Study on the *in vivo* toxic mechanism of xixin based on trace elements determination by inductively coupled plasma-mass spectrometry

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ABSTRACT

Background: Xixin has been widely used as a traditional Chinese medicine for headache, toothache and inflammatory diseases. Clinical investigation indicated that adverse drug reactions occurred with an overdose of xixin, but the toxic mechanism of xixin *in vivo* based on trace elements has not been researched yet. **Objective:** To explore the *in vivo* toxic mechanism of xixin induced by trace elements. **Materials and Methods:** The contents of trace elements in the serum and liver of mice were determined by inductively coupled plasma-mass spectrometry (ICP-MS) after obtaining xixin extracts. Principal component analysis (PCA) and cluster analysis (CA) were performed between the trace elements' content and dosage using the software GeneSpring 12.1 to analyze the main toxic elements *in vivo*. **Results:** Trace elements' contents were obviously raised after xixin extracts were taken as a dosage of 150 mg/mL and 50 mg/mL, respectively. Na, Ca, Cu and Cd in serum and Ca and Zn in liver were the main trace elements inducing the toxic reaction of xixin. **Conclusion:** Xixin possesses the potential function of indirectly upregulating trace elements *in vivo*. This study, for the first time, elucidated the *in vivo* toxic mechanism of xixin based on trace elements. This method could also be utilized in the research of corresponding aspects.

Key words: *Asarum heterotropoides* Fr. Schmidt var. *mandshuricum* (Maxim.) Kitag, cluster analysis, inductively coupled plasma-mass spectrometry, principal component analysis, toxicity

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INTRODUCTION

Xixin, *Asarum heterotropoides* Fr. Schmidt var. *mandshuricum* (Maxim.) Kitag, a herb belonging to the family Aristolochiaceae, has been widely used as a traditional Chinese medicine for headache, toothache and inflammatory diseases (rheumatic arthritis, gingivitis). Volatile oils, the main active components in xixin, have been reported to have functions of analgesia and immunosuppression and to act as anti-inflammatory, anti-myocardial ischemia and rising blood.[1] Alkaloids, fatty acids and lignans were also isolated in previous research.[2] According to the Chinese pharmacopoeia (2010 edition), the maximum dose of xixin is 3 g/d. Clinical investigation indicated that adverse drug reactions, such as arrhythmias and respiratory paralysis, could occur when an overdose of xixin was taken.[3]

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Trace elements play an important role in metabolism in the body. Too much or too less of essential trace elements in the body could induce metabolic disorder, and toxic elements could also cause the damage of tissues and organs. Excess copper (Cu) *in vivo* could induce Wilson disease (hepatolenticular degeneration)[4-5] and renal injury could also occur due to the association of nickel (Ni), chrome (Cr) and cobalt (Co).[6]

In this study, the contents of trace elements in the serum and liver of mice were assayed by inductively coupled plasma-mass spectrometry (ICP-MS) for the first time after obtaining xixin extracts. Principal component analysis and cluster analysis between dosage and content of trace elements were achieved to explain the toxic mechanism of metabolism of microelements *in vivo*.

MATERIALS AND METHODS

General

An Agilent 7500a ICP-MS (Agilent Technologies Co. Ltd.

Santa Clara, State of California, CA, USA) was used for the determination of microelement with a quantitative analysis. The Agilent 7500 ICP-MS Chem Station software was used for data acquisition. A MDS-6 digester/extractor, including a microwaver and PTFE vessels, was from Shanghai Xinyi Microwave Chemical Scientific and Technology Co. Ltd. (Shanghai, China).

The instrument was optimized daily in terms of sensitivity (Li, Y and Tl), level of oxide (CeO/Ce) and doubly charged ion (Ce^{+2}/Ce) using a tuning solution containing 10^{-9} g/ml of Li, Y, Tl, Ce and Co in 2% HNO_3 to meet the demands of the trace element determination. The operating conditions of the ICP-MS instrument are summarized in Table 1.

