, 4, 8, 12, and 24 h prior to analysis. FT stability was evaluated by initially analyzing three aliquots each of the high and low QC concentrations (i.e., the same day of sample preparation) and then freezing and storing all standards at −80 °C for 24 h. The standards were then thawed unassisted at RT, analyzed and compared with the initial analysis. The remaining standards were again frozen, thawed, and analyzed. In total, this process was performed for three FT cycles. It should be noted that internal standards were added to the QCs directly prior to sample preparation, exclusive of autosampler stability, to correct for variations due to sample preparation and instrumental errors.

Both low and high QC standards were also used for long-term stability studies. The QC standards were stored at −80 °C, −20 °C, 4 °C, and RT. These standards were analyzed in triplicate on the day they were prepared, and after 1, 2, 5, 10, 20, and 30 days. Cyanide and thiocyanate were considered stable if the calculated concentrations were within ±10 % of the original concentration.

The assay recovery of each compound was determined from spiked swine plasma and spiked aqueous samples at low, medium, and high QC concentrations. Recoveries of cyanide and thiocyanate were determined as a percentage by comparing peak areas obtained from the spiked swine plasma

with spiked aqueous samples at the same concentrations. All recovery experiments were performed in triplicate.

## Results and discussion

### HPLC-MS-MS analysis of CN and $SCN^-$

The method presented includes the chemical modification of CN and $SCN^-$ with a mixture of NDA/taurine and monobromobimane (MBB), respectively (Figs. 1 and 2), in a one-pot sample preparation method. The mass spectra of cyanide (as CBI) and thiocyanate (as SCN-bimane) produced by ESI(−)-MS are shown in Fig. 3a, b, respectively, with the major abundant ions identified. The *m/z* ratios of 298.6 and 248.0 correspond to the molecular ion of the CBI complex and SCN-bimane product of cyanide and thiocyanate, respectively ($[M–H]^-$). For cyanide, the 298.6→190.7 and 298.6→80.9 transitions were selected as the quantification and identification transitions, respectively, using the corresponding transitions for isotopically labeled cyanide as internal standard signals, 300.6→192.7 and 300.6→80.9. For thiocyanate, the 248.0→111.0 and 248.0→124.1 transitions were selected as the quantification and identification transitions, respectively, while the corresponding transitions for labeled thiocyanate internal standard were 250.0→111.0 and 250.0→126.1. The optimized declustering potentials (DPs) and collision energies (CEs) for the detection of CBI were −70 and −25 V, respectively. For SCN-bimane, the optimized DPs and CEs were −185 and −19 V, respectively. Identical DPs and CEs were used for the applicable isotopically labeled internal standards.

