Simultaneous Quantification and Pharmacokinetics of Alkaloids in Herba Ephedrae-Radix Aconiti Lateralis Extracts

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The combination of Herba Ephedrae (Mahuang in Chinese) and Radix Aconiti Lateralis (Fuzi in Chinese) is a classical preparation in traditional Chinese medicine and used for treating colds and rheumatic arthritis. However, herbal medicines containing ephedrines and Aconitum alkaloids are strictly regulated because of the potential for adverse effects on the cardiovascular system and the central nervous system. We aimed to investigate the pharmacokinetics of 11 alkaloids in the Mahuang-Fuzi combination and single-herb extracts after oral administration in rats. The alkaloids were norephedrine, norpseudoephedrine, ephedrine, pseudoephedrine, methylephedrine, aconitine, mesaconitine, hypancotine, benzoylcoaconine, benzoylmesaconine and benzoylhypaconine. Simultaneous determination of the alkaloids, including two pairs of diastereomers, was achieved in 14.5 min by a simple, rapid and sensitive ultra-performance liquid chromatography–tandem mass spectrometry method. The separation was performed on a Zorbax SB-Aq column (100 mm × 2.1 mm, 3.5 μm) at a flow rate of 0.3 mL/min using acetonitrile-0.1% formic acid aqueous solution as the mobile phase. The validated method demonstrated adequate sensitivity, selectivity and process efficiency for the quantitative analysis of complex herbal components. Compared with single-herb extracts, alkaloids in plasma (except methylephedrine, benzoylmesaconine and benzoylhypaconine) showed slower elimination (the mean residence time or half-life was longer), although the maximum plasma concentration and area under the plasma concentration curve values decreased. Accumulation may occur with continuous drug intake. These results suggest that drug monitoring may be essential for the safe use of the Mahuang-Fuzi combination.

Introduction

The Herba Ephedrae-Radix Aconiti Lateralis extracts is the aqueous extract of the Herba Ephedrae-Radix Aconiti Lateralis combination used in traditional Chinese medicine (TCM) and is effective in the treatment of common cold symptoms, rheumatic arthralgia (1), bradyarrhythmias (2) and allergic rhinitis (3).

Herba Ephedrae (Mahuang in Chinese) consists of the dried herbaceous stems of Ephedra sinica Stapf., Ephedra intermedia Schrenk & C. A. Mey. or Ephedra equisetina Bunge (4). Ephedra sinica, the primary commercial species (5), has been widely used in China for the treatment of asthma and the common cold. The established pharmacological effects appear attributable to its ephedrine-type alkaloids (Figure 1A), mainly ephedrine (E) and pseudoephedrine (PE) (6). However, these alkaloids can also cause adverse effects on the cardiovascular (CV) system and central nervous system (CNS) (7). Dietary supplements containing ephedrine alkaloids have been banned in the United States (8).

Radix Aconiti Lateralis (Fuzi in Chinese) is the lateral root of Aconitum carmichaelii Debeaux, which is frequently used as an herbal medication in Japan, Korea and other countries for rheumatoid arthritis (9) and heart failure (10). However, it is also the herb most commonly associated with toxicity in China and Hong Kong (11). Aconitum alkaloids (Figure 1B), including diester alkaloids (aconitine [AC], hypaconitine [HA] and mesaconitine [MA]) and monoester alkaloids (benzoylcoaconine [BAC], benzoylhypaconine [BHA] and benzoylmesaconine [BMA]), have been identified as the main pharmacologic and toxic components (12, 13). These alkaloids have narrow therapeutic indices (14). Their potential toxicity, which is caused by hyperpolarization and activation of voltage-dependent sodium and calcium channels, can result in fatal cardiac poisoning and neurotoxicity (15–17).

In our acute toxicity test of Mahuang-Fuzi extract, the combination index (CI, calculated using Calcsyn 2.0, Biosoft, Cambridge, UK) was <1 when the dose was ≤76.69 g of crude aqueous extract/kg. A CI of <1 suggested that the toxicity of this combination was synergistic to some extent (Supplementary data, Figure S1). In addition, this extract showed an apparent advantage in an analgesic effect over the individual extracts (Supplementary data, Figures S1 and S2). These data may indicate that the combination of Mahuang and Fuzi could increase their efficacy and toxicity.