Chemicals

Ultrapure water was prepared with a Milli-Q deionization unit (Millipore, Bedford, MA, USA). Nitric acid used for sample digestion was of high-purity grade and purchased from Kermel Chemical Reagent Co. Ltd. (Tianjin, China). Wash-Nitric Acid Blank: Part# G1820-60258 (5% HNO_3), Wash-water blank: Part# G1820-60259, tuning solution of MS optimization: 10^{-8} g/ml of Li, Y, Ce, Tl and Co (2% HNO_3) (Part# G5184-3566) was used to perform external calibration, mixed standard solution including 10^{-3} g/ml of Fe, K, Ca, Na, Mg and 10^{-5} g/ml of Ag, Al, As, Ba, Be, Cd, Co, Cr, Cu, Mn, Mo, Ni, Pb, Sb, Se, Tl, V, Zn, Th, U, (Part # G5183-4688) and the internal standard solutions including six elements of 10^{-5} g/ml of Li, Sc, Ge, Y, In and Bi (5% HNO_3) (Part# G5183-4680) used to reduce matrix effect and compensate for instrument drift during the analysis were purchased from Agilent (NJ, USA) and diluted to approximately 8 ng/g by 5% HNO_3 before the experiment.

Animals

Adult female Kunming mice weighing 18-22 g were purchased from Dalian Medical University (Grade II).

Table 1: Instrumental operating conditions for ICP-MS

ICP system	
Carrier gas	1.14 L/min
RF power	1300 W
Atomizer chamber	2°C
Sample uptake rate	0.1 rps
Points per spectral peak	6
Number of replicate	3
Sampling depth	8.2 mm
Mass spectrometer	
Sampling cone	Nickel, −96.2 V
Skimmer cone	Nickel, −22 V
Vacuum	5×10-7 Mba
Mass range	2-260 amu
Total acquisition	181 s

ICP-MS: Inductively coupled plasma-mass spectrometry

The animals were housed in an air conditioned room (temperature, 25°C; relative humidity, 55%) and fed *ad libitum* with standard feed and water in the course of the study. At the end of the experiment, pentobarbital sodium was used for euthanasia of the animals. The investigation conforms to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996). The study protocol was approved by the ethics regulations of Liaoning University of Traditional Chinese Medicine (131/2010).

Extraction

The leaves of xixin were extracted as per the water decoction technology,[7] which is as follows: Adding 10 times quantity of water, decocting twice (90 min for each time). Evaporation of the water under reduced pressure gave a condensed aqueous extract of different concentrations (150 mg/mL, 50 mg/mL and 16.7 mg/mL).

Sample collection

Take 48 healthy female Kunming mice, weighing 18-22 g, divided into four groups randomly ($n = 12$). Xixin extracts of 0.3 ml with the dosage of 150 mg/ml (H), 50 mg/ml (M) and 16.7 mg/ml (L) were taken orally for up to 7 days, while water solution of 0.3 ml was taken as the negative control group. The blood samples were obtained via orbital vein on the 7^{th} day after administration and serum was collected when the blood samples were centrifuged at 3500 rmp for 10 min at room temperature after standing for 1 h. Liver of mice were also collected and stored at -20°C before use.

Digestion of samples

To the 50 ml closed polytetrafluoroethylene (PTFE) vessel, 200 μL of serum or 0.2 g liver, together with 3 mL of conc. HNO_3, was added and then digested using the MDS-6 microwave digestion system. The optimized digestion conditions were a four-step procedure for 6 vessels including steps 1-3 (pressures were 0.3, 0.6 and 1.0 MPa for 4 min, respectively) and step 4 (pressure was set at a maximum pressure of 1.5 MPa for 10 min); the microwave power was 600 W for every step. After cooling, the decomposed sample solutions were heated almost to dryness to remove any excess HNO_3. Finally, the residue was dissolved in deionized water and made up to 10 ml in a volumetric flask with deionized water; the solution was then stored at 4ºC and subjected to analysis within 48 h.

RESULTS AND DISSCUSION

In vivo analysis of trace elements by ICP-MS

Before the analysis, wash-nitric acid blank and wash-water blank were used to rinse the system and flow path,

respectively. Then, tuning solution including10^{-8} g/ml of Li, Y, Ce, Tl and Co was utilized to modulate the conditions of the system. Samples were directly introduced by a peristaltic pump at the flow rate of 0.3 ml/min following the sample diluted with 5% HNO_3. The internal standard of Li, Sc, Ge, Y, In and Bi (approximately 8 ng/g) was added on-line as the reference solution to alleviate matrix effect and compensate for signal drift in each individual run on the ICP-MS.

