Determination of Trace Cantharidin in Plasma and Pharmacokinetic Study in Beagle Dogs Using Gas Chromatography–Mass Spectrometry

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Abstract

The blister beetle is traditional Chinese medicine that was first discovered and used as an anticancer drug in China, and cantharidin proved to be its principal active ingredient. Cantharidin-based pharmaceutical preparations are now widely used in clinics in China with good therapeutic efficacy. As a toxic anticancer drug, the therapeutic dose of cantharidin is low, and no method to determine the blood cantharidin concentration under the therapeutic dose has so far been reported. Here, we present a simple, sensitive, and reliable gas chromatography–mass spectrometry (GC–MS) method to monitor the plasma cantharidin and perform the pharmacokinetic study of cantharidin in beagle dogs. After protein precipitation by hydrochloric acid, a liquid–liquid extraction procedure using ethyl acetate was applied to extract cantharidin from plasma. An elastic quartz capillary GC column DB-5MS was used in GC–MS, the temperature was kept at 60°C for 1 min, then increased to 220°C at the rate of 6°C/min, held there for 1 min, and then to 280°C at the rate of 20°C/min, held for 3 min. The extraction recovery was over 80% for all the tested specimens. The linearity ranged from 2.14 to 314.2 ng/mL, the intra- and interday precisions were both below 20%, the limit of detection was 0.5 ng/mL, and the limit of quantification was 2.14 ng/mL. Cantharidin in plasma proved to be stable during the whole period of storage, treatment, and analysis. Cantharidin demonstrated as one-compartment model after i.v. administration with an elimination half-life of 0.69 ± 0.03 h and area under curve of 204 ± 24 h·ng/mL. This GC–MS assay proved to have high precision, accuracy, reliability, and sensitivity, and it was suitable for determination of trace cantharidin in plasma.

Introduction

As described in China Pharmacopeia (1), Mylabris is the dry body of Mylabris phalerata Pallas and Mylabris cichorii Linnaeus. According to the record in Shen Nong’s Herbal Classic, a very famous book about medicine written in ancient China, Mylabris had the biological action of eliminating toxic material, eroding mycosis, removing blood stasis, and dispersing obstructions and lumps, among others, and thus could be used to treat such diseases as carbuncle, boils, ulcer, and mycosis. Modern research indicated that cantharidin (CA) (2,3-dimethyl-7-oxabicyclo[2.2.l]heptane-2,3-dicarboxylic acid anhydride) (Figure 1) is the principal active ingredient among many kinds of compounds in Mylabris. The first CA crystalline was isolated from Lytta vesicatoria by a French pharmacist (Robiquet) in 1810.

As traditional Chinese medicine that has an anticancer effect, Mylabris was first discovered and applied in practice in China; its use can be traced back 2000 years (2). CA has demonstrated particular therapeutic efficacy in the treatment of cancer and some refractory diseases. Modern pharmacologic studies prove that CA can interfere with the metabolism of nucleic acids and the metabolism of proteins in cancer cells, significantly inhibit the growth of various implanted tumors in animal models, and has an inhibitory effect on primary hepatoma and certain other carcinomas, such as uterine cervical cancer, nasopharyngeal carcinoma, cutaneous cancer, and leukemia, among others (3–5).

There are now many kinds of Mylabris-based pharmaceutical preparations in the Chinese market, such as compound Mylabris injection (Aidi injection, State Medical Permit No. Z52020236) and compound Mylabris capsules (State Medical Permit No. Z19993294; State Medical Permit No. Z20003270; State Medical Permit No. ZP20000427); all proved to have a good anticancer effect.

