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Determination of bulleyaconitine A in human plasma by liquid chromatography–electrospray ionization tandem mass spectrometry

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Abstract

A sensitive and specific high-performance liquid chromatography (HPLC)–electrospray ionization tandem mass spectrometry (MS–MS) was developed for the determination of bulleyaconitine A (BLA) in human plasma. BLA and internal standard (I.S.) ketoconazole were extracted from the plasma by a liquid–liquid extraction. The supernatant was evaporated to complete dryness and reconstituted with acetonitrile containing 0.1% acetic acid before injecting into an ODS MS column. The gradient mobile phase was composed of a mixture of acetonitrile (containing 0.1% acetic acid, v/v) and 0.1% acetic acid aqueous solution eluted at 0.3 ml/min. BLA and I.S. were determined by multiple reaction monitoring using precursor → product ion combinations at *m/z* 644.6 → 584.3 and 531.2 → 81.6, respectively. Linearity was established for the concentration range of 0.12–6 ng/ml. The recoveries of BLA ranged from 96.93 to 113.9% and the R.S.D. was within 20%. The method is rapid and applicable to the pharmacokinetic studies of BLA in human.

Keyword: Bulleyaconitine A

1. Introduction

Bulleyaconitine A (BLA, Fig. 1), a diester–diterpene type *Aconitum* alkaloid extracted from *Aconitum longtounense* T.L. Ming, is a potent analgesic and anti-inflammatory agent, and can be used for the treatment of rheumatoid arthritis, osteoarthritis, periarthritis humeroscapularis, lumbar muscle strain, sprain, etc. [1]. The analgesic effect of BLA might be related to the 5-HT level in brain, and no physical dependence has been observed. The relative analgesic effect of BLA was found to be as much as 3, 65 and 7200 times as potent as 3-acetylaconitine, morphine and aspirin, respectively [2]. In clinical, BLA is administered as low dose intramuscular injections (0.2 mg) or as oral tablets/soft-gel capsules (0.4 mg).

The potential toxicity of the diester–diterpene type *Aconitum* alkaloids such as BLA is severe. The LD_{50} of BLA, injected subcutaneously, is 0.92 mg/kg for mouse and 0.51 mg/kg for rat, respectively [1]. In animal studies, toxicity reactions such as breath depression, hydrocephalus and ventricular arrhythmia have been reported when large dose of BLA was administrated [1]. Although the major side effects of BLA injections in clinical application are nausea, numbness, palpitation, pain feeling and anaphylaxis at the site of injection [1,3], and no cases of death have been reported, it may be desirable to monitor the plasma concentration of BLA during clinical use. However, due to the low dose and rapid metabolism, the concentration of BLA in the biological matrix is extremely low. This poses great challenges in the analytical method development. Several methods including high-performance liquid chromatography (HPLC) with UV detection and mass spectrometry (MS) were developed for

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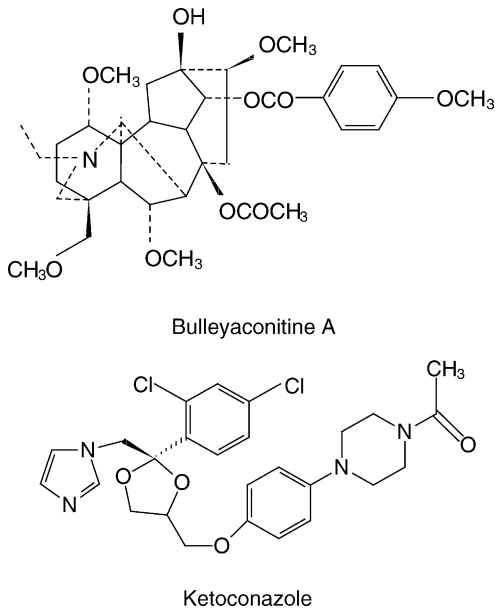

Fig. 1. Chemical structures of bulleyaconitine A and ketoconazole (internal standard).

the determination of *Aconitum* alkaloids including aconitine, mesaconitine and hypaconitine etc. in blood or urine [4–7]. Gas chromatography (GC) with MS detection that needed tedious derivatization has also been reported [8]. But none of the above methods were validated for the pharmacokinetic study in humans. To our best knowledge, no author has reported the analysis method of BLA in biological fluids or pharmacokinetic data on BLA in humans.