The ICP-MS method was validated as follows. The calibration curves (correlation coefficient) and limits of detection (LOD) for 17 elements are shown in Table 2. The variation coefficients of elements were less than 10%, which showed good precision, and the accuracy of the method was examined by performing recovery experiments. The recoveries of trace elements were in the range of 92.76-111.98%, which presented good accuracy for the analysis.

Quantitative determination of trace elements in sample solutions of serum and liver were measured by the ICP-MS as mentioned above. The semiquantitative method enables us to automatically determine the concentrations of up to 71 elements in sample solutions of serum and liver after obtaining the xixin extracts. This method affords an approach to characterize sample solutions. Trace elements' contents in sample solutions were quantified by the semiquantitative method before the quantitative analysis. Trace elements, whose content of administration groups showed significant difference with that of blank group in the semiquantitative analysis, was picked for quantitation *in vivo*. The contents of trace elements (Be, Na, Mg, K, Ca, V, Cu, As, Se, Ag and Cd in serum and Na, Mg, Al, K, Ca, V, Cr, Mn, Fe, Co, Zn, Cu, As and Se in liver) were quantitated subsequently and the results are shown in Tables 3 and 4.

Table 2: Regression equations and LOD of determined elements

Trace elements	Regression equations	*r*	LOD (ng/ml)	Linear range (μg/L)
Be	$Y=2.961\ X-2.888$	0.9997	0.5869	0-100
Na	$Y=13.16\ X+5.145\times10^{3}$	0.9990	2.1754	0-500
Mg	$Y=7.645\ X-7.745\times10^{2}$	0.9995	0.0684	0-500
K	$Y=0.9543\ X+5.981\times10^{2}$	0.9998	1.0916	0-500
Al	$Y=1.007X+1.367$	0.9989	1.0546	0-100
Ca	$Y=1.489\times10^{-3}\ X+0.1718$	0.9999	1.7225	0-500
Cr	$Y=9.083\times10^{-2}\ X+0.7256$	0.9998	0.8635	0-100
V	$Y=0.9985\ X-1.470$	0.9991	0.1525	0-100
Mn	$Y=1.003\ X-1.015$	0.9994	0.0267	0-100
Fe	$Y=0.8332\ X+1.226\times10^{2}$	1.0000	0.2234	0-100
Co	$Y=5.957\ X-3.559$	0.9998	0.6928	0-100
Cu	$Y=2.896\ X+7.996$	0.9997	0.1356	0-100
Zn	$Y=0.5391\ X+1.736$	1.0000	0.2084	0-100
As	$Y=0.5543\ X+18.68$	0.9999	0.6786	0-100
Se	$Y=6.733\times10^{-3}\ X+9.745\times10^{-3}$	0.9999	0.1961	0-100
Ag	$Y=0.3725\ X-2.842\times10^{-2}$	1.0000	0.5769	0-100
Cd	$Y=7.868\times10^{-2}\ X-1.907\times10^{-2}$	0.9999	0.2554	0-100

x: Concentration of elements (ppb); y: The ratio of response of tested elements to internal standard elements. LOD: Limits of detection

Table 3: Content of 10 elements in serum (average±SD, *n*=6, ng/ml)

Trace elements	Dosage			Blank group (BG)
	150 mg/mL (H)	50 mg/mL (M)	16.7 mg/mL (L)	
Na	70710.0333±5031.7733***	68561.6667±7528.5946***	31488.3333±4743.5785	34883.3333±3137.8124
Mg	488.3333±47.9701***	368.0001±37.3153***	249.7583±18.8226	252.1500±40.7928
K	5016.5000±362.63***	4828.3001±296.1906***	2544.0333±337.9931	2662.8333±362.6328
Ca	2023.8667±148.8219**	1924.8167±218.3320*	832.6333±130.8708***	1674.8333±281.5531
V	0.0568±0.0105	0.0575±0.0058	0.0594±0.0077	0.0583±0.0067
Cu	13.4283±0.9990***	10.9518±1.0587***	8.4455±0.9673***	5.7628±0.8724
As	1.5553±0.1238	1.4958±0.0854	1.4978±0.1093	1.4534±0.0933
Se	1.9170±0.2248	1.9192±0.2470	1.9234±0.2389	1.9430±0.1702
Ag	0.0253±0.0017	0.0244±0.0023	0.0252±0.0018	0.0273±0.0031
Cd	0.0424±0.0103***	0.1548±0.0256***	0.1750±0.0246***	0.5605±0.0560

