AKADÉMIAI KIADÓ

Acta Chromatographica

35 (2023) 2, 139–143

DOI:
10.1556/1326.2022.01014

ORIGINAL RESEARCH PAPER

A simple and sensitive HPLC method for simultaneous quantification of macrocyclic spermidine alkaloids in root, stem and leaf of *Tripterygium wilfordii*

LIANG ZHANG[1,2], WEIWEI XU[1], HONGXIA JIANG[1] and JIANQUN LIU[1]*

[1] Key Laboratory of Modern Preparation of TCM, Ministry of Education, Jiangxi University of Chinese Medicine, Nanchang, 330004, China

[2] College of Animal Pharmaceutical Sciences, Jiangsu Agri-animal Husbandry Vocational College, Taizhou, 225300, China

Received: January 9, 2022 • Accepted: February 28, 2022
Published online: March 23, 2022

ABSTRACT

There has been a lively interest on macrocyclic polyamine alkaloids due to their remarkable pharmacological activities such as anti-tumor, anti-inflammatory, anti-Alzheimer's disease and anti-parasitic. *Tripterygium wilfordii* is a widely used traditional Chinese medicine, which is abundant in alkaloids including macrocyclic polyamine alkaloids. However, there are rarely studies on macrocyclic spermidine alkaloids of *T. wilfordii* so far. In this article, we use three known macrocyclic spermidine alkaloids celafurine, celabenzine and celacinnine, and successfully develop a simple and sensitive HPLC method for simultaneous quantification of macrocyclic spermidine alkaloids in root, stem and leaf of *T. wilfordii*.

KEYWORDS

macrocyclic spermidine alkaloids, *Tripterygium wilfordii*, HPLC, simultaneous quantification

INTRODUCTION

Spermidine alkaloids are polyamine heterocycles, biosynthetically derived from L-Orn or L-Arg via putrescine. The interests in the isolation and synthesis of these alkaloids result from the structural complexity and therapeutical properties attributed to these macrocycles [1]. A broad biological activity has been revealed by members of the polyamine family, such as anti-tumor [1], anti-inflammatory [2], anti-Alzheimer's disease [3] and anti-parasitic [4, 5]. However, although possessing remarkable activities, few macrocyclic polyamines have been isolated from natural products so far.

Tripterygium wilfordii is a widely used traditional Chinese medicine with a long history. Alkaloids, the main active components of *T. wilfordii*, possess many beneficial pharmacological activities, such as immunosuppression, anti-inflammation, analgesia, anti-tumor, anti-HIV, insecticidal, neuroprotection and so on [6]. The alkaloids of *T. wilfordii* include sesquiterpene alkaloids and macrocyclic polyamine alkaloids. In previous study, we found a new macrocyclic spermidine alkaloid of *T. wilfordii*, which possessed significant anti-inflammatory activity [2]. In addition, we also found other new unknown macrocyclic spermidine alkaloids existing in *T. wilfordii* [7]. Up to now, there are rarely studies on macrocyclic spermidine alkaloids of *T. wilfordii* and no researches on spermidine alkaloids analysis are reported. Therefore, it is meaningful to develop a simple and sensitive method for identify and determine macrocyclic spermidine alkaloids in *T. wilfordii*.

*Corresponding author.
E-mail: liu5308@sina.com

The macrocyclic polyamine alkaloids of *T. wilfordii* have 13 membered macrocyclic mother nucleus, with a unique structure of three nitrogen atoms. In this study, we use three known macrocyclic spermidine alkaloids celafurine, celabenzine and celacinnine (Fig. 1), which have been isolated and identified in root of *T. wilfordii* by our research group, to develop a simple and sensitive HPLC method for simultaneous quantification of macrocyclic spermidine alkaloids in root, stem and leaf of *T. wilfordii*.

