Pharmacokinetics of next generation cyanide antidote sulfanegen in rabbits

Aim: Sulfanegen has been shown to be an effective next generation cyanide antidote in multiple animal studies. Sulfanegen detoxifies cyanide by acting as a sulfur donor, converting cyanide to thiocyanate through the enzyme 3-mercaptopyruvate (3-MP) sulfurtransferase. The current study was performed to determine the PK behavior of sulfanegen in rabbits and compare it to current US FDA-approved cyanide therapeutics.

Methods: Plasma sulfanegen concentrations, as 3-MP (i.e., sulfanegen is a prodrug that converts to the active sulfur donor, 3-MP, upon administration), were monitored using LC–MS/MS following intramuscular administration of sulfanegen in rabbits.

Results: Concentrations of 3-MP rapidly increased following sulfanegen administration, indicating rapid absorption and distribution of 3-MP throughout the body. Elimination of 3-MP was also relatively rapid; the calculated half-life was approximately 114 min. A one-compartment model with first-order distribution and elimination was used to describe the PK behavior of 3-MP.

Conclusion: Overall, the PK characteristics of sulfanegen were found to be well suited for the rapid treatment of cyanide poisoning.

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Exposure to cyanide can occur from a number of sources, including occupational exposure from industrial operations, cigarette and fire smoke inhalation, terrorist activities and catastrophic events involving the release of cyanide (e.g., the warehouse explosion in Tianjin, China) [1-5]. Following exposure, cyanide acts rapidly to inhibit cytochrome c oxidase, disrupting the electron transport system, leading to anaerobic metabolism, hypoxia and eventually death. Although current treatments for cyanide poisoning can be effective, they each have limitations, especially for mass casualty situations. Therefore, the development of novel cyanide antidotes that are faster acting, safer and/or allow for timely administration is important.

Currently, there are three US FDA-approved cyanide antidotes: hydroxocobalamin (Cbl, a direct sequestering agent), sodium nitrite (an indirect sequestering agent) and sodium thiosulfate (a sulfur donor). The current sulfur donating cyanide antidote, sodium thiosulfate, detoxifies cyanide by converting it to thiocyanate. Rhodanese, a sulfurtransferase enzyme, catalyzes this reaction [6-8]. Since rhodanese is primarily found in the mitochondria of liver and kidney cells, sodium thiosulfate must distribute into the liver and kidneys, then into the mitochondria of these cells. Furthermore, the rate at which thiosulfate traverses cell membranes is relatively slow [9]. This results in a slow onset of action. Thiosulfate also has a relatively short half-life [10,11], indicating it is rapidly metabolized [12]. Therefore, sodium thiosulfate has kinetic restrictions that mitigate its ability to rapidly reverse severe cyanide toxicity [13].
Currently, a novel cyanide antidote, sulfanegen, is being developed as an alternative sulfur donor. Suf-
anegen is a dimer of 3-mercaptopyruvate (3-MP) that is converted to two 3-MP molecules upon entering the body. Similar to sodium thiosulfate, 3-MP acts as a sulfur donor to produce thiocyanate, but is instead catalyzed by 3-MP sulfurtransferase (3-MST) [7,13,14]. Utilization of 3-MST is advantageous to using rhodane-
se because 3-MST is present in most organs, including the heart, lungs and CNS, the primary targets of cyanide toxicity, where rhodanese is found at low concentra-
tions [16]. Also, 3-MST is found in the cytosol and mitochondria, where rhodanese is only found in the mitochondria [12].

Multiple studies [13,14,17] of sulfanegen’s effective-
ness have been performed. Sulfanegen was first intro-
duced in 2007, when prototype prodrugs of 3-MP were eval-
uated for their antidotal efficacy against a toxic, but nonlethal, cyanide dose [14]. Each prodrug studied was adminis-
tered intraperitoneally at 5 min prior to cyanide exposure and the righting reflex in mice was observed. Sulfanegen was found to be a fast and effective antidote with mice righting at 8.7 ± 1.0 min, whereas sodium nitrite/thiosulfate and Cbl pro-
duced righting times of 16.4 ± 2.2 and 12.6 ± 3.3 min, respectively [14]. Therefore, sulfanegen was superior to both a sodium nitrite–thiosulfate combination and Cbl at reducing righting times [14].

