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Measurement and pharmacokinetic study of plumbagin in a conscious freely moving rat using liquid chromatography/tandem mass spectrometry

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Abstract

The aim of the present study is to develop an automated blood sampling (ABS) method coupled to a liquid chromatography–tandem mass spectroscopy (LC–MS/MS) method to evaluate the oral bioavailability of plumbagin in a conscious freely moving rat. Plumbagin, an herbal ingredient, was isolated from *Plumbago zeylanica* L. The separation was performed using a reversed phase C18 (150mm × 4.6 mm I.D.; 5 μm) column and was eluted with the mobile phase of water–acetonitrile (40:60, v/v) at a flow-rate of 0.8 ml/min. Multiple reaction monitoring (MRM) was used to monitor the transition of the deprotonated molecule *m/z* 187 $[M-H]^-$ to the product ion *m/z* 159 $[M-H-CO]^-$ for the plumbagin analysis. The calibration curve was linear over the concentration range of 10–2000 ng/ml with a coefficient estimation of 0.995. The intra- and inter-day variations (% relative standard deviation; RSD and % bias) of the assay for rat plasma samples were less than 17%. The limit of detection and the limit of quantification were 5 and 10 ng/ml, respectively. Recovery of plumbagin from the rat plasma was about 80%. This LC–MS/MS method has been validated to study the pharmacokinetics of plumbagin in rats. The oral bioavailability $(AUC_{PO}/Dose_{PO})/(AUC_{IV}/Dose_{IV})$ of plumbagin was about 38.7 ± 5%.

Keywords: Bioavailability; Herbal medicine; Pharmacokinetics; Plumbagin; *Plumbago zeylanica* L

1. Introduction

Plumbagin (5-hydroxy-2-methyl-1,4-naphthoquinone) (Fig. 1A) is a natural naphthoquinone and a major component of the herb *Plumbago zeylanica* L. This plant is distributed in thickets or grassland at low elevations of Taiwan [1]. In traditional Chinese medicine, the whole plant and its root are used for the treatment of rheumatic pain, menostasis, carbuncle, and injury due to bumping [2]. Some pharmacological effects of plumbagin have been investigated on anticancer [3], anti-leishmanial [4], anti-bacterial [5], anti-fungal properties [6], anti-immediate allergic reaction [7] and anti-*Helicobacter pylori* infection [8]. Although naphthoquinones are widely distributed in nature and are extensively used by human for various purposes, there have been few reports about their toxicological properties [9,10].

Several chromatographic methods have been reported for the measurement of plumbagin from herbal extract [11–14]. Recently, an LC–MS/MS method has been reported for the quantification of plumbagin from the herbal extracts [15]. A previous report indicated that the limit of quantification analysis for plumbagin derived from *P. zeylanica* L. using LC-UV method was found to be 0.06 μg/ml. The retention time for plumbagin standard solution was 22.92 min [14]. In our previous report with LC–MS/MS, it was indicated that the run time was within 8 min.

In 1981, Chandrasekaran and Nagarajan indicated a colorimetric method to determine the metabolism of echitamine and plumbagin in rat [16]. However, the pharmacokinetics of plumbagin from *P. zeylanica* L. has not been clarified so far. Therefore, the aim of this study is to develop an LC–MS/MS method to investigate the pharmacokinetics of plumbagin following oral and intravenous administrations. The

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oral bioavailability of plumbagin will be performed by an automated blood sampling (ABS) method in a conscious and freely moving rat.

2. Experimental

2.1. Materials and reagents

The authentic plumbagin (purity 99.8%, by HPLC) was confirmed by using UV, IR, NMR and HPLC with a photodiode array detector and compared the data and melting point with previous reports [17,18]. The internal standard honokiol (Fig. 1B) was extracted and purified from *Magnoliaceae* in our laboratory. Plumbagin and internal standard were dissolved and stocked in methanol. HPLC-grade methanol and acetonitrile were purchased from Tedia Company Inc. (Fairfield, OH, USA). Milli-Q grade (Millipore, Bedford, MA, USA) water was used for the preparation of solution and mobile phase. All other reagents were of analytical grade.