Although pharmacokinetic studies on the alkaloids of Fuzi (18–21) and Mahuang (22–26) have been reported, no data on the Mahuang-Fuzi extract are presently available. Given the potential adverse effects of this combination, determination of the alkaloid concentrations in plasma may be essential for its safe use. The objectives of this work were to develop a simple UPLC–MS-MS analytical method for simultaneous determination of the 11 alkaloids in plasma and to study the pharmacokinetic behavior of these alkaloids in rats after oral administration of Mahuang, Fuzi and Mahuang-Fuzi extracts.

Experimental

Materials and reagents

Standards for NE and NPE (purity, ≥98%) were provided by Aike Pharmaceutical Technology Co., Ltd (Chifeng, China). E hydrochloride, PE hydrochloride, ME hydrochloride, AC, MA, HA, BAC, BMA, BHA, diphenhydramine hydrochloride (DP, used as internal standard-1, IS-1) and lappaconite hydrobromide (LA, IS-2) were supplied by the National Institutes for Food and Drug...
Control (Beijing, China). *Radix Aconiti Lateralis* (batch number: 20130801) and Herba *Ephedrae* (batch number: 20110901) were purchased from Guangzhou Zhixin Chinese Medicine YinPian Co., Ltd. (Guangzhou, China). These plant materials were authenticated through macroscopic and microscopic examination by Professor Ji Ma (Department of Chinese medicine authentication, School of TCM, Southern Medical University, Guangzhou, China). HPLC-grade acetonitrile (Merck, KGaA, Darmstadt, Germany) and formic acid (Kermel Chemical Reagent Co., Ltd.) were used for HPLC analysis. Distilled deionized water was freshly generated using a MilliQ Ultra-Pure Water System (Millipore, Billerica, MA, USA).

**Animals**

Male Wistar rats (200 ± 20 g) were supplied by the Lab Animal Center of Southern Medical University (license No. 44002100002118). The experimental protocol was approved by the University Ethics Committee for the use of experimental animals, and animal studies were performed according to the Guide for Care and Use of Laboratory Animals.

**Preparation of herb extracts**

*Mahuang* (96 g) was immersed in water (1:20, w/v) for 30 min and boiled for 30 min. *Fuzi* (176 g) was then added and boiling continued for 70 min. The extraction method was described in the *Formula of Traditional Chinese Medicine* (27). Water in the extracts was evaporated under reduced pressure and the extracts were concentrated to 0.6, 1.1 and 1.7 g/mL of *Mahuang*, *Fuzi* and *Mahuang-Fuzi* extract, respectively. Single-herb extracts were prepared in the same way.

**Preparation of standard solution and quality control samples**

Stock solutions were prepared by accurately weighing and dissolving the 13 standard and reference compounds either in methanol for the ephedrines, IS-1 and IS-2, or in acetonitrile containing 0.1% hydrochloric acid for the *Aconitum* alkaloids. A mixed stock solution was prepared by mixing the five stock solutions of the ephedrine compounds to yield these final concentrations: NE 20.2 μg/mL, NPE 20.1 μg/mL, E 202.4 μg/mL, PE 101.2 μg/mL and ME 41.4 μg/mL. Mixed *Aconitum* alkaloids and internal standards were prepared by similar methods to yield the following final concentrations: AC 40.4 μg/mL, MA 40.2 μg/mL, HA 40.6 μg/mL, BAC 80.3 μg/mL, BMA 80.0 μg/mL, BHA 79.9 μg/mL, IS-1 400.0 ng/mL and IS-2 100.0 ng/mL. Serial dilutions were prepared for quality control (QC) samples and calibration curves. The standards and QC samples were extracted on each analysis day with the procedures described below for plasma samples.

**Sample preparation**

A solution of IS-1 and IS-2 (40 μL) and 80 μL of methanol was added to an Eppendorf tube and evaporated to dryness under a stream of nitrogen. Rat plasma (200 μL) was transferred to the tube and vortex-mixed for 1 min. A mixture of dichloromethane and methyl tert-butyl ether (3:7) (1 mL) was added to the plasma and vortexed for 2 min. After centrifugation at 17,760 × g for 15 min, the supernatant was transferred to a new tube. An additional 1 mL of extractant was added to the plasma and the extraction procedure was repeated. The supernatants were combined and dried under a stream of nitrogen. The residue was reconstituted with 200 μL of 10% ACN: 90% water (containing 0.1% formic acid) and centrifuged at 20,385 × g for 20 min. A 2-μL aliquot of supernatant was injected into the UPLC–MS-MS system for quantitative analysis.