*$P<0.05$; **$P<0.01$; ***$P<0.001$

Table 4: Content of 16 elements in liver (average±SD, *n*=6, ng/g)

Trace elements	Dosage			Blank group
	150 mg/ml	50 mg/ml	16.7 mg/ml	
Be	0.0298±0.0070	0.0259±0.0034	0.0224±0.0027*	0.0286±0.0082
Na	52433.3333±7902.5734***	62533.3333±4747.0693	65900.0000±5942.3901	63583.3333±6533.1207
Mg	13033.3333±550.1515***	17750.0000±1164.0447	17301.6667±2471.3593	17916.6667±2181.2077
Al	83.7533±13.0788***	84.0883±8.6215***	59.5883±8.5653	54.0717±7.2966
K	201166.6667±15917.4956***	243666.6667±15357.9513***	322333.3333±54456.0985	286666.6667±23695.2879
Ca	12644.8333±1350.7495***	21045.0000±916.6842***	3968.3333±943.1737***	1381.6667±140.9137
Cr	9.9123±0.7099*	9.9933±0.6376**	13.9000±0.9022***	9.0027±0.7948
V	0.7722±0.0643	0.7980±0.0935	0.8588±0.0222	0.7803±0.0616
Mn	83.8983±7.2773	90.8283±5.2690	88.2167±7.1518	88.2167±7.1518
Fe	6091.6667±200.8399*	6088.3333±182.6928**	5618.3333±666.0756	5576.6667±498.9856
Co	1.1365±0.1152***	1.3272±0.1115***	1.5455±0.0739	1.5942±0.0733
Cu	221.2667±30.7106***	314.6167±39.7550	343.7167±61.0277	341.1167±44.7892
Zn	3095.6333±200.8243***	6601.6667±339.5536***	2268.3333±247.3392*	2026.6667±161.8229
As	1.3736±0.1516***	1.4002±0.2087***	2.1473±0.3577*	2.5423±0.3039
Se	60.0283±3.2935***	61.5500±3.1981***	41.0433±2.9614	40.8817±2.7668
Ag	0.0363±0.0041***	0.0366±0.0026	0.0352±0.0027	0.0385±0.0048