Our preliminary results showed that neither HPLC–UV nor HPLC–MS was sensitive enough to detect the plasma concentration of BLA after intramuscular injection of 0.2 mg in healthy volunteers. Therefore, a more sensitive method is needed for the determination of BLA in human plasma. High-performance liquid chromatography–electrospray ionization tandem mass spectrometry (MS–MS) is well known for its high specificity and sensitivity for the determination of drugs and metabolites in biological fluids. It provides an enhanced signal-to-noise ratio compared with single stage MS. In addition, the sample preparation can be simplified with no or little chromatographic separation required. The purpose of this work was to establish a rapid, specific and sensitive method for the determination of BLA in human plasma using HPLC–MS–MS and to apply this new method to a clinical pharmacokinetic study of BLA in healthy volunteers.

2. Experimental

2.1. Materials

BLA standard (the purity is 99.3%) was donated by Kunming Pharmaceutical Co. Ltd., and internal standard (I.S.) ketoconazole (99.6%, Fig. 1) was obtained from Nanjing 2nd Pharmaceutical Factory. BLA injections (0.2 mg/2 ml, Lot20010619) were purchased from Yunnan Pinbian Pharmaceutical Factory. Acetonitrile of HPLC grade was purchased from Merck (Darmstadt, Germany). Other reagents used were of analytical grade. Water was deionized, filtered and purified by a Milli-Q Reagent Grade Water System from Millipore (Millipore, Bedford, MA, USA). Drug-free human heparinized plasma was obtained from Shanghai Blood Center (Shanghai, PR China). Nitrogen (99.999%) and argon were purchased from Shanghai BOC Gas Industry (Shanghai, PR China).

2.2. Instrumentation

The mobile phase was delivered by an Alliance 2690 HPLC system (Waters, Milford, MA, USA) consisted of a quaternary pump, a column heater, an autosampler, a 7725i injector and a Millennium chromatographic work station. The detection was performed by a Quattro-LC ion trap mass spectrometer (Micromass, Manchester, UK) using electrospray ionization (ESI) for ion production and a Masslynx work station.

2.3. Liquid chromatography–ESI–mass–mass spectrometry

The chromatography separation was performed using Waters Xterra MS C_{18} reversed-phase column (3.5 μm, 2.1 mm × 50 mm) equipped with a guard column of symmetry C_{18} (3.9 mm × 20 mm). The column temperature was maintained at 25 °C. The mobile phase composed of a premix of solvent A (0.1% (v/v) acetic acid aqueous solution) and solvent B (0.1% acetic acid acetonitrile solution). The mixture was eluted at 0.3 ml/min by a gradient method: 40% B from 0 to 1.5 min, 40% B to 95% B from 1.5 to 4 min, and 95% B from 4 to 6 min. The temperature of the sample cooler in the autosampler was 10 °C.

Electrospray ionization was performed in the positive ion mode with nitrogen as the nebulizer and drying gas. Collision-induced dissociation (CID) was achieved using argon as the collision gas. The exact source conditions were: drying gas temperature, 450 °C; drying gas flow, 294 l/h; nebulizer gas flow, 51 l/h; source block temperature, 100 °C; capillary voltage, 3.85 kV; cone voltage, 42 V; RF lens voltage, 0.08 V; gas cell pressure, 7.2×10^{-4} mbar. The collision energies for both BLA and I.S. were 20.0 eV. The analyzer vacuum was set at 2.4×10^{-5} mbar.

The tandem-in-time MS was operated at unit resolution in the multiple reaction monitoring (MRM) mode. Sample control, data acquisition and processing were performed by Masslynx software (version 3.1). The ions for MRM analysis of the two channels of BLA and the I.S. were selected at *m*/*z* 644.6 and 531.2 as the precursor ions, and at *m*/*z* 584.3 and 81.6 as the product ions, respectively. The dwell time was 0.2 s.