2.2. Animals

Adult, male Sprague–Dawley rats (300 ± 20 g body weight) were provided by the Laboratory Animal Center at National Yang-Ming University (Taipei, Taiwan). These animals were specifically pathogen-free and were allowed to adapt in their environmentally controlled quarters (24 ± 1 °C and 12:12 h light–dark cycle). The diet (Laboratory Rodent Diet no. 5P14, PMI Feeds Inc., Richmond, IN, USA) and water were available *ad libitum*.

The rat was initially anesthetized with pentobarbital (50 mg/kg, i.p.), and remained anesthetized during a series of surgeries and its body temperature of rats was maintained at 37 °C with a heating pad. After surgery, the rat was placed in an experimental cage and allowed to recover for one day. During the period of anesthetic recovery, the rat was kept warm with a light.

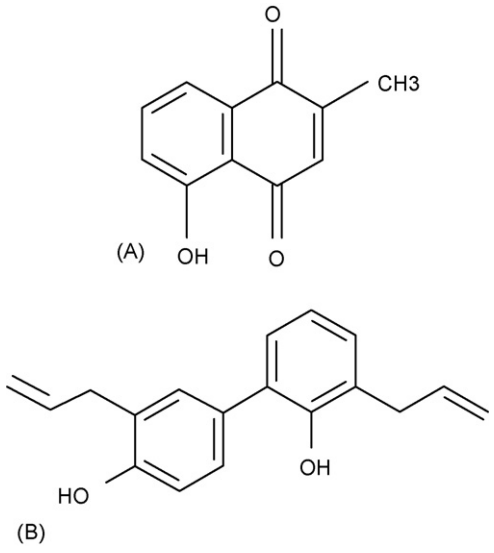

Fig. 1. Chemical structures of (A) plumbagin and (B) internal standard honokiol.

2.3. LC–MS/MS

LC–MS/MS analysis was performed using a Waters 2690 chromatography system with a 996 photodiode array detector and an autosampler (Bedford, MA, USA) coupled to a Micromass Quattro Ultima tandem quadrupole mass spectrometry (Micromass, Manchester, UK) equipped with an electrospray interface (ESI). The separation was performed on a ZORBAX Extend-C18 column (150mm × 4.6 mm I.D.; 5 μm, Agilent, Palo Alto, CA, USA) using water–acetonitrile (40:60, v/v) at a flow-rate of 0.8 ml/min. The mobile phase was filtered through a Millipore 0.45 μm filter. The injection volume was 20 μl. The infusion experiment was performed using a Mode 22 multiple syringe pump (Harvard, Holliston, MA, USA).

Analyses were performed in negative ionization mode by multiple reaction monitoring (MRM) and the following precursor to product ion transitions was used: m/z 187 → 159 for plumbagin and m/z 265.3 → 244.1 for the internal standard. The following parameters were optimized for plumbagin analysis: capillary voltage of 2.13 kV for negative ionization mode, desolvation gas (nitrogen) heated to 370 °C, and source block temperature of 80 °C. The cone voltage was set to 40 V and the collision energy voltage was set to 20 eV. The nebuliser and desolvation gas flows were 130 and 550 l/h, respectively. The collision gas was Argon 99.998% (Sanfu Chem., Taipei, Taiwan) with a pressure of 2.18×10^{-3} mbar in the collision cell. Total data were acquired using a dwell time of 0.3 s and interchannel delay of 0.1 s. All LC–MS/MS data were processed by the MassLynx version 3.5 NT Quattro data acquisition software.

2.4. Method validation

The linearity of the method was evaluated using freshly prepared spiked plasma samples in the mass concentration range of 10–2000 ng/ml. All calibration curves of plumbagin were constructed prior to the experiments with correlation values of at least 0.995. The intra- and inter-day variabilities for plumbagin were assayed (six replicates) at mass concentrations of 10, 50, 100, 500, 1000 and 2000 ng/ml on the same day and on six sequential days, respectively. The limit of detection was defined by the concentration with a signal-to-noise ratio (SNR) of 3. The limit of quantitation is defined as the lowest analyte concentration that can be measured with a stated level of concentration. The accuracy (% bias) was calculated from the nominal mass concentration (C_{nom}) and the mean value of the observed mass concentration (C_{obs}) is calculated as follows: bias (%) = $[(C_{obs} - C_{nom})/(C_{nom})] \times 100$. The relative standard deviation (RSD) was calculated from the observed mass concentrations as follows: %RSD = [standard deviation (SD)/C_{obs}] × 100.