Most of the literature about the analysis of CA uses gas chromatography (GC) (6,7) to determine CA in extracts or in its pharmaceutical preparations, all with low limit of quan-
tification. CA is a toxic anticancer drug, and it is stated in 2005 version of China Pharmacopoeia (1) that the lethal dose of CA is about 30 mg in humans. In clinics, CA is administered in the dose range of 0.5–4 mg/d, so it is hard to measure CA in blood specimens using GC alone because of the low concentration. The acute LD₅₀ of mice is 1.71 mg/kg. It is reported that its effective dose is very close to the toxic dose (3). Steyn et al. (8) reported the determination of human blood CA in a toxic patient and even in that case the blood CA was found to be only 76 ng/mL with a lower limit of quantification (LOQ) of 15 ng/mL. The LOQ of CA in biological specimens is a great barrier for research in vivo. So far, no method has been reported to measure CA in the biological samples after administration of clinic dose, and no literature has reported the pharmacokinetic study of CA in vivo.

Our aim is to develop and validate a sensitive and simple GC–mass spectrometry (MS) method to solve the problem of blood CA determination under the clinical dose and utilize this method as a monitoring tool to ensure safe CA use, such as therapeutic drug monitoring of the CA-based pharmaceutical preparations release in vivo. In this paper, the pharmacokinetic study of CA was performed, after intravenous CA administration to beagle dogs, using the established GC–MS analysis, and the basic pharmacokinetic parameters were obtained, which will provide the theoretical basis for the proper use of CA.

Experimental

Chemicals and reagents
The reference standard of CA (part number: 110783-200503) was purchased from National Institute for Control of Pharmaceutical and Biological Products (Beijing, China). The CA (the purity suggested to be above 98%) that was given to the dogs was purchased from Nanjing Qingze Medical Technological Development (Nanjing, China). The internal standard, clofibrate (IS) (purity above 99%) (Figure 1), was purchased from Alexis Biochemicals (Lausanne, Switzerland). Chromatography-grade ethyl acetate was obtained from Yong Da (Tianjin, China), and analytical-grade methanol was obtained from Tianjin Sijou (Tianjin, China). Hydrochloric acid was analytical-reagent grade and from Beijing Shiji (Beijing, China), and all other reagents were of analytical grade.

Six beagle dogs (male, healthy, weighing 10.8 ± 1.11 kg) were purchased from Tongli Laboratory Animals Center (Beijing, China) for the animal experiment. Animals were kept in a normally controlled breeding room with standard laboratory food and water for one week prior to the experiments. The dogs were maintained in accordance with internationally accepted principles for laboratory animal use, and the study was approved by the Beijing Animal Care Committee.

Chromatographic system and conditions
The GC–electron ionization (EI)–MS system consisted of TRACE GC with a THERMO DSQ MSD (ThermoFinnigan, San Jose, CA). Samples were injected in splitless mode on DB-5MS analytical column (30 m × 0.25-mm i.d., 0.25-µm film thickness, J&W Scientific, Folsom, CA). The carrier gas was helium with 99.999% purity at a flow rate of 1 mL/min. Injector temperature was set at 250°C. The column temperature was started at 60°C, held for 1 min, initially increased to 220°C at 6°C/min, and then to 280°C at 20°C/min. Temperature of the source and MS Quad were both 250°C. The MS was operated in single ion monitoring (SIM) mode with electron impact ionization. CA and IS ion fragments monitored were m/z 128 (Figure 2). Electron ionization (70 ev) was used.

Preparation of standard solution
Stock solutions of CA (0.1 mg/mL) were prepared in ethyl acetate and stored at 4°C until use. The initial stock solution was further diluted in ethyl acetate to produce a series of working standard solution of CA (2.14–342.4 ng/mL).

IS stock solution was made at an initial concentration of 280 µg/mL. The IS working solution (2.8 µg/mL) was made from the stock solution by methanol dilution.

Preparation of calibration standard and quality control samples
Calibration standard of CA at concentrations of 2.14, 4.28, 8.56, 21.4, 42.8, 64.2, 85.6, 171.2, and 342.4 ng/mL were prepared by spiking the appropriate amount of CA standard solutions in blank plasma obtained from healthy beagle dogs.

