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RESEARCH ARTICLE

Organ-distribution of the metabolite 2-aminothiazoline-4-carboxylic acid in a rat model following cyanide exposure

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Abstract

The reaction of cyanide (CN^-) with cystine to produce 2-aminothiazoline-4-carboxylic acid (ATCA) is one of the independent detoxification pathways of cyanide in biological systems. In this report, *in vivo* production of ATCA and its distributions in plasma and organs were studied after a subcutaneous sublethal dose of 4 mg/kg body weight potassium cyanide (KCN) administration to rats. At this sublethal dose of KCN, ATCA concentration was not significantly increased in the plasma samples, however, it was found significantly increased in liver samples. These results suggested that ATCA might not be a good diagnostic biomarker in plasma for sublethal cyanide exposure; however, liver could serve as the right organ for the detection of ATCA in post-mortem examinations involving cyanide exposure in military, firefighting, industrial and forensic settings.

Keywords: Diagnostic biomarker, forensic biomarker, cyanide poisoning, 2-aminothiazoline-4-carboxylic acid (ATCA), LC-MS/MS

Introduction

Human metabolism of cyanide and detection of its biomarkers has been described in detail by Baskin et al. (1997, 2006). Briefly, thiocyanate (SCN^-), 2-aminothiazoline-4-carboxylic acid (ATCA), and cyanide-protein adducts in biological fluids and tissues are alternative biomarkers for cyanide exposure and poisoning. Although SCN^- is the major cyanide metabolite found in blood (Baskin et al. 2004), it is also a natural metabolite of non-cyanide mediated pathways and thus it is not a specific marker for cyanide poisoning (Ballantyne et al. 1977). On the other hand, ATCA has been identified and suggested as an alternative chemically stable biomarker for cyanide exposure (Logue et al. 2010). Unfortunately, ATCA half-life and distribution of ATCA due to cyanide exposure have not yet been studied or published. The purpose of this study was to evaluate the potential use of ATCA as a diagnostic biomarker for cyanide poisoning by: (1) *in vivo* measurements of ATCA concentrations in plasma, and (2) post-mortem measurements of the distribution of ATCA amongst organs following cyanide exposure in a rat system.

Cyanide exposure occurs in military, firefighting, industrial and forensic settings. Although potassium cyanide (KCN) can be produced from a non-toxic source, such as cooking potassium ferrocyanide (Musshoff et al. 2011), cyanide exposures most commonly originate from smoke inhalation or direct exposure to either cyanide salt or hydrogen cyanide (HCN). In an ambient environment, cyanide salts usually appear as white crystalline powders, while HCN is present as colorless (or pale blue) liquid or as gas with a bitter almond-like odor (Lv et al. 2005). In the investigation of deaths, a bitter almond odor emanating from the victim, and the presence of pink lividity from post-mortem examination are two common indicators of acute cyanide poisoning (Gill et al. 2004). Alkali burns

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of the gastrointestinal tract tract can often be observed during autopsy in cases where cyanide salts have been ingested. Since cyanide salts are solid crystalline, their presence in a crime scene or in the areas near victim's nose or mouth can be easily discovered, collected and preserved for further forensic testing. In cases where no suspicious substances are observed at the scene of death, the presence of cyanide in the victim's body can be confirmed chemically using a colorimetric test, followed by laboratory analysis using gas chromatography mass spectrometry (GC–MS). Forensic evidences, such as stomach contents and whole blood of the victims, are usually collected and analysed in order to confirm the cause of death (Laforge et al. 1994).

The toxicological detection of cyanide involves extraction and measurement of HCN from biological extracts (Darr et al. 1980; Shiono et al. 1991). Blood or urine can be collected from the victim for laboratory analysis (Lundquist et al. 1989; McAuley et al. 1983; Zamecnik et al. 1987). Due to the relatively short half-life of cyanide (from minutes to hours depending on the matrix), toxicological detection of cyanide to confirm cyanide poisoning may only be feasible within the first few hours following exposure (Calafat & Stanfill 2002; Moriya & Hashimoto 2001). Moreover, the volatility and reactivity of cyanide give direct measurements highly susceptible to errors introduced during the sample collection and separation step (Lindsay et al. 2004). Cyanide levels in blood samples taken at autopsy the next day have been reported to decrease by approximately 79% (Curry 1963). Post-mortem formation of cyanide may also occur and complicates the interpretation of cyanide results (McAllister et al. 2008). Therefore, the presence of cyanide becomes less feasible when the detection window is passed or the victims' body has been damaged, such as might be the case when autopsies are delayed or when tissues have been damaged by fire or advanced decomposition. The detection of stable biomarkers of cyanide is a promising approach to extend the time in which cyanide exposure can be reliably assayed in a post-mortem examination.

