Global Journal of Life Sciences
2020; 1(1): 24-33.
ISSN Online: 0000-0000; ISSN Print: 0000-0000
Website: http://naturescholars.com
Email: Glo_J_Ls@126.com
Publisher: Scholars Publishing, LLC

Research Article

The Effect of Terminalia Chebula on Metabolism of Radix Aconiti Kusnezoffii

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Received: January 5, 2020; **Accepted**: March 29, 2020; **Published online:** June 7, 2020.
Cite this paper: Lulu Zhao, Shengnan Wang (2020) The Effect of Terminalia Chebula on Metabolism of Radix Aconiti Kusnezoffii. *Global Journal of Life Sciences*, 1(1): 24-33. http://naturescholars.com/gjls.010105.
https://doi.org/10.46633/gjls.010105.
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Abstract

Objective At present, the domestic and foreign research on Terminalia chebula (HZ) and Radix Aconiti Kusnezoffii (CW) is mainly in the toxicity, but how HZ affects the metabolism of CW is not very clear. Therefore, this paper will focus on the study of whether the tannic acid in HZ has an effect on the metabolism of three kinds of double ester aconitine in CW. **Methods** CW is mainly composed of three components, namely aconitine (AC), mesaconine (MA) and hypaaconitine (HA). Metabolism of AC, MA and HA were studied *in vitro* in rat liver microsomes. Samples were taken at different time points. The residual concentration of three aconitines at different time points (0, 30, 60, 90, 120, 150, 180 min) was obtained. The metabolic rate was non-linear regression to the substrate concentration, thus, the curve of reaction speed to the substrate concentration was obtained. Finally, the metabolic parameters the maximum reaction rates (V_{max}), the micron constants (K_m) and the internal removal rates (CL_{int}) of three aconitines were obtained. At the same time, HZ and CW were coincubated to determine whether HZ had any effect on the metabolism of CW. **Results** V_{max} of AC, MA and HA are 387.59 ng·min^{-1}·mg protein^{-1}, 337.83 ng·min^{-1}·mg protein^{-1} and 450 ng·min^{-1}·mg protein^{-1}; K_m are 1124 ng·L^{-1}, 962.8 ng·L^{-1} and 1278 ng·L^{-1}. CL_{int} are 0.34 μL·min^{-1}·mg protein^{-1}, 0.35 μL·min^{-1}·mg protein^{-1} and 0.352 μL·min^{-1}·mg protein^{-1}, respectively. **Conclusion** HZ has a small inhibitory effect on the metabolism of CW.

Key words: Radix Aconiti Agrestis; Terminalia Chebula Retz; Compatibility ratio; Detoxification; Metabolism.

Introduction

The Radix Aconiti Kusnezoffii (CW) , which is spicy, bitter and hot，is the dry root of the Aconitum kusnezoffii Reichb (1). The 2015 edition of the Chinese Pharmacopoeia records that it has the effects of expelling wind and dehumidification, warming and relieving pain, so it is often used to treat wind cold and dampness paralysis, joint pain, abdominal cold pain, cold hernia pain and relief of anesthesia pain (2). Modern pharmacological studies have shown that it has anti-inflammatory, anesthetic, analgesic, anti-tumor and blood pressure-lowering effects (3). However, many studies have suggested that CW is highly toxic, mainly due to its toxic components: aconitine (AC), mesaconine (MA) and hypaaconitine (HA) (4). In clinical applications, it can often cause cardiac dysfunction, dizziness, language difficulties, genotoxicity and reproductive toxicity (5). Terminalia Chebula (HZ), the "king of Mongolian medicine" (6), also known as He Lile, He Li and Sui Fengzi, is the mature fruit of the Combretaceae plant, mainly distributed in the southwestern part of China. The "Mongolian Pharmacopoeia" points out that the formula containing the CW must be matched with HZ (7), In the clinical practice of Mongolian medicine, the commonly used compatibility ratio of CW to HZ is 1: 1 and 3: 1.

