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Short communication

Plasma persistence of 2-aminothiazoline-4-carboxylic acid in rat system determined by liquid chromatography tandem mass spectrometry

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ABSTRACT

2-Aminothiazoline-4-carboxylic acid (ATCA) was intravenously injected to rats in order to investigate its plasma distribution. ATCA was extracted from plasma samples by solid phase extraction (SPE) and molecularly imprinted polymer stir bar sorption extraction (MIP-SBSE). Detection and quantification of ATCA were achieved by using liquid chromatography–tandem mass spectrometry (LC–MS/MS). It was found that the intravenously injected ATCA concentration quickly decreased to half within 2.5 h in the rat system. However, after 2.5 h, the concentration of ATCA in plasma stayed constant at least 5 folds above the endogenous ATCA level for more then 48 h. This finding can be used for evaluating ATCA's diagnostic and forensic value as a biomarker for cyanide exposure.

1. Introduction

Formation of thiocyanate (SCN^-) in the presence of the sulfurtransferase, also known as rhodanese, is a well-known cyanide metabolic pathway and has been well studied and published [1–5]. Wood and Cooley reported in the early fifties that 2-aminothiazoline-4-carboxylic acid (ATCA) is formed in the body from cystine and cyanide *via* beta-thiocyano-alanine [6]. The roles of any enzymes in ATCA formation in the body has not been ruled out. Under normal condition (pH 7.4), 56% of cyanide is transformed to SCN^-, and just 23% is converted to ATCA [7]. Cyanide also reacts with certain disulfides within human serum albumin (HSA) *via* a stable cysteine-SCN adduct [8,9]. Although formation of SCN^- is regarded as the main pathway of cyanide detoxification, the alternative biotransformation pathway of ATCA formation becomes more important under some conditions, such as chronic cyanide exposure when cyanide depletes sulfur donors preventing the formation of SCN^- [10,11], and during metabolic acidosis (the optimal pH for rhodanese is 8.5).

In order to study the *in vivo* behavior of ATCA, it was necessary to develop suitable and sensitive analytical methods to detect it in biological samples. Recent analytical method developments for ATCA determination made it possible to establish the endogenous level of ATCA in the studied biological matrices and detect cyanide exposure [12]. Baskin et al. reported a spectrophotometric method, and indicated a demand for more sensitive and specific analytical techniques for ATCA determination [13,14]. Logue et al. determined ATCA in human urine and plasma for smokers and non-smokers using a GC–MS [15,16]. The methods were sensitive and required a derivatization step with MSTFA ((N-methy-N-(trimethylsilyl) trifluoroacetamide) in hexane. Jackson et al. reported a new, simple analytical method without derivatization for analyzing ATCA in biological samples by employing molecularly imprinted polymer stir bar sorption extraction (MIP-SBSE) and electrospray ionization tandem mass spectroscopy [17].

For this present study, ATCA samples were analyzed by a modified LC–MS/MS analytical method with a solid phase extraction (SPE) sample preparation, compared to the MIP-SBSE extraction technique. To explore the potential that ATCA might serve as a diagnostic biomarker, it is important to carry out more detailed studies of its *in vivo* behavior and stability. The purpose of this study was to

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measure ATCA plasma distribution after direct intravenous injection of ATCA into rat, and to examine its persistence in the plasma by utilizing our latest LC–MS/MS analytical technique with SPE sample preparation.

2. Materials and methods

2.1. Chemicals and samples

All solvents used in this study were HPLC grade. Trifluoroacetic acid (TFA) was purchased from EMD Chemicals (Gibbstown, NJ, USA) and used to prepare 0.5% (v/v) TFA in methanol as the mobile phase. 2-Aminothiazoline-4-carboxylic acid (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) and used as an internal standard for calibration. Potassium cyanide (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). Molecularly imprinted polymer stir bar was prepared in house [17].