Materials and methods

Chemicals and samples

All solvents used in this study were at least HPLC grade. Trifluoroacetic acid (TFA) was obtained from EMD Chemicals (Gibbstown, NJ, USA) and used to prepare 0.5% (v/v) TFA in methanol as the mobile phase. ATCA was obtained from Chem-Impex International (Wood Dale, IL, USA). 2-Aminothiazole-4-carboxylic acid (ATZA) was obtained from Synthonix (Wake Forest, NC, USA). KCN was purchased from Sigma-Aldrich (St. Louis, MO, USA). For *in vivo* study, serial dilutions were used to produce aqueous KCN solutions of systematically decreasing concentration. Oasis® MCX (mixed-mode cation exchange) cartridges were obtained from Waters Corporation (Milford, MA, USA).

Animals

Male CD rats weighing 250–300 grams with catheters implanted were purchased from Charles River (Charles River Breeding Laboratories, Inc., Wilmington, MA, USA) The experimental animals were housed in temperature and light controlled rooms (22 ± 2°C, 12 h light/dark cycle). They were furnished with water and Teklad Rodent Diet (W) 8604 (Teklad HSD, Inc., WI, USA) ad libitum. All animal procedures were conducted in accordance with the guidelines in The Guide for the Care and Use of Laboratory Animals (National Academic Press, 1996). The research facility was accredited by American Association for the Assessment and Accreditation of Laboratory Animal Care, International and this animal study was approved by the Institutional Animal Care and Use Committee at Sam Houston State University (SHSU).

In vivo production of ATCA in plasma

Three rats were received 4 mg/kg body weight KCN solution subcutaneously. Before injection, blood was drawn to establish baseline endogenous ATCA levels at the zero time point. After exposing the rats by injecting the KCN solutions subcutaneously, blood samples were taken through the catheters at the following time intervals: 5, 15, 30, 60 min, and 2, 4, 6, 12, 15, 50.5 h. For each withdrawal of blood, lock solutions were drawn out till blood appeared in the catheter; 320 μL blood was collected from each rat; 320 μL of isotonic saline was injected back into the rat and the catheter was resealed with 100 μL of the lock solution. Individual samples were deposited into a 15 mL tube (15 mL/tube) that had been pre-washed with heparin to prevent coagulation. The heparinized blood samples were than centrifuged at 2000 rpm for 10 min at +4°C (VWR Model 5810R, VWR International, Dallas, TX 75267) to prepare plasma samples for ATCA analysis.

Organs distribution study

Following the plasma measurements discussed above, different groups of animals were used in a series of measurements (Table 1) to determine organ-distribution of ATCA following sublethal cyanide exposure. Organs (liver, heart, kidneys, spleen, brain, and lungs) of the rats were collected after subcutaneous injection of KCN at sublethal dose of 4 mg/kg body weight. KCN was injected with a 25 G × 1½ needle. Rats were terminated at 30 and 60 min, 4 and 12 h after cyanide exposure and organs were collected. Organ samples were placed into plastic 3 mL tubes and stored in the freezer until they were thawed for analysis.

Sample preparations and ATCA measurement

Cation exchange solid-phase extraction (SPE) columns and individual pre-treatment steps for the extraction and analysis of ATCA from biological samples have been reported (Bradham et al. 1965; Lundquist et al. 1995). In our work, ATCA was extracted from biological samples by SPE. Detection and quantification of

ATCA was accomplished by using a liquid chromatography-tandem mass spectrometer (LC-MS/MS). Sample preparation and instrumentation details of electrospray ionization/tandem mass spectrometer (ESI/MS/MS) for the detection of ATCA can be found in our previous report (Jackson et al. 2010). Briefly, tissue samples were homogenized with ready-to-use Precellys® lysing kits on a Precellys-24 tissue homogenizer (Bertin Technologies, France). ATCA was extracted from homogenates by SPE, which was performed in a glass manifold equipped with Teflon needle inserts and evacuated with a Buchi V-700 Vacuum Pump (Mallinckrodt Baker, Inc., Phillipsburg, NJ, USA). After SPE, Pierce Reacti-Therm II Heating Module was used to stream air to dry elution solvent in borosilicate glass disposable culture tubes (13 × 100 mm). A Shimadzu liquid chromatograph (LC-20AT, Shimadzu, Columbia, MD, USA) coupled to a tandem mass spectrometer (API 3200 ESI/MS/MS system, Applied Biosystems, Foster City, CA, USA) was employed for the LC-MS/MS separation, detection and quantification of ATCA. A Luna CN column (3 micron, 100 × 2 mm; Phenomenx; Torrance, CA, USA) was used for the separation. A 5 µL aliquot of sample after SPE was injected to the LC-MS/MS by an auto-sampler, and eluted isocratically at a 0.5 mL/min flow rate. Electrospray ionization (ESI) was used at the LC and MS/MS interface. Transition ions of ATCA (m/z 147+ → 101+) and ATZA (m/z 145+ → 127+) were monitored under multiple reaction monitoring mode. The conditions of ESI were as follows: Ion spray voltage: +5500 volts, temperature: 450°C, curtain gas 50 psi, gas 1: 70 psi, gas 2: 20 psi. The MS/MS parameters were as follows: Collision gas (collision-activated dissociation): 6 psi, collision cell entrance potential: 14 volts, and the collision cell exit potential: 4 volts.