At present, research on HZ and CW at home and abroad is mainly about the evaluation of toxicity. Research of Du Hong et al shows that the tannin in the HZ can inhibit the dissolution of alkaloids, causing alkaloids to slowly hydrolyze and reduce the toxicity (8). Song Lin et al. demonstrated that HZ has a palliative effect on arrhythmia caused by aconitine, suggesting a protective effect of HZ against aconitine on the heart (9). Li et al. demonstrated that the compatibility of different proportions of HZ-CW has a great influence on the content of three diester alkaloids in decoction. On one hand, it promotes the dissolution of three diester alkaloids, on the other hand, when the proportion of HZ is high, the hydrolysis of alkaloids is accelerated (10).

In daily life, the body is exposed to foreign substances, such as food, drugs and harmful substances in the environment. At the same time, the body also has its own defense system to dispose these foreign substances. After drugs and exogenous substances entering the body, most of them are metabolized to be excreted (11-13). The most important organ to metabolize the drug is the liver. The cytochrome P450 enzyme system (CYP450), a very important family of oxidases that catalyze the phase I metabolic reaction in the body, is called the liver microsomal mixed function oxidase system. The CYP450 catalyzes a wide range of oxidation reactions and is the main pathway for drug metabolism in the body. Most drugs entering the body undergo biotransformation through this enzyme system (14-15).

AC, MA and HA are the main active ingredients and toxic ingredients of CW. According to the metabolism of AC in liver microsomes of male rats (16), the main metabolic pathways are: 18-O-demethylation, 16-O-demethylation, 18-O, 16-O dedimethylation, N-deethylation, 3-dehydrogenation, 8-O-demethylation; the main sub-enzymes involved in this metabolic pathway are CYP3A4 and CYP1A1/2. When one drug accelerates the biotransformation of another drug through the same or different enzyme pathways, this increases the concentration and activity of the CYP450 enzyme system, thereby accelerating the metabolism of many drugs. This effect is also called enzymatic effect. Conversely, enzyme inhibition is when the CYP450 enzyme system is inhibited (17). In this paper, we will focus on whether tannins in gardenia have an effect on the metabolism of three diester aconitines in Radix Aconiti.

1. Materials and methods

1.1 Reagents and materials

1.1.1 Instrument

Desktop high speed refrigerated centrifuge (SIGMA, 3-18K, CHRIST, Germany); Electronic PH meter (METTLER, Z23906212A2); Homogenizer (Gene Co., Ltd., BBY-8515SR); Digital thermostatic water bath (Guohua Electric Co., Ltd., HH-6); Ultra-pure water machine (Sichuan Youpu Chaochun Technology Co., Ltd., UPHW-II-90T); Dionex UltiMate 3000 Rapid Ultra Performance Liquid Chromatograph (Thermo-Fisher, USA); Q Exactive Quadrupole - MCO-15AC type (SANYO Corporation).

1.1.2 Reagents and drugs

HZ (batch number: 20140121) was purchased from the medicinal materials market in Hohhot, and was identified by the relevant professional professor as the dried fruit of the family Gentianaceae (Terminalia chebula Retz.); CW (lot number: 20140101) was purchased from the medicinal materials market in Hohhot, and was identified by the relevant professional professors as dry roots of Aconitum kusnezoffii Reichb.; AC standard (batch number: 110797-201108, purity: 98%), MA standard (batch: 110799- 201107, purity: 98%) and HA standard (110798-201307, purity: 98%) were purchased from China National Institute for Food and Drug Control; sodium carboxymethyl cellulose (Shanghai Yuanye Company, article number: 11615C). Other reagents were analytical reagents.