2.5. Recovery of rat plasma

The stock solution of plumbagin was added to yield final concentrations of 0.5, 1 and 2 μg/mL in 50 μg/ml rat plasma. The individual experiment was repeated three times. Meanwhile plumbagin stock solution was diluted to 0.5, 1 and 2 μg/mL in methanol. Both the plasma sample and the diluted solution

were processed using the sample preparation method described above. The ratio of peak area (plasma sample/diluted solution) for plumbagin was used to calculate the recovery in plasma.

2.6. Pharmacokinetic application

Plumbagin (100 mg/kg) was given orally and the femoral vein was exposed for plumbagin injection (3 mg/kg). The automated blood sampling system DR-II (Eicom Kyoto, Japan) has been applied for blood sampling in conscious and freely moving rats. A 150 μl blood sample was withdrawn from the jugular vein into a heparin rinsed tube with a fraction collector according to a programmed schedule at 1, 5, 10, 20, 30, 40, 50 min, 1, 1.5, 2, 2.5, 3, 3.5, 4, 4.5, 5, 6, 7, 8, 9, 10, 12, 24 and 48 h after oral administration. In the case of the intravenous administration, blood samples were withdrawn from the jugular vein at 1, 5, 10, 20, 30, 40, 50 min, 1, 1.5, 2, 2.5 and 3 h following plumbagin administration using a heparinized tube and centrifuged at 6000 rpm for 10 min. The resulting plasma (50 μl) was mixed with 10 μl of internal standard (honokiol) at 5 ng/ml and 1 ml of ethyl acetate into a clean tube, vortex for 10 min and centrifuged at 10,000 rpm for 10 min. The upper organic layer was transferred to a new tube and evaporated to dryness under nitrogen flow. The dried residue was reconstituted with 100 μl of the mobile phase. A 20 μl aliquot of the solution was injected to the LC–MS/MS.

Pharmacokinetic parameters were calculated using the pharmacokinetic software WinNonlin Standard Edition Version 1.1 (Pharsight, Mountain View, CA, USA) by the non-compartmental method. The area under the concentration–time curve (AUC) and that under the first moment curve (AUMC) were calculated according to the log linear trapezoidal method. The clearance (Cl) was calculated as the total administered dose/AUC. Bioavailability was calculated according to the equation: bioavailability $(\%F) = 100 \times (AUC_{PO}/Dose_{PO})/(AUC_{IV}/Dose_{IV})$.

3. Results and discussion

3.1. LC–MS/MS

A reversed-phase C18 column with the mobile phase of water–acetonitrile (40:60, v/v) provided a good separation of plumbagin eluted within 8 min. No significant interfering peaks were present near the analyte peak. Fig. 2A shows a chromatogram of plasma blank, illustrating a clean and stable baseline without any interfering endogenous peaks. Fig. 2B shows the chromatogram of a plasma sample containing plumbagin (0.35 μg/ml) collected from rat plasma 2.5 h after plumbagin administration (100 mg/kg, p.o.). Fig. 2C shows the chromatogram of a plasma sample containing plumbagin (0.19 μg/ml) collected from rat plasma 1 min after plumbagin administration (3 mg/kg, i.v.). The analytes were well separated using the present chromatographic conditions. The retention times were 5.2 and 6.5 min for plumbagin and internal standard, respectively, without any visible peak distortions. After plumbagin administration, an unidentified peak was observed in blood

