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Screening and verification of linearly dependent biomarkers with acute toxicity induced by Radix Aconiti based on liquid chromatography–mass spectrometry-based metabolite profiling †

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Radix Aconiti, with its unique anti-inflammatory and analgesic effects, is a well-known form of traditional medication; however, improper use of the aconite drug often leads to severe acute toxicity. Raw aconite ethanol extraction is the most toxic ingredient, followed by raw aconite water extraction; processed products have less toxic ingredients. Current clinical examinations primarily use biochemical tests and histopathological examination, but such approaches lack specificity, are time-consuming, and have low sensitivity, which can easily lead to false positive results. Therefore, a fast and accurate way to evaluate acute toxicity is needed. We have established a method that combines metabonomics with trend analysis of a gavage concentration series to find and validate acute toxicity biomarkers of Radix Aconiti. The purpose of this study is to identify Radix Aconiti acute toxicity biomarkers based on UPLC-Q-TOF-MS metabonomics technology. We use relative amounts of biomarkers with dosage and degree of toxicity to determine a dose-dependent trend; these substances may be exclusive aconite acute toxicity biomarkers. These exclusive biomarkers were validated both in water extraction of aconite and drug incompatibility with processed Radix Aconiti-Pinellia ternata couple medicines; ultimately, the acute toxicity biomarkers (shikimic acid, acetyl-L-carnitine, LysoPC (22:5), valine) were determined. This new method provides a better way to discover and validate specific metabonomics endogenous small molecule compounds.

Introduction

Radix Aconiti, the mother root of Aconitum (Ranunculaceae), is a famous traditional Chinese medicine (TCM) commonly used in the treatment of rheumatic diseases, joint pain, cancer and other diseases[1]. However, abuse of the Radix Aconiti tincture or its improper combination in a prescription (such as processed Radix Aconiti-Pinellia ternata) often causes severe toxicity, including major cardiac toxicity, central nervous system injury and embryotoxicity[2-6]. Alkaloids (such as aconitine) are the major components for the toxicities of Radix Aconiti[7]. The aconite toxicity varied according to the processing and extracting methods: The ethanol extraction of aconite was more toxic than water extraction, and particular processing methods can reduce its toxicity greatly. Considering the wide application of Radix Aconiti in clinics, it is necessary to build a fast, sensitive and reliable method to assess the safety and toxicity of Radix Aconiti. Biochemical and histological examination are the main methods for conventional drug safety evaluation; however, they often lack sensitivity and specificity, and thus, it is difficult to assess the toxicity of TCM due to the complexity of the components[8,9]. Given the above issues, we believe a specific evaluation is needed for the detection of acute toxicity caused by Radix Aconiti, as well as a more sensitive and proprietary evaluation tool for the evaluation of drug safety.

First proposed by Nicholson et al[10], metabonomics not only characterizes the body's response at the metabolism level but also can be applied to diagnose diseases and clarify the toxicity mechanism. An LC-MS-based metabonomics method can quickly obtain comprehensive information about endogenous metabolites in biological samples[11,12]. Metabonomic study of aconite is not rare, but few studies have addressed the correlation between metabonomics biomarkers and the severity of toxicity. Biomarkers that change consistently with the degree of toxicity can function as traditional biochemical parameters.

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In this study, as shown in Figure 1, ultra-performance liquid chromatography coupled with quadrupole-time-of-flight mass spectrometry (UPLC-Q-TOF-MS) was used for the metabolic profiling analysis of plasma samples of rats treated with different concentrations of Radix Aconiti to find the biomarkers for the acute toxicity of Radix Aconiti. In addition,

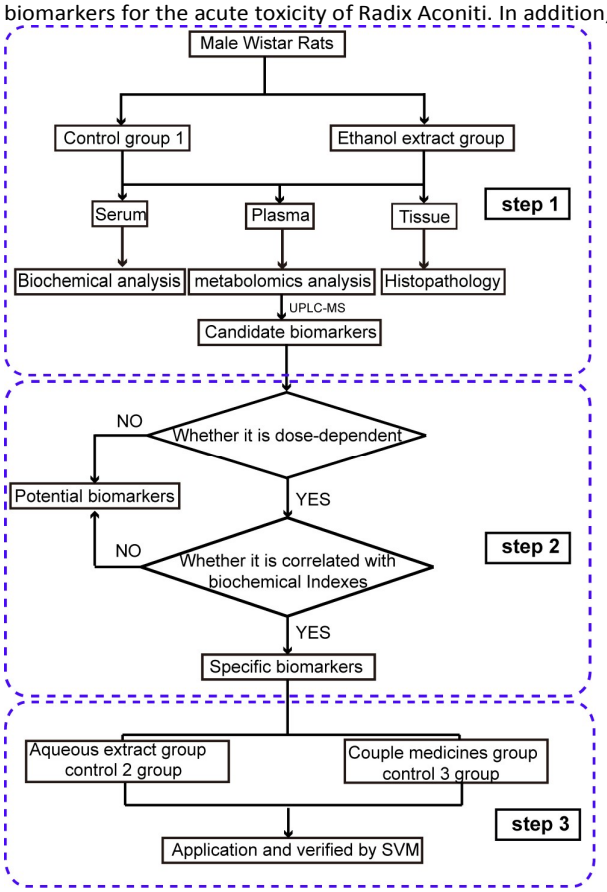

Figure 1. The experimental design flow chart

the variation trends of these toxicity biomarkers caused by different concentrations of Radix Aconiti ethanol extraction were investigated to screen small molecule compounds that changed regularly with dosage, which were closely related to the toxicity index trends. Finally, according to the results of Radix Aconiti extracted by water and in consideration of the prohibited processed Radix Aconiti-Pinellia ternata combination, we used SVM to validate the acute toxicity biomarkers that were found. Considering the toxicity degree of different aconite productions, we choose high toxicity Radix Aconiti ethanol extraction in the discovery stage and low toxicity Radix Aconiti water extraction and processed Radix Aconiti-Pinellia ternata couple medicines in the verification stage. With this approach, biomarkers with high specificity can be easily detected and compensate for the disadvantages of traditional biochemical indexes.