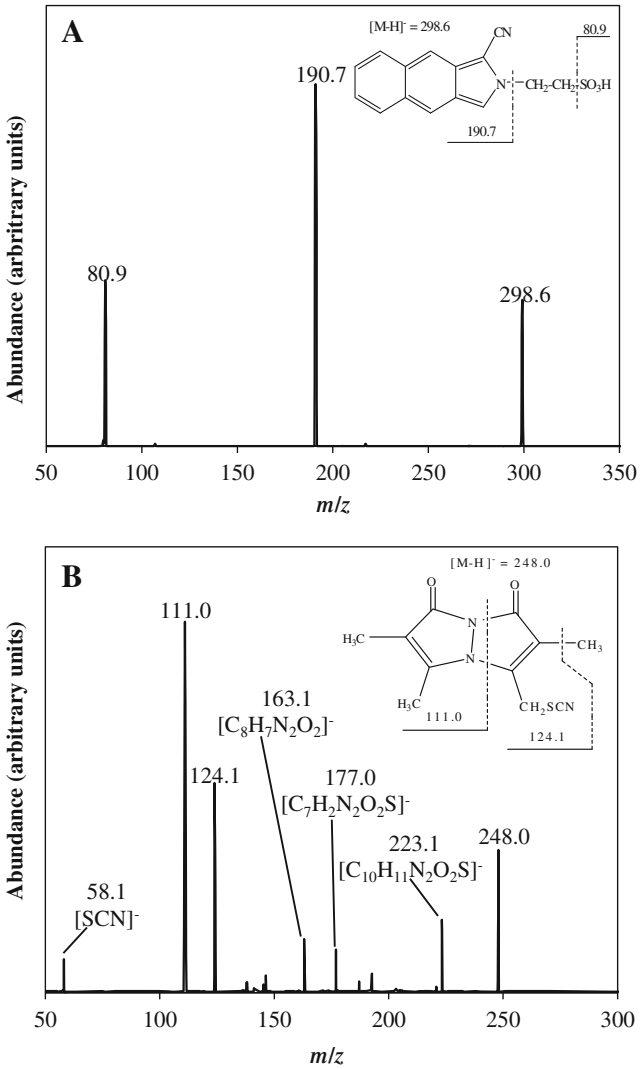


**Fig. 3** ESI(-) product ion mass spectra of CBI (**a**) and SCN-bimane (**b**) with identification of the abundant ions. Molecular ions of CBI and SCN-bimane $[M–H]^-$ correspond to 298.6 and 248.0, respectively. *Insets*, structures of CBI (**a**) and SCN-bimane (**b**) with abundant fragments indicated

Representative HPLC-MS-MS chromatograms of cyanide and thiocyanate, as CBI and SCN-bimane, are depicted in Fig. 4. Initially, the analysis of $SCN^-$ following MBB addition was not possible because MBB reacted with abundant thiol groups present in plasma, which competed with the MBB-SCN reaction [21, 22]. Thus, Ellman's reagent was added in excess to react with the free thiols in plasma, prior to MBB addition, to allow increased production of the SCN-bimane complex. As seen in Fig. 4, the peak shapes for both thiocyanate (1.7 min) and cyanide (2.1 min) were sharp and symmetrical with peak asymmetries of 1.0 and 1.1, respectively.

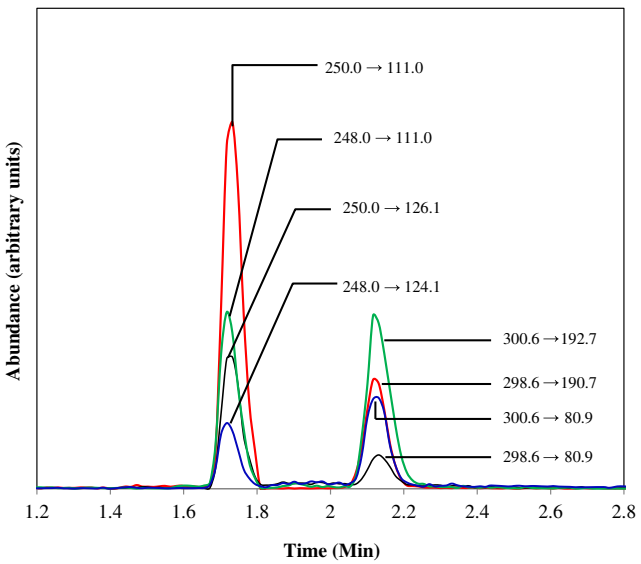


**Fig. 4** HPLC-MS-MS chromatograms of 10 μM cyanide and 20 μM thiocyanate spiked into swine plasma with internal standard (50 μM each). The chromatograms represent signal response to the MRM transitions of cyanide (298.6→190.7, 298.6→80.9, 300.6→192.7, and 300.6→80.9) and thiocyanate (248.0→111.0, 248.0→124.1, 250.0→111.0, and 250.0→126.1). Thiocyanate and cyanide (as SCN-bimane and CBI) eluted from the column at approximately 1.7 and 2.1 min, respectively

**Table 2** Comparison of the stability of the slope, $R^2$, accuracy and precision for cyanide, and thiocyanate analysis from spiked swine plasma over 3 days

| Analyte | Day | $R^2$ | Slope | Accuracy (%) | Precision (%RSD) |
|---|---|---|---|---|---|
| CN | 1 | 0.9997 | 0.019 | 100±8.5 | <7.5 |
| | 2 | 0.9999 | 0.018 | 100±8.4 | <5.4 |
| | 3 | 0.9996 | 0.019 | 100±8.8 | <6.5 |
| $SCN^-$ | 1 | 0.9994 | 0.022 | 100±5.9 | <5.6 |
| | 2 | 0.9997 | 0.021 | 100±5.3 | <6.8 |
| | 3 | 0.9998 | 0.020 | 100±6.1 | <7.3 |

Overall, the sample preparation and analysis was rapid and simple. The duration of sample preparation was approximately 40 min, with the chromatographic analysis lasting approximately 8 min (including equilibrium for the following sample), for a total analysis time of approximately 50 min. Therefore, using conservative estimates, it is estimated that approximately 170 parallel samples could be processed and analyzed within a 24-h period. The duration of analysis for this method is shorter than previous methods for simultaneous analysis of CN and $SCN^-$ (Table 1), and although the duration of the Imanari et al. [14] and Paul and Smith [17] methods are certainly comparable, these two methods were not used for the analysis of plasma or blood.

