Journal of Chromatography A, 1218 (2011) 1131–1138

Contents lists available at ScienceDirect

Journal of Chromatography A

journal homepage: www.elsevier.com/locate/chroma

Qualitative and quantitative determination of polyacetylenes in different *Bupleurum* species by high performance liquid chromatography with diode array detector and mass spectrometry

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ARTICLE INFO

Article history:
Received 28 May 2010
Received in revised form 1 December 2010
Accepted 2 December 2010
Available online 10 December 2010

Keywords:
Polyacetylenes
HPLC-DAD–MS
Bupleuri Radix
Bupleurum
Bupleurum longiradiatum
Acute toxicity

ABSTRACT

Polyacetylenes are main toxic ingredients in *Bupleurum longiradiatum*, a poisonous plant that has ever been misused as substitutes for *Chaihu* (Bupleuri Radix). For the first time, a high-performance liquid chromatography method coupled with diode array detector and mass spectrometry (HPLC-DAD–MS) was developed for qualitative and quantitative analysis of nine polyacetylenes in *Bupleurum* species. All references, including two new polyacetylenes, were isolated from *B. longiradiatum* and purified using a semi-preparation HPLC chromatography. The analysis was performed on a TSKgel ODS-100 V C18 column (3 μm, 150 mm × 4.6 mm i.d.) using a gradient system of acetonitrile and water, with diode array detection (254 nm). The method was validated for linearity, precision, accuracy, limit of detection and quantification. A total of 27 *Bupleurum* samples were examined with this method, which showed a great variety in the distribution and contents of the polyacetylenes. It was found that polyacetylenes (**1**–**8**) were the main ingredients in *B. longiradiatum*, while a few kinds of polyacetylenes (**5**–**8**) were also identified in *B. smithii*, *B. smithii* var. *parvifolium*, *B. bicaule* and *B. angustissimum*. However, no polyacetylenes (**1**–**9**) were detected in the authentic *Chaihu* samples and the other *Bupleurum* species. The results indicated that the toxic *B. longiradiatum* could readily be distinguished from other medicinal *Bupleurum* species based on the polyacetylene profiles, and the guidelines and quality control of polyacetylenes for *Chaihu* are useful. The acute toxicity of the ethanol extract of *B. longiradiatum* and its fractions was also investigated.

1. Introduction

Traditional Chinese medicine (TCM) herbs and their manufactured products have been used for thousands of years for prevention and treatment of disease in China. When prescribed according to Chinese medical philosophy and practice, those medications and crude materials recorded in the Chinese Pharmacopoeia have been approved for safety and efficacy [1]. However, increasing concerns and fears have been expressed to the efficacy, toxicity and quality of TCM herbs, because many popular and expensive TCM herbs are in short supply and inferior substitutes or fake crude herbs have been found in the market [2–4]. Moreover, misuse and confusion of names of certain herbs enhances difficulty in identifying the correct herbs [3,4]. This can be dangerous to consumers because some substitution involve poisonous material. Examples of the substitution of indicated material for poisonous alternatives are described throughout the literature [3–5]. A famous case was the substitutions of *Stephania tetrandra* with poisonous *Aristolochia fangchi* that resulted in cases of rapidly progressive renal fibrosis found in young Belgian women [6–8]. Despite the growing interest in TCM herbs, there are still insufficient scientific data on the safety and efficacy of some herbs, and sometimes the toxicity of a particular plant and its constituents have not been well characterized. Therefore, reliable methods are in great demand for the efficient detection and rapid characterization of specific active or toxic components, and to ensure that the TCM herbs are safe and their labeling is truthful and not misleading.