Similarly, quality control samples (QC) at concentrations of 2.8, 84, and 280 ng/mL were also prepared as described.

Sample preparation
To 0.5-mL aliquots of beagle plasma samples in disposable Eppendorf tubes were added with 200 µL hydrochloric acid (6 M), 28 ng of IS (10 µL of the 2.8 µg/mL), and 2 mL ethyl acetate. After being vortex mixed for 90 s, the tube was centrifuged at 15,000 × g for 15 min. An aliquot (1.4 mL) of the supernatant was transferred to a clean tube and evaporated to dryness under stream of nitrogen gas at 30°C. The residue was dissolved by 100 µL ethyl acetate. One microliter was injected into the GC system for analysis.
Validation of the method

The method was fully validated for specificity, linearity, LLOQ, accuracy, and precision.

Specificity and linearity

Blank plasma and plasma spiked with CA and IS were assessed by the procedure as described to evaluate specificity of the method. The retention time of the drug and IS under the chromatographic conditions for the CA assay was determined. The calibration samples (2.14, 4.28, 8.56, 21.4, 42.8, 64.2, 85.6, 171.2, and 342.4 ng/mL) were prepared in duplicates and assayed as described. The calibration curve was constructed by plotting the peak-area ratio of CA/IS versus CA nominal concentration. The calibration equation was obtained by linear least-squares regression analysis with the aid of Microsoft Excel.

Accuracy and precision

QC samples (2.8, 84, and 280 ng/mL) in five replicates were analyzed on the same day to determine the intraday precision and accuracy, and on five consecutive days to determine the interday precision and accuracy. The concentration of each sample was determined by using the calibration standards prepared on the same day.

Recovery

The extraction recovery at three different concentrations (2.8, 84, and 280 ng/mL) of CA was determined. The absolute recovery was determined in five replicates by comparing the peak areas of the extracted samples to the peak areas obtained from the organic solutions of CA at the same concentration.

Sensitivity

Sensitivity was determined by the detection limit (LOD, three times of the value of the background noise signal, S/N = 3) and the lowest quantification limit (LLOQ, 10 times the value of the baseline noise signal, defined as the lowest concentration at which both the accuracy and precision were less than 20%).

Stability

The stabilities of CA and IS solution were also tested at room temperature and 4°C, respectively, for 24 h. For freeze-thaw stability testing, the samples (three replicates at each QC concentration) were determined after three freeze (−20°C) and thaw (room temperature) cycles, and the concentration was compared to their nominal concentration. For the short-term stability, the samples (three replicates at each QC concentration) were extracted, stored at room temperature for 24 h, and then injected into the GC–MS system for analysis.

Pharmacokinetic study

CA was intravenously administered in a single dose of 34 µg/kg to dogs deprived of food but with free access to water for 12 h prior to the experiment. Each blood sample was drawn into a heparinized tube at 5, 10, 20, 40, 80, 120, 240, or 300 min after administration and immediately centrifuged at 4000 × g for 10 min at room temperature. The supernatant was then transferred to another tube and stored at −20°C until analyzed.

Results and Discussion

CA extraction from the blood specimen

CA is easily dissolved into lipophilic solvents such as ethyl acetate, chloroform, and toluene. Chloroform and toluene were once used as the extraction solvent for CA, but the strong toxicity of these two solvents often brings the safety consideration. It is said in the recent study (9) that hydrochloric acid help precipitate the plasma protein and leave little interference with the further analysis. In addition, the acidic environment will be beneficial in CA extraction from plasma (10) because CA has an anhydride moiety in its structure. Hydrochloric acid was used in this study to precipitate proteins from plasma, the supernatant collected after centrifugation was then extracted by less toxic ethyl acetate for further GC–MS analysis. Different concentrations of hydrochloric acid were tested, and the addition of 200 µL hydrochloric acid to 500 µL plasma for protein precipitation (the final concentration 6 M for hydrochloric acid) and the addition of 2 mL ethyl acetate into the collected supernatant for CA extraction gave the best CA extraction (over 80% in recovery rate). The procedure is simple and reliable.

