



SHORT COMMUNICATION

Use of liquid chromatography-tandem mass spectrometry for determination of higenamine in urine following oral administration of traditional Chinese medicine

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Abstract

Since higenamine (HG) was first included in the World Anti-doping Agency (WADA) 2017 Prohibited List, an increasing number of plants have been found to contain this ingredient. As a result, doctors are hesitant to prescribe traditional Chinese medicine (TCM) to athletes. Thus, it is very important to assess the risks of doping violations due to HG following the oral administration of TCM. We determined the drug concentration-time curves for HG in urine by liquid chromatography-tandem mass spectrometry (LC-MS/MS) after single or multiple administrations of lotus seed powder on volunteers, the single dose was equivalent to 750 µg of HG, and the multiple doses were equivalent to 90 µg of HG each, 3 times daily for 5 consecutive days. For the single-dose group, the HG could be detected in urine 0.5 h after administration and reached a maximum concentration of 16.5 ng/mL 1 h after administration. For the multiple-dose group, the HG concentrations in urine showed two peaks at 29 and 77 h post-administration with 22.6 and 23.1 ng/mL, respectively. At the dosage used in this study, the maximum concentration of HG in some urine samples exceeded the WADA limit of 10.0 ng/mL; the risk was still very high, so athletes must avoid this amount of HG when using TCM. In addition, our study provided further data supporting the presence of sulfonated metabolites of HG in urine samples.

KEYWORDS

anti-doping, higenamine, traditional Chinese medicine (TCM), UPLC-MS/MS, WADA

1 | INTRODUCTION

Higenamine (HG), also known as norcoclaurine or demethylcoclaurine (Figure 1), is a nonselective β_2 agonist.^{1,2} Pharmacological studies have shown that the compound has an obvious cardiotoxic effect³ and appears to control cardiac electrophysiology through its predominant effect on sinoatrial node cells.⁴ A recent study confirmed that the

functional groups of HG, except for the 4'-hydroxy group, are required to enhance glucose uptake and that the S-isomer shows a greater ability to enhance glucose uptake than the R-isomer.⁵ HG was also identified as a novel α_1 -adrenergic receptor antagonist.⁶ Due to the above physiological activity, this compound has been added to a large number of dietary supplements and weight-loss products, and there is also a trend of abuse in athletes.⁷ The World Anti-doping

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Agency (WADA) clearly listed HG under the S3 (β_2 agonist) group in the International Standard Prohibited List since 2017 and banned HG use both in- and out-of-competition.⁸ However, in the TD2022MRPL document, an exception was made for HG; that is, if the concentration in urine is less than 10 ng/mL, adverse analytical findings (AAFs) are not reported.⁹ Currently, an increasing number of plants, such as Gnetum, lotus, *Annona squamosa*, aconite, and *Lindera aggregata*, have been determined to contain HG, and the reported content of HG in most natural plants ranges from 0.2 to 70 $\mu\text{g/g}$ ^{10,11}; many of these plants are commonly used in traditional Chinese medicines (TCMs). As a result, doctors are hesitant to prescribe TCMs to athletes, even though it has proven very useful in treating sports injuries. Thus, it is very important to assess the risks of doping violations due to HG following the oral administration of TCMs. Although similar application of lotus seeds with known content of HG to assess the risk of AAFs has been reported in the literature, the dosage of HG in the literature is much larger than the actual content of HG in commonly used TCMs.^{12,13} In this study, the content of HG in the self-made lotus seed powder used is closer to that in commonly used TCMs, which should have more practical reference value.

2 | MATERIALS AND INSTRUMENTATIONS

2.1 | Standards and reagents

Methanol and acetonitrile (LC-MS grade) were supplied by Thermo Fisher Scientific. HG (LOT: Z28J11B116887) was purchased from Shanghai Yuanye Bio-Technology. Dobutamine hydrochloride (Dobu, internal standard [IS]) (LOT:101185-201202) was supplied by the China Institute of Food and Drug Test (Figure 1). Formic acid (LC-MS grade) was supplied by Shanghai Macklin Biochemical. Purified water was prepared in the laboratory (RO-MB water purifying instrument, conductivity: $\leq 0.01 \mu\text{S/cm}$).