Results

Plasma ATCA concentration after cyanide exposure

As shown in Figure 1, the endogenous level of ATCA in these experimental rats was 222 ± 19 (n = 3) ng/mL. The mean of ATCA level in plasma after KCN exposure within the experimental period was 203 ± 26 (n = 30) ng/mL. This result suggests that ATCA concentrations in plasma samples were not increased when the experimental animals (rats) were exposed to the sublethal dose of 4 mg/kg body weight of KCN.

Table 1. Organ collection for ATCA measurement.

Organ sampling groups					
		30 min	60 min	4 h	12 h
4 mg/kg	Rat #4	Terminate			
4 mg/kg	Rat #5	Terminate			
4 mg/kg	Rat #6	Terminate			
4 mg/kg	Rat #7		Terminate		
4 mg/kg	Rat #8		Terminate		
4 mg/kg	Rat #9		Terminate		
4 mg/kg	Rat #10			Terminate	
4 mg/kg	Rat #11			Terminate	
4 mg/kg	Rat #12			Terminate	
4 mg/kg	Rat #13				Terminate
4 mg/kg	Rat #14				Terminate
4 mg/kg	Rat #15				Terminate

Note: Rats #1–#3 were used for the measurement of endogenous ATCA levels in organs.

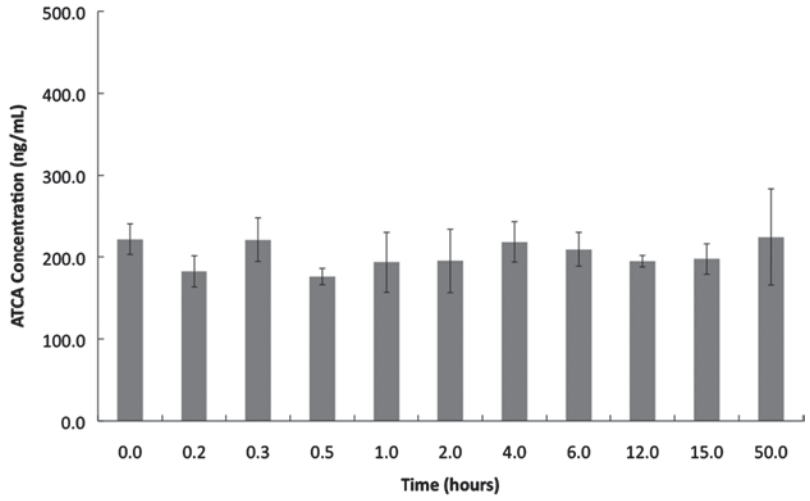

Figure 1. Plasma ATCA concentration after cyanide exposure (4 mg/kg body weight KCN administration (sc) to rats).

ATCA concentrations in organs after cyanide exposure

As shown in Figure 2, ATCA concentration levels in organs were all increased after injection of KCN. The increase in ATCA concentration was most significant in the liver, rising from an endogenous level of 0.85 ± 0.4 (n = 3) μg/g to a level of 33 ± 7 (n = 3) μg/g at 12 h after exposure.

Discussion

The primary purpose of this toxico-kinetics study, funded by National Institute of Health (NIH), was to determine the role of ATCA to serve as a diagnostic biomarker for detecting cyanide exposure. Based on the results of this study, ATCA was not proved to be a good diagnostic biomarker when analysing plasma in the species of rat after sublethal dose cyanide exposure. Ongoing studies are focusing on higher, but still sublethal doses of KCN (8 mg/kg body weight) exposures, and collecting urine as well as an alternative biological matrix to analyse ATCA level as a part of the search for diagnostic biomarkers for cyanide exposure.