Experimental

Reagents and materials

High pressure liquid chromatography (HPLC)-grade acetonitrile and formic acid were purchased from Oceanpak (Gothenburg, Sweden) and ROE (USA), respectively. Distilled water was obtained from Wahaha (Hangzhou, China). Normal saline (NS) was purchased from Queensland Technology Co., Ltd. (Tianjin, China) and dissolved in saline solution prior to use. Raw Radix Aconiti, processed Radix Aconiti and Pinellia ternata were purchased from AnGuo, HeBei province and had been identified by pharmacognosy experts

Extraction of traditional Chinese medicine

In this study, the ethanol extract of raw aconite was investigated first to excavate biomarkers, which were then validated by the water extract and processed Radix Aconiti-Pinellia ternata couple medicines. For the preparation of aconite extract, 50 g of crushed herb was extracted twice with 10 and 8 times the volume of 70% ethanol or water, respectively, under reflux for 60 minutes, and the extracting solutions were filtered, combined, and concentrated to 1 g/ml (equivalent to the raw herb) to supply 50 ml ethanol extraction samples. For couple medicine extraction, processed Radix Aconiti and Pinellia ternate, each 150 g, were crushed and extracted twice under reflux for 60 minutes with 10 and 8 times the volume of water, respectively. The extracting solutions were combined, filtered and concentrated to 1 g/ml (equivalent to aconite raw herb), i.e., 150 ml extraction samples.

Animal treatment

The experimental animals were purchased from the Academy of Military Medical Sciences experimental animal centre (Beijing), with the license number "SCXK (Jun) 2009-003". The animal study was performed at the Institute of Radiation Medicine, Chinese Academy of Medical Sciences (Tianjin, China). Sixty-four male Wistar rats weighing 200±20 g were raised in an SPF-level lab. The rats were housed under the following conditions: 12 h day and 12 h night, ambient temperature of 23±2 °C and humidity of 35±5%. The groups, doses, administration modes and sampling times are shown in Table 1.

Sample collection and preparation

Before sample collection, all animals were fasted for 12 h with access to water. After each group of rats was fed with the corresponding concentrations of different drugs, ca. 2 ml blood was taken from the intraocular canthus venous. One aliquot (1 ml) of blood had heparin added to it and was centrifuged at 3500 rpm for 10 minutes to afford the supernatant, which was stored in a -80 °C freezer before the metabonomic study. Another aliquot (1 ml) of whole blood was handled and stored in the same conditions as those for the biochemical parameters test of CK, LDH, AST, ALT, Cr and Urea. At the end of this experiment, all animals were disposed of, and the hearts, livers and kidneys were immediately

removed and fixed in 10% formalin solution. The pathological features of the tissues were examined by haematoxylin and eosin (H&E) staining.

To reduce the suffering of animals, all the experiments were performed in strict accordance with China's national laws and local standards. The animal study was approved by the Animal Ethics Committee of Tianjin University of Traditional Chinese Medicine with ethical approval number TCM-2012-078F01

Chromatographic and mass spectrometric conditions

HPLC analysis was performed in a Waters UPLC-Q-TOF-MS system (Waters, USA). Plasma samples (5 μL) were injected into an ACQUITY UPLC HSS C18 column (2.1 × 100 mm, 1.7 μm, Waters). The column temperature was set to 40 °C, and the flow rate was set to 0.3 mL/min. The UPLC separation system includes a binary solvent system with mobile phase A (0.1% formic acid in water) and mobile phase B (0.1% formic acid in acetonitrile). The gradient profiles for the plasma samples were as follows: 99% A; followed by 0-0.5 min, A: 99%-99%; 0.5-2 min, A: 99%-50%; 2-9 min, A: 50%-1%; 9-10 min, A: 1%-1%; 10-10.5 min, A: 1%-99%; and 10.5-12 min, A: 99% - 99%. Q-TOF-MS was equipped with electrospray ionisation in positive mode and negative mode. The MS parameters were as follows: drying gas temperature, 325 °C; drying gas flow, 10 mL/min; desolvation gas flow, 600 L/h; capillary voltage, 3.5 kV; fragmentor voltage, 6 kV; collision energy, 20-30 kV; nebulizer pressure, 350 psi; and evaporative gas and auxiliary gas, high purity nitrogen; reference ions ([M+H]+=556.2771, [M-H]-=554.2615) were used to ensure accuracy during spectral acquisition. The range of data acquisition was 50 - 1000 Da. All samples were randomly injected. The samples were singled out from each group and mixed together to make quality control (QC) samples. Containing all plasma information, the QC samples were used to optimize and supervise the analysis process[13,14] Blank and QC samples were injected regularly every 10 samples to test the stability of the samples and the system during acquisition.

Data process

To validate the LC/MS analysis, the precision, reproducibility and stability of the specimen were determined according to the QC samples. The retention time and the relative content of the metabolites differed within the spectrum. Twenty of them were randomly selected to evaluate the RSD of precision and reproducibility. Masslynx (Waters, USA) software was used to collect the original data of rats with intragastric administration of ethanol extract and water extract. The data processing system Makerlynx software (Version 4.1) was used for the processing of spectra, such as ion-pair extraction, peak alignment, peak matching and peak intensity correction. The data were exported after normalization. Then, after rounding with 80% rules (Excel format), the data were imported into SIMCA-P11.5 software (Sweden Umetrics company) for multivariate statistical analyses. The model established by supervised Partial Least Squares-discriminant analysis (PLS-DA) was validated. Based on the model, substances with VIP over 1 were selected as candidate markers in the result of the ethanol extract intragastric administration. The quality of the PLS-DA model could be assessed by the parameters R2 (cum) and Q2 (cum), which reflect the stability and reliability of the constructed model. Statistical significance was taken as $p < 0.05$ (student's t-test). Relatively accurate m/z values of ions obtained were used to retrieve in the HMDB database (http://www.hmdb.ca) and Massbank database (http://www.massbank.jp). after the removal of xenobiotics and non-existent compounds in the blood. The endogenous substances that were screened were used to further determine biomarkers; ultimately, biomarkers were determined by two-stage mass spectrometry. The variation trends of toxicity biomarkers with the dosage used were investigated. The metabolites that changed regularly with the dosages should be closely related to the toxicity and could be used to denote the severity of the toxicity. Finally, according to the results of raw Radix Aconiti extracted by water and processed Radix Aconiti-Pinellia ternata couple medicines, we used SVM to verify the specific biomarkers that were found.