**Pharmacokinetic study**

Eighteen Wistar rats were randomly divided into three groups (six rats per group), i.e., a *Mahuang* group, a *Fuzi* group, and a *Mahuang-Fuzi* group. Rodents in these groups were adminis-tered an oral dose of 10 mL/kg (equivalent to 6 g crude *Mahuang*/kg, 11 g crude *Fuzi*/kg). Blood samples (0.5 mL) were collected from the orbital plexus at 13 time-points after dosing (5, 15, 30, 45, 60, 90 min, 2, 3, 4, 5, 7, 12 and 24 h) into heparinized micro-centrifuge tubes. Plasma was prepared by centrifuging each blood sample at ≈9,060 × g for 10 min, and the resulting plasma was stored in polypropylene tubes at −20°C until analysis. Data from these samples were used to construct
the pharmacokinetic profiles by plotting the drug concentration over time. A non-compartmental analysis that does not require the assumption of a specific compartmental model for the drug of interest was used in this study, DAS 3.2 (Mathematical Pharmacology Professional Committee of China, Shanghai, China), was used to calculate the pharmacokinetic parameters for each rat. The pharmacokinetic parameters were performed using SPSS (SPSS for Windows, Version 16.0. Chicago, SPSS Inc.). Comparisons between two groups were performed using the unpaired Student’s t-test. The value of \( P \leq 0.05 \) was considered statistically significant.

**Instrumentation**

**Liquid chromatography**

Chromatographic analysis was performed on an Agilent 1,290 Infinity LC system (Agilent Technologies, Wilmington, Delaware, USA) equipped with an online degasser, a binary pump, a high-performance SL autosampler and a temperature-controlled column compartment. Separation of the analytes was achieved on a Zorbax SB-Aq column (100 mm × 2.1 mm, 3.5 μm; Agilent Technologies, USA) using a mobile phase that consisted of acetonitrile (A) and 0.1% formic acid aqueous solution (B) in a gradient program: 0%A→0%A at 0–2.0 min; 0%A→5%A at 2.1–5.0 min; 5%A→35%A at 8.0–9.5 min; 35%A at 9.5–12.5 min; 35%A→0%A at 12.5–13.0 min. The flow rate was 0.3 mL/min, the injection volume was 5 μL and the column temperature was set at 25°C.

**Mass spectrometry**

The detector system for the UPLC system was a 6410B triple-quadrupole mass spectrometer (Agilent Technologies, USA). Analytes were detected by using multiple reaction monitoring with an electrospray source in positive mode. An Agilent Mass Hunter workstation was used for data acquisition and analysis. The source parameters were set as follows: capillary voltage, 4,000 V; MS heater temperature, 100°C; drying-gas flow, 10 L/min; drying gas temperature, 350°C; nebulizer pressure, 40 psi.

**Method validation**

The current method was validated for selectivity, linearity, precision, accuracy, extraction recovery, matrix effect and stability according to the FDA guidelines for the validation of bioanalytical methods (28).

**Specificity**

The specificity of the method was evaluated by comparing the chromatograms of six blank rat plasma samples, plasma samples spiked with the analytes and IS and plasma samples after a single oral dose from different individuals. Blank rat plasma samples were analyzed for endogenous interference. Blank plasma samples were spiked with internal standards to assess whether any peak may interfere with the quantification of analytes.

**Linearity and the lower limit of quantification**

Calibration plasma samples were prepared by adding ephedrines, Aconitum alkaloids and mixed IS working solution (40 μL) to an Eppendorf tube. The solution was evaporated to dryness with a nitrogen stream, and then samples were prepared according to the methods described in the section “Sample preparation.” The linearity of each calibration curve was determined by plotting the peak area ratio of the analytes to IS against the nominal concentration of analytes with weighted (1/x) least square linear regression. A least squares linear regression equation with a correlation coefficient of 0.990 or better was required. The lower limit of quantification (LLOQ) was defined as the lowest concentration on the calibration curve with an acceptable accuracy and precision, i.e., a relative error (RE) of ± 20% and a relative standard deviation (RSD) lower than 20%.