*P<0.05; **P<0.01; ***P<0.001

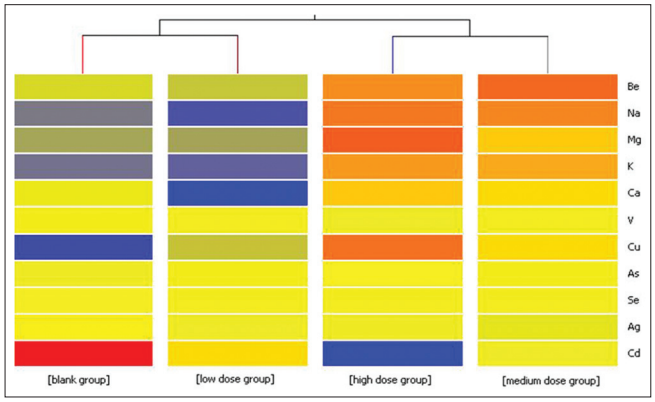

Figure 1: Cluster analysis of trace elements in serum

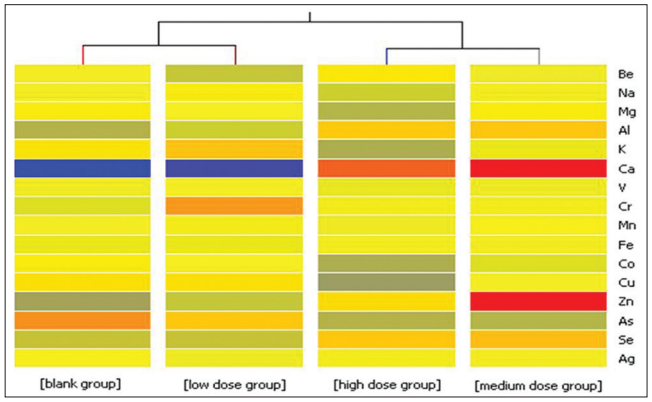

Figure 2: Cluster analysis of trace elements in liver

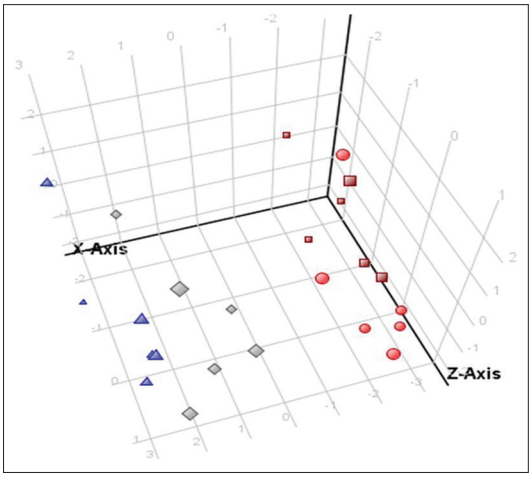

Figure 3: Principal component analysis of trace elements in serum

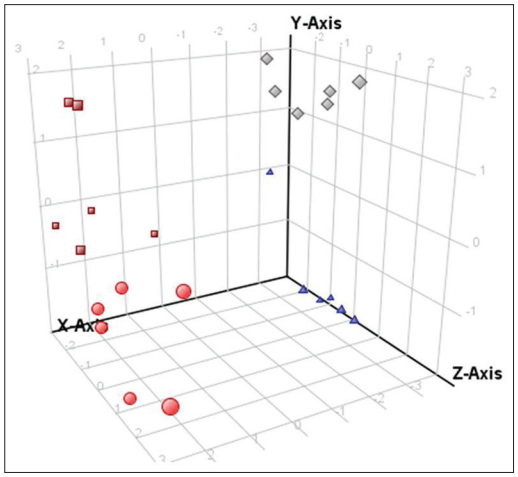

Figure 4: Principal component analysis of trace elements in liver

Statistical analysis

Quantitative analysis of trace elements in sample solutions of serum and liver were accomplished by ICP-MS after oral administration of xixin extracts. Clustering analysis (CA) and principal component analysis (PCA) between dosage and trace elements

content were confirmed using the GeneSpring 12.1 software [Figures 1-4].

The result showed that trace elements *in vivo* could be obviously raised after xixin extracts were taken as the dosage of H and M group above. And, xixin could possess the potential function of upregulating trace elements in vivo indirectly. Therefore, the adverse reactions caused by xixin were probably related to the high concentration of trace elements *in vivo*.

Statistical analysis (one-way ANOVA) was also completed and the result indicated that Na, Ca, Cu and Cd in serum and Ca and Zn in liver were the main elements affected by xixin extracts *in vivo*. The variation of elements' content in serum and liver are shown in Figures 5 and 6.

CA and PCA between dosage and trace elements' content were also confirmed using the Gene Spring 12.1 software [Figures 7-10]. The results of ANOVA were in accordance with that of CA and PCA. The result also indicated that the variation of elements' content (Na, Ca, Cu and Cd in

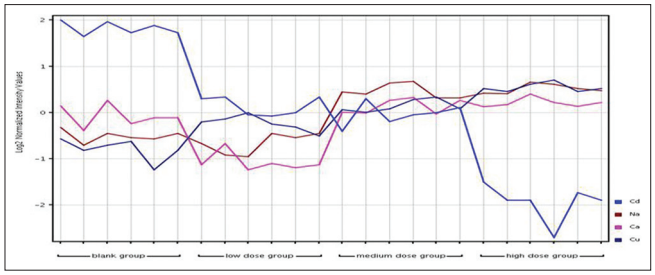

Figure 5: Content of Cd, Na, Ca and Cu in serum

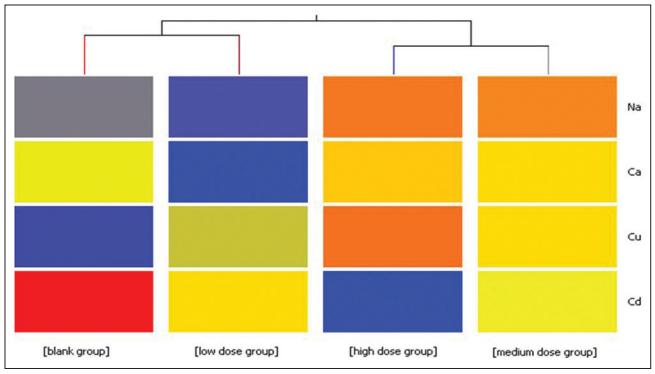

Figure 7: Cluster analysis of Cd, Na, Ca and Cu in serum (distance Meric: Manhattan)