EXPERIMENTAL

Materials

T. wilfordii was collected from Pingxiang City, Jiangxi Province in July 2020. The root, stem and leaf were identified by professor Jianqun Liu, key laboratory of modern preparation of traditional Chinese medicine, ministry of education, Jiangxi university of Chinese medicine. Celafurin, celabenzine and celacinnine were isolated from *Tripterygium Wilfordii* in our laboratory. The structures were identified by nuclear magnetic resonance and UPLC-Q-TOF-MS/MS, and which were consistent with the literature [8, 9]. The purities were more than 99% by HPLC.

Preparation of standard and sample solutions

Stand solutions. Celafurine, celabenzine and celacinnine were accurately weighed to 10 mL volumetric flask and dissolved in methanol to make a mixed standard solution 1 of 111.6, 130.9 and 129.8 $\mu g\,mL^{-1}$, respectively. The solutions were stored at 4°C and warmed to room temperature before use. The stock solutions were diluted with methanol to get a series of mixed standard solutions. In addition, the solutions of each compound are prepared to determine the elution order.

Root, stem and leaf extract solutions. 10 g powder of root, stem and leaf of *T. wilfordii* were accurately weighed to conical bottle with stopper, respectively. Ultrasonic extraction was carried out with 100 mL methanol twice. Filtered and collected the extracting solutions, then concentrated up to dryness, the residue was dissolved in 20 mL 2% H_2SO_4 solution. The acid water extracts were further refined to pH10.0 with 3 mL aqueous ammonia. Filtered and extracted with 20 mL chloroform solution three times, then concentrated up to dryness, the residue was dissolved in 2 mL methanol. Filtered with 0.22-μm microporous membrane, then diluted 1–10 mL with methanol. The solutions were stored at 4°C and warmed to room temperature before use. Besides, the effects of different aqueous ammonia concentrations on the contents of macrocyclic polyamine alkaloids were studied. The acid water extracts were further adjusted to pH 9.0 and 11.0 with 2 and 4 mL aqueous ammonia, respectively.

Chromatographic conditions

HPLC analysis was performed on Agilent 1260 liquid chromatography system (Agilent Technologies, Palo Alto, CA, USA), equipped with a thermostated-column device, a DAD detector and an auto-sample injector. Chromatographic conditions were as following, the analytical column Diamonsil®5 μm C18(250 × 4.6 mm) was purchased from Dikma Technologies Inc. (Beijing, China), equipped with a guard column. We used water and acetonitrile as the mobile phase, gradient elution condition (water: acetonitrile): 0–20 min, 70:30–60:40; 20–30 min, 60:40; 30–40 min, 60:40–30:70. Column temperature was set as 30°C. The spectrums were scanned by DAD detector, and finally 210 nm was chosen as the detection wavelength for all analytes. Injection volume was 20 μL.

Method validation

The method for the determination of celafurine, celabenzine and celacinnine in stem of *T. wilfordii* was validated in terms

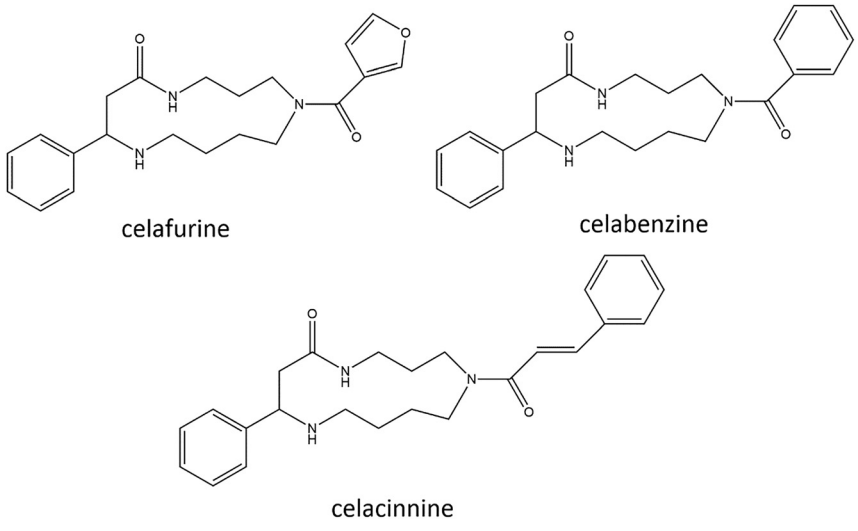

Fig. 1. Chemical structures of celafurine, celabenzine and celacinnine

of specificity, linearity, limit of detection and quantification, precision, repeatability, stability and accuracy.