When analysing various organs, such as liver, kidney, heart, spleen, brain and lungs, these results suggest that ATCA potentially can serve as a forensic biomarker for post-mortem examination of victims of cyanide exposure. The purpose of forensic toxicological test for cyanide is to provide quantitative analysis of cyanide from samples collected from a victims' body. Usually, whole blood is tested. Natural dietary and pulmonary intake of cyanide from the environment, such as cyanogenic glycosides in food, vehicle exhaust, cigarette, smoke from fires, leads to a non-zero cyanide background level in the body (Noguchi et al. 1988). For example, the burning of nitrogen-containing polymeric materials can introduce sufficient HCN to be lethal for those enhaling the smoke (Ishii et al. 1998). Human endogenous levels of cyanide are typically less than 40 ng/mL in plasma. Cyanide concentrations greater than 2500 ng/mL are fatal in humans. Thus, the lethal dose of HCN of human is about 1 mg/kg body weight. In certain environments such as fires, cyanide can continue to diffuse into a body following death (Karhunen et al. 1991). When victims are removed from such cyanide enriched environments, the rapid decomposition of cyanide in biological matrices results in a systematic decrease in measurable cyanide (McAllister et al. 2008). Therefore, a complementary metabolic biomarker would be useful for opening a larger window for post-mortem analysis, and a more accurate assessment of the causal relationship between cyanide exposure and cause of death.

In the field of cyanide research, it has been well known that detoxification of cyanide by cystine produces ATCA *in vivo* (Wood & Cooley 1956). The formation mechanism of stable cyanide-protein adducts, such as the reaction of cyanide with the C-terminal Cys^{558} Cys^{567} disulfide bond of human serum albumin (Fasco et al. 2011), is similar to that of ATCA. It has been suggested in an *in vivo* study that the reaction of an oxidized disulfide with a sulfur nucleophile from glutathione could be a plausible origin for ATCA (Zottola et al. 2009). It is likely that endogenous levels of ATCA in each organ reflect the availability of disulfide and the concentration of glutathione in those organs. Thus, endogenous ATCA levels may give an estimate of each organ's capacity for cyanide detoxification. For example, the results showing lower endogenous ATCA levels in the brain may be consistent with lower capacity for cyanide detoxification in this organ. This lower capacity might be partially due to the absence of rhodanese enzymes in the brain.

The pathway producing ATCA was estimated approximately 20% of cyanide metabolism (Baskin & Brewer 1997). The quantity of ATCA produced has been found to be directly proportional to the amount of cyanide metabolized. ATCA is metabolically inert in experimental rats and is stable in urine matrix for months in the freezer (Logue et al. 2005). Therefore, ATCA seems a promising candidate as a chemically stable biomarker of cyanide. Although ATCA production is not significant in plasma at sublethal doses of cyanide, its concentration in liver samples was significantly increased. Future studies will also focus on the determination of ATCA concentrations in human plasma and liver samples, and stability of

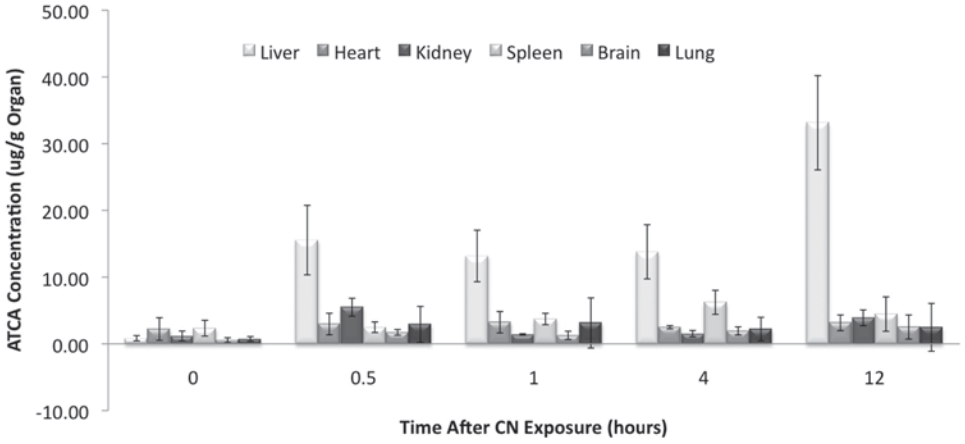

Figure 2. TCA concentrations in organs after cyanide exposure (4 mg/kg body weight KCN administration (sc) to rats).

ATCA in the post-mortem matrix as a part of future forensic research on ATCA. These studies will be supported by the College of Criminal Justice of SHSU, and will be independent from the present NIH–supported diagnostic biomarker studies. In an investigation of death in a fire scene, one of the important questions is whether the victim was alive at the beginning of fire. An interesting project for future study is to see whether post mortem HCN diffusion can produce ATCA in liver samples.

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Declaration of interest

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