Results and discussion,

Animal behavior status

Intragastric administration with different concentrations of raw Radix Aconiti ethanol extraction: After 0.5-1 h, the animals exhibited varying degrees of abnormal motion, drowsiness, ataxia, convulsions, opisthotonos and other symptoms. Meanwhile, with the increase in drug concentration, the effects of toxicity were pronounced. Obvious and severe toxicity occurred early in the high dose group. As the dose decreased, the occurrence of toxicity grew less. However, there was no abnormal behaviour in the control group. The water extract of raw Radix Aconiti showed a similar but weaker toxicity than the ethanol extract with a later toxic response of approximately 1-2 h. The processed Radix Aconiti-Pinellia ternata couple medicines group showed no severe symptoms even after seven days of consecutive administration.

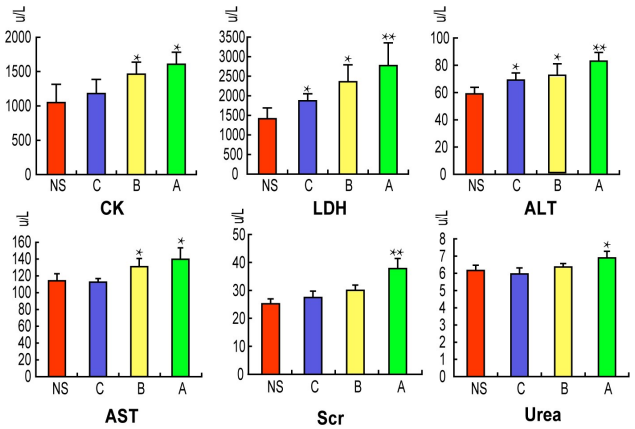

Figure 2. Effect of different concentrations of Radix Aconiti (ethanol extract group) on various biochemical parameters in rats. Significant difference from control: *p<0.05, **p<0.01. NS: Normal Saline C: 2 g/kg ethanol extraction B: 5 g/kg ethanol extraction A: 10 g/kg ethanol extraction

Biochemical indicators results in ethanol extraction

To evaluate the toxicity of aconite, the levels of CK and LDH (heart damage detection index), ALT and AST (liver damage monitoring indicators), and urea and Cr (kidney damage detection index) were determined (Figure 2). All the increases in these biochemical parameters were dose dependent. Compared with control group 1, CK, AST, Cr and LDH significantly increased in the middle dose (5 g/kg) and high dose (10 g/kg) of aconite extracts; ALT significantly increased in all dose groups. The histopathology of tissues and descriptions are shown in Figure S1

Screening and identification of potential biomarkers

The typical plasma UPLC-Q-TOF-MS fingerprint spectrum of the rats after intragastric administration with raw Radix Aconiti is shown in Figure S2. The QC result met the requirement for testing endogenous metabolites and are shown in supplementary information Table S1. The three dosed groups and the control group were well separated and dose dependent in the score plot of the PLS-DA model (Figure 3). The R2 (cum) and Q2 (cum) values of the PLS-DA model in this experiment were high and are listed in supplementary information Table S2, ensuring the accuracy of the obtained results. In the model, VIP> 1, p <0.05 for the follow-up screening of substances.

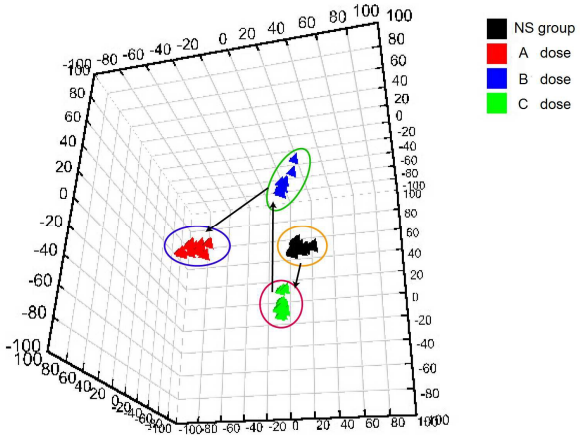

Figure 3 Score plot of PLS-DA performed on samples from the 4 ethanol extraction groups: NS: Normal Saline, A dose (10 g/kg), B dose (5 g/kg), C dose (2 g/kg)

The process of the identification of compounds is shown in supplementary information part 1. The biomarkers obtained and their assignments are shown in Table 2

Screening linearly dependent with *Radix Aconiti* acute toxicity biomarkers

With the dosage as the abscissa and the contents of the biomarkers as the ordinates, a histogram was created (see Figure S3). In the histogram, we found that the trend in biomarker variation has four classes: (I) Class substances that totally render fluctuations in shape, e.g., cytosine, and these substances have no material relationship with the administration dosage; compared with the control group, the substance variation of the treatment groups either increased or decreased, and the trends are not uniform. (II) Class materials that appear to be partially fluctuating, although the administered group showed obvious differences in relation to the blank group; however, there is no significant correlation with dosage, and this class mainly includes estrone, 3-indole indole propionic acid, acetylcholine, 5-dodecenoic acid, Taurodeoxycholic acid and other LPCs. (III) Class substances presented as dose dependent; the relative content of these substances exhibits an upward trend with increasing dosage, e.g., shikimic acid. (IV) Class substances that are also dose dependent, but the relative content of these substances decreases with increasing dose administration; acetylcarnitine, LysoPC (22:5), and valine are representative materials. Changes in substances may present fluctuating trends that have nothing to do with dosage; however, the class III and IV biomarkers in the figure appear to be dose dependent (Figure 4); at the same time, the content of these two types of biomarkers were subjected to linear fitting, and the fitting results as shown in Figure 5 also indicate that they were both dose dependent.