1.2 Experimental animals

40 SD rats, ♂, male, clean grade, weight 200±20g, were purchased from Speyford (Beijing) Biotechnology Co., Ltd. Quality certificate number: SCXK (military) 2016-0002, housed in the Experimental Animal Center of Inner Mongolia Medical University, at room temperature (20 ± 2 °C), in 72% humidity, 12 hours day and night shift, provided with rodent diet and water *ad libitum.*

1.3 Experimental methods (Figure 1.)

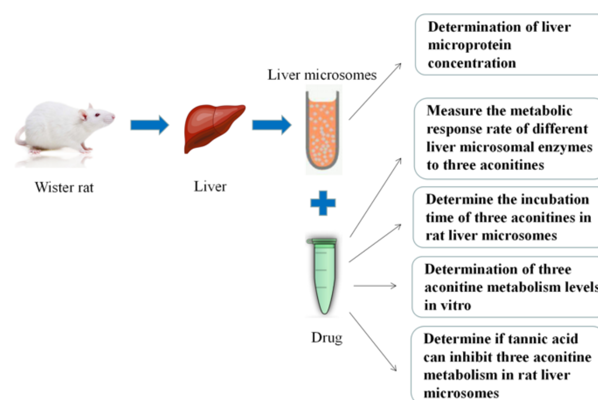

Figure 1. Flow chart of the experimental method.

1.3.1 Preparation of Standards

Dry AC、MA、HA and tannic acid was accurately weighed (1.00 mg standard) in a brown volumetric flask and dissolved in absolute ethanol to obtain 1% solution of each compound. These solutions were diluted with neutral phosphate buffer solution（KPI）KPI solution to obtain 1 mg/mL AC、MA、HA and tannic acid standard stock solution and stored at 4 °C for later use.

1.3.2 Preparation of related solutions

(1) KPI (50 mM) solution is formulated: Weigh accurately 1.20 g KH_2PO_4 and 9.74 g $K_2PO_4 \cdot H_2O$ dissolved in 100 mL of deionized water, add 900 mL of deionized water, adjust the pH of the solution to 7.4, and store at 4 °C until use.

(2) Sucrose (250 mM) solution is formulated: Weigh accurately 1.72 g of sucrose, dissolved in 20 mL of deionized water to obtain a 250 mM sucrose solution, and stored at 4 °C until use.

(3) 88 mmol/L $CaCl_2$ solution is formulated: Weigh accurately 0.9768 g of anhydrous $CaCl_2$, dissolve it in distilled water, dilute to a 100 mL volumetric flask, and place in a refrigerator at 4 °C for use.

1.3.3 Preparation of liver particles

Before the experiment, the rats were fasted

but not water, and the rats were anesthetized with acetic acid (the following are performed on an ice bath). The abdominal cavity was quickly opened, and the liver was lavaged through the hepatic portal vein with ice-cold saline. At the same time, the inferior vena cava was cut open and released. When the liver is lavaged to earthy yellow, the liver is quickly cut, dry before weighing. 4 mL of ice-cold saline is added per gram of liver. The liver homogenate is prepared by homogenizer on an ice bath. The liver homogenate was centrifuged at 10000 g for 20 min at 4 °C. The supernatant was added with ice-cold 88 mmol/L $CaCl_2$ solution (0.1 mL per mL of supernatant), mixed and ice bathed for 5 min. After centrifugation at 13 000 rpm for 3 h at 4 °C, the supernatant was discarded, and the precipitate was resuspended with sucrose (250 mM) and stored at -80 °C until use.

1.3.4 Determination of protein concentration of the liver microparticle

The concentration of the liver enzyme microsomal protein was determined according to the procedure of the BCA kit. The specific operation steps are shown in Table 1.

Table 1: Operation steps of the BCA protein kit.