2.4. Preparation of standard and quality control samples

The stock solution of BLA (600 ng/ml) was prepared in acetonitrile. The stock solution was further diluted with water to obtain a series of standard working solutions with concentrations in the range of 1.2–60 ng/ml. The samples for calibration or quality control (QC) during validation and pharmacokinetic study were prepared by spiking 1.0 ml drug free plasma with 100 μl standard working solutions.

The stock I.S. solution was a 200 ng/ml ketoconazole aqueous solution.

All the solutions were stored at 4 °C and were brought to room temperature before use.

2.5. Sample preparation

On the day of analysis, 50 μl of I.S. solution (200 ng/ml) was added to 1.0 ml of each plasma sample. This mixture was extracted with 5 ml of ether by vortex for 1 min. The organic and aqueous phases were separated by centrifugation at 1500 × *g* for 10 min. The upper organic phase was transferred to another tube and evaporated to dryness at 35 °C under a gentle stream of nitrogen. The residue was reconstituted with 100 μl of 0.1% acetic acid acetonitrile solution. The injection volume was 10 μl.

2.6. Method validation

2.6.1. Specificity

The chromatograms of the samples prepared from 11 human blank plasma were inspected visually to assess the potential interferences from endogenous substances. The apparent response at the retention times of BLA and I.S. was compared to the response at the lower limit of quantitation (LLOQ) for BLA and to the response at the working concentration for I.S.

2.6.2. Extraction recovery

The extraction recovery for BLA was determined by comparing the peak areas obtained from QC samples with the un-extracted standard working solutions at the same concentration in the same solvent.

2.6.3. Calibration and sample quantification

The calibration standards at levels of 0.12, 0.6, 1.2, 3.6, 6 ng/ml were extracted and assayed by above-mentioned method. Each concentration was analyzed in triplicate. The calibration curves ($y = ax + b$) were constructed by the plots of the peak-area ratios (y) of BLA to I.S. versus the concentration (x) of the calibration standards, and the linearity was established by least-squares regression. BLA concentrations in unknown samples were determined by interpolation from the calibration curve.

2.6.4. Accuracy and precision

The intra-day accuracy and precision were evaluated by the analysis of QCs at levels of 0.12, 1.2 and 6 ng/ml ($n = 5$ for each level) on the same day. These levels were chosen to demonstrate the performance of the method and to determine the LLOQ of the method. The upper limit of quantitation (ULOQ) was given by the highest level of the calibration curve. To assess the inter-day accuracy and precision, the assays were repeated on five different days. The recovery (%) represents the accuracy, while the relative standard deviation (R.S.D.) indicates the precision.

2.6.5. Evaluation of matrix suppression effect

The matrix effect (co-eluting, undetected endogenous matrix compounds that may influence analyte ionization) was investigated by extracting blank plasma from five different sources. The residues were reconstituted with 100 μl of 0.1% acetic acid acetonitrile solution containing a known amount of BLA. The reconstituted samples were analyzed and the peak areas of the BLA were compared with that in BLA standard at the same concentration.

2.6.6. Stability

The stability of reconstituted plasma samples after preparation was evaluated for every 6 h up to 24 h at 10 ± 0.5 °C. To assess the stability of BLA in plasma during frozen storage at −20 °C, the plasma samples were analyzed on day 1 and day 7.

3. Results

3.1. ESI mass spectra and MS–MS conditions

The full-scan ESI-positive mass spectra of BLA and I.S. are shown in Fig. 2. The pseudo-molecular ions $[M + H]^+$ were identified at *m*/*z* 644.6 and 531.2, respectively. The product ion spectra of the two compounds were acquired with these pseudo-molecular ions as precursors. The assay of BLA showed the predominant fragment ion at *m*/*z* 584.3, and for I.S. at *m*/*z* 81.6.