Bupleuri Radix, with a Chinese name *Chaihu*, is recorded as the roots of *Bupleurum chinense* and *B. scorzonerifolium* (Apiaceae) in the Chinese Pharmacopoeia, which has been widely practiced to treat influenza, fever, inflammation, malaria and menstrual disorders [9,10]. In previous chemical studies on *Bupleurum* plants, saponins, flavonoids, coumarins, fatty acids, steroids, polysaccharides and polyacetylenes were identified [11,12]. Among them,

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doi:10.1016/j.chroma.2010.12.007

saponins were known to be the major bioactive compounds, which were commonly used as chemical standards for quality evaluation of *Chaihu* in the current Chinese Pharmacopoeia and recent publications [9,13–17]. However, a systematic study on the authentication and assessment on related *Bupleurum* species was not well defined. There are at least ten species of the genus *Bupleurum* in the family Apiaceae that are also used under the name of *Chaihu* locally [11]. Even worse, *B. longiradiatum*, widely distributed in northeastern mainland China, is a poisonous plant but sometimes has been found in herb markets [9,11]. The misuse of *B. longiradiatum* as *Chaihu* had caused several cases of human poisoning, and at least three people died after administration of this plant, which showed symptoms such as serious nausea, vomiting, twitching, opisthotonus, and so on [11,18]. Following investigation on the acute toxicity of extracts of *B. longiradiatum* also found its strong toxicity against mice, which was attributed to its high content of hydrophobic compounds [11,19]. Six polyacetylenes were then isolated from the roots of *B. longiradiatum*, among which bupleurotoxin and acetylbupleurotoxin were toxic, with LD_{50} values of 3.03 mg/kg and 3.13 mg/kg, respectively [18]. Another study also found that bupuleurotoxin showed strong toxicity to mice at high doses [28].

Our preliminary surveys on *B. longiradiatum* demonstrated that polyacetylenes were of particular abundance in root of this plant and proved to be responsible for the toxicity of *B. longiradiatum* [12]. Naturally occurring polyacetylenes were previously reported to be cytotoxic and neurotoxic at high concentrations, and they also to be potent skin sensitizers [20–22]. The well known polyacetylene toxin is oenanthotoxin, isolated from *Oenanthe fistulosa*, a toxic plant in the family Apiaceae [23,24]. As major toxic ingredients of *B. longiradiatum*, however, the contents of polyacetylenes in *B. longiradiatum* have not been studied yet. Moreover, their distributions in other related *Bupleurum* species are still unknown.

In general, the differences in constituents in TCM herbs will affect the efficacy and safety of pharmaceutical products and the standardization of this herbal medicine. Therefore, it is important to quantify the content of active constituents in herbal medicines, to ensure its efficacy. As to the clinic security, it is crucial to determine the toxic compounds, and to confirm the absence or presence of toxic ingredients in any drug products. Up until now, the quality control of *Chaihu* and related *Bupleurum* species mainly emphasized on the determination of bioactive saponin compounds [13–17], but not considered the scrutiny of various toxic ingredients in *Bupleurum*. To the best of our knowledge, no method for the determination of polyacetylenes in *Bupleurum* species has been reported so for.

Recently, HPLC-DAD–MS has shown its wide application in TCM research because the combination of DAD and MS can provide online UV and MS information at the same time for each individual peak in a chromatogram, while HPLC–UV is a convenient and effective method to control the quality of TCM for its rapid separation and quantitation [25].

This work focuses on developing a simple, effective and reliable method to analyze polyacetylenes in *B. longiradiatum* and related *Bupleurum* species. A high-performance liquid chromatography (HPLC) method coupled with diode array detection (DAD) and mass spectrometry (MS) was established to the qualitative and quantitative determination of nine polyacetylenes (**1**–**9**, Fig. 1). The structure of the constituents isolated was determined on the basis of UV, IR, NMR and MS data and the complete structural elucidation of compounds **1** and **3** is reported herein for the first time. The acute