	Blank hole	Standard hole	Measuring hole
Double distilled water (uL)	10		
563 ug/mL standard (uL)		10	
Sample to be tested (uL)			10
Working solution (uL)	250	250	250

After adding the reagents according to the above method, mix well, incubate at 37 ℃ for 30 minutes, read the absorbance at 562 nm with a microplate reader, and calculate the protein concentration according to the following formula. Total protein concentration = (measured OD value - blank OD value) / (standard OD value - blank OD value) × standard concentration × dilution factor

1.3.5 Establishment of *in vitro* incubation system

(1) The entire system was carried out in a 50 mM KPI buffer solution having a pH of 7.4 and a volume of 3.6 mL.

(2) 80 μL rat liver enzyme microsomes was added (final concentration in the incubation system is 0.4 mg/mL) to the KPI buffer solution.

(3) NADPH regeneration system (200 μL of solution A, 400 μL of solution B) was added to the KPI buffer solution and incubated for 5 min at KPI buffer solution.

(4) Different concentrations of the drug were added, and samples were collected at 0, 30, 60, 90, 120, 150, 180 min.

1.3.6 Sample processing

300 μl of the sample was placed in a 1.5 mL centrifuge tube, and 3 times the amount of acetonitrile was added to precipitate the protein. 5 μl of internal standard reserpine was added for each 1 ml sample (the final concentration of reserpine in the sample was 0.25 μg/mL). The samples were centrifuged at 12 000 RPM at 4 ℃ for 10 min.

1.4 Test conditions

(1) Chromatographic conditions: the column was a Hypersil Gold C18 column (specification 100 mm × 2.1, 1.9 μm, Thermo-Fisher, USA); mobile phase A (0.1% formic acid-water): mobile phase B (acetonitrile) = 65 : 35, flow rate of 0.2 mL / min, column temperature of 30 ° C, injection volume of 3 μL.

(2) Mass spectrometry conditions: A positive ion scanning mode under an electrospray ion source was used. The ionization source (ESI) temperature was 300 °C; the capillary voltage was 3.5 kV; the S-lens RF level was 50; the ion transport tube temperature was 300 °C; the sheath gas pressure was 40 arb; the auxiliary gas pressure

was 2 arb. The scan mode is Full MS/SIM and the ions used for quantification are m/z: $[M+H]^+$ 646.322 (AC), m/z: $[M+H]^+$ 616.311 (HA) and m/z: $[M+H]^+$ 632.307 (MA).

1.5 Statistics

All data are expressed as mean ± standard deviation (X ± S). Statistical analysis was performed using SPSS 20.0 software. One-way analysis of variance was used for the multiple groups. $P<0.05$ was considered to be significant.

2. Results

2.1 Preparation of standard curve

Standard stock solutions of the three aconitines were added to the KPI buffer to prepare standard solutions with the corresponding concentration of 125, 200, 500, 1000, 2000, 4000, 6000 ng/mL, and quantified by internal standard method. Taking the sample concentration as the abscissa (X), the ratio of the sample peak area to the internal standard peak area is plotted on the ordinate (Y) and the standard curve equation is obtained by linear regression analysis. In addition, the lowest quantitative concentration was calculated as signal to noise ratio (S/N) >10, and the results are shown in Table 2. The results showed that the linear relationship of sample concentration and ratio of sample peak area to internal standard peak area was good within the measurement range. The lowest quantitative concentration of AC MA and HA was 12.5 ng/mL.

Table 2: Standard curve equation and lower limit of quantification of three aconitines in KPI buffer solution.

Compound	Regression equation	Correlation coefficient（r^2）	Linear range (ng/mL)	Quantification limit (ng/mL)
AC	Y=0.0115X + 0.8251	0.999	125-6000	12.5
MA	Y=0.005X + 0.8067	0.997	125-6000	12.5
HA	Y=0.0154X - 0.829	0.997	125-6000	12.5

2.2 Methodological investigation

The HBSS buffer solution of 1mL was precisely absorbed, and three standard aconitine solutions with concentration of 1 g/mL were prepared, respectively. The retention time of AC, MA and HA were 4.23 min, 3.17 min and 4.18 min respectively under the selected chromatographic and mass spectrometry conditions. The components were well separated, no impurity peak interference was observed and the analytical method was feasible (Fig. 2).