Stock solutions of standard substances were prepared in methanol to a concentration of 1 mg/mL and stored at -25°C .

2.2 | Sample preparation

2.2.1 | Drug preparation

Lotus seeds and plumules were mixed and prepared as a fine powder, temporarily called lotus seeds powder, and its HG content

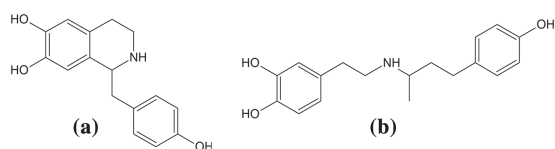


FIGURE 1 Chemical structure of higenamine (C₁₆H₁₇NO₃), MW: 272.1 Da (a); chemical structure of dobutamine (internal standard [IS]) (C₁₈H₂₃NO₃), MW: 301.4 Da (b)

was determined by UPLC–tandem mass spectrometry (MS/MS) (value: 75 $\mu\text{g/g}$).

2.2.2 | Healthy subjects

This study was approved by the hospital's ethics committee and complied with the ethical principles of the "Declaration of Helsinki." Each subject signed an informed consent form. The selection criteria for subjects were as follows:

1. Age: 19 ~ 50 years;
2. Body mass index (BMI): 18 ~ 25 kg/m²;
3. Bodyweight: ≥ 50 kg (male) or ≥ 45 kg (female);
4. Diastolic blood pressure (dbp): ≤ 12 kPa, systolic blood pressure (sbp): ≤ 18.7 kPa, heart rate (Hr): 45 ~ 90 bpm;
5. Routine urine and blood test results were required to be within the normal range before administration;
6. No recent medical history, no serious illness; and
7. No history of allergies or of serious adverse drug reactions.

2.2.3 | Dosing and urine sample preparation

Single-dose regimen

A total of 10 g of lotus seed powder was administered orally with warm drinking water on an empty stomach. Five milliliters of mid-stream urine sample was collected before administration (0 h) and post administration at 0.5, 1.5, 2, 4, 6, 8, 12, 24, 48, and 72 h, and the urination time and volume of all subjects were recorded. During the collection of urine samples, the subjects were given a unified standard diet. The urine samples were sealed and stored at -25°C for subsequent analysis.

Multiple-dose regimen

Lotus seed powder was taken orally on an empty stomach, 1.2 g each time, 3 times daily, for 5 consecutive days. Five milliliters of mid-stream urine sample was collected every 12 h from pre-dosing (Day 0) to 3 days after drug discontinuation (day 7), and the urination times and volumes of all subjects were recorded. There were no specific dietary restrictions during urine sample collection. The urine samples were sealed and stored at -25°C for subsequent analysis.

Sample preparation

In our experiment, Dobu, similar in structure to HG, was added as the IS by referring to the reported test method,¹⁴ and the urine samples were prepared by the direct dilution method (dilute-and-shoot, DaS) reported previously.¹⁵ A 500 μL urine sample was transferred precisely to an Eppendorf 1.5 mL tube, and 100 μL of 1 $\mu\text{g/mL}$ IS solution was added and diluted with 600 μL of 24% methanol solution containing 0.2% formic acid. The diluted urine sample was vortexed and centrifuged for 10 min (12,000 r/min). The supernatant was filtered with a 0.22 μm filter membrane, transferred into a vial, and injected.

2.3 | Instrumentation

2.3.1 | Liquid chromatography

The ultra-high performance liquid chromatography (UPLC) system was an Agilent 1260 Infinity II (Agilent Technologies Inc., CA, USA) instrument equipped with a ZORBAX Eclipse Plus C₁₈ column (1.8 μm, 2.1 × 50 mm, Agilent). The mobile phase consisted of 0.1% formic acid in methanol (A) and 0.1% formic acid in water (B). The ratio of A remained constant at 12% from 0 to 5 min, then increased to 90% in 1 min in a linear manner, was held at 90% for another 3 min, and then decreased linearly from 90% to 12% in 1 min. Then, the column was re-equilibrated for 6 min. The flow rate was constant at 200 μL/min at a temperature of 40°C, and the injection volume was fixed at 1 μL.