Chan et al. [18] studied sulfanegen in combination with cobinamide. Cobinamide is another novel cyanide antidote, similar to Cbl, that is currently being developed and works via direct sequestration of cyanide. Sulfanegen, with and without cobinamide, was adminis-
tered to mice pre and post-cyanide exposure. When administering sulfanegen precyanide exposure, sulfanegen doses of 0.08 to 0.24 mmol/kg were used to treat cyanide (0.24 mmol/kg) poisoning. For treated mice, the closer the sulfanegen dose was to 0.24 mmol/kg (an equimolar ratio to cyanide), the greater the survival rate, with 0.24 mmol/kg yielding 100% survival. When sulfanegen (0.06 mmol/kg) was administered up to 3 min following the administration of cyanide (0.16 mmol/kg), it also rescued 100% of the mice. When sulfanegen (0.05 mmol/kg) was adminis-
tered with cobinamide (0.02 mmol/kg), the simultane-
ous administration rescued twice the amount of mice than when sulfanegen (0.05 mmol/kg) was adminis-
tered alone, indicating additive effects for cobinamide and sulfanegen [18]. All control (saline-treated) mice died following cyanide exposure.

Sulfanegen has also been shown to be effective in larger mammalian species. Brenner et al. [17] tested sulfanegen in rabbits with sublethal doses of cyanide using diffuse optical spectroscopy and continuous wave near-IR spectroscopy to monitor physiological changes associated with cyanide exposure and survival. When administered either intravenously or intramus-
cularly, sulfanegen was shown to reverse the effects of cyanide on oxyhemoglobin and deoxyhemoglobin as compared with control animals [17].

Swine have also been used to determine the effectiveness of sulfanegen against cyanide toxicity. A high-
dose (5 mg/kg/h) intravenous infusion of sodium nitroprusside (SNP), which produces cyanide when metabolized [10,19–21], was administered and survival was evaluated [13]. The infusion of SNP resulted in lactic acidosis and increase in cyanide levels. All the animals that received sulfanegen following the administration of SNP survived with reversal of lactic acidosis and decrease in cyanide levels, whereas all the animals receiving a placebo died. The lactate and cyanide levels were high and generally consistent after the administration of placebo. All the animals that survived had no observable neuro-
ological deficits (p = 0.0286) [13]. The effectiveness of sulfanegen against cyanide toxicity in this study was also evaluated against direct cyanide administration. The administration of sulfanegen resulted in survival of the swine and reversal of blood cyanide levels, whereas nontreated animals died rapidly.

Although sulfanegen shows promise in treating cyanide toxicity, its PK behavior is currently unknown. This information is vital to the continued development of sulfanegen as a cyanide antidote. Therefore, the objective of this study was to evaluate the PK behavior of sulfanegen in rabbits.

**Experimental Reagents & materials**

All solvents were LC–MS grade unless otherwise noted. Ammonium formate and 3-MP (HSCH$_2$COOH) were purchased from Sigma-Aldrich (MO, USA). Acetone (HPLC grade, 99.5%) was purchased from Alfa Aesar (MA, USA). Isotopically labeled 3-MP (HS$^{13}$CH$_2$$^{13}$COOH) was synthesized and provided by the Center for Drug Design, University of Minnesota (MN, USA). Millex tetrafluoropolyethylene syringe filters (0.22 µm, 4 mm, MA, USA) and LC-MS water were obtained through Fisher Scientific (PA, USA). Monobromobimane (3-[bromomethyl]-2,5,6-
trimethyl-1H,7H-pyrazolo[1,2-a]pyrazole-1,7-dione; C$_{19}$H$_{13}$BrN$_2$O$_2$) was obtained from Fluka Analytical (Buchs, Switzerland) and a standard solution (500 µM) was prepared in LC–MS grade water and stored at 4°C. 3-MP calibration and QC standards were prepared from a 10 mM stock solution by serial dilution with swine plasma. The internal standard solution was prepared from a stock solution of 1 mM isotopically labeled 3-MP in LC–MS grade water and stored at 4°C.
Animal study
Treatement of animals with sulfanegen was performed at the Department of Anesthesiology at the University of Minnesota. New Zealand White (Oryctolagus cuniculus) male rabbits (n = 6), 6–8 months old, weighing between 3 and 5 kg were anesthetized by an infusion of ketamine (5–12 mg/kg). Baseline blood samples were drawn from the rabbits just before sulfanegen was administered. A single dose of sulfanegen (149 mg/kg) \( [12] \) was administered (0.6–0.9 ml im. in the thigh) and blood was drawn (1.5 ml) at 0.5, 1, 1.5, 2, 3, 4, 5, 10, 15, 30, 45, 60, 90, 120, 150, 180, 210 and 240 min after sulfanegen administration. Blood was collected from an artery into EDTA-containing collection tubes, centrifuged at 1500 \( \times \) g for 15 min at 4°C, and plasma was immediately separated from blood. Plasma samples were then frozen at -80°C and shipped on dry ice to South Dakota State University for analysis. Upon arrival, the plasma samples were stored at -80°C until analyzed. All rabbits survived and recovered from anesthesia 380 min after commencement of anesthesia. Rabbits were euthanized (by an overdose of anesthesia) one week after recovery from anesthesia according to the Institutional Animal Care and Use Committee (IACUC).