RESULTS AND DISCUSSION

Wavelength

The wavelengths of analytes were scanned from 210 to 400 nm by DAD detector (Fig. 2). Celafurine and celabenzine both have highest absorption at 210 nm. Celacinnine has maximum absorptions at 210 and 280 nm. When at 210 nm, the solvents did not interfere with the baseline. Therefore, we chose 210 nm as detection wavelength for the analytes.

Method validation

Specificity. Specificity of the assays was determined by comparing the chromatograms of mixed standard solutions and samples solutions of root, stem and leaf of *T. wilfordii*. Celafurine, celabenzine and celacinnine were all base line separated with a resolution over 1.5, and the number of theoretical plates shall not be less than 4,000 according to the peak of celafurine. The chromatograms of mixed standard solutions and samples solutions of root, stem and leaf are shown in Fig. 3. Besides, we found an interesting phenomenon that one compound exists in stem and leaf, while not in root of *T. wilfordii*, at least lower than the limit of

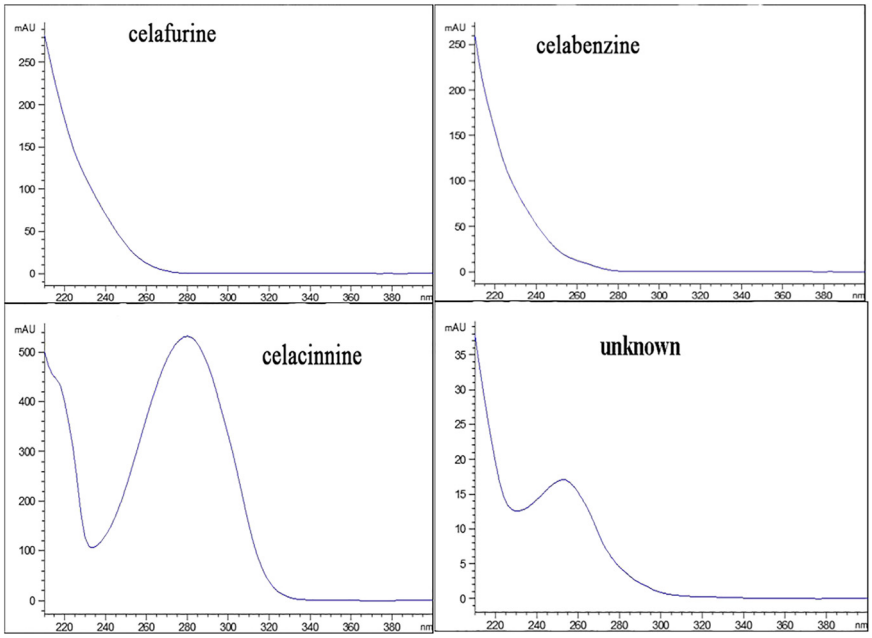

Fig. 2. The UV wavelengths of celafurin, celabenzine, celacinnine and an unknown compound