2.3.2 | Mass spectrometry

HG was analyzed in multiple reaction monitoring (MRM) mode with an Ultivo-QQQ 6465B (Agilent Technologies Inc., CA, USA) mass

spectrometer equipped with a jet stream electrospray ion source (Agilent). The analytes were detected in positive electrospray ionization (ESI) mode. The drying gas flow rate was set at 10 L/min at 325°C. The sheath gas flow rate was set at 10 L/min at 200°C. The capillary voltage was 4000 V. In MRM, quantification of target analytes was accomplished by tracing specific ion transitions from precursor ion to product ions; that is, the precursor/product ion pairs of m/z 272.1 ≥ 107.0 (quantifier, CE: 22 V), 272.1 ≥ 161.0 (CE: 18 V), and 272.1 ≥ 255.0 (CE: 12 V) were traced for HG and that of m/z 302.2 ≥ 137.0 (quantifier, CE: 22 V) and 302.2 ≥ 107.0 (CE: 25 V) for the IS.

3 | METHODS AND RESULTS

3.1 | Method validation

The selectivity, sensitivity, precision, stability, linearity, matrix effects, and recovery of the method were validated according to the Chinese Pharmacopeia and TD2019MRPL guidelines issued by WADA.¹⁶ The chromatograms of all six blank urine samples obtained by the MRM

TABLE 1 Results of method's validation in higenamine determinations ($n = 6$)

Items		Mean ± SD	RSD (%)	Matrix effect (%)
Matrix effect	LOQ	0.187 ± 0.024	13.00	96.7
	50%	4.967 ± 0.144	2.91	103.2
	100%	9.729 ± 0.376	3.86	101.0
	150%	14.871 ± 0.548	3.69	103.0
Reproducibility ^a		10.301 ± 0.134	1.30	
Recovery ^b		105.03 ± 5.53	5.26	

Abbreviation: LOQ, limit of quantity; RSD, relative standard deviation.

^aReproducibility was obtained by statistical results of six parallel determinations of data by two experimenters in the same laboratory, respectively.

^bRecovery = (Content of HG measured – Content of HG in sample) × 100%/amount of HG spiked.

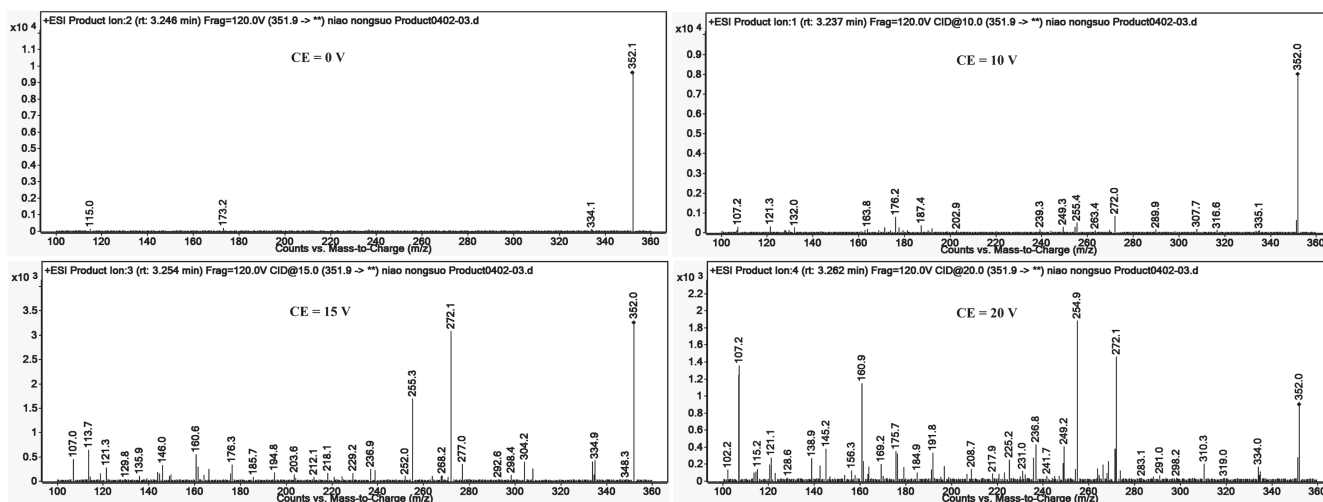


FIGURE 2 The product ion mass spectra extracted at the peak of sulfo-higenamine (HG) on the chromatogram obtained from different collision voltages