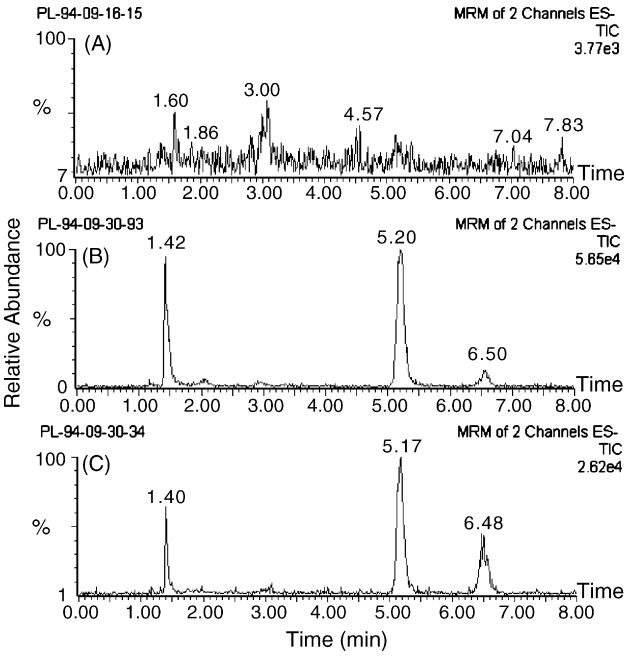

Fig. 2. Typical chromatograms of (A) plasma blank; (B) blood sample containing plumbagin (0.35 μg/ml) collected from the jugular vein at 2.5 h after plumbagin administration (100 mg/kg, p.o.); and (C) blood sample containing plumbagin (0.19 μg/ml) collected from the jugular vein at 1 min after plumbagin administration (3 mg/kg, i.v.): 1, plumbagin; 2, internal standard (honokiol).

at a retention time of 1.4 min (Fig. 2B and C). We suggested that it was an unidentified compound from biotransformation.

A colorimetric method has been reported for the determination of plumbagin in blood and urine samples. However, plumbagin was not detected in blood up to 24 h when it was administered into the rats [16]. For some reasons, it may be an unidentified compound present in the blood which could possibly interfere with the detection of plumbagin in the blood. One possibility is that the limit of detection may be greater than plumbagin in blood concentration and thus its detection in blood would not be determined using the colorimetric method. We therefore developed a more selective and reliable LC–MS/MS method for the determination of plumbagin in biological samples.

3.2. Linearity and limit of quantification

The linearity of calibration graphs was demonstrated by the good determination coefficients (r^2) obtained for the regression line. Good linearity was achieved over the range 10–2000 ng/ml, with all coefficients of correlation greater than 0.995. All samples were freshly prepared including the standard solution from the same stock solution (50 μg/ml). The limit of quantification of 10 ng/ml was defined the lowest concentration on the calibration curve that could be measured routinely with acceptable % bias and RSD.

3.3. Precision and accuracy

The intra- and inter-assay precision and accuracy values are presented in Table 1. The overall mean precision, defined by

Table 1
Intra- and inter-assay accuracy and precision values of the LC–MS/MS method for the determination of plumbagin in rat plasma

Nominal mass concentration (ng/ml)	Observed mass concentration (ng/ml)	Precision (RSD%)	Accuracy (% bias)
Intra-assay ($n=6$)			
10	10.20 ± 1.18	11.5	2.0
50	52.55 ± 2.96	5.63	5.1
100	97.97 ± 4.74	4.83	−2.0
500	493.5 ± 43.9	8.89	−1.3
1000	1017 ± 71.1	6.99	1.7
2000	2000 ± 10.7	0.54	0
Inter-assay ($n=6$)			
10	11.12 ± 1.89	17.0	11
50	47.91 ± 3.99	8.32	−4.1
100	100.2 ± 5.00	4.99	0.2
500	469.3 ± 24.9	5.30	−6.1
1000	977.4 ± 46.4	4.74	−2.2
2000	2040 ± 12.7	0.62	2.0

Observed mass concentrations are expressed as mean ± SD.

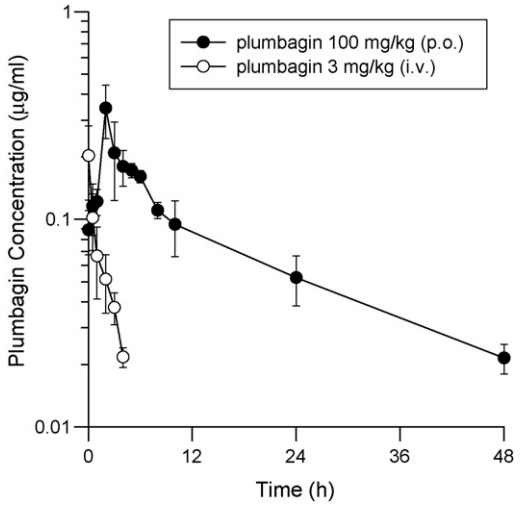

Fig. 3. Concentration–time profiles for plumbagin in blood after plumbagin i.v. and p.o. administration at dosage of 3 and 100 mg/kg, respectively. The data are mean ± SE from six individual experiments.

the RSD, ranged from 0.535 to 17.0%. Analytical accuracy, expressed as the percentage difference of the mean observed values compared with known concentration varying from −6.1 to 11%.