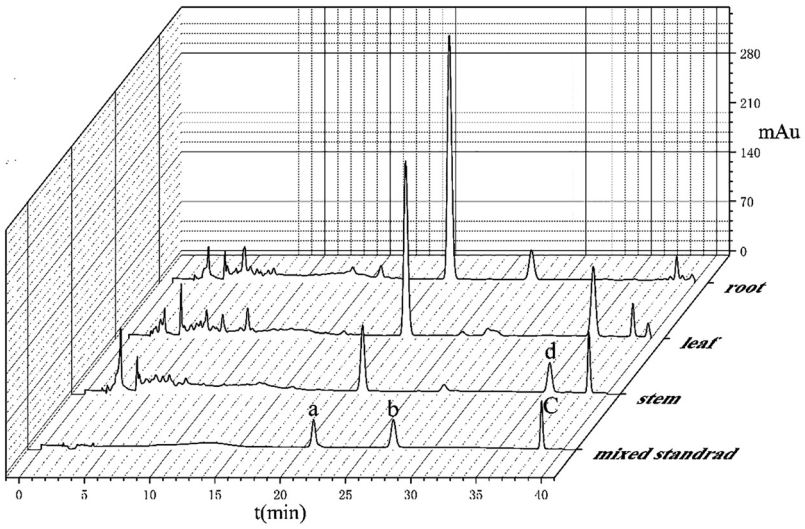

Fig. 3. The baseline separated chromatograms of mixed standard solutions and samples solutions of root, stem and leaf of *Tripterygium wilfordii* (a-celafurine, b-celabenzine, c-celacinnine, d-unknown compound)

detection. The compound is separated between celabenzine and celacinnine (Figs 2–3), and suspected to be a new macrocyclic polyamine which had not been reported in *T. wilfordii*.

Linearity. Calibration curves were prepared by using methanol spiked with known concentrations of celafurine, celabenzine and celacinnine. The calibration curves of each compound were linear over the concentration by performing a regression linear analysis of the peak area (A) of each compound vs the concentrations (C). Celafurine, celabenzine and celacinnine were linear over concentrations 1.744–111.6 μg mL^{-1}, 2.045–130.9 μg mL^{-1} and 2.027–129.8 μg mL^{-1}, respectively. The regression equation of the calibration curves was $A = 57.688C + 44.567$, with regression coefficient (r^2) of 1 for celafurine, $A = 62.53C - 13.374$ ($r^2 = 1$) for celabenzine, and $A = 55.865C - 13.286$ ($r^2 = 1$) for celacinnine, respectively.

Limit of detection and quantification. The limit of detection (LOD) and the lower limit of quantification (LOQ) were determined as 3 and 10 times the signal to noise ratios, respectively. LODs were 0.436, 2.046 and 2.028 ng, LOQs were 0.872, 4.090 and 4.054 ng for celafurine, celabenzine and celacinnine, respectively.

Precision. Precision of the methods were studied in terms of repeatability by determining mixed standard solution 1 in "*Stand solutions*" section, and which was assayed in six times. RSD values of the peak area was 0.15 for celafurine, 0.18 for celabenzine, and 0.17 for celacinnine, which indicated that the instrument had good precision.

Repeatability. Accurately weigh six 10 g stem powder, and prepare the sample solutions according to the method in "*Root, stem and leaf extract solutions*" section. The average contents of celafurine, celabenzine and celacinnine in stem were 0.070 mg g^{-1}, 0.012 mg g^{-1} and 0.050 mg g^{-1}, with RSD values of the peak area 4.08, 3.67 and 3.89, respectively.

Stability. One sample solution of "*Repeatability*" section was injected at 0, 2, 4, 8, 12, and 24 h respectively. RSD values of the peak area for celafurine, celabenzine and celacinnine were 0.82, 2.55 and 0.62, respectively. The results showed that the solution remained stable within 24 h.

Recovery. The recoveries were carried out by adding known amounts of celafurine, celabenzine and celacinnine to 5 g stem powder at three different levels, prepare the sample solutions according to the method in "*Root, stem and leaf extract solutions*" section. Each level was repeated three times and the amount of each compound was found by the assay method in "*Chromatographic conditions*" section. The recovery was calculated by dividing the amount of found by the added, then multiplied by 100%. The results presented in Table 1 indicated that the method was accurate.