method showed no interference at the retention time of HG. The linearity, ranging from 0.193 to 192.9 ng/mL was validated for HG; the coefficient of determination (R^2) for calibration was determined to be 0.9995 (nonweighted). The limit of quantitation (LOQ) was 0.2 ng/mL, for the HG concentration at which the S/N ratio of the chromatographic peak was approximately 10, and the bias of the peak area was $\pm 8.95\%$ ($n = 6$). The matrix effect values for four different concentrations of blank urine samples spiked with HG of LOQ; 5, 10, and 15 ng/mL were determined to be 96.7%, 103.2%, 101.0%, and 103.0%

($n = 6$), respectively. Reproducibility was determined by analysis of samples by two different analysts with six urine samples collected from the same subject after administration of the medicine. The relative standard deviation (RSD) of all 12 values from the two analysts was 1.30%. The recovery of HG was verified by the method of half-volume sample spiked with the standard, the recovery means was 105.0%, and the RSD of all six recovery values was 5.26%. The stability of the working solution at room temperature for 24 h and the stability of urine samples at -25°C for 7 days were investigated, and the

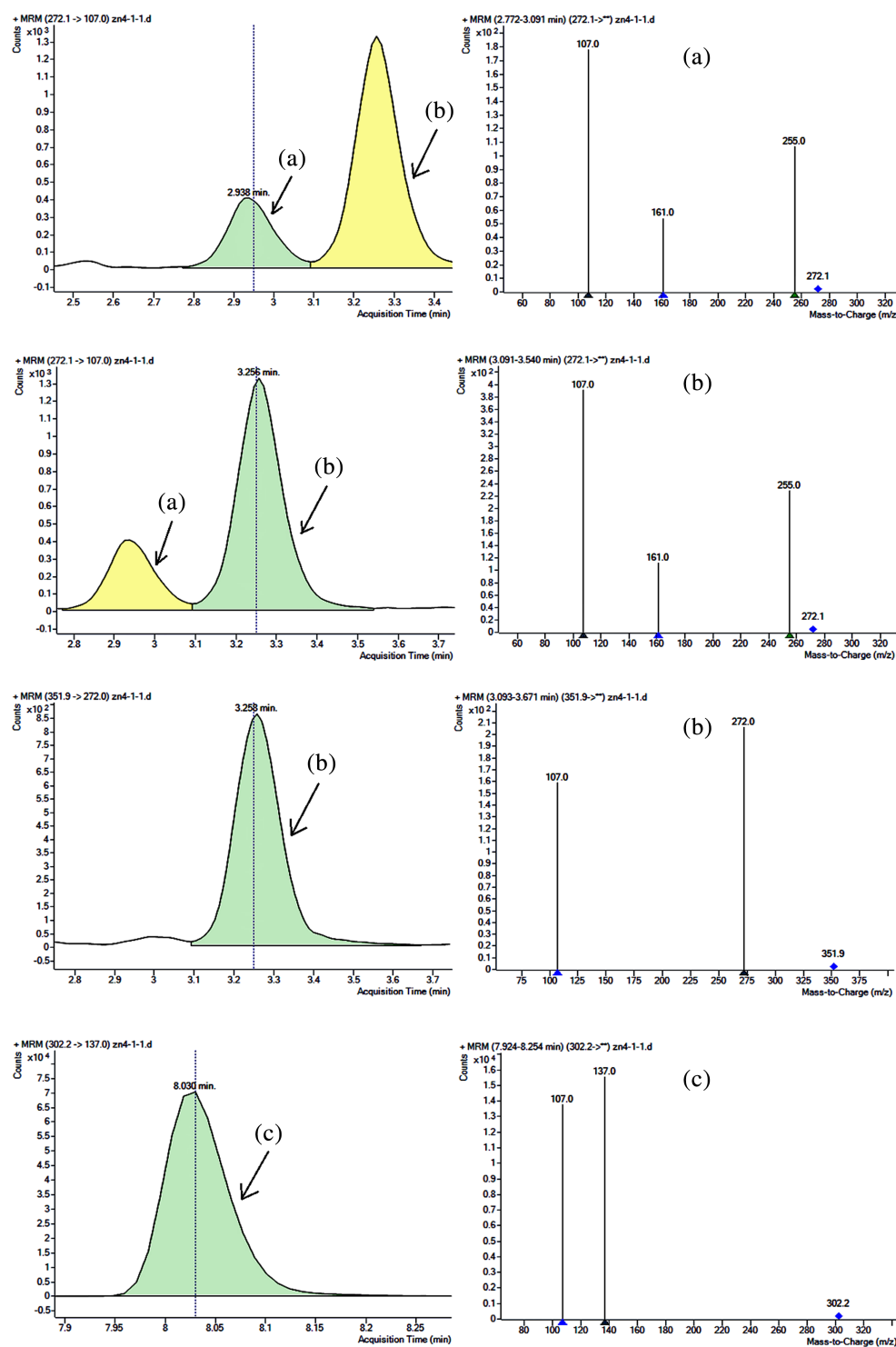


FIGURE 3 Multiple reaction monitoring (MRM) chromatograms and mass spectra of the urine sample collected from post-dosing subject. The peak of higenamine (HG) (a); the peak of sulfo-HG (b); the peak of Dobu. internal standard (IS) (c) [Colour figure can be viewed at wileyonlinelibrary.com]

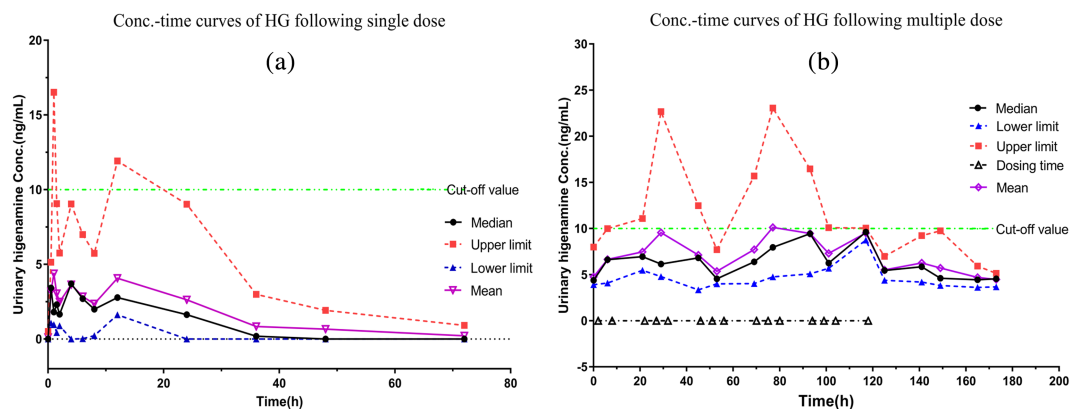


FIGURE 4 Urinary concentration-time curve of higenamine (HG) following single and multiple dose. The medium, upper limit, lower limit, and mean concentration-time curves of HG following single dose (a); the medium, upper limit, lower limit, and mean concentration-time curves of HG following multiple dose (b) [Colour figure can be viewed at wileyonlinelibrary.com]

