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Determination and identification of plumbagin from the roots of *Plumbago zeylanica* L. by liquid chromatography with tandem mass spectrometry

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Abstract

Plumbagin (5-hydroxy-2-methyl-1,4-naphthoquinone) is an herbal ingredient which is isolated from the root of *Plumbago zeylanica* L. This herb is a semi-climbing subshrub distributed in thickets or grassland at low elevations of Taiwan. The crushed roots of *P. zeylanica* L. were ground from lumps to powder and boiled with H_2O, 50% EtOH, or 95% EtOH. Chromatographic separation of plumbagin from the herb was carried out using a ZORBAX Extend-C18 column (150 × 4.6 mm I.D.; 5 μm) that was eluted with the mobile phase of water–methanol (10:90, v/v). 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 plumbagin analysis. The limit of quantification was determined to and accuracy of 1 ng/ml. Furthermore, the mass fractions of plumbagin in *P. zeylanica* L. for H_2O, 50% EtOH and 95% EtOH were 0.24 ± 0.04, 3.92 ± 0.87 and 13.4 ± 1.59 g/kg, respectively. These results present a reliable liquid chromatography coupled with tandem mass spectrometric (LC–MS/MS) method for the determination of plumbagin form herbal medicines.

Keywords: Electrospray ionization tandem mass spectrometry; Plumbagin; *Plumbago zeylanica* L

1. Introduction

Plumbagin (5-hydroxy-2-methyl-1,4-naphthoquinone) (Fig. 1) is a naturally occurring yellow pigment derivative from the herb *Plumbago zeylanica* L., a semi-climbing subshrub distributed in thickets or grassland at low elevations of Taiwan [1]. The whole plant and its root have been used as a folk medicine in Taiwan for the treatment of rheumatic pain, menostasis, carbuncle, and injury due to bumping [2]. Plumbagin has been shown to have anticancer, anti-leishmanial, anti-bacterial and anti-fungal properties [3–5], as well as a contraceptive effect [6]. The ethanol extract of *P. zeylanica* stems has been investigated for its immediate allergic reaction, which is probably mediated by reducing the release of mediators such as histamine from mast cells by elevating intracellular cAMP level and weakening the inflammatory action of mediators [7].

Conventional extraction methods with organic solvents, as maceration or hot extraction in Soxhlet apparatus, are widely used to obtain plant extracts. However, prolonged heating time has been shown to promote plumbagin degradation [8]. The amount of dried plant extract depends on the analytical technique that will be employed. Other parameters should be considered such as: type of solvent, the organic solvent volume, the temperature and the duration of extractive process duration [9]. In order to develop a fast LC–MS/MS method, low toxic solvent extraction with water, 50% ethanol and 95% ethanol were used for the extraction in this study.

A normal-phase liquid chromatographic separation with *n*-hexane–chloroform–2-propanol (30:70:2) and UV detec-

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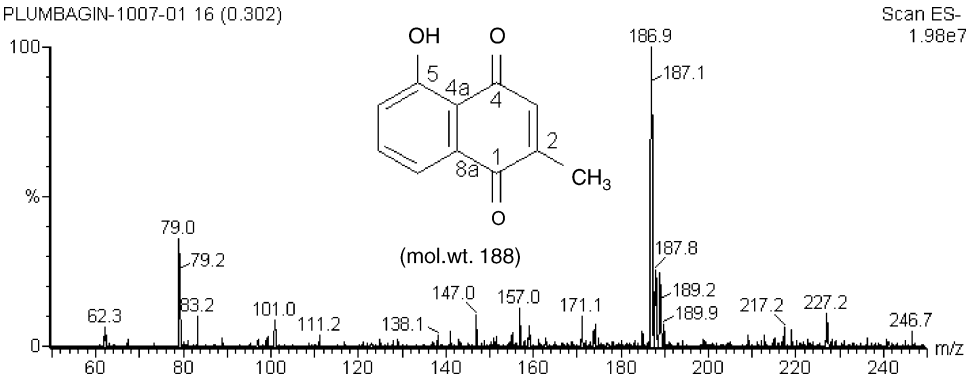

Fig. 1. Chemical structure of plumbagin (molecular weight 188) and full scan mass spectrum of plumbagin.