3.4. Recovery

The one-step extraction procedure was fairly rapid. Initially, simple protein precipitation by the organic solvent, methanol was tried to prepare the plasma samples for LC–MS/MS, but gave a poor recovery of 20–25% for plumbagin using external standard linear regression (data not shown). Ethyl acetate was selected as the solvent for our liquid–liquid extraction method. Our extraction method was slightly modified from Cheng et al. [19]. Ethyl acetate is a very popular extracting solvent because of its high polarity and volatility. The solvent ethyl acetate resulted in about 74–80% recovery of plumbagin from plasma. Hence, the liquid–liquid extraction procedure might be more advantageous as compared with protein precipitation in order to avoid the influence of unknown matrix constituents on the analyte signal.

3.5. Pharmacokinetic application

A typical plasma concentration–time profile is shown in Fig. 3. We collect 12 times of blood samples in the group of intravenous administration and collect 24 times of samples for the group of oral administration for pharmacokinetic calculation. It is due to the greater lipophilicity of the analyte, which may extensively distribute into the body tissue and causing slow elimination. Pharmacokinetic parameters such as half-life, volume of distribution, clearance and area under the plasma concentration–time graph were calculated by standard formulas [20]. The developed LC–MS/MS method was applied to evaluate the pharmacokinetic parameters of plumbagin from *P. zeylanica* L. The pharmacokinetic parameters are summarized in Table 2.

The oral bioavailability $(AUC_{PO}/Dose_{PO})/(AUC_{IV}/Dose_{IV})$ of plumbagin in a freely moving rat was 38.7 ± 5%. Due to its greater lipophilicity, plumbagin has been extensively distributed in the body tissue creating a deep compartment model causing slow elimination [16].

The traditional pharmacokinetic method of manually drawing blood from the animal requires the animal to be anesthetized or restrained with some device, both of which cause stress to the animal. It is known that stress affects the pharmacokinetics by reducing the absorption and altering the metabolism [21]. The LC–MS/MS method is a sensitive and convenient instrument to measure biological samples especially for the slowly excretion of plumbagin. The blood samples were immediately collected by an automated blood sampling system, which has been proven to be a powerful device involving a freely moving rat with low stress for pharmacokinetic study [22]. This approach should be reflected on the pharmacokinetics of plumbagin from a conscious animal. Therefore, we demonstrated an automated blood sampling system for serial blood sampling from conscious and freely moving rats for pharmacokinetic studies. In contrast with the previous report [16], this approach coupled with LC–MS/MS method provides more advantage and confidence for pharmacokinetic study.

Since the quinone pigments are widely distributed in nature and extensively used by human further work is necessary to

Table 2
Pharmacokinetic data after plumbagin administration (3 mg/kg, i.v. and 100 mg/kg, p.o.) in rat

Parameters	3 (mg/kg, i.v.)	100 (mg/kg, p.o.)
AUC (min μg/ml)	18.76 ± 5.44	271.9 ± 66.2
$t_{1/2}$ (min)	108 ± 64	1028 ± 323
C_{max} (μg/ml)	0.19 ± 0.08	0.35 ± 0.10
MRT (min)	166 ± 89.2	1318 ± 324
T_{max} (min)	0	150 ± 46

Data are expressed as mean ± SE from six individual experiments for each group.

clarify such as biotransformation, toxicological properties, and long-term effects of plumbagin from *P. zeylanica* L.

4. Conclusions

We developed an automated blood sampling method coupled to an LC–MS/MS method to evaluate the oral bioavailability of plumbagin in a conscious freely moving rat. To our knowledge, this is the first description of plumbagin pharmacokinetics in rat plasma by an LC–MS/MS method in the literature. The assay system and pharmacokinetic results could be useful for the application of plumbagin in the pre-clinical research.

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