2.3 Metabolism of AC、MA and HA

2.3.1 Effect of liver microsomal protein concentration on metabolism of three aconitines

The protein concentration measured by the BCA kit was 18.97 mg/mL. In the KPI buffer solution, the effects of different concentrations of liver microsomal enzyme protein on the metabolic rate of three aconitines were investigated. Based on the pre-experiment, the concentration of the three aconitines was set as 3 μg/mL and the rat liver microparticles homogenate with a concentration of 0.1, 0.2, 0.8, 1.0 and 2.0 mg/mL was sequentially used. The concentration of liver microsomal enzyme protein was plotted on the abscissa and the metabolic rates of three aconitines were plotted on the ordinate (Fig. 3). When the concentration of rat liver microsomal enzyme protein is 0.4 mg/mL, the reaction rate tends to be the maximum, so the final mass concentration of rat liver microsome incubation enzyme protein was set as 0.4 mg/mL.

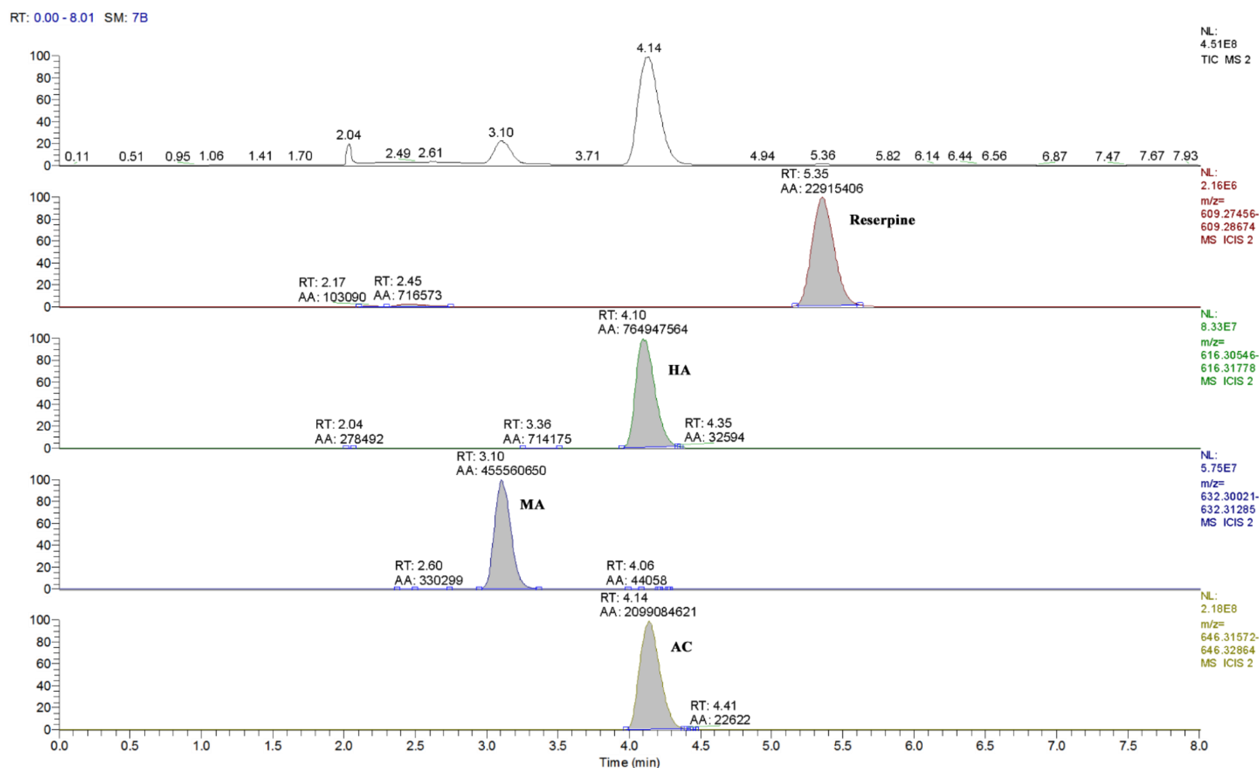

Figure 2 Flow chromatograms of three aconitine and internal standard (reserpine) in HBSS buffer solution.
Note: AC is aconitine; MA is mesaconine; HA is hypaaconitine.