## Calibration and quantification

Calibration curves for cyanide and thiocyanate were constructed in the range of 0.01–100 μM in swine plasma. For cyanide, calibration standards at 0.01, 0.02, 0.05, and 100 μM were found to be outside the LLOQ or ULOQ, while calibration standards at 0.01, 0.02, 0.05, 0.1, and 100 μM were found to be outside the LLOQ or ULOQ for thiocyanate, resulting in linear dynamic ranges from 0.1 to 50 to 0.2 to 50 μM, for cyanide and thiocyanate, respectively. The linear ranges for both cyanide and thiocyanate are comparable to typical bioanalytical LC-MS-MS methods, which generally span at least two orders of magnitude [24–26]. For both cyanide and thiocyanate, the calibration curves were found to be highly stable over 3 days in terms of slopes and correlation coefficients (Table 2).

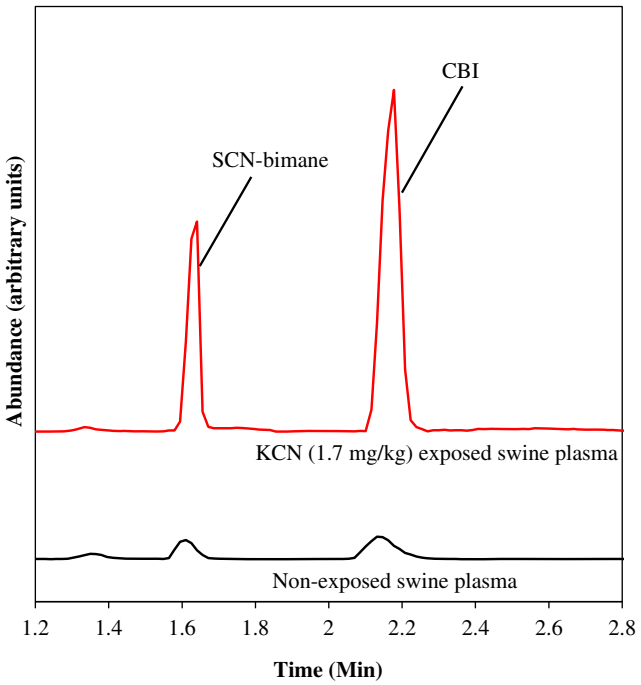


**Fig. 5** Chromatograms of potassium cyanide-exposed (1.7 mg/kg) swine plasma (*upper trace*) and nonexposed swine plasma (*lower trace*), both without internal standard. The chromatograms represent the signal response of the MRM transition 298.6→190.7 and 248.0→111.0 *m/z* transition for CBI and SCN-bimane, respectively

## LOD, accuracy, and precision

The accuracy, precision, and LOD for CN and $SCN^-$ are reported in Table 3. The LODs found for cyanide and thiocyanate are in the nM range; lower than methods previously reported for simultaneous analysis of CN and $SCN^-$ (Table 1). While the significantly lower LODs for cyanide and thiocyanate in plasma are not necessarily essential (i.e., significant endogenous CN and $SCN^-$ concentrations mitigate the need for extremely low LODs), they should allow for quantification of cyanide and thiocyanate concentrations in other biological matrices where they may be present at extremely low levels.

**Table 3** The accuracy, precision, LOD, and recovery of cyanide and thiocyanate analysis from spiked swine plasma by HPLC-MS-MS

| Analyte | LOD (μM) | QC Concentration (μM) | Recovery (%) | Intraassay | | Interassay | |
|---|---|---|---|---|---|---|---|
| | | | | Accuracy (%)[a] | Precision (%RSD)[a] | Accuracy (%)[b] | Precision (%RSD)[b] |
| CN | 0.01 | 0.3 | 72.9 | 100±7.5 | 1.1 | 100±7.2 | 1.5 |
| | | 3 | 81.6 | 100±8.4 | 7.3 | 100±9.4 | 5.4 |
| | | 15 | 83.1 | 100±7.3 | 2.2 | 100±4.2 | 4.1 |
| $SCN^-$ | 0.05 | 0.7 | 73.1 | 100±4.4 | 4.2 | 100±5.3 | 6.8 |
| | | 4 | 78.6 | 100±5.9 | 3.4 | 100±5.9 | 3.4 |
| | | 15 | 80.8 | 100±1.9 | 5.6 | 100±1.9 | 3.9 |