2.2. Animals

250–300 g male rats (CD® IGS) with catheters implanted were purchased from Charles River (Charles River Breeding Laboratories, Inc., Wilmington, MA). The experimental animals were housed in room temperature and light controlled rooms ($22 \pm 2\,^{\circ}C$, 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 AAALAC (American Association for the Assessment and Accreditation of Laboratory Animal Care, International) and this animal study was approved by the IACUC (Institutional Animal Care and Use Committee) at Sam Houston State University (SHSU).

2.3. Animal treatments and sample plasma preparation

To measure the persistence of ATCA in plasma 100 mg/kg-bodyweight of ATCA standard was injected by tail vein in a group of 3 rats. Blood samples were taken at the following time intervals: 5, 15, 30, 60 min, and 2, 4, 6, 12, 24, 48 h after ATCA injection. The average volume of blood obtained was 0.25 mL, and the blood was placed into Eppendorf tubes with 40 μL of 10 mg/mL of heparin in water. With this design of experiment, 2.5 mL of blood (10×0.25 mL) was taken from each rat in 2 days. Since we did not want to exceed the limitation of the blood amounts what can be taken from each animal, more data points could only be obtained if we setup a new group of animals with other time intervals. For the purpose of determining ATCA persistence in the circulation, we thought that sampling for two days should be enough. The Eppendorf tubes were vortexed, then centrifuged at 13,000 rpm for 5 min. Plasma was then collected. ATCA concentrations in plasma were measured by LC–MS/MS after SPE. Note that if sample is gelled and it is not possible to pipette, use 95 mg of sample and add 600 μL of 1% HCl in acetone in a 15 mL polypropylene conical tube. Cap the tube and vortex it for 2 min and centrifuge the tube for 4 min at 10,000 rpm. Then, take 350 μL of supernatant liquid and add 3.15 mL of 0.1 M HCl for SPE. For the purpose of determining ATCA persistence in the circulation, we thought that sampling for two days should be enough.

2.4. SPE extraction

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 [18,19]. In our work, the MCX SPE cartridges were activated by adding 1 mL of methanol followed by 1 mL DI water. Then the plasma samples were loaded into the cartridges. The cartridges were washed with 1 mL of 0.1 M HCl followed by 1 mL of methanol. After new test tubes were placed into SPE manifold, the ATCA was eluted with 1 mL of NH_4OH. After all solution went through, the vacuum was turned on for 1 min to drain through the eluate completely. The samples were dried with an air stream then 200 μL of mobile phase was added to reconstitute the extracts waiting for LC–MS/MS analysis.

2.5. Molecularly imprinted polymer stir bar extraction

The original extraction method was published by Jackson et al. [17]. Briefly, the glass stir bar with the screw in place was added to a small vial with 0.5% acetic acid in methanol and sonicated for 5 min to clean the stir bar. In a 8 mL vial with the molecularly imprinted polymer stir bar (MIP-SB), 1 mL of 1% aqueous acetic acid and 200 μL plasma sample was added. The 8 mL vial on positioned on the stir plate, set at 700 rpms, and allow sample to run for 30 min to extract the ATCA from the solution. After 30 min of extraction, the magnetic rod was used to put the MIP-SB into a sample vial with 200 μL of mobile phase. The solution and the stir bar were sonicated for 5 min to desorb ATCA. After the 5 min the MIP-SB was removed from the sample solution. The sample solution was analyzed by LC–MS/MS and the MIP-SB was washed with 1 mL 0.5% acetic acid in methanol for regeneration.

2.6. ATCA measurement

In this work, detection and quantification of ATCA was accomplished by using a LC–MS/MS. Sample preparation and instrumentation details of ESI/MS/MS for the detection of ATCA can be found in our previous report [17]. Briefly, a Shimadzu liquid chromatograph (LC-20AT, Shimadzu, Columbia, MD) coupled to a tandem mass spectrometer (API 3200 ESI/MS/MS system, Applied Biosystems, Foster City, CA) was employed for the LC–MS/MS separation, detection and quantification of ATCA. A Luna CN column (3 μm, 100×2 mm; Phenomenex; 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 $147+ \rightarrow 101+$) and ATZA (m/z $145+ \rightarrow 127+$) were monitored under multiple reaction monitoring (MRM) mode. The conditions of ESI were as follows: ionspray voltage: +5500 V, temperature: 450 °C, curtain gas 50 psi, gas 1: 70 psi, gas 2: 20 psi. The MS/MS parameters were as follows: collision gas (CAD): 6 psi, collision cell entrance potential: 14 V, and the collision cell exit potential: 4 V.