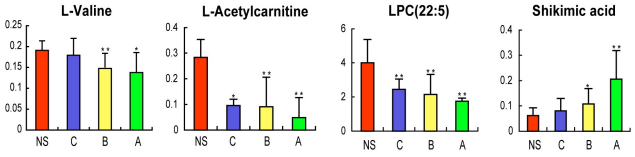

Figure 4 The tendency of biomarkers' relative content (peak area intensity) changed with different concentrations of administration (ethanol extraction group). Significant difference from control: *p<0.05, **p<0.01. NS: Normal Saline C: 2 g/kg; ethanol extraction B: 5 g/kg; ethanol extraction A: 10 g/kg ethanol extraction

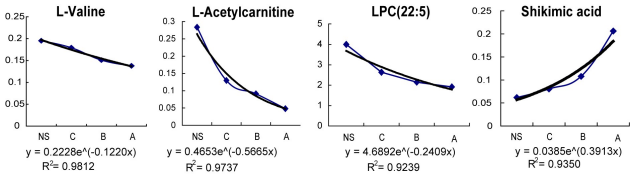

Figure 5 Fitting results of (III) and (IV); gavage concentration and relative content (peak area intensity) of biomarkers L-valine, L-acetylcarnitine and LPC (22:5) decrease as dosage is increased; shikimic acid increases as dosage is increased. NS: Normal Saline C: 2 g/kg; ethanol extraction B: 5 g/kg; ethanol extraction A: 10 g/kg

We conduct Pearson correlation analysis in SPSS 17 for combined dose-dependent biomarkers with a degree of toxicity, and the results are shown in Table 3. The results of Pearson correlation analysis show good correlation between biomarkers such as shikimic acid, acetylcarnitine, LysoPC (22:5) and valine creatinine and the measured biochemical indicators. These biomarkers appear to be significantly dose dependent and have obvious relevance to the degree of toxicity; meanwhile, the biomarkers with a low dose administration of the extraction showed significant changes and presented positive and negative relations with increasing dosage; such biomarkers not only provide the basis for clinical diagnosis and treatment similar to traditional biochemical indicators but are also useful in the early detection of toxicity. Acetylcarnitine, LysoPC (22:5), valine, and shikimic acid are dose dependent in relation to the Radix Aconiti extraction; in addition, there is significant correlation between the changes in the biomarkers and the degree of toxicity. Therefore, we believe that these four biomarkers can be used as acute toxicity biomarkers of Radix Aconiti.

Verification results

To verify the compounds that had more specificity, we validated the acute toxicity biomarkers of Radix Aconiti in the oral administration experiments, in which Radix Aconiti was extracted by water as well as the processed Radix Aconiti-Pinellia ternate couple medicines. Although the content of different products and extraction methods are different, the toxic components are the same. Thus, the toxic mechanisms in rats caused by the aconite water extraction and couple medicines are the same as those in the ethanol extraction, according to the verification results, in the dose sequence D group: 10 g/kg and E group: 5 g/kg with the water extraction administered orally and the ethanol extraction method. We found that the time of emerging toxicity caused by water extraction was longer than that of ethanol extraction, and the symptoms were not as violent after water extraction gavage compared to ethanol extraction administration. We found these four biomarkers in the metabolic profiling of the plasma of the water extract intragastric administration rats, and we have drawn the histogram of the content trends for these biomarkers in the water extraction intragastric administration group (Figure S4). The trend chart indicates that the four biomarkers are dose dependent. For the water extract group, CK for all the water extraction groups trended upward but did not change significantly. The LDH of all the Radix Aconiti administered group exhibited a significant increase. Regarding the indicators of liver damage, in addition to the ALT of the 10 g/kg water extract intragastric administration group exhibiting a significant increase, the ALT and AST of the other group did not show a significant change. Urea and Cr of the 10 g/kg water extract intragastric administration group increased significantly, and Cr of the 5 g/kg water extract intragastric administration group also increased significantly. These results showed that the water extraction of Radix Aconiti caused toxicity damage to the heart, liver and kidney and presented a positive relationship with dose. (Figure S5).The histopathology of tissues is shown in Figure S6. In the field of traditional Chinese medicine, some drugs used together can cause toxicity. In this study, we verified our biomarkers by the toxicity of aconite contained in couple medicines with processed Radix Aconiti-Pinellia ternata. On the one hand, we can verify the accuracy of the biomarkers we found, and on the other hand, the study provides new metabonomics research methods for compatibility toxicity monitoring. The trend in the specific biomarkers of the couple medicine is consistent with the searching stage, as shown in Figure S7. For biochemical changes, the CK of the couple medicines trended upward, although not significantly. The LDH of the couple medicine group increased slightly. The indicators of liver damage, the ALT and AST, of the couple medicine groups did not change significantly. Urea and Cr of the couple medicine groups increased slightly (Figure S8). These results showed that the processed Radix Aconiti-Pinellia ternata couple medicine induced only slight toxicity in the heart and kidney. The histopathology of the tissues is shown in Figure S9. We randomly selected data from every control group; the ethanol extraction and couple medicine groups were used as the training set and test set together for support vector machine prediction, and the prediction rate was 85.30%. The Bestc, Bestg and CV accuracy parameters from the cross-verification method are shown in Figure 6. These results suggest that the specific biological markers we screened have a higher toxicity discriminating ability.

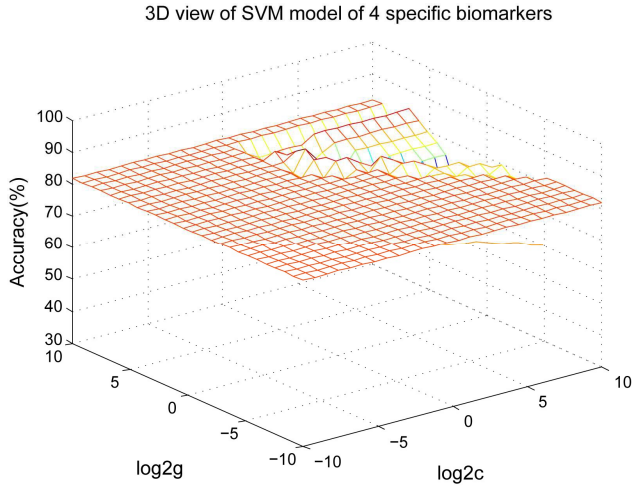

Figure 6 3D view of SVM model of 4 specific biomarkers (the parameters are as follows: Best c=2.2974; g=21.1121; CV Accuracy=86.3636%).