tion at wavelength 267 nm was used for the determination of plumbagin in the *P. zeylanica* L. [10]. Another normal-phase liquid chromatographic method was reported by Marston and Hostettmann who demonstrated the separation of plumbagin from six different naturally occurring naphthoquinones using μBondapak CN column. The mobile phase was *n*-hexane with 1% acetic acid with UV detection at 254 nm [11]. Subsequently, Stensen and Jensen used reversed-phase liquid chromatography for separation of plumbagin and its monomeric analogues [12]. To the best of our knowledge, there are no published methods for the determination of plumbagin from *P. zeylanica* L. with LC–MS/MS. The aim of this study is to develop a validated LC–MS/MS with negative selected ion monitoring mode method for the determination of plumbagin from *P. zeylanica* L. extract. The method was optimized and validated so that it might be applied to assay biological samples.

2. Experimental

2.1. Materials

Roots of *P. zeylanica* L. were collected in Wulai, Taipei county, Taiwan, during June, 2001. A voucher specimen (No. 1732) has been deposited in the herbarium of the Department of Botany of the National Taiwan University [13]. The authentic plumbagin (purity 99.8%, by HPLC) was extracted from *P. zeylanica* L. Triple deionized water from Millipore (Bedford, MA, USA) was utilized for all preparations, while methanol (HPLC grade), and chromatographic solvents were obtained from Tedia Company, Inc. (Fairfield, OH, USA).

2.2. Sample extraction

The crushed roots of *P. zeylanica* L. were ground from lumps into powder. Dried powder (1 g) of *P. zeylanica* L. was boiled with H_2O, 50% EtOH, and 95% EtOH (10 ml) for 3 min and the least volume of solvent was added to 10 ml. The herbal extracts were centrifuged at 8000 × *g* for 10 min and the supernatant was filtered with a syringe filter of 0.45 μm. The filtrate was then injected into LC–MS/MS.

2.3. Liquid chromatography–tandem mass spectrometry

LC–MS/MS analysis was performed using a Waters 2690 chromatography system with a 996 photodiode array detector (PDA) 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 of LC–MS/MS was achieved using a ZORBAX Extend-C18 column (150 × 4.6 mm I.D.; 5 μm, Agilent, Palo Alto, CA, USA). The mobile phase was comprised of water–methanol (10:90, v/v). The mobile phase was filtered through a Millipore 0.45 μm filter. The injection volume was 10 μl.

For operation in MS/MS mode, a mass spectrometer fitted with an orthogonal Z-spray ion interface was used for all analyses. Ionization was achieved using electrospray in the negative ionization mode. The infusion experiment was performed using a Mode 22 multiple syringe pump (Harvard, Holliston, MA, USA). The following parameters were optimized for plumbagin analysis: capillary voltage of 2.93 kV for negative ionization mode, desolvation gas (nitrogen) heated to 250 °C, and source block temperature of 80 °C. The cone voltage was set to 40 V and the collision energy voltage to 20 eV. The nebuliser and desolvation gas flows were 110 and 530 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 0.2 s and inter-channel 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, precision and accuracy were evaluated by analysing seven samples at each mass concentration. The precision of the method at each mass concentration was expressed as a coefficient of variation (CV) by calculating the standard deviation as a percentage of the mean calculated mass concentration, and the accuracy of the assay was determined by expressing the mean calculated mass concentration as a percentage of the added mass concentration.

Table 1
Intra- and inter-assay accuracy and precision values of the LC–MS/MS method for the measurement of plumbagin

Nominal mass concentration (ng/ml)	Observed mass concentration (ng/ml)	Precision (RSD %)	Accuracy (% bias)
Intra-assay ($n = 6$)			
1	1.0 ± 0.2	17	3.5
5	5.2 ± 0.2	4.7	3.8
10	9.7 ± 0.2	2.5	−2.5
50	50.0 ± 0.1	0.06	0.05
500	489 ± 17	3.4	−2.2
1000	1055 ± 44	4.1	5.5
5000	4989 ± 9.2	0.18	−0.2
Inter-assay ($n = 6$)			
1	1.1 ± 0.2	19	9.18
5	5.2 ± 0.2	2.3	1.75
10	9.8 ± 0.2	2.1	−2.07
50	49.7 ± 0.9	0.07	0.06
500	472 ± 22.7	4.8	−5.66
1000	1038 ± 49	4.8	3.76
5000	4994 ± 10.4	0.21	−0.12

Observed mass concentrations are expressed as mean ± SD.