All animals were handled in accordance with the Guide for the Care and Use of Laboratory Animals \( [22] \) and accredited by the Association for the Assessment and Accreditation of Laboratory Animal Care International. The University of Minnesota IACUC at the respective institution approved the study.

Preparation & analysis of 3-MP in rabbit plasma
Plasma was prepared and analyzed for 3-MP using the previously established method of Stutelberg et al. \( [23] \). Briefly, isotopically labeled 3-MP internal standard (100 µl) was initially added to rabbit plasma. Proteins were then precipitated with acetone (300 µl), samples were centrifuged, and the supernatant was dried under \( \text{N}_2 \). The resulting sample was reconstituted in two solutions, 10% methanol with 5 mM ammonium formate solution (100 µl) and monobromobimane (500 µM, 100 µl). Samples were heated at 70°C to produce the 3-MP-bimane product for analysis. The 3-MP-bimane was analyzed by LC–MS/MS, using the 311–223.1 \( \text{m} / \text{z} \) transition for quantification. Rabbit plasma samples were diluted 1:10 to fit on the calibration curve and concentrations were back-calculated from the calibration curve equation.

Pharmacokinetics & data analysis
PK analysis of 3-MP was completed to determine the most appropriate distribution model and to evaluate standard PK parameters, such as \( C_{max} \), \( t_{max} \), \( t_{1/2} \), AUC and the elimination constants (\( K_e \)). Microsoft Excel was used to calculate parameters (e.g., [AUC] determine using the trapezoidal rule) \( [24] \). These parameters were obtained from evaluating the plasma concentration of 3-MP over time.

Results
PK behavior of 3-MP
Figure 1 shows the calculated plasma concentrations of 3-MP from rabbits treated with sulfanegen. The inter-assay accuracy (100 ± 5%) and precision (<6%), and intra-assay accuracy (100 ± 9%) and precision (<7% RSD) for quality control samples were previously reported for the method \( [23] \). As seen in Figure 1, the plasma 3-MP concentration rapidly increased to a maximum (\( C_{max} \)) of about 1.775 mM at approximately 60 min postadministration (\( t_{max} \)). The concentrations then decreased slowly as 3-MP was cleared. A one-compartment distribution model was used to describe the behavior of 3-MP (Figure 1 inset). Using this model and the data represented in Figure 1, the \( K_e \), \( t_{1/2} \) and AUC were 7.30 × 10\(^{-3}\)/min, 114 min and 2.963 × 10\(^5\) µmol × min/l, respectively \( [22,24] \).

Discussion
The PK parameters that describe the behavior of sulfanegen in rabbits are presented alongside other currently approved cyanide antidotes in Table 1. (Note: some PK parameters reported were evaluated in the presence of cyanide.) The main difference between 3-MP and currently approved cyanide antidotes is the type of PK model. 3-MP is the only cyanide antidote that is modeled by one-compartment behavior. The one-compartment behavior of 3-MP indicates its rapid equilibration throughout the body. Furthermore, the absorption of 3-MP, following intramuscular administration of sulfanegen is rapid, with detection of 3-MP in the first blood sample collected at 0.5 min. When comparing \( \alpha \)-phase half-lives in Table 1, the half-life of 3-MP is relatively high. This is because the other \( \alpha \)-phase half-lives are associated with two-compartment models, whereas the \( \alpha \)-phase likely only accounts for distribution in the circulatory system. The one-compartment \( \alpha \)-phase half-life for 3-MP accounts for distribution of 3-MP into the cytosol and mitochondria, where 3-MST is located. When 3-MP arrives in the cytosol or mitochondria, the catalyzed conversion of 3-MP to pyruvate by 3-MST is quick \( [6] \), which explains, in part, why 3-MP follows a one-compartment model (Note: 3-MP concentration levels had not reached baseline at 240 min, so longer duration PK studies are necessary to confirm one-compartment behavior for 3-MP). In contrast, a two-compartment model (as for sodium thiosulfate, Cbl and sodium nitrite) generally
indicates that a compound does not rapidly equilibrate throughout the body. One-compartment behavior of 3-MP, its rapid appearance in the plasma, and the rapid conversion of 3-MP to pyruvate indicate it should have a rapid onset of action [6].