Biological significance of biomarkers for the prediction of acute toxicity of Radix Aconiti

Glycerophospholipid metabolism In the discovery of biomarkers, carnitine substances can increase the oxidation rate of fatty acids and reduce the consumption of glycogen[15,16]. Mitochondrial damage occurs, creating metabolism disorder in the carnitine substances; therefore, carnitine substances are a reliable energy metabolism biomarker of plasma[17]. LPC substances accomplish signal transduction by lysophospholipid receptors (LPL-R). The LPL-R messengers in the body play an important role in life activities[18]. We infer that certain factors may activate phospholipase A2, which leads to damage of the phospholipid membranes, and LPC reduction, which increases heart damage[19].

Fatty acid oxidation and Amino acid metabolism When the heart is damaged, β-oxidation and the Krebs cycle may be inhibited; this is followed by a lack of ATP function, and branched-chain amino acids such as valine that are derived from protein can be used as an alternative source of energy for the heart[20]. At the same time, amino acids, as fundamental materials of the body, participate in various forms of energy metabolism, which may be involved in signal transduction or enzymes during toxicity development. 5-Dodecenoic acid is an intermediate metabolite of unsaturated fatty acid oxidation. It is well known that oxidative catabolism of fatty acids is an important source of energy in the body. When carnitine substances are reduced, myocardial fatty acid oxidation is suppressed, resulting in reductions in fatty acid metabolism and sugar aerobic oxidation; therefore, as an intermediate in the oxidation of unsaturated fatty acids, the 5-dodecenoic acid content also decreases.

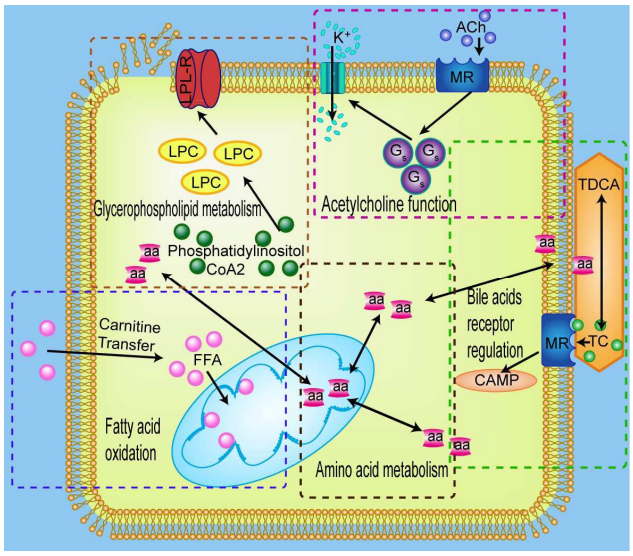

Figure 7 Different metabolites and corresponding pathways in Radix Aconiti-treated rats.

Acetylcholine function and ion channels Cardiovascular physiological activity is regulated by the autonomic nervous system. The sympathetic and parasympathetic nervous systems are two important components of the autonomic nervous system. Acetylcholine is a major neurotransmitter that is released by the peripheral nervous system. With parasympathetic nervous excitement, acetylcholine is combined with myocardial cell membrane muscarinic receptors (M) through G protein-coupled receptors; this results in the acetylcholine-activated inwardly rectifying potassium channel opener, which has a direct inhibitory effect on atrial muscle[21,22]. Acetylcholine is decreased because of the affection on the ion channels; thus, the inhibition of the atrial muscle is weakened, leading to arrhythmia.

Bile acid metabolism Cholic acid is a byproduct of cholesterol metabolism, regulating the digestion of lipids and the cholesterol from metabolism and other functions. In the early 1980s, Bogin discovered bezoar deoxycholic acid, which can affect the voluntary contraction of the myocardial cells of mice[23]. Bezoar deoxycholic acid is increased in this experiment; it becomes taurocholic acid by in vivo chemical conversion. Taurocholic acid can be considered as part of an agonist of the M2 receptor; it can combine with M2 receptors, thus reducing cAMP and resulting in arrhythmia[24]. Related mechanisms are shown in Figure 7. Because knowledge is limited in this area, the biology of the remaining biomarkers is unclear, pending further study.

Significance of biomarkers for the prediction of acute toxicity of Radix Aconiti

Aconite poisoning can produce shortness of breath and arrhythmia, and it can also cause cardiac arrest, respiratory centre paralysis and even death if not treated immediately. Thus, determining the toxicity of aconite has important significance for the development of Chinese medicine containing aconite class ingredients, as well as the safety assessment and clinical diagnosis of the drug. At present, drug-induced body toxicity or damage is typically detected by biochemical tests and histopathological analysis in the traditional sense, but these approaches lack specificity and sensitivity[25]. In contrast, specific biomarkers can determine the degree of intrusion into the body in a short period of time and even have a more predictive effect than biochemical tests. By searching and screening the aconite biomarkers of acute toxicity, we can judge the toxicity caused by aconite accurately and also can provide a reference for the proper use of aconite. In recent years, metabonomics technology has played an important role in the evaluation of drug toxicity[26-28]. However, it is still in its infancy with respect to the research on acute exclusive aconite biomarkers. In this study, we established the acute toxicity model with different doses of ethanol extraction of aconite; we then screened the metabonomics biomarkers and eventually confirmed that they are more exclusive than biochemical tests, which supports the application of metabonomics in drug toxicity evaluation. At the same time, we performed correlation analysis with respect to administration dosage and toxicity and found exclusive acute toxicity biomarkers for aconite. These biomarkers may exhibit significant differences when toxicity has not been clearly identified, which nevertheless shows the potential for early drug toxicity detection. Compared to current detection methods, metabonomics technology offers advantages such as low trauma, specificity, and low cost. The application of metabonomics in toxicity studies has more broad application prospects for toxicity evaluation of traditional Chinese medicine, clinical medicine supervision, and the detection of clinical disease. It also provides a feasible platform to explain the mechanism for toxicity in traditional Chinese medicine.