All calibration curves (peak-areas versus mass concentrations) of plumbagin (external standards method) were made prior to the experiments with correlation values of at least 0.998. The intra-day and inter-day variabilities for plumbagin were assayed at mass concentrations of 1, 5, 10, 50, 500, 1000 and 5000 ng/ml (six replicates) on the same day and on six sequential days, respectively (Table 1). The accuracy (% bias) was calculated from the nominal mass concentration (C_{nom}) and the mean value of the observed mass concentration (C_{obs}) 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

The plumbagin was extracted with 95% EtOH from *P. zeylanica* L. and the herbal extracts were divided into four portions (one portion as control group). All herb extracts except the control group were spiked one-fold plumbagin standard solution at three different mass concentrations (0.1, 0.5 and 1 μg/ml). All samples were votex-mixed for further 50–60 s and filtered through a 0.45 μm Millipore filter. This assay was repeated three times and three replica analyses were performed. The recoveries for the plumbagin were calculated by subtracting the mass concentrations of non-spiked herb extracts using external standard linear regression.

3. Results and discussion

The sliced roots of *P. zeylanica* L. (2.2 kg) were extracted with 95% EtOH (40 L × 3). The concentrated extract was successively partitioned between H_2O (1 L) and *n*-hexane (1 L × 3), followed by EtOAc (1 L × 3) and *n*-BuOH (1 L × 3). The EtOAc extract (21 g) was subjected to silica gel column chromatography with a gradient of EtOAc in *n*-hexane, and 10 fractions were collected. Fraction 1 was rechromatographed over Silica gel, eluting with *n*-hexane–EtOAc (9:1) yield a solid, which on crystallization from an *n*-hexane–EtOAc mixture gave plumbagin (1.54 g) [13]. Plumbagin was obtained as orange needles, mp 72–73 °C, with the molecular formula $C_{11}H_8O_3$ ($[M–H]^-$ *m*/*z* 187). The IR spectrum (KBr) indicated the presence of quinone carbonyl groups at 1660 and 1650 cm^{-1} and a phenyl group at 1610, 1450 cm^{-1}. ^1H and ^{13}C NMR ($CDCl_3$) spectra showed the following signals: ^1H: δ 2.19 (3H, s, Me-2), 6.81 (1H, s, H-3), 7.25 (1H, m, H-6), 7.62 (2H, m, H-7, 8), 11.97 (−OH); ^{13}C: δ 16.5 (2-$\underline{C}H_3$), 115.1 (C-4a), 119.3 (C-8), 124.1 (C-6), 132.0 (C-8a), 135.4 (C-3), 136.1 (C-7), 149.6 (C-2), 161.2 (C-5), 184.8 (C-1), 190.2 (C-4). On the basis of the above spectroscopic analysis and compared with the published data [13,14], plumbagin structure was deduced.

A reversed-phase C18 column with the mobile phase of water–methanol (10:90, v/v) provided good separation of plumbagin from the herbal extracts eluted within 5 min. No other interfering peaks were present near the analyte peak. Method development started with full scan experiments (scan range from *m*/*z* 50 to *m*/*z* 250, scan time 1 s) of 1 μg/ml solutions of the intended plumbagin in negative ion modes. A

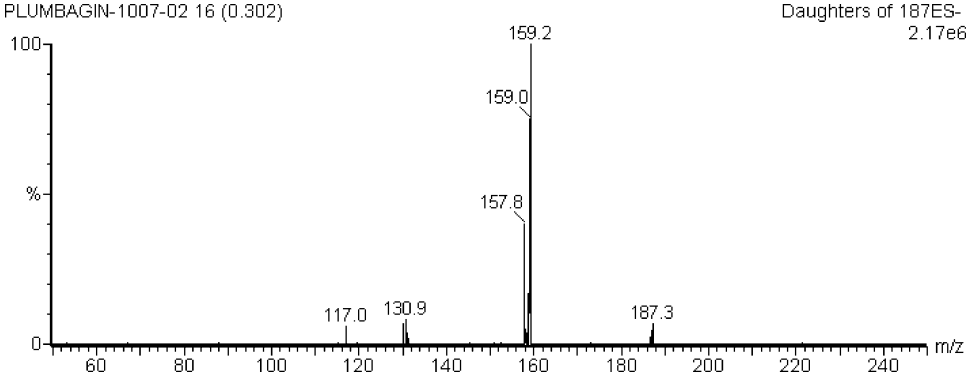

Fig. 2. Negative product ion mass spectrum of the deprotonated molecule of plumbagin (*m*/*z* 187).

typical mass spectrum of standard plumbagin is shown in Fig. 1. The *m*/*z* of the precursor ion of plumbagin is 187 $[M-H]^-$. After colliding with reagent gas, the selected precursor ion *m*/*z* 187 produced the product ion spectrum shown in Fig. 2.