results showed that the *RSD* of the ratio of HG peak area to Dobu peak area was 2.87% and 4.62% ($n = 6$), respectively (Table 1).

3.2 | Urine sample analysis

Grucza et al. pointed out that a component peak with a molecular weight of 80 Da larger than HG was found in the urine sample of athletes, which was presumed to be a sulfonated metabolite of HG (sulfo-HG).¹⁵ We referred to the literature and added the precursor/product ion pairs of m/z 351.9 \geq 272.0 (quantifier, CE: 15 V) and 351.9 \geq 107.0 (CE: 20 V) to the MRM data acquisition. There was a distinct peak at a position immediately adjacent to the HG peak on the extracted MRM chromatograms. We performed product ion scans with different collision voltages on the urine samples of subjects post administration without changing the chromatographic conditions and extracted the product ion mass spectra at the retention time of sulfo-HG. The mass spectra showed that when the collision voltage was 0 V, almost only a precursor ion at m/z 351.9 was found, attributed to the $[M + H]^+$ ion. With increasing collision voltage, the ion intensities at m/z 272.1, 255.0, 161.0, and 107.0 gradually increased, and the ion intensity at m/z 351.9 was correspondingly reduced (Figure 2). Further experimental data analysis showed that the sulfo-group of the component was very easily separated from the HG skeleton after entering the mass spectrometer, and a considerable proportion of sulfo-HG was decomposed before entering the MS1, and the remaining skeleton was HG. All the above data provided more evidence for the presence of sulfonated metabolites of HG. However, it was tentatively identified, with the location of the sulfate moiety being unknown. The related chromatograms and mass spectra are shown in Figure 3.