Conclusions

Using UPLC-Q-TOF/MS metabonomics methods, this study attempts to find biomarkers of aconite ethanol extraction; in addition, by means of biochemical detection indicators, it builds a method to detect specific toxicity biomarkers and investigate Radix Aconiti acute toxicity biomarkers with aconite water extraction. Ultimately, we identify the exclusive biomarkers (shikimic acid, acetyl-L-carnitine, LysoPC (22:5), valine) of Radix Aconiti toxicity. Compared to conventional biochemical indicators for evaluating the toxicity of aconite, these biomarkers are more exclusive and effective in the early stages of toxicity exposure, which helps the rapid diagnosis and treatment of toxicity caused by Radix Aconiti. At the same time, this study used metabonomics for the analysis and evaluation of complex traditional Chinese medicine toxicity; this helps to promote metabonomics analysis of the mechanisms of complex toxic components, which also promotes the development of metabonomics. In addition, this study is a useful reference for evaluating the safety of other medicines and the proper use of drugs; it is also a source of reference for the study of biomarkers, providing trend analysis of drug concentration sequences.

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Notes and references

1 J. Singhuber, M. Zhu, S. Prinz and B. Kopp, J Ethnopharmacol, 2009, **126**, 18-30.
2 T. Y. Chan, Toxins, 2014, **6**, 2605-2611.
3 C. C. Lin, T. Y. Chan and J. F. Deng, Ann Emerg Med, 2004, **43**, 574-579.
4 A. Ameri, Prog Neurobiol, 1998, **56**, 211-235.
5 Y. T. Tai, P. P. But, K. Young and C. P. Lau, Lancet, 1992, **340**, 1254-1256.
6 K. Xiao, L. Wang, Y. Liu, C. Peng, G. Yan, J. Zhang, Y. Zhuo and H. Li, Birth Defects Res B Dev Reprod Toxicol, 2007, **80**, 208-212.
7 N. G. Bisset, J Ethnopharmacol, 1981, **4**, 247-336.
8 Y. Tonomura, Y. Mori, M. Torii and T. Uehara, Toxicology, 2009, **266**, 48-54.
9 Y. Tan,J. Ko, X. Liu, C. Lu, J. Li, C. Xiao, L. Li, X. Niu, M. Jiang, X. He, H. Zhao, Z. Zhang, Z. Bian, Z. Yang, G. Zhang, W. Zhang and A. Lu, Mol Biosyst, 2014, **10**, 2305-2316.
10 J. K. Nicholson, J. C. Lindon and E. Holmes, Xenobiotica, 1999, **29**, 1181-1189.
11 E. J. Want, I. D. Wilson, H. Gika, G. Theodoridis, R. S. Plumb, J. Shockcor, E. Holmes and J. K. Nicholson, Nat Protoc, 2010, **5**, 1005-1018.
12 A. Sreekumar, L. M. Poisson, T. M. Rajendiran, A. P. Khan, Q. Cao, J. Yu, B. Laxman, R. Mehra, R. J. Lonigro, Y. Li, M. K. Nyati, A. Ahsan, S. Kalyana-Sundaram, B. Han, X. Cao, J. Byun, G. S. Omenn, D. Ghosh, S. Pennathur, D. C. Alexander, A. Berger, J. R. Shuster, J. T. Wei, S. Varambally, C. Beecher and A. M. Chinnaiyan, Nature, 2009, **457**, 910-914.
13 Gika, H. G.; Theodoridis, G. A.; Wingate, J. E.; Wilson, I. D, J. Proteome Res. 2007, **6**, 3291–3303
14 Y. B. Li, L. Ju, Z. G. Hou, H. Y. Deng, Z. Z. Zhang, L. Wang, Z. Yang, J. Yin and Y. J. Zhang, J. Proteome Res, 2015, **14**, 2437-2445.
15 M. A. Arsenian, Prog Cardiovasc Dis, 1997, **40**, 265-286.
16 M. Kivilompolo, L. Ohrnberg, M. Oresic and T. Hyotylainen, J Chromatogr A, 2013, **1292**, 189-194.
17 H. Hong and W. Tong, Biomark Med, 2014, **8**, 143-146.
18 J. Chun, E. J.Goetzl, T. Hla, Y. Igarashi, K. R. Lynch, W. Moolenaar, S. Pyne and G. Tigyi, Pharmacol Rev, 2002, **54**, 265-269.
19 S. Al Makdessi, J. L. Andrieu, H. Herilier and G. Faucon, J Mol Cell Cardiol, 1987, **19**, 141-149.
20 P. H. McNulty, R. Jacob, L. I. Deckelbaum and L. H. Young, Metabolism, 2000, **49**, 1365-1369.
21 W. J. Zang, X. J. Yu, H. Honjo, M. S. Kirby and M. R. Boyett, J Physiol, 1993, **464**, 649-679.
22 W. J. Zang, X. J. Yu and M. R. Boyett, Pflugers Arch, 1995, **430**, 348-357.
23 E. Bogin, O. Better and I. Harari, Experientia, 1983, **39**, 1307-1308.
24 S. H. Sheikh Abdul Kadir, M. Miragoli, S. Abu-Hayyeh, A. V. Moshkov, Q. Xie, V. Keitel, V. O. Nikolaev, C. Williamson and J. Gorelik, PLoS One, 2010, **5**, 1-10.
25 I. Andreadou, M. Papaefthimiou, A. Zira, M. Constantinou, F. Sigala, A. L. Skaltsounis, A. Tsantili-Kakoulidou, E. K. Iliodromitis, D. T. Kremastinos and E. Mikros, NMR Biomed, 2009, **22**, 585-592.
26 Y. Gao, Z. Ma and B. Zhang , Zhongguo Zhong Yao Za Zhi, 2012, **37**, 1-4.
27 E. Holmes, A. W. Nicholls, J. C. Lindon, S. C. Connor, J. C. Connelly, J. N. Haselden, S. J. Damment, M. Spraul, P. Neidig, J. K. Nicholson, Chem Res Toxicol, 2000, **13**, 471-8.
28 J. Sun, L. K. Schnackenberg, R. D. Holland, T. C. Schmitt, G. H. Cantor, Y. P. Dragan and R. D. Beger, J Chromatogr B, 2008, **871**, 328-430.