The analyte was detected in negative ionization mode by monitoring the precursor–product combination in multiple-reaction monitoring (MRM) mode. After optimizing, the detecting channels were *m*/*z* 187 → *m*/*z* 159 for plumbagin with good symmetry and high intensity. The retention time of plumbagin was 2.8 min. Figs. 1 and 2 show the MS and MS/MS spectra, respectively, obtained from a plumbagin standard. For multiple reaction monitoring (MRM), which offers both sensitivity and selectively, the transition *m*/*z* 187 to *m*/*z* 159 was chosen and mass spectrometric parameters were optimized.

Table 1 represents the validation data on accuracy and precision of plumbagin in each standard mass concentration. The limit of quantification (LOQ) for plumbagin was 1 ng/ml and % RSD was below 20%, which has been defined as LOQ [15]. The limit of detection (LOD) was 0.5 ng/ml for a signal-to-noise ratio of 3. A good linear relationship was obtained for

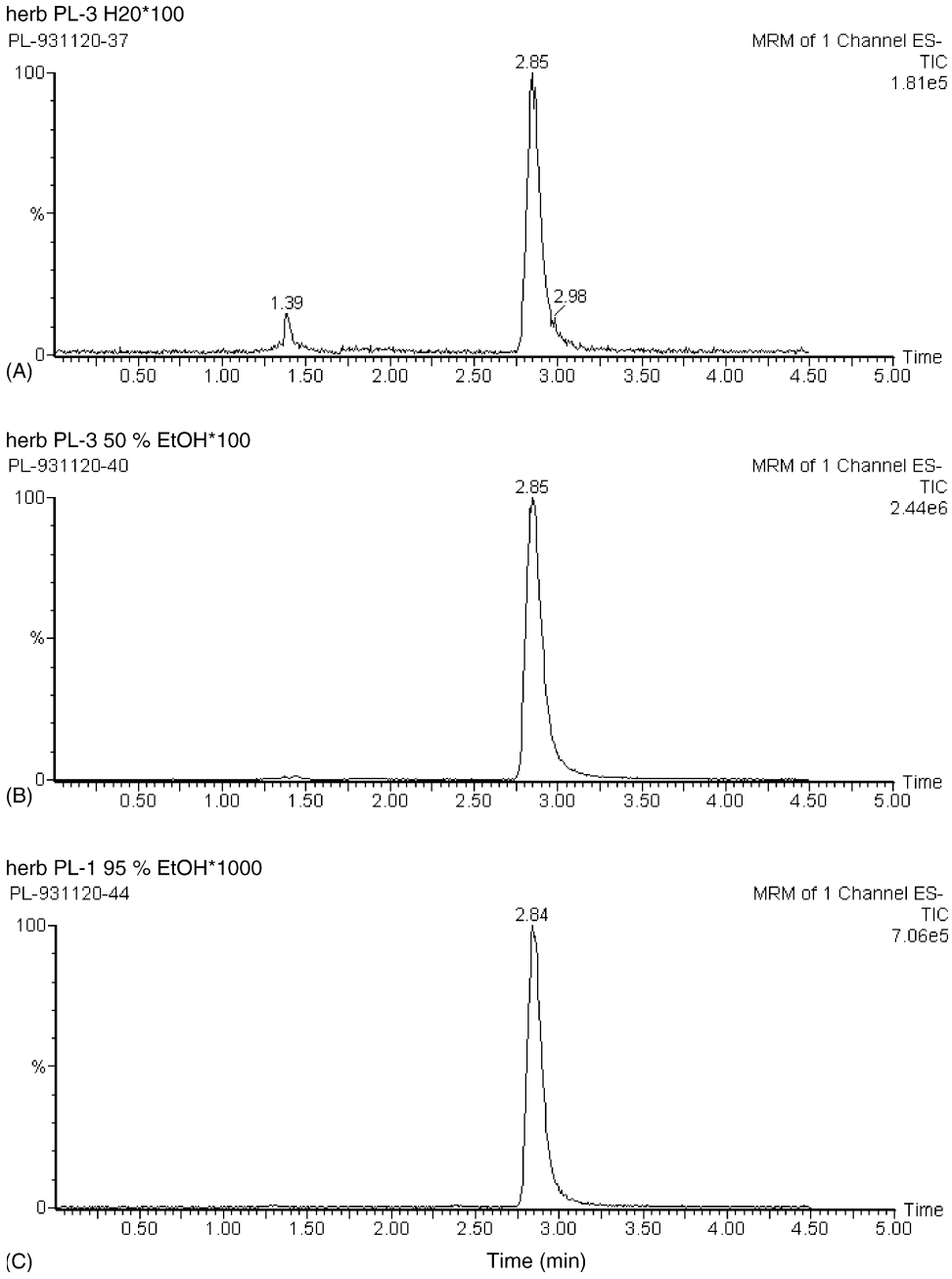

Fig. 3. LC–MS/MS chromatogram shows (A) a chromatogram of plumbagin (1 μg/ml) extract with H_2O, (B) extract with 50% EtOH, (C) extract with 95% EtOH.

Table 2
Extractive recovery (%) spiked with plumbagin in *Plumbago zeylanica* L.

Added plumbagin mass concentration (μg/ml)	Observed mass concentration (μg/ml)	Recovery (%)	RSD (%)
0.1	0.11 ± 0.01	95.40 ± 0.05	9.1
0.5	0.48 ± 0.01	94.04 ± 0.01	2.1
1	0.96 ± 0.02	93.25 ± 0.03	2.1

The basal plumbagin mass concentration was 0.016 ± 0.002 μg/ml. Data expressed as mean ± SD, $n = 3$. Recovery = [(observed mass concentration − basal plumbagin mass concentration)/added plumbagin mass concentration] × 100.

Table 3
Mass fractions of plumbagin (g/kg) in various extracts of the herb *Plumbago zeylanica* L. ($n = 3$, individual extraction)

Extracted solvent	Individual samples			Mean ± SD
	1	2	3	
H_2O	0.22	0.21	0.29	0.24 ± 0.04
50% ethanol	3.0	4.03	4.73	3.92 ± 0.71