3.2. Retention times and specificity

High specificity was found in MRM mode for the determination of BLA in human plasma samples. The typical MRM chromatograms of blank plasma and spiked plasma samples are depicted in Fig. 3. The retention times of BLA and the I.S. were about 1.8 and 1.6 min, respectively. No additional peaks due to endogenous substances that could have interfered with the detection of the compounds of interest were observed. The chromatogram of an extracted plasma sample from a healthy volunteer who received BLA administration is also shown in Fig. 3.

3.3. Extraction recovery

The extraction recoveries under the liquid–liquid extraction conditions were 76.35 ± 6.874, 88.38 ± 5.931 and

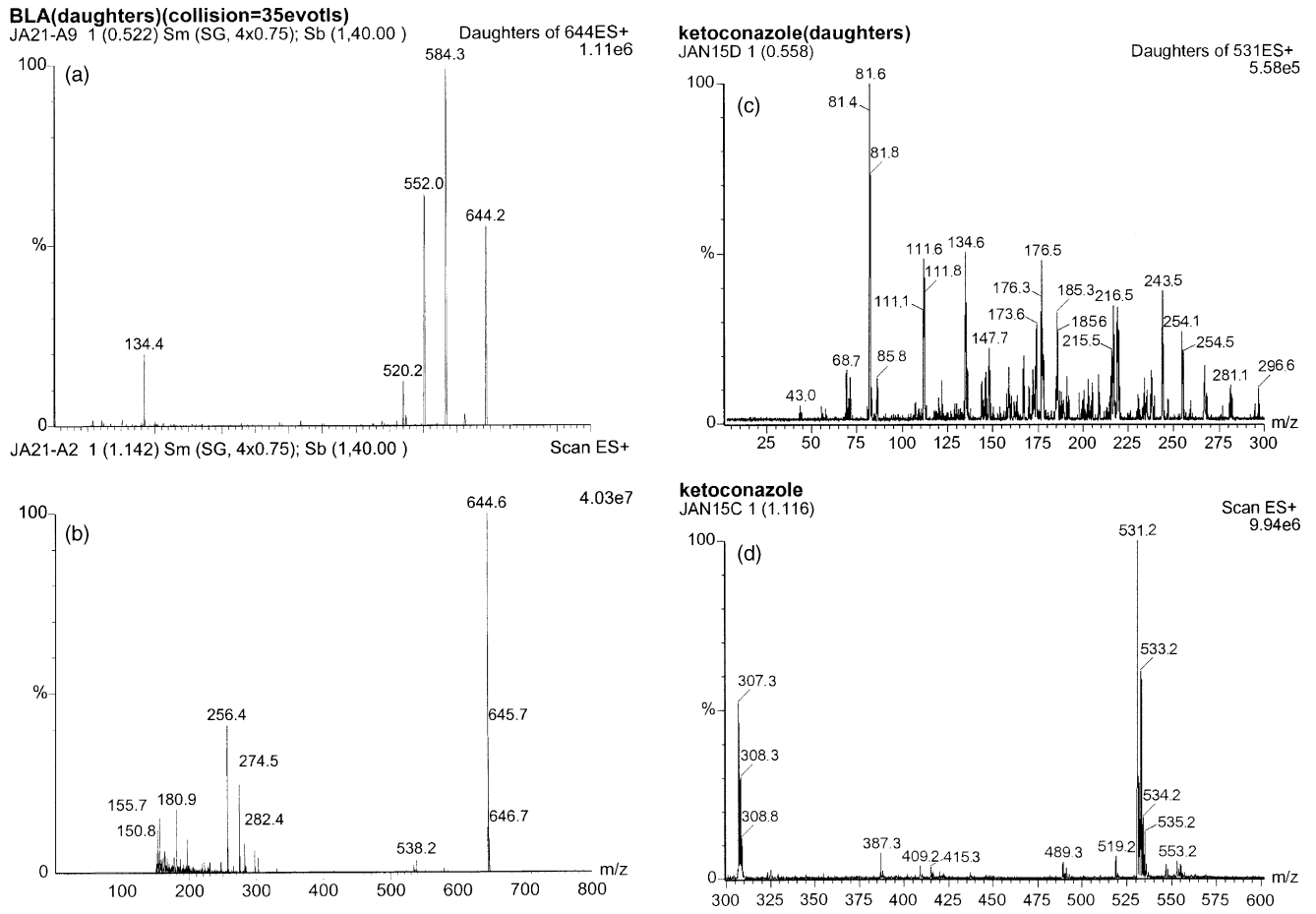

Fig. 2. ESI mass spectra of BLA (b) and I.S. (d) showing *m/z* 644.6 and 531.2 as the pseudo-molecular ions. Product-ion spectra of the two pseudo-molecular ions (a, c) showing *m/z* 584.3 and 81.6 as their predominant fragment ions, respectively.