3. Results and discussion

3.1. Extraction method comparison

Two extraction techniques, solid phase extraction (SPE) cation exchange and molecular imprinted polymer stir bar (MIP-SB), were compared for ATCA extraction. SPE and MIP-SBSE both have strengths and weaknesses. Using SPE, as shown in Fig. 1, the results show that there are some interferences near the ATCA

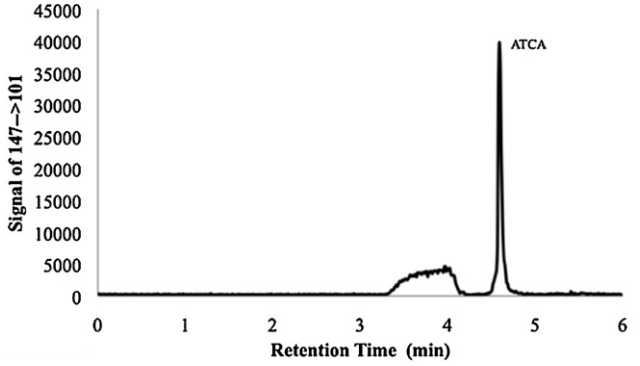

Fig. 1. LC–MS/MS chromatogram for ATCA with SPE extraction (the spiked level of ATCA was 500 ng/mL).

peak due to the biological nature of the extraction. As seen in Fig. 2, when MIP-SBSE was used, ATCA was the only peak appeared in the chromatogram. It suggested MIP-SBSE was more selective. Unfortunately, MIP-SB could not offer enough capacity and suffered from low recoveries when compared to regular SPE technique [17]. Moreover, the SPE–LC–MS/MS offered a good precision and reproducibility. With the use of internal standard calibration, calibration curves yielded an average calibration equation $y = (0.018 \pm 0.00007) \times$ (n=4, RSD of slope = 4%, between days). For a 250 ng/mL sample, %RSD = 5.9% (n = 3) was obtained in a within day analysis. Limit of detection (LOD) and limit of quantitation (LOQ) of the SPE–LC–MS/MS method were 12 ng/mL and 15 ng/mL, respectively. More details of the traditional validation parameters of the SPE–LC–MS/MS method can be found from our previous report [20]. Therefore, SPE was selected for this study as a better extraction method for plasma samples.

3.2. ATCA persistence

The persistence of ATCA is an important factor to determine whether ATCA is a good diagnostic biomarker for cyanide poisoning. A long-lasting biomarker will also offer a better forensic value in most of the criminal cases. In other words, if ATCA is quickly eliminated from the biological system, it will not be a good biomarker for forensic applications. In this study, ATCA was injected to rats in order to see how ATCA concentration changed in plasma samples after intravenous administration. As shown in Fig. 3 (when 100 mg ATCA/kg-body-weight injected), ATCA concentration in plasma showed an initial rapid decrease with a 1/e time constant of 1.26 min that we postulate corresponds to an organ equilibration time. After this equilibration, ATCA concentrations

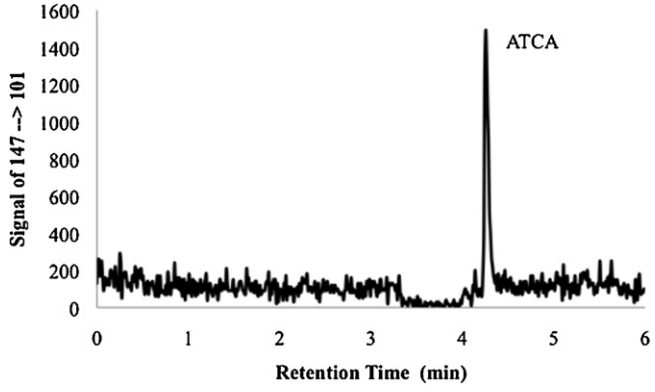

Fig. 2. LC–MS/MS chromatogram for ATCA with MIP-SBSE extraction (the spiked level of ATCA was 500 ng/mL).