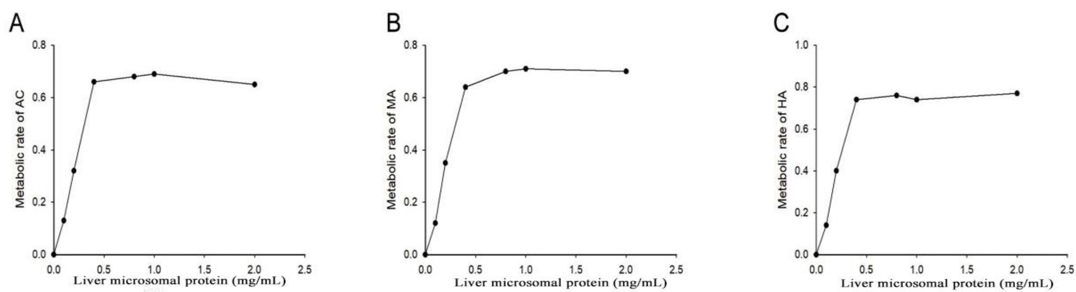

Figure 3. Effect of liver microsomal protein concentration on metabolism of three aconitines.
The effects of different liver microsomal protein concentrations on the metabolism of AC (A)、MA (B) and HA (C).

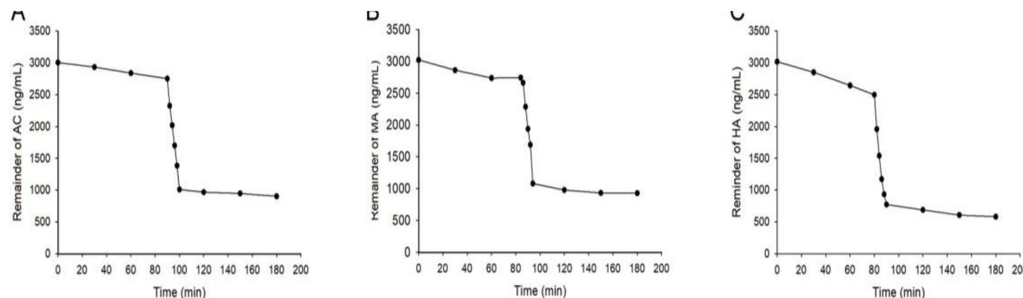

Figure 4 Effect of different incubation times on the metabolism of three aconitines.
A, B, C correspond to the effects of different incubation times of AC、MA and HA on metabolism.

2.3.2 Effect of time on the metabolism of three aconitines

The concentration of three aconitines used in this study was 3 μg/mL and the concentration of liver microsomes was 0.4 mg/mL. What was/were incubated in a 37 °C water bath for 0-180 min. AC was found at 90-100 min. MA was found at 84-94 min, HA was linearly eliminated at 80-90 min. Therefore, the incubation times of AC, MA, and HA in rat liver microsomes were 90-100 min, 84-94 min, and 80-90 min, respectively (Fig. 4).