Table 1 Dose, mode of administration and sampling time in the process of screening and validating biomarkers

Grouping	Number	Dose(g/kg)	Administration mode	Sampling time
NS 1	6	10 ml/kg	Intragastrically, single-dose	30 min
high dose ethanol extraction group	6	10	Intragastrically, single-dose	30 min
middle dose ethanol extraction group	6	5	Intragastrically, single-dose	30 min
low dose ethanol extraction group	6	2	Intragastrically, single-dose	30 min
NS 2	8	10 ml/kg	Intragastrically, single-dose	60 min
high dose water extraction group	8	10	Intragastrically, single-dose	60 min
low dose water extraction group	8	5	Intragastrically, single-dose	60 min
NS 3	8	10 ml/kg	Intragastrically, single-dose	7 days
couple medicines	8	10	Intragastrically, single-dose	7 days

Table 2 The 30 identified metabolites were changed by Aconite root in plasma.

No	t_R (min)	Obsd m/z	Calcd m/z	Error (ppm)	Metabolite	Formula	MS/MS	C vs NS	B vs NS	A vs NS
1	2.2074	204.1232	203.1157	-1.97	L-Acetylcarnitine	$C_9H_{17}NO_4$	204.1$[M+H]^+$ 145.0$[M+H-N^+(CH_3)_3]^+$ 85.0$[M+H-N^+(CH_3)_3-CH_3COOH]^+$	0.46	0.32	0.17
2	8.7678	548.371	547.3637	-1.10	LysoPC(20:2)	$C_{28}H_{54}NO_7P$	548.4 $[M+H]^+$ 471.3 $[M+H-C_3H_8O_2]^+$ 370.2 $[M+H-C_8H_{19}NO_5P]^+$ 184.0 $[M+H-C_{23}H_{41}NO]^+$ 125.0 $[M+H-C_{26}H_{48}NO_3]^+$ 104.0 $[M+H-C_{24}H_{46}NO_4P]^+$	0.72	0.73	0.61
3	4.6713	271.17	270.1619	0.74	Estrone	$C_{18}H_{22}O_2$	271.2$[M+H]^+$ 293.2$[M+Na]^+$ 147.1$[M+H-C_8H_{12}O]^+$	4.94	9.13	5.36
4	8.0129	570.355	569.3481	-1.58	LysoPC(22:5)	$C_{30}H_{52}NO_7P$	570.4 $[M+H]^+$ 552.3 $[M+H-H_2O]^+$	0.66	0.54	0.48

							184.1 $[M+H-C_{21}H_{41}NO_3P]^+$ 125.0 $[M+H-C_{28}H_{47}NO_3]^+$			
5	1.6095	175.0589	174.0528	-9.77	Shikimic acid	$C_7H_{10}O_5$	175.1$[M+H]^+$ 95.1$[M-2H_2O-2CO]^+$	1.30	1.75	3.34
6	7.9257	568.3374	545.3481	-0.88	LysoPC(20:3)	$C_{28}H_{51}NO_7PNa$	546.4 $[M+H]^+$ 528.4 $[M+H-H_2O]^+$ 184.1 $[M+H-C_{18}H_{36}NO_4P]^+$ 125.1 $[M+H-C_{26}H_{46}NO_3]^+$	0.42	0.52	0.30
7	4.291	190.0864	189.0789	-2.12	3-Indolepropionic acid	$C_{11}H_{11}NO_2$	190.1$[M+H]^+$ 173.1$[M+H-NH_3]^+$ 172.0$[M+H-H_2O]^+$	0.71	0.90	0.67
8	1.61	156.0423	117.0789	-2.58	L-Valine	$C_5H_{10}NO_2K$	156.0$[M+K]^+$ 118.1$[M+H]^+$ 72.0$[M+H-HCOOH]^+$	0.92	0.77	0.70
9	1.6458	114.0665	113.0589	-1.77	Creatinine	$C_4H_7N_3O$	114.1$[M+H]^+$ 86.0$[M+H-CO]^+$	1.16	1.09	1.05
10	2.2098	132.1017	131.0946	-5.34	L-Norleucine	$C_6H_{13}NO_2$	132.0$[M+H]^+$ 86.1$[M+H-HCOOH]^+$	0.18	0.64	0.64

Please do not adjust margins

11	3.1349	169.0984	146.1181	-55.92	Acetylcholine	$C_7H_{15}NO_2Na$	68.0[M+H-HCOOH-H_2O]$^+$ 169.1[M+Na]$^+$ 147.1[M+H]$^+$ 87.1[M-C_3H_9N]$^+$ 43[M-$C_5H_{13}NO$]$^+$	0.72	0.84	0.67
12	8.309	221.1516	198.1619	-0.45	5-Dodecenoic acid	$C_{12}H_{21}O_2Na$	221.2[M+Na]$^+$ 199.0[M+H]$^+$ 181.0[M+H-H_2O]$^+$	0.59	0.69	0.62
13	8.9237	510.3559	509.3481	0.00	LysoPC(17:0)	$C_{25}H_{52}NO_7P$	510.4 [M+H]$^+$ 492.4 [M+H-H_2O]$^+$ 433.4 [M+H-$C_3H_{10}NO$]$^+$ 285.2 [M+H-$C_5H_{13}NO_4P$]$^+$	0.83	0.82	0.82
14	3.5107	231.0209	230.0191	-26.52	D-Ribose 5-phosphate	$C_5H_{11}O_8P$	231.0[M+H]$^+$ 213.0[M+H-H_2O]$^+$	0.30	0.72	0.25
15	6.92	522.2846	499.2967	-3.64	Taurodeoxycholic acid	$C_{26}H_{44}NO_6SNa$	522.3[M+Na]$^+$ 482.2[M+H-H_2O]$^+$ 464.2[M+H-2H_2O]$^+$	2.56	2.85	3.83

						454.2[M+H-H_2O-CO]$^+$ 438.2[M+H-H_2O-CO_2]$^+$			
16	0.8799	115.0023	116.0109	-7.76	Maleic acid	$C_4H_4O_4$	115.0[M-H]$^-$ 71.0[M-COOH]$^-$	1.22	1.35	1.50
17	0.9382	180.0652	181.0738	-4.97	L-Tyrosine	$C_9H_{11}NO_3$	180.0[M-H]$^-$ 163.0[M-NH3]$^-$ 119.0[M-NH3-COOH]$^-$	0.72	0.71	0.74
18	2.0808	112.0508	111.043	-2.70	Cytosine	$C_4H_5N_3O$	112.1[M+H]$^+$ 95.0[M+H-NH_3]$^+$ 69.0[M+H-HNCO]$^+$	1.11	0.93	1.11
19	4.672	391.2829	390.2751	-4.87	Nutriacholic acid	$C_{24}H_{38}O_4$	391.3[M+H]$^+$ 373.3[M+H-H_2O]$^+$ 355.3[M+H-2H_2O]$^+$ 347.2[M+H-CO_2]$^+$ 345.2[M+H-CO-H_2O]$^+$ 337.3[M+H-3H_2O]$^+$	0.32	1.47	0.45