84.93 ± 5.048% at the concentration of 0.12, 1.2 and 6 ng/ml (QC samples), respectively.

3.4. Calibration and sample quantification

Acceptable linearity was observed over the range of concentration from 0.12 to 6 ng/ml for BLA in human plasma ($r = 0.993$–0.999). The inter-day repeatability of calibration curves on three different days is shown in Table 1.

3.5. Accuracy and precision

The data from QC samples were calculated to estimate the intra- and inter-day precision and accuracy of the method. The results are presented in Table 2. The intra- and inter-day R.S.D. of BLA detection ranged from 5.514 to 16.49 and from 6.829 to 19.36, respectively. The % recoveries of the method for BLA ranged from 98.28 to 106.8 for intra-day, and from 96.93 to 113.9 for inter-day, respectively. These results were within the acceptable criteria for precision and accuracy.

The LLOQ, defined as the lowest concentration at which the analyte can be quantitated with an accuracy of 80–120% and a precision ≤20%, was 0.12 ng/ml for BLA in plasma.

3.6. Matrix suppression effect

The peak area of BLA in acetonitrile solution was 3047. Meanwhile, the mean peak area of BLA from the five differ-

Table 1
Inter-day precision in the slope and intercept of standard curves

Day	Slope	Intercept	Correlation
1	1.087	−0.1103	0.9990
2	1.349	−0.1582	0.9990
3	1.161	−0.06711	0.9948

Table 2
Summary of the precision and accuracy of the method ($n = 5$)

Spiked concentration (ng/ml)	Intra-day		Inter-day	
	Recovery (%)	R.S.D. (%)	Recovery (%)	R.S.D. (%)
0.12	106.8	16.49	113.9	19.36
1.2	101.3	13.44	100.2	15.28
6.0	98.28	5.514	96.93	6.829