95% ethanol	12.1	13.0	15.2	13.4 ± 1.30

plumbagin in the mass concentration range of 1–5000 ng/ml with a correlation coefficient >0.998.

Plumbagin has been detected in linear ranges of 0.2–10 μg/ml by a normal phase LC-UV separation with *n*-hexane–chloroform–2-propanol (30:70:2) in the *P. zeylanica* L. [10] by a μSpherogel column. Marston and Hostettmann determined plumbagin from six different naturally occurring naphthoquinones using a μBondapak CN column [11]. And Stensen and Jensen [12] reported a reversed-phase liquid chromatography with UV detection at 415 nm used for the determination of plumbagin with separations performed on a Waters Nova-Pak C18 reversed-phase column from 15 naphthoquinone derivatives. In this study, LC–MS/MS provides a higher sensitivity and specificity for the determination of plumbagin from the medicinal herb. The extractive recoveries of plumbagin from the herb were 92–94%, and the average% RSD was below 5% (Table 2). LC–MS/MS has been allowed a powerful and reliable analytical approach for determination and identification on active components in Chinese medicines.

Samples of the crushed roots of *P. zeylanica* L. were ground from lumps into powder and boiled for 3 min with H_2O, 50% EtOH, or 95% EtOH. A typical LC-tandem mass chromatogram of plumbagin extracts with the extracted solvents of H_2O, 50% EtOH, and 95% EtOH are shown in Fig. 3(A)–(C), respectively. The mass fractions of plumbagin in *P. zeylanica* L. for H_2O, 50% EtOH and 95% EtOH were 0.24 ± 0.04, 3.92 ± 0.87 and 13.4 ± 1.59 g/kg, respectively (Table 3).

In summary, we developed a LC–MS/MS method for the determination of plumbagin from *P. zeylanica* L. extract. The analytical liquid chromatographic separation of plumbagin with tandem mass spectrometric detection demonstrated an accurate and reproducible quantitation of this compound.

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