Specificity

The base peak of CA (i.e., m/z 128) was selected for the quantification of peak area. Retention time (RT) was 18.35

![Figure 3. Representative SIM (m/z 128) chromatograms of CA and IS: blank dog plasma (A); dog plasma spiked with CA (85.6 ng/mL) and IS (28 ng/mL) (B); and plasma sample obtained at 80 min after intravenous dosing of 34 µg/kg of CA (C).](https://academic.oup.com/jat/article-abstract/33/7/384/756779)
min under set GC conditions, and no interfering peak was found at the same RT.

As Figure 3 shows, under the GC–MS conditions, no interference peaks were observed from the blank plasma as well as the pre-dose plasma samples from the beagle dogs which participated in the pharmacokinetic study of CA. This method provided a sufficient specificity.

**Linearity, precision, accuracy recovery, and stability**

Calibration curves of CA were linear over the concentration range of 2.14–342.4 ng/mL. Good linearity with a correlation coefficient $r = 0.9999$ was observed.

The absolute recovery of CA from plasma was determined to 80–87% (Table I). This method showed good precision and accuracy as summarized in Table II. The intraday precisions were measured to be less than 14.1%, and the interday precisions were measured to be less than 17.8%.

Stock solutions of CA and IS were stable for at least 30 days when stored at 4°C. No significant change in CA and IS was found after three freeze-thaw cycles and storage at room temperature for 24 h, for which the absolute recovery of the QC samples at three different concentrations ranged from 96.0 to 101.6% with an RSD of less than 5.0%.

**Sensitivity**

The LLOQ, defined as the lowest quantification concentration of CA, which can be detected in plasma was 2.14 ng/mL, which is 7 times lower than the LLOQ (15 ng/mL) (9) reported. Similarly the LLOD was 0.5 ng/mL. With very high sensitivity, the established method could be used to monitor the blood CA concentration under therapeutic dose so as to ensure the safety in clinic practice.

**Pharmacokinetic study**

The validated method was successfully applied to determine the plasma concentration of CA in beagle dogs following an intravenous administration of CA (34 µg/kg) (Figure 4). Descriptive statistical analysis was performed using Microsoft Excel. Individual time-course concentration data was analyzed for pharmacokinetic parameters using WinNonLin (Version 4.0, Pharsight Corporation) pharmacokinetic computer modeling program.

The one-compartment model best described the blood level data for CA after intravenous administration. The detailed pharmacokinetic parameters are shown in Table III.

It was evident that CA was distributed quickly into the organs and tissues after intravenous administration with the elimination half-life ($t_{1/2}$) of 0.69 ± 0.03 h.

In this paper, the dynamic course of CA in vivo after clinical dose in beagle dogs could provide much more reliable and valuable data that may help to understand the activity of CA in vivo, guide the rational use in treatment and instruct the design of more effective and reliable CA preparations dosage forms. In the next research, we will utilize this analytical method to study the tissue distribution of CA after administration, to reveal the accumulation activity in different tissues.

**Conclusions**

To the authors’ knowledge, this is the first study for the determination of plasma CA after clinical dose and its pharmacokinetic study. This method employed liquid–liquid extraction for sample preparation and was convenient for the quantification. The validation data demonstrated good precision and
accuracy. This method was successfully applied to the pharmacokinetic study of CA in beagle dogs followed a single intravenous dosing and the important pharmacokinetic characteristics were obtained.

In conclusion, the present study provides a simple and sensitivity method for the analysis of CA in blood to monitor the safe use of CA, and also provides the pharmacokinetic parameters for the rational use of CA in the future research.

References


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