[a] QC method validation ($N$=5) for day 3

[b] Mean of three different days of QC method validation ($N$=15)

Our method produced excellent accuracy and precision for all the conditions tested. The accuracy and precision reported in Table 2 is the aggregate of all QC standards for 3 days. The accuracy was within 8.8 and 6.1 % of the nominal concentration for CN and $SCN^-$, respectively, and the precision was not higher than 7.5 % relative standard deviation (RSD) for either CN or $SCN^-$. Moreover, the absolute values of the accuracy and precision were very consistent for each analyte. The accuracy and precision reported in Table 3 was calculated in aggregate for low, medium, and high QC standards analyzed on three different days. The intra- and inter-assay precision and accuracy were below 8 % RSD and within ±10 % of the nominal concentrations for all intra- and inter-assay analyses.

## Stability and recovery

The short-term stabilities of cyanide and thiocyanate in swine plasma were evaluated in the autosampler and on the bench-top over 24 h. In the autosampler, both cyanide and thiocyanate demonstrated excellent stability for prepared samples, with the measured concentrations within 10 % of the initial concentration at all times tested. On the bench-top, cyanide and thiocyanate concentrations were stable for up to 1 and 8 h, respectively. In addition, the concentrations of cyanide and thiocyanate were within 10 % of the original concentration for both low and high QC standards for only one FT cycle.

For long-term stability investigations, both cyanide and thiocyanate were evaluated for 1 month at −80, −20, and 4 °C. Cyanide was stable for 2 days at −80 and −20 °C but was quickly eliminated from plasma at 4 °C for both the low and high QC standards. Thiocyanate was stable for 5 days at −80 and −20 °C, and for 2 days at 4 °C. The results from investigations of long-term stability suggest that both cyanide and thiocyanate should be analyzed immediately. If this cannot be done, the plasma samples should be frozen and analyzed within 2 days.

The limited stability of cyanide under typical storage conditions may be due to its volatile nature with rapid loss of hydrocyanic acid from biological samples at pH values below 7–8 (HCN $pK_a$=9.2). Alternatively, cyanide can be produced or utilized through single-carbon metabolism [27, 28]. Other studies have implicated microbial metabolism for alteration in CN levels [29–31]. It has been suggested that additives, such as addition of silver ions or ascorbic acid, may increase the stability of cyanide [29, 32], which may be an area of future investigation. The instability of $SCN^-$ could be due to thiocyanate protein binding, resulting in the loss of free thiocyanate in plasma samples [8, 33].

The recoveries of cyanide and thiocyanate are reported in Table 3 and ranged from 72 to 83 % for cyanide and 73–81 % for thiocyanate. The recoveries for this method are similar to previous reports [16, 17, 34].

## Application of the method

Potassium cyanide-exposed swine plasma samples were collected and analyzed for plasma cyanide and thiocyanate using the method presented here. Figure 5 shows representative chromatograms of potassium cyanide-exposed (1.7 mg/kg; upper trace) and non-exposed (lower trace) swine. The peaks for thiocyanate and cyanide were observed around 1.7 and 2.1 min, respectively, with the presence of endogenous concentrations detected in the nonexposed swine. In Fig. 5, the non-spiked swine plasma contained small amounts of cyanide (3.58 μM) and thiocyanate (4.35 μM). These levels were attributed to endogenous concentrations which likely come from multiple sources, such as diet [3, 13, 35, 36]. The assignment of endogenous CN and $SCN^-$ was verified by identical retention times as compared with spiked plasma possessing the quantitation and identification ions. Overall, the method performed well for the diagnosis of cyanide exposure in swine.

## Conclusions

A highly selective method featuring simple sample preparation with excellent accuracy and precision was developed and validated in swine plasma. The reported method has the ability to simultaneously detect cyanide and thiocyanate at low concentrations and proved useful for their detection from the plasma of cyanide-exposed swine. To our knowledge, this is the first description of an HPLC-MS-MS method for the simultaneous analysis of cyanide and thiocyanate from any matrix.

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