Compared with sulfanegen, sodium thiosulfate must distribute into the mitochondria of the liver and kidney to be effective [10], leading to a slow rate of reaction (~5 min in guinea pigs, with a return to normal heart activity in 100 min) [25]. Furthermore, its half-life is relatively short (Table 1) as compared with Cbl, although it is longer than nitrite. The slow rate of action and short half-life are not ideal for a cyanide antidote because of the fast onset of action of cyanide. The outcomes of the PK and therapeutic behaviors of this antidote are evident in multiple studies of sodium thiosulfate effectiveness against cyanide poisoning. For example, for a sodium cyanide continuous infusion dog model, sodium thiosulfate was not effective if administered after the start of apnea [26]. However, when sodium thiosulfate was administered with sodium nitrite or Cbl, this combination was effective at later phases of cyanide poisoning. Other studies confirm the slow onset of action of sodium thiosulfate. Therefore, it is currently used in tandem with Cbl or, more commonly, sodium nitrite. In fact, sodium thiosulfate and sodium nitrite have been FDA approved to be packaged together as Nithiodote™ [27-29].

Sodium nitrite detoxifies cyanide by indirectly sequestering cyanide through the conversion of hemoglobin to methemoglobin, which has a strong affinity toward cyanide [9]. Nitric oxide, converted from nitrite, has recently been proposed as an alternative prominent method of cyanide detoxification [30-32]. Nitric oxide displaces cyanide bound to cytochrome c oxidase allowing cyanide to be metabolized or treated (by sulfur donors or sequestration). This can occur in the absence of appreciable methemoglobin formation [31,32]. Many studies have proven the effectiveness of sodium nitrite against the effects of cyanide poisoning. For example, sodium nitrite, at a minimum dose of 5 mg/kg, was shown to be an effective antidote for cyanide poisoning in dogs, and 200 mg/kg was efficacious in rabbits [33,34]. Although sodium nitrite is a proven cyanide antidote, reduction in the hemoglobin reduces the oxygen carrying capacity in the blood. Therefore, sodium nitrite alone is not ideal for treating smoke inhalation victims [17,31,32,35]. PK data for sodium nitrite are limited, since the reaction of nitrite with hemoglobin to produce methemoglobin and nitric oxide occurs rapidly [4,30,36]. In one PK study, humans (n = 5) were administered with sodium nitrite doses of 7 and 110 μg/kg x min (120 ml/h iv.) [30]. The biological terminal half-lives of nitrite and methemoglobin were approximately 42 and 78 min (Table 1), respectively [30], which are more rapid than sulfanegen. The half-life of nitric oxide in blood is also very rapid (~1 s) [37]. In another study, sodium nitrite (300 mg) was administered to smoke inhalation patients. The half-life was 168 min (as methemoglobin), which is

Figure 1. Plasma 3-mercaptoppyruvate concentrations after intramuscular sulfanegen administration. Error bars represent standard error of the mean (n = 6).
longer than sulfanegen [38]. Although sodium nitrite is an effective antidote for cyanide poisoning, it has a limited therapeutic efficacy, is generally thought of as a short-acting antidote, and is typically administered in tandem with other antidotes.

When comparing sulfanegen to Cbl, it is important to understand that they work via disparate mechanisms. Cbl detoxifies cyanide through direct sequestration forming cyanocobalamin. The cyanocobalamin is then transported in blood by globulin, actively metabolized by the liver, processed by the kidney, and excreted through the urinary tract [39]. Therefore, sulfanegen, as with thiosulfate, may be utilized with Cbl to produce additive or synergistic effects. Individually, Cbl shows two-compartment PK behavior, producing both $\alpha$- and $\beta$-phase half-lives, as reported in Table 1. While the $\alpha$-phase half-life of Cbl is short (averaging 15–40 min), the $\beta$-phase (terminal) half-life was much longer than sulfanegen (averaging >360 min) [40–43]. The relatively long half-life is combined with a fast onset of action. When 300 mg/kg Cbl was administered in guinea pigs after cyanide infusion (4.1 µM/kg), de La Coussaye et al. [43] found rate of reaction of <1.0 min. Others also found that the heart returns to normal activity within approximately 18.0 min [2,25,26,43]. Although Cbl has excellent PK properties, its large MW and poor solubility make it difficult to rapidly administer in therapeutic doses.

Overall, sulfanegen appears to have the excellent PK properties for the early onset symptoms of cyanide exposure, without the complications inherent for sodium nitrite. Although sulfanegen is rapidly acting, it also has a relatively short half-life. Therefore, larger doses of cyanide may necessitate multiple sulfanegen doses or the combination of sulfanegen with a longer acting antidote, such as Cbl, similar to the sodium thiosulfate–sodium nitrite combination.