**Assay precision and accuracy**

The intra- and inter-batch precision and accuracy were assessed by quantifying three concentrations of QC samples (six samples for each concentration) on the same analytical run and on three different analytical runs, respectively. The precisions and accuracies were evaluated by RSD and RE, respectively.

**Matrix effect and extraction recovery**

The extraction recovery and matrix effect were assayed at three QC concentrations in sets of six replicates. The extraction recovery was calculated by comparing the peak area of analytes added to plasma from untreated rats and then extracted with that of analytes added into pre-extracted plasma from untreated rats. The matrix effect was evaluated by comparing the peak area of analytes added into pre-extracted plasma from untreated rats with that of analytes dissolved in matrix-free solvent.

**Stability**

Stability was evaluated under conditions that were present during sample analysis. Short-term stability was evaluated by keeping QC samples at room temperature for 12 h. Long-term stability was assessed by storing QC plasma samples at low temperature (−20°C) for 14 days. Post-preparation stability was measured by placing QC samples under auto sampler conditions (15°C) for 12 h. Freeze and thaw stability was tested by analyzing QC samples that were subjected to three freeze–thaw cycles (−20°C to room temperature) on three consecutive days.

**Results**

**Quantification of alkaloids in extracts of Mabuанг, Fuzi and their combination**

The identification, quantification and chromatographic separation of ephedrines and Aconitum alkaloids were performed using our validated HPLC method (29) and the Chinese Pharmacopoeia (30), respectively. The content of alkaloids in the Mabuанг-Fuzi aqueous extract was (in μg/mL): NE 130.4 ± 4.2, NPE 139.3 ± 1.1, E 1,772 ± 31.9, PE 607.6 ± 13.7, ME 107.1 ± 8.8, HA 36.7 ± 2.7, BAC 38.6 ± 4.1, BMA 149.1 ± 7.6 and BHA 106.4 ± 4.1. The alkaloid content of Mabu앙 aqueous extract was (in μg/mL) NE 241.4 ± 18.0, NPE 326.3 ± 14.2, E 3,320 ± 136.4, PE 1,428.9 ± 8.4 and ME 331.4 ± 17.7. None of the Aconitum alkaloids was detected in the Mabu앙 extract. The alkaloid content in the aqueous Fuzi extract was HA 46.7 ± 3.1 μg/mL, BAC 68.3 ± 2.9 μg/mL, BMA 258.5 ± 9.3 μg/mL and BHA 191.2 ± 14.9 μg/mL. The concentrations of AC and MA were lower than the limit of detection because...
Figure 2. Representative multiple reaction monitoring chromatograms of (1) Norephedrine/Norpseudoephedrine/Methylephedrine (2) Ephedrine/Pseudoephedrine/Internal standard-1, (3) Aconitine/Mesaconitine/Hypaconitine/Benzoyleaconine/Benzoylemesaconine/Benzoylehypaconine (4) Internal standard-2. (A) Blank plasma, (B) blank plasma spiked with standard solutions in LLOQ, (C) plasma sample after oral administration of Mahuang extract, (D) plasma sample after oral administration of Fuzi extract and (E) plasma sample after oral administration of Mahuang-Fuzi extract.
of hydrolyzation (31). None of the Mahuang alkaloids was detected in the Fuzi extract.

**UPLC–MS-MS conditions**

Satisfactory separation of diastereomers (Supplementary data, Figure S2) and high sensitivity were achieved in these studies. Proper transitions between the precursor and product ions of the analytes and IS (Supplementary data, Table SI) played an important role in sensitivity and accuracy.

**Specificity and selectivity**

Representative chromatograms of blank plasma, blank plasma spiked with a standard solution at the LLOQ level and plasma from rats following oral administration of extracts are shown in Figure 2. Under the established chromatographic conditions, there were no endogenous substances in plasma producing interference with the analytes of the IS. All 11 analytes and IS were well separated from each other.

**Linearity of calibration curves and LLOQ**

The weighted calibration curves for all analytes exhibited good linearity (Supplementary data, Table SII). The LLOQs for the analytes were (in ng/mL) NE 0.2, NPE 0.2, E 2.0, PE 1.0, ME 0.4, AC 0.05, MA 0.05, HA 0.05, BAC 0.10, BMA 0.10 and BHA 0.10.