For the single-dose group, a relatively large single dose of HG was administered, and the concentration of HG in the urine samples collected from the subjects was determined. A total of 10 subjects were selected; two of them did not eat the unified diet during urine collection, which resulted in abnormal data, and they were excluded

as unqualified subjects. The data of the remaining eight urine samples were statistically processed. The results showed that HG was rapidly absorbed and metabolized after oral administration, and HG was detected in the urine only 0.5 h after administration. Few subjects reached the maximum concentration of 16.5 ng/mL 1 h after administration (Figure S1), and the maximum mean conc. of 4.4 ng/mL also occurred 1 h after administration. The maximum median conc. was 3.7 ng/mL, which appeared 4 h after administration. HG was almost complete excretion in urine at 48 h and could not be detected at 72 h. The renal excretion rate of HG as a free parent compound within 72 h post-dosing was $10.25 \pm 6.07\%$. The related concentration-time curves were shown in Figure 4.

For the multiple-dose group, the urine samples were collected from pre-dosing (Day 0) to the third day after stopping dosing (Day 7). The subjects did not specially arrange a unified diet during the administration period in order to simulate the treatment course of TCMs. The results showed that trace amounts of HG were detected in the urine samples of some subjects before taking the drug, with a concentration of 4.7 ± 1.2 ng/mL. The concentrations of HG in urine samples showed that the mean concentration had a maximum value on the third day (77 h), reaching 10.1 ng/mL, and the median concentration had a maximum value on the fifth day (117 h), reaching 9.6 ng/mL. However, the HG concentration of a few subjects showed double peaks at the 29th and 77th hours, reaching 22.6 and 23.1 ng/mL, respectively (Figure S1). The renal excretion rate of HG as a free parent compound within 7 days was $8.30 \pm 4.43\%$. The related concentration-time curves of HG were shown in Figure 4.

4 | DISCUSSION AND CONCLUSION

In this study, we assessed the risk of HG concentration in urine reaching an illegal dose by taking lotus seed powder with known HG content and determined the drug concentration-time curve of HG in urine after single and multiple administrations. Meanwhile, HG sulfo-conjugate was also detected.

From the results, HG is rapidly absorbed and metabolized after oral administration, and it can be detected in the urine half an hour post administration and does not drop below the detection limit until approximately 72 h later. Under the condition of a single dose, equivalent to 750 µg of HG, or multiple doses, equivalent to 270 µg of HG daily, for 5 consecutive days, the highest concentration of HG in some urine samples exceeded the WADA reporting cut-off of 10.0 ng/mL, the risk of AAFs was still very high, and athletes must avoid this amount of HG when taking Chinese herbal medicines or condiments.

The WADA clearly stipulates in the currently enforced documents that the detection of HG currently only involves the free parent compound,⁹ but the sulfo-HG still deserves to be concerned. This study provided further data to support the presence of sulfonated metabolites of HG in urine.

Okano et al. assessed the AAF risk of HG post administration of Nanten-nodo-ame throat lozenge based on supplementary criteria in 2017, the total dose in 1 day was equivalent to 19.8 µg of HG, and the concentrations of HG in all four volunteers were much lower than the threshold of 10 ng/mL.¹⁷ Based on the data of this study and Okano et al., if the daily dosage of TCM does not exceed an equivalent of 50 µg of HG, the risk of doping violation may be very low, but further studies are needed to ensure that athletes can take TCMs for injury treatment under absolutely safe conditions. In addition, trace amounts of HG can be detected in some pre-dosing subjects, which deserves more study in the future.

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DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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SUPPORTING INFORMATION

Additional supporting information may be found in the online version of the article at the publisher's website.

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