**Conclusion**

This study demonstrated that sulfanegen is rapidly absorbed and cleared after administration. Sulfanegen also has a short half-life (~114 min) relative to the terminal half-life of Cbl and sodium thiosulfate, but has a comparable half-life to sodium nitrite, a similar rapidly absorbed and rapidly acting antidote. The PK parameters found in this study are helpful in identifying the time for onset of action, duration of effectiveness, necessary concentrations of sulfanegen needed to prevent cyanide poisoning, design of follow-on PK studies, and will ultimately aid in approval of sulfanegen as a cyanide antidote. Further PK studies of sulfanegen where 3-MP concentrations return to baseline, and evaluation of sulfanegen in the presence of cyanide, need to be conducted.

**Future perspective**

Preliminary studies show great promise for sulfanegen as a cyanide antidote. There is a need for rapidly acting

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**Table 1. The PK parameters of US FDA-approved and novel cyanide therapeutics.**

<table>
<thead>
<tr>
<th>Therapeutic</th>
<th>Animals</th>
<th>Dose</th>
<th>$C_{\text{max}}$ (µM)</th>
<th>$t_{1/2 \alpha}$ (min)</th>
<th>$t_{1/2 \beta}$ (min)</th>
<th>Study Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sulfanegen</td>
<td>Rabbits</td>
<td>149 mg/kg</td>
<td>1775</td>
<td>114</td>
<td>N/A</td>
<td>This study</td>
</tr>
<tr>
<td>Sodium thiosulfate</td>
<td>Humans</td>
<td>1 g, 150 mg/kg</td>
<td>6400</td>
<td>15–20*</td>
<td>182</td>
<td>Schulz et al. [10,11]</td>
</tr>
<tr>
<td>Sodium nitrite</td>
<td>Humans</td>
<td>550 µg/kg</td>
<td>320</td>
<td>N/A</td>
<td>42.1 ± 10.2*, 78<em>5, 168</em>5</td>
<td>Dejam et al. [36]</td>
</tr>
<tr>
<td>Hydroxocobalamin</td>
<td>Humans</td>
<td>5 g</td>
<td>212.4 ± 30.9</td>
<td>36</td>
<td>954, 1572 ± 162*</td>
<td>Becker et al. [40]</td>
</tr>
<tr>
<td></td>
<td></td>
<td>N/A</td>
<td>111.6 ± 20.4*</td>
<td></td>
<td></td>
<td>Houeto et al. [41]</td>
</tr>
<tr>
<td></td>
<td>Dogs</td>
<td>70 mg/kg, 140 mg/kg</td>
<td>316 ± 47, 604, 366 ± 66</td>
<td>18.6 ± 12, 15.6 ± 12.6</td>
<td>441.6 ± 47.4, 360 ± 34.8</td>
<td>de La Coussaye et al. [43]</td>
</tr>
</tbody>
</table>

*PK analysis in the presence of cyanide.
*These studies reported a ‘terminal half-life’ and a two-phase distribution model. The table lists the terminal half-life as the $t_{1/2 \beta}$-phase.
*Methemoglobin formed from administration of sodium nitrite.

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**Executive summary**

**Aim**
- Investigate the pharmacokinetics of sulfanegen in rabbits.

**Results**
- We observed that the pharmacokinetics of sulfanegen appears to follow a one-compartment model.

**Conclusion**
- Sulfanegen is rapidly absorbed and cleared with a short half-life in rabbits.
antidotes that can be quickly administered to large number of people. The continued development of sulfanegen will ultimately aid in approval of sulfanegen as a cyanide antidote. The FDA approval of sulfanegen will add a necessary antidote, which can be rapidly administered intramuscularly. We believe that sulfanegen will add breadth to the assortment of current antidotes.

Financial & competing interests disclosure

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No writing assistance was utilized in the production of this manuscript.

Ethical conduct of research

Study protocols were approved by the IACUC. All animals were handled in accordance with the Guide for the Care and Use of Laboratory Animals and accredited by the Association for the Assessment and Accreditation of Laboratory Animal Care International.

References

Papers of special note have been highlighted as:
• of interest;  • of considerable interest
• Review of the analysis and effects of cyanide.
• Mechanism of metabolism with 3-mercaptopyruvate and cyanide by 3-mercaptopyruvate sulfurtransferase.
• Pharmacokinetics of current cyanide antidotes.
• Summary on the development of sulfanegen as an antidote.
• Use of sulfanegen to reverse cyanide toxicity.
Pharmacokinetics of sulfanegen

Research Article


24 Method for the quantitation of 3-mercaptopyruvate by LC–MS-MS.