**Accuracy and precision**

The reproducibility of the method was evaluated by examining both intra- and interday variability. The intra- and interday RSD (%) of the 11 analytes at low-to-high concentrations was lower than 12.3%. Assay accuracy ranged from $-11.8$ to $+11.8\%$. These data indicate that the LC–MS-MS method was reliable and reproducible. The results of these studies are summarized in Table I.

**Matrix effect and extraction recovery**

The extraction recoveries of the three level QC samples varied from 94.3 to 103.7%. The matrix effect of the blank plasma for all the analytes was found to be within the acceptable range, and all values were in the range from 97.6 to 102.3%. The results for the recovery and matrix effect for all of the analytes are shown in Table I. These assays demonstrate that the extraction recoveries were consistent and reproducible. Neither signal suppression nor enhancement via matrix effects was significant.

**Stability**

The stability studies are summarized in Table II. These studies show that the RSD values for short-term stability, long-term stability and freeze–thaw stability of analytes for high and medium concentration QC samples ($n = 6$) were within 6.6%, whereas those for the low-concentration QC samples ($n = 6$) were

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**Table I.**

Recovery, matrix effect, precision and accuracy of the analytes at three different concentrations in rat plasma ($n = 6$)

<table>
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<th>Analytes</th>
<th>Spiked conc. (ng/mL)</th>
<th>Recovery (%)</th>
<th>Matrix effect (%)</th>
<th>Precision (RSD%)</th>
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$^a$Relative standard deviation.
$^b$Relative error.
within 13.8%. All analyses were stable under conditions designed to replicate experimental conditions during sample preparation (in plasma at room temperature for 12 h), sample detection (three freeze–thaw cycles in at −20°C) and storage (in plasma at −20°C for 15 days).

Pharmacokinetic data analysis

This validated method was successfully applied to the pharmacokinetic study of the 11 alkaloids in rat plasma after oral administration of Mahuang, Fuzi, and Mahuang-Fuzi aqueous extracts. The mean plasma concentration–time profiles (n = 6) are shown in Figures 3 and 4, and the calculated pharmacokinetic parameters are shown in Table III.

Compared with samples from groups given single-herb extracts, the time achieved peak concentration (T\text{max}) for ME, AC, MA, HA, BMA and BHA were shorter in the plasma profile in the Mahuang-Fuzi group. Therefore, other components in the extract may contribute to the enhancement of its absorption. The peak plasma concentration (C\text{max}) for all constituents except BAC decreased and the area under the plasma concentration–time curve (AUC\text{0−\text{t}}) were decreased compared with the single-herb groups. These results are consistent with the variation in the contents of the main active components in aqueous extracts. C\text{max} and AUC\text{0−\text{t}} were closely related to drug concentration.

There were also increases in half-life (t\text{1/2}) and mean residence time (MRT\text{0−\text{t}}) for many of the alkaloids. Longer MRT\text{0−\text{t}} values and longer t\text{1/2} values were obtained in the Mahuang-Fuzi group compared with the single-herb groups, indicating that the complex interaction of ingredients in the combination may have delayed the elimination of some alkaloids.

The pharmacokinetic parameters of the 11 alkaloids showed statistically significant differences (P < 0.05) in some parameters including T\text{max}, C\text{max}, t\text{1/2} and AUC\text{0−\text{t}} between the single-herb and herb-combination groups, suggesting that drug–drug interactions may have occurred in this herb combination.

Discussion

Optimization of UPLC–MS–MS conditions

When attempting reverse-phase liquid chromatography separation of basic, hydrophilic compounds, it is difficult to achieve symmetrical peaks and acceptable retention factors. E and PE are diastereomers that are highly basic (pKa values, >9.3) and hydrophilic (log P values <1.74), similar to their demethylated analogs, NE and NPE. MS has high specificity, making it suitable for biopharmaceutical analysis. However, MS presents an additional challenge because diastereomers share very similar fragmentation patterns, and each analyte must be separated chromatographically prior to detection.