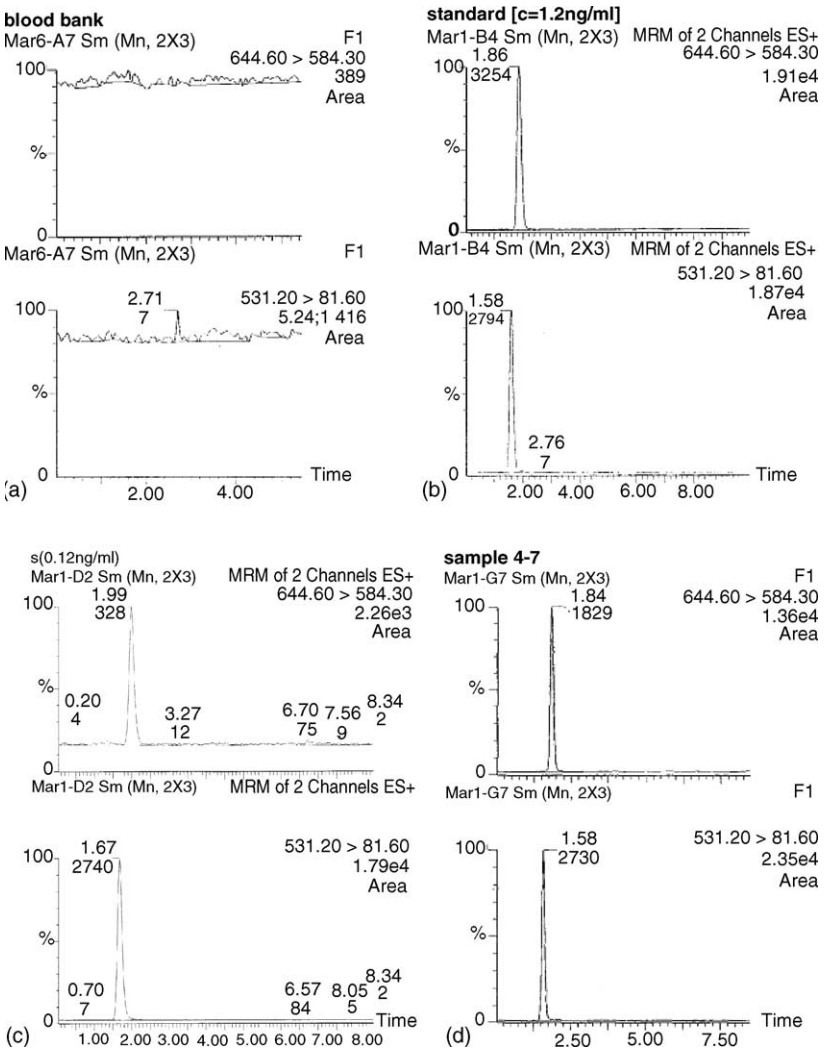

Fig. 3. Typical chromatograms of (a) blank plasma; (b) quality control sample (1.2 ng/ml); (c) quality control sample (0.12 ng/ml); (d) actual human plasma sample after single intramuscular injection of 0.2 mg BLA (2 h after administration).

ent reconstituted extracts was 3158 (R.S.D. = 14.33%). The results indicated that the extracts had little or no detectable co-eluting compounds that could influence ionization of the BLA.

3.7. Stability

The analytes were found stable in plasma after 7 days of storage at −20 °C. The recovery calculated from the QC samples ranged from 86.08 to 107.6%. BLA was stable in the reconstitution solution at 10 °C for at least 24 h (R.S.D. = 12.26%).

3.8. Application of analytical method in pharmacokinetic studies

After a single intramuscular injection of 0.2 mg BLA to 11 healthy males, venous blood samples (3 ml) were collected for the measurement of plasma BLA concentrations by the above-mentioned method. This pharmacokinetic study was an open-label, uncontrolled, single-dose, single-center study. The study protocol, amendments and subject consent forms were reviewed and approved by Ethics Committee of

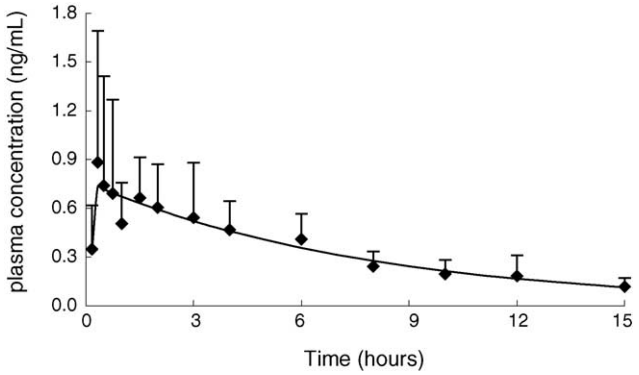

Fig. 4. Mean plasma concentration–time profile of 11 healthy male volunteers after receiving a single intramuscular injection of 0.2 mg bulleyaconitine A.

Base for Drug Clinical Trial of SFDA, Shanghai Zhongshan Hospital, PR China. Fig. 4 displays the mean plasma concentration–time curve ($n = 11$). It can be seen that the concentrations of BLA in human plasma were detectable for at least 12 h after the intramuscular administration. The results of pharmacokinetic study show that BLA is absorbed rapidly after intramuscular injection. Some pharmacokinetic parameters are as follows: t_{max} is 0.8890 ± 0.6766 h, C_{max} is 1.134 ± 0.7576 ng/ml, and $t_{1/2}$ is 4.877 ± 0.9714 h.

4. Conclusion

In this study, a highly sensitive and specific method for the determination of BLA in human plasma was developed using high-performance liquid chromatographic separation with tandem mass spectrometric detection. The method is rapid and practically applicable to the pharmacokinetic studies of BLA in human.

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