2.3.3 *In vitro* metabolism of three aconitines

The results obtained were taken as the 100% ratio of the three aconitines and the internal standard reserpine peaks detected at time 0. The ratio of the peak area of the samples at other times was compared with the results obtained to obtain the remaining substrate level and the natural pair of remaining substrate levels were taken. The graph is plotted against time, and the slope of the line is the substrate elimination parameter (K_{dep}) of the concentration, as in equation (1). K_{dep} is plotted by substrate concentration, and equation (3) is linearly regressed. Combining equation (2), the micron constants （K_m） and the maximum reaction rates （V_{max}）are finally obtained. The results are shown in Figure 5 and Table 3.

$$[C_S]=[C_S]e^{-k_{dep}t} \quad (1)$$

$$-\frac{1}{K_{dep}}=\frac{1}{V_{max}}C_S+\frac{K_M}{V_{max}} \quad (2)$$

$$\frac{V_{max}}{K_m}=CL_{int} \quad (3)$$

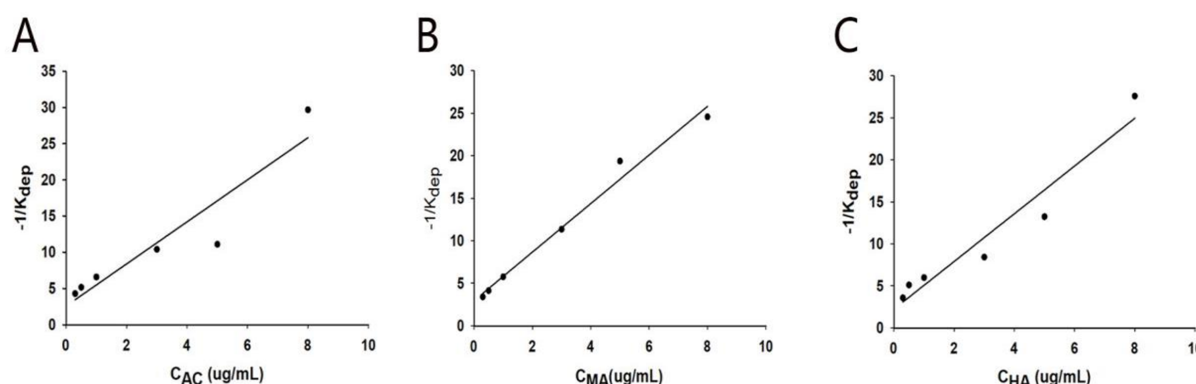

Figure 5. Linear relationship of elimination parameters of three aconitine metabolizing substrates.
Note: AC is aconitine; MA is mesaconine; HA is hypaaconitine.

Table 3: Related metabolic parameters of three aconitines.

Group	K_m（ng/mL）	V_{max}（ng/min/mg）	CL_{int}（mL/min/mg）
AC	1124	387.59	0.34
MA	962.83	337.83	0.35
HA	1278	450	0.352

2.3.4 Effects of Tannin on the Metabolism of The Three Aconitines

The metabolic rate was calculated using a negative control （only tannic acid was given and no drugs were given.） as a reference. The results showed that tannic acid inhibited the metabolism of three aconitines in rat liver microparticles in a concentration-dependent manner, but the inhibition was not significant difference. When the

concentration of tannic acid was 5 μg/mL and the concentration of three aconitines was 1 μg/mL, the metabolic inhibition rate of tannic acid on AC, MA and HA were 13%, 20% and 13%, respectively. Combining references and the compatibility ratios of practical applications, we conclude that the effect of HZ on the metabolism of CW is small. As shown in Figure 6.