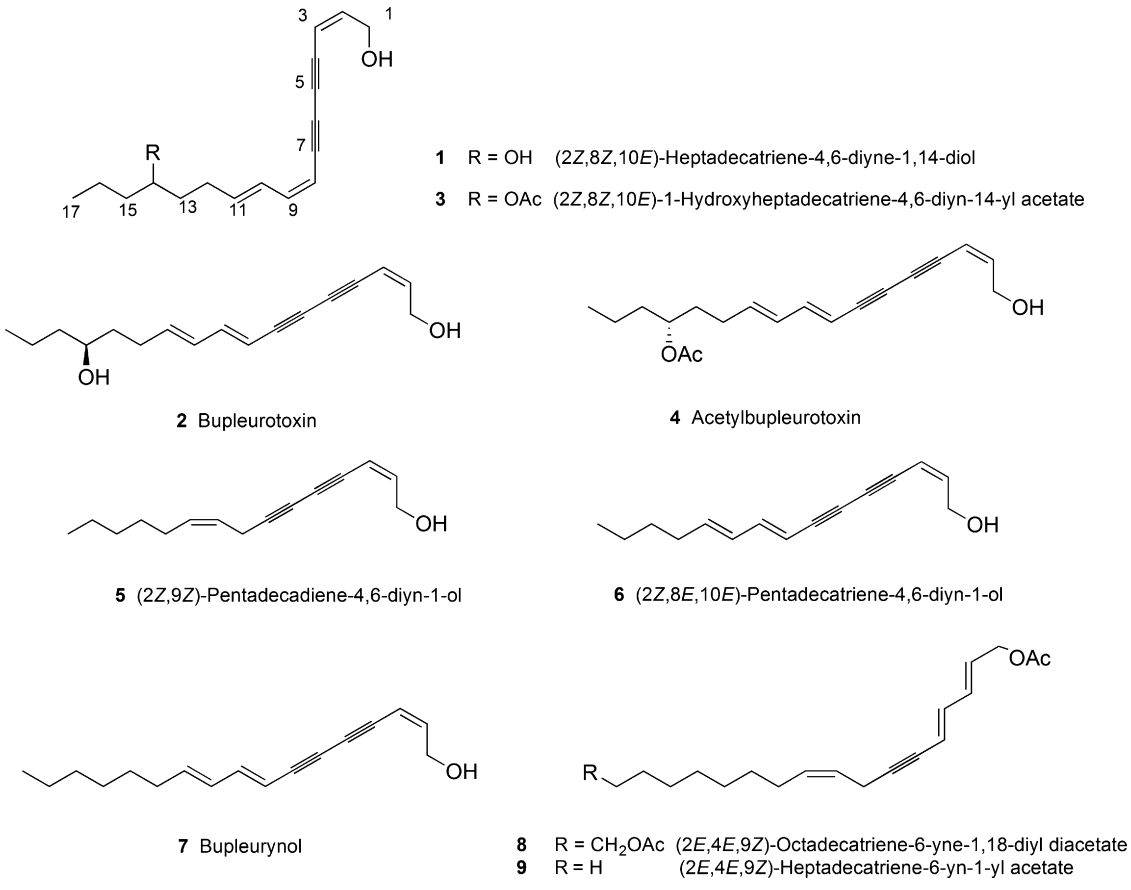

Fig. 1. Chemical structures of the nine standard compounds in the study.

Table 1
Summary for the tested samples roots of *Bupleurum*.

Species	Sampling parts	Site of collection	Code
B. longiradiatum	Aerial part	Heilongjiang Province	LO-1A
	Root	Jilin Province	LO-2
	Aerial part	Jilin Province	LO-2A
B. smithii	Root	Inner Mongolia Autonomous Region	SM-1
	Root	Shaanxi Province	SM-2
B. smithii var.	Root	Qinghai Province	PA-1
parvifolium	Root	Gansu Province	PA-2
B.bicaule	Root	Inner Mongolia Autonomous Region	BI-1
B. angustissimum	Root	Shanxi Province	AN-1
B. scorzonerifolium	Root	Anhui Province	SC-1
	Root	Shanxi Province	SC-2
	Root	Gansu Province	SC-3
	Aerial part	Gansu Province	SC-3A
B. chinense	Root	Shanxi Province	CH-1
	Root	Jilin Province	CH-2
	Root	Henan Province	CH-3
	Root	Anhui Province	CH-4
	Aerial part	Anhui Province	CH-4A
B. marginatum var.	Root	Yunnan Province	ST-1
stenophyllum	Root	Shaanxi Province	ST-2
	Aerial part	Yunnan Province	ST-2A
B. wenchuanense	Root	Sichuan Province	WE-1
B. komarovianum	Root	Jilin Province	KO-1
	Aerial part	Jilin Province	KO-1A
B. rockii	Root	Yunnan Province	RO-1
	Aerial part	Yunnan Province	RO-1A
B. candollei	Aerial part	Yunnan Province	CA-1A

toxicity the ethanol extract of *B. longiradiatum* and its fractions was also investigated.