Table. 1. Results of recovery

Compound	Amount of sample (g)	Content of the sample (μg)	Added (μg)	Measured (μg)	Recovery (%)	Average recovery (%)	RSD (%)
celafurine	5.0158	291.07	103.6	419.26	106.2	107.4	1.6
	5.0618	293.74	103.6	434.91	109.4		
	5.0077	290.60	103.6	420.33	106.6		
	5.0577	293.50	207.2	531.88	106.2	110.6	4.0
	5.0540	293.28	207.2	575.48	115.0		
	5.0028	290.31	207.2	550.62	110.7		
	5.0785	294.70	414.2	789.50	111.4	106.3	4.9
	5.0314	291.97	414.2	712.78	100.9		
	5.0104	290.75	414.2	750.84	106.5		
celabenzine	5.0618	67.85	57.96	137.11	109.0	110.5	3.6
	5.0158	67.23	57.96	144.04	115.0		
	5.0077	67.12	57.96	134.59	107.6		
	5.0577	67.79	115.92	190.51	103.7	105.3	5.2
	5.0540	67.74	115.92	204.71	111.5		
	5.0028	67.06	115.92	184.51	100.8		
	5.0785	68.07	231.84	305.47	101.8	98.4	3.5
	5.0314	67.44	231.84	284.15	94.9		
	5.0104	67.16	231.84	294.83	98.6		
celacinnine	5.0618	65.84	60.3	136.69	108.4	104.5	4.5
	5.0158	65.24	60.3	132.95	105.9		
	5.0077	65.14	60.3	124.59	99.3		
	5.0577	65.79	120.6	180.62	96.9	94.9	2.2
	5.0540	65.74	120.6	176.83	94.9		
	5.0028	65.08	120.6	172.25	92.8		
	5.0785	66.06	241.2	302.39	98.4	96.9	1.5
	5.0314	65.45	241.2	292.60	95.4		
	5.0104	65.18	241.2	296.91	96.9		

The effect of different ammonia concentrations

In the experiment, we adjusted the acid water extracts to pH 9.0, 10.0, and 11.0 with 2, 3, and 4 mL aqueous ammonia, respectively. According to the chemical structures, the macrocyclic spermidine alkaloids celafurine, celabenzine and celacinnine all have three nitrogen atoms, possessing the structure with two amide bonds and secondary amine, leading to weak alkalinity. The role of aqueous ammonia was to free the alkaloids, and then the alkaloids could be extracted by chloroform easily. There were no significant differences of the contents of macrocyclic polyamines at pH 10.0 and 11.0. However, when at pH 9.0, the contents of macrocyclic polyamines were significantly lower (10%–20%) than pH 10.0 and 11.0. In addition, when at pH 11.0, there was interference at the peak position of celabenzine, which affected the specificity. Thus, finally we adjusted the acid water extracts to pH 10.0.

Macrocyclic spermidine alkaloids in root, stem and leaf of *T. wilfordii*

The established method was used to determine macrocyclic spermidine alkaloids in root and leaf of *T. wilfordii* as well. The contents of celafurine, celabenzine and celacinnine are 0.138 mg g^{-1}, 0.019 mg g^{-1} and 0.010 mg g^{-1} in dry root powder, and 0.095 mg g^{-1}, 0.009 mg g^{-1} and 0.012 mg g^{-1} in dry leaf powder, individually. Thus it can be seen, macrocyclic spermidine alkaloids are also distributed in stem and leaf, not only in root. *T. wilfordii* has a long growth cycle, with alkaloids grows year by year [10]. Moreover, root is non-renewable, while stem and leaf are renewable. Consequently, stem and leaf could be better alternatives for root considering macrocyclic spermidine alkaloids.

CONCLUSION

In this article, we established a simple and sensitive HPLC method for simultaneous quantification of macrocyclic spermidine alkaloids celafurine, celabenzine and celacinnine in root, stem and leaf of *T. wilfordii*. The macrocyclic spermidine alkaloids all have conjugate systems, leading to high UV responses. However, the contents of these macrocyclic spermidine alkaloids are very low, which limit further researches. We also find that stem and leaf contain comparable macrocyclic spermidine alkaloids with root. Besides, other new macrocyclic spermidine alkaloids may exist in stem and leaf of *T. wilfordii*, while not in root. These findings might have guiding significances for the phytochemical study of *T. wilfordii*.

ACKNOWLEDGMENTS

This research was financially supported by the Project for Academic and Technical Leaders of Major Disciplines in Jiangxi (No. 20182BCB22004) and the National Natural Science Foundation of China (No. 81860686).

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