Please do not adjust margins

20	1.67	136.0484	135.0406	38.51	Homocysteine	$C_4H_9NO_2S$	136.0[M+H]$^+$ 119.0[M+H-NH_3]$^+$ 118.1[M+H-H_2O]$^+$ 90.0[M+H-HCOOH]$^+$	1.15	1.02	1.05
21	6.5692	490.2905	489.2827	-0.82	LPC(14:0)	$C_{22}H_{45}NO_7PNa$	468.3 [M+H]$^+$ 450.3 [M+H-H_2O]$^+$ 391.3 [M+H-$C_3H_{10}NO$]$^+$ 285.2 [M+H-$C_5H_{13}NO_4P$]$^+$ 184.1 [M+H-$C_{17}H_{33}NO_2$]$^+$ 125.0 [M+H-$C_{20}H_{40}NO_3$]$^+$ 104.1 [M+H-$C_{18}H_{38}NO_4P$]$^+$	0.94	0.99	0.83
22	4.2907	172.0759			UN			0.71	0.89	0.63
23	4.2913	130.0653			UN			0.74	0.91	0.63
24	6.3899	346.3311			UN			1.17	0.97	1.70
25	6.6844	502.2917			UN			0.60	1.04	0.66
26	5.5171	432.31			UN			1.00	0.76	1.57
27	7.7747	468.3449			UN			0.69	0.81	0.59

28	1.6986	132.0771	UN	1.13	0.88	1.06
29	5.9688	299.2	UN	1.16	1.21	0.41
30	5.3207	279.2329	UN	0.27	0.75	0.54

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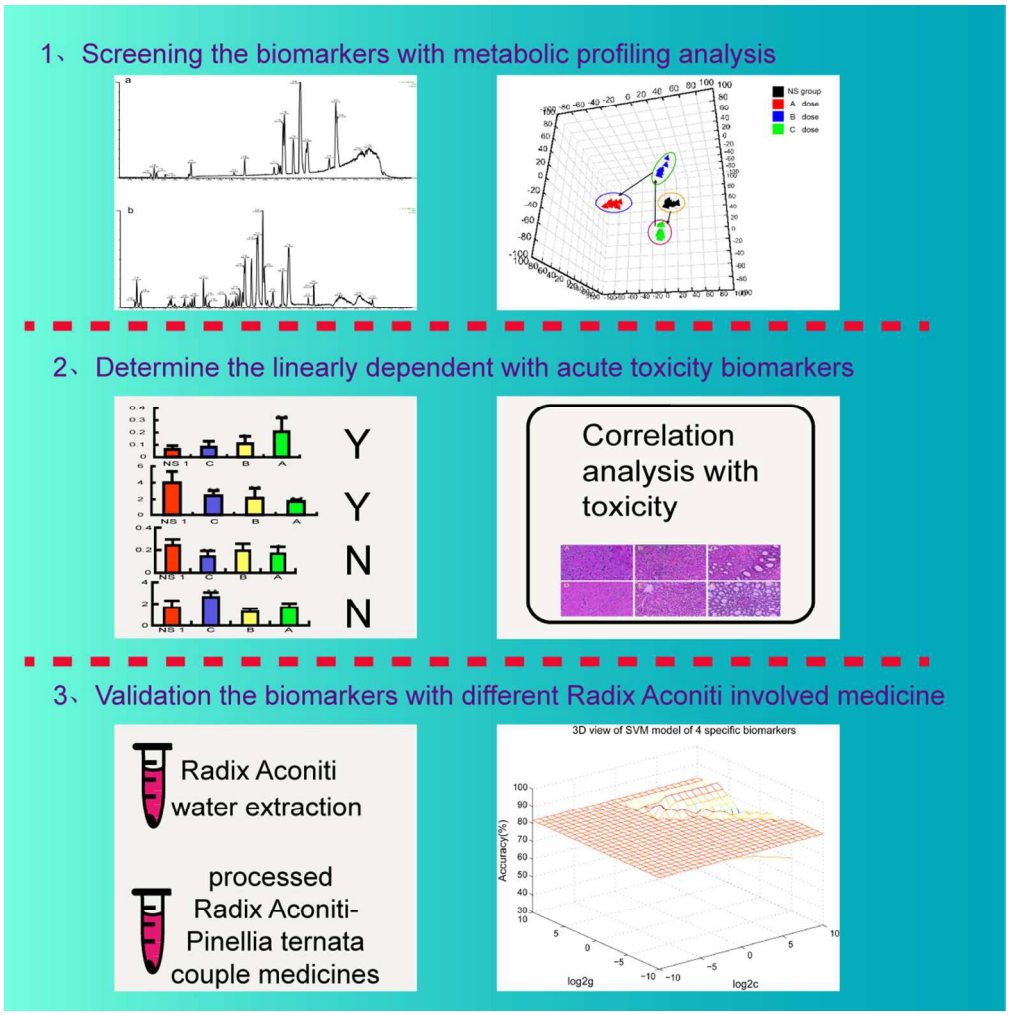
Table 3 The correlation between candidate metabolites and biochemical indicators

Metabolites	CK	LDH	ALT	AST	SCR	UREA
L-Valine	-0.981*	-0.957*	-0.844	-0.947	-0.842	-0.790
L-Acetylcarnitine	-0.991**	-0.974*	-0.877	-0.952*	-0.827	-0.808
LPC(22:5)	-0.993**	-0.985*	-0.903	-0.935	-0.883	-0.791
Shikimic acid	0.979*	0.977*	0.956*	0.969*	0.982*	0.928

The significance of the Pearson correlation analysis: *p<0.05; **p<0.01.



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