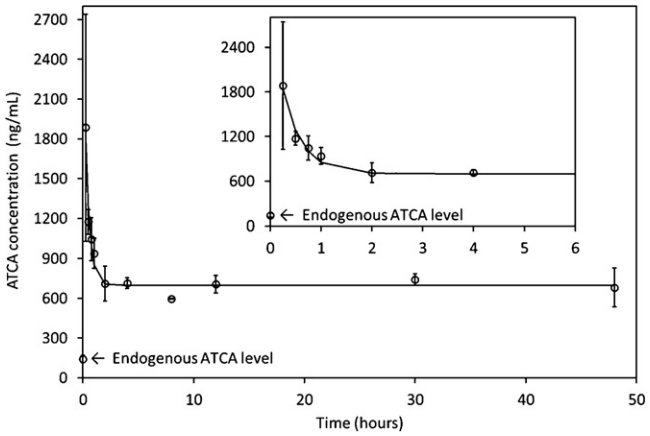

Fig. 3. ATCA concentrations in the plasma samples after intravenously injection of ATCA (100 mg/kg-body-weight) in rats.

stabilize. Specifically, the stabilized ATCA concentration from 2 h to 48 h was 718 ± 78 (n = 6) ng/mL, which is significantly higher then the endogenous level, 141 ± 26 (n = 3) ng/mL. This result demonstrated that ATCA is stable in plasma and metabolically inert for at least 48 h.

3.3. ATCA as a biomarker for CN exposure

Increased concentration of ATCA in human urine were observed in urine [15] and plasma were observed when comparing smokers to non smokers, which suggested that ATCA could be a good biomarker for CN exposure [16]. It is obviously true that if ATCA is quickly eliminated from the biological system, it will not be a good biomarker for cyanide poisoning. The objective of this study was setup for determining the persistence of ATCA in blood, after injecting ATCA directly to the blood stream. The result of this study showed that after an initial loss of ATCA, the remaining amounts of ATCA in blood (600 ng/mL) stayed stable over 48 h. The remaining amount of ATCA was significantly (5×) higher than the endogenous ATCA level without CN exposure. This 48 h persistence in the circulation declares ATCA as a biologically stable CN antidote, with a potential importance to be considered as a CN biomarker. Unfortunately, it has been shown recently that ATCA concentrations in plasma samples were not increased when rats were exposed to a sublethal dose of KCN [20]. Since ATCA blood concentration did not increase after sublethal dose of KCN exposure, it suggested that blood is not a good matrix to detect ATCA as a diagnostic marker for CN exposure. However, ATCA concentration levels in organs, such as liver, were all increased after injection of KCN. These findings were suggesting that ATCA could serve as a biomarker for forensic purposes. It is also known that different species has different metabolizing enzyme systems [1,21,22], what can drive to contradictions in results with metabolites. The role of ATCA as a biomarker for CN exposure should become clear as future investigations of the behavior of ATCA are completed.

4. Conclusion

Our studies focused on determining plasma distribution of ATCA after administering intravenously to rat. From the results, the initial rapid loss of ATCA in plasma is estimated as the equilibrium with the organs in the body. After this equilibrium time ATCA showed a stable persistence over 48 h what confirms that it is a metabolically stable CN antidote. As reported by Petrikovics et al. [20], ATCA has been proved as a forensic diagnostic biomarker, what enhances the forensic importance of ATCA as a chemically stable biomarker.

New studies with prolonged time intervals are needed to determine the actual half-life for ATCA in the circulation following AAALAC guidelines for the practical animal handling issues, such as the limitations of the volume of blood drawn *vs.* body weight.

Conflicts of interest

The authors declared no conflicts of interest with respect to the authorship and/or publication of this article.

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