Solutions to this challenge may involve the use of highly aqueous mobile phases to retain polar analytes, and/or the use of chaotropic salts or ionic liquids as mobile phase additives. However, such approaches are unsuitable for a conventional stationary phase and interfacing with electrospray ionization mass spectrometry.

The Zorbax SB-aq column (2.1 mm × 100 mm, 3.5 μm), which used 0.1% formic acid in water as the initial gradient, was...
Figure 3. Mean concentration–time profiles of ephedrine-type alkaloids in rat plasma after oral administration of Mahuang and Mahuang-Fuzi extracts. Each point represents the mean ± SD (n = 6).
Figure 4. Mean concentration–time profiles of Aconitum alkaloids in rat plasma after oral administration of Fuzi and Mahuang-Fuzi extracts. Each point represents the mean ± SD (n = 6).
validated for the separation of the two pairs of diastereomers. In summary, the 11 active components and two internal standards were separated and quantified within 14.5 min, which provides a rapid and sensitive method compared with previous studies (23, 32).

Choice of internal standard

Choosing the appropriate internal standard is also an important aspect in achieving acceptable performance, especially with LC–MS–MS, where matrix effects can lead to poor analytical results. Isotopically labeled internal standards can provide excellent performance but may not be cost-effective. Several compounds that were investigated to find a suitable IS, DP and LA were found to be suitable for quantification of Ephedra and Aconitum alkaloids, respectively.

Pharmacokinetic study

As shown in Figures 3 and 4 and Table III, $t_{1/2}$ and MRT$_{0-0}$ values for all alkaloids except BMA and BHA were significantly higher in the Mahuang–Fuzi group, even though the alkaloid content in this aqueous extract decreased compared with that in the single-herb extracts. This demonstrated that elimination of the alkaloids in the Mahuang–Fuzi group was slower than that in the single-herb groups, which may have resulted from the drug–drug interactions. These results may explain why Mahuang–Fuzi extracts at a lower concentration, had a significant effect in acute toxicity tests (unpublished observations).

We hypothesized that the competition for or inhibition of drug-metabolizing enzymes by the constituents in Mahuang and Fuzi may reduce the metabolism and increase the residence time of the alkaloids in plasma. It has been reported that pretreatment with an Ephedra water decoction induced CYP1A2 activity, which accelerated the metabolism of theophylline and decreased the effect of theophylline in rats (33). Wang (34) found that an inhibitor of CYP3A decreased aconitine metabolism in a concentration-dependent manner. Liu (35) found that AC neither inhibits nor induces CYP3A in rats, indicating that AC does not cause CYP3A-related drug–drug interactions in the liver. However, the impact that changes in the activity of the CYP450 enzymes involved in ephedrine metabolism may have on the metabolism of Aconitum alkaloids has not been reported.

Chronic oral administration is a common method in the practice of TCM. According to the guidance by the FDA, CYP enzyme activity can be inhibited by a single dose of an inhibitor or induced by repeated exposure to an inducer in 3–5 days (36). The mechanism of drug accumulation seen with the combination of Mahuang and Fuzi remains an open question. Further investigation of the risk of Ephedra–Aconitum combinations and the effects of the CYP enzyme on these combinations are planned. Given the currently available data, careful therapeutic drug monitoring is essential for the concomitant use of Fuzi and Mahuang.

Conclusion

In this study, a rapid and reliable UPLC–MS–MS method was developed for simultaneous analysis of 11 alkaloids [NE, NPE, E, PE (two pairs of diastereomers), ME, AC, MA, HA, BAC, BMA and BHA] in rat plasma. The method was successfully applied to a pharmacokinetic study in rats after intragastric administration of Mahuang–Fuzi and single-herb extracts.
significant differences \( (P < 0.05) \) in the pharmacokinetic parameters (MRT, \( t_{1/2} \) and \( C_{\text{max}} \)) of the 11 alkaloids were observed between the rats orally administered the Mabuаng extract or Fuzi extract compared with the Mabuаng-Fuzi extract. These pharmacokinetic differences showed that combination of Mabuаng and Fuzi could decrease the plasma concentration of the alkaloids except for BAC and at the same time delay the elimination of the alkaloids except for ME, BMA and BHA. These results would be applicable to the safe administration of the combination of Mabuаng and Fuzi in the clinic.

Supplementary data

Supplementary data are available at Journal of Analytical Toxicology online.

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