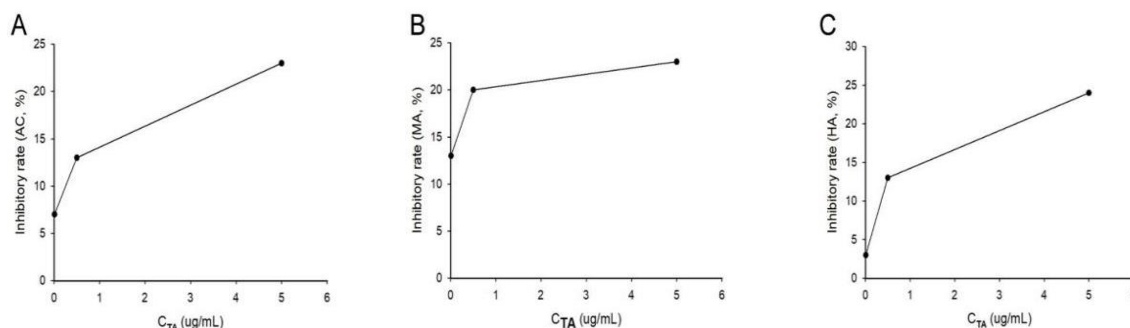

Figure 6. Effect of tannin on metabolism of the three aconitines.

Note: AC is aconitine; MA is mesaconine; HA is hypaaconitine; TA is tannic acid.

3. Discussion

In this study, we studied the effects of HZ on the metabolism of CW from the perspective of pharmacokinetics, and obtained the relevant metabolic parameters of three aconitines. The metabolic effects of noptine are minimal. At present, there are two ways in which the enzyme reaction rate is expressed by the amount of product produced per unit time and the amount of substrate elimination per unit time. We use the amount of substrate elimination, without knowing the metabolic pathways of the substrate, the types of metabolites and the standard products of metabolites. The kinetic parameters are calculated according to the corresponding mathematical formulas. Therefore, it is a convenient and fast method for determining enzyme kinetic parameters (18). This method has great value in the early development of drugs. We measured the concentration of liver microsomal protein and also used CO reduction differential spectroscopy to investigate the activity of CYP450 enzyme. The results showed that the prepared rat liver enzyme microsome activity was higher than control group (19), so, the rat liver enzyme microsomes can be used for experiments. However, due to the instability of the prepared CO, the results of this part of the experiment are not shown.

Domestic and foreign studies on the toxicity of CW found that it can cause four aspects including cardiotoxicity, neurotoxicity, genetic toxicity and reproductive developmental toxicity. Among them, the most fatal and most common toxic reaction is cardiotoxicity. It can be seen that obvious cardiac dysfunction appears. In severe cases, it can cause circulatory system failure and shock death (20). By reading the relevant domestic and foreign literature reports, the main clinical manifestations of CW poisoning are rapid supraventricular tachycardia, cardiac toxicity with accelerated heart rate, atrioventricular block, and ventricular tachycardia (21). Vomiting, chest tightness, numbness of the limbs, blurred vision, and abdominal pain and diarrhea. After aconitine poisoning, the glutamine and creatinine content in the body decreased significantly, which proved to be more toxic. But so far, it is not particularly clear how HZ affects the metabolism of CW. Our results indicate that HZ has little effect on the metabolism of CW. The possible reason is that the main component of HZ is neither an inhibitor nor a promoter of CYP450 enzyme.

In general, based on the previous efficacy, we started from pharmacokinetics and studied the effect of HZ on the metabolism of CW, and the results showed that the effect of HZ on the metabolism of CW was small. This provides a scientific explanation for the classic compatibility between HZ and CW, and provides a basis for how HZ can solve CW poisoning, which is helpful for scholars to start related research from other aspects.

Declarations

1) Consent to publication

We declare that all authors agreed to publish the manuscript at this journal based on the signed Copyright Transfer Agreement, and followed publication ethics.

2) Ethical approval and consent to participants

Not applicable.

3) Disclosure of conflict of interests

We declare that no conflict of interest exists.

4) Funding

This work was supported by grants from the National Natural Science Foundation of China (grant 81560685).

5) Availability of data and material

We declare that the data supporting the results reports in the article are available in the published article.

6) Authors' biography

Authors contributed to this paper with the design (Lulu Zhao), literature search (Lulu Zhao), revision (Lulu Zhao and Shengnan Wang), editing (Lulu Zhao and Shengnan Wang) and final approval (Lulu Zhao).

7) Acknowledgement

None

8) Authors' biography

None

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