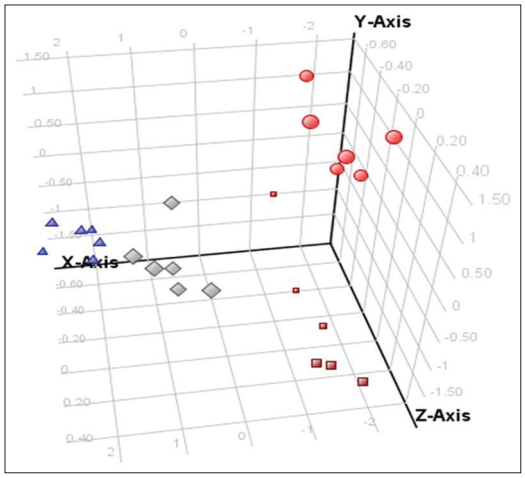

Figure 9: Principal component analysis of Cd, Na, Ca and Cu in serum

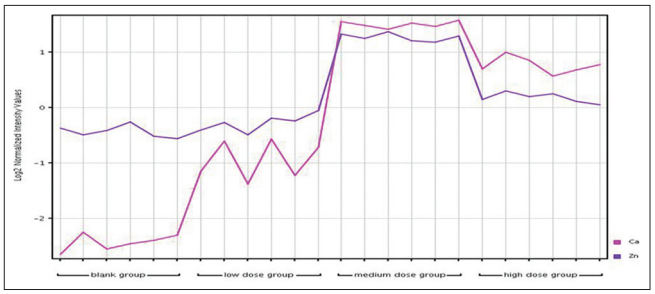

Figure 6: Content of Zn and Ca in liver

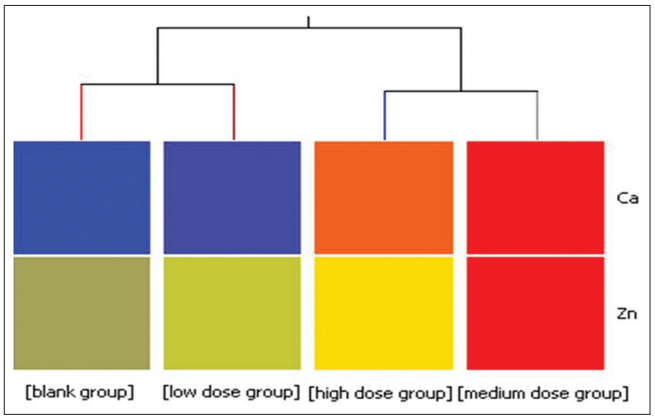

Figure 8: Cluster analysis of Ca and Zn in liver (distance Meric: Manhattan)

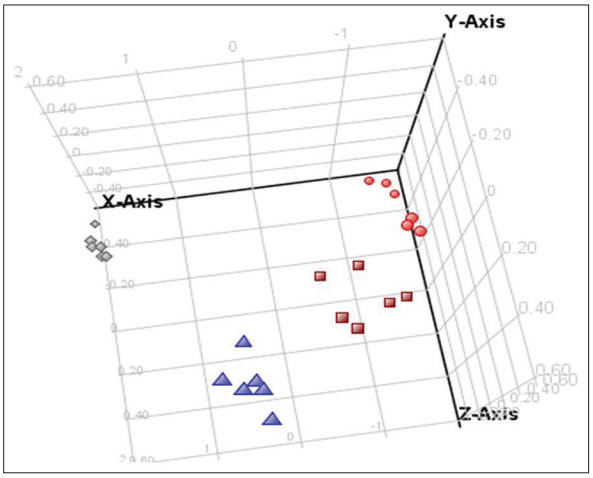

Figure 10: Principal component analysis of Ca and Zn in liver

serum and Ca and Zn in liver) could be reflected by the dosage of xixin indirectly.

CONCLUSION

This study is the first to elucidate the *in vivo* toxic mechanism of xixin based on trace elements. The main trace elements in serum and liver, which could reflect the toxicity of xixin, were also identified in this study. The adverse reactions of these elements were almost the same as that of xixin, and the tendency of elements contents in serum and liver were in accordance with the toxic symptoms when xixin was taken.[8] Thus, this method could also be utilized in the research of the corresponding aspects, and the relation between trace element content and dosage of xixin should be investigated further.

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