2. Experimental

2.1. Plant materials

A total of 27 samples of *Bupleurum* species were collected from various habitats of China (Table 1). All the samples were authenticated by Prof. Sheng-li Pan, School of Pharmacy, Fudan University, and by Prof. Han-ming Zhang, Department of Pharmacognosy, Second Military Medical University. The voucher specimens are deposited in the Herbarium of Second Military Medical University, Shanghai, PR China. Optimization of extraction and chromatographic conditions, method validation and acute toxicity assays were tested with samples of *B. longiradiatum*, collected from Jilin Province (code: LO-2).

2.2. Chemicals

HPLC-grade acetonitrile and methanol (MeOH) were purchased from Merck (Darmstadt, Germany). Ultra pure water was obtained in a Milli-Q system from Millipore (Bedford, MA, USA). $CDCl_3$ were obtained from Sigma Chemical Co., (St. Louis, MO, USA). Other organic solvents and chemical reagents used were of analytical grade and were purchased from Shanghai Chemical Reagent Co. (Shanghai, China). Materials for column chromatography were silica gel (100–200 mesh; Huiyou Silical Gel Development Co. Ltd. Yantai, China), Sephadex LH-20 (40–70 μm; Amersham Pharmacia Biotech AB, Uppsala, Sweden), and YMC-gel ODS-A (50 μm; YMC, MA, USA). Preparative TLC (0.4–0.5 mm) was conducted with glass plates precoated silica gel GF_{254} (Yantai).

2.3. Instruments

Optical rotations were recorded using a Perkin-Elmer 341 polarimeter. UV spectra were obtained by Shimadzu UV-2550 UV–Vis spectrophotometer. IR spectra were recorded on a Bruker Vector 22 spectrometer with KBr pellets. NMR spectra were recorded on a Bruker Avance 500 NMR spectrometer in $CDCl_3$ with TMS as internal standard. EIMS and HREIMS were acquired on a Thermo DSQ II and a Finnigan MAT 95 mass spectrometer, respectively. Chromatography was performed on an Agilent-1100 HPLC system (Agilent Technologies, MA, USA), comprising a quaternary pump, a vacuum degasser, an autosampler, a column compartment and a diode array detector, and coupled with a LC/MSD Trap XCT electrospray ion mass spectrometer (Agilent Technologies, MA, USA).

2.4. Extraction and isolation of polyacetylene standards

Polyacetylene standards were isolated in the same manner as described in our previous paper [12], but using a semi-preparation HPLC chromatography for further purification. The HPLC system used for the separation was a LC2010AHT HPLC Series (Shimadzu Corporation, Kyoto, Japan) coupled with DAD detector set to 254 nm. A TSKgel ODS-100 V C18 (5 μm, 150 mm × 21.5 mm i.d., Tosoh Co., Tokyo, Japan) column, with a column temperature at 25 °C, was used for the separation. MeOH aqueous solutions or acetonitrile aqueous solutions were used as the mobile phase.

Powdered whole plants of *B. longiradiatum* (2.0 kg) were extracted in a Soxhlet apparatus with CH_2Cl_2 (5 L). The extract (60 g) was separated into six fractions (A–E) by column chromatography on silica gel using gradient mixtures of hexane–EtOAc (100–0%). Each fraction was purified using reversed-phase MPLC in a gradient system of H_2O–MeOH (50–100%), and column chromatography on silica gel and Sephadex LH-20, and then finally applied to a semi-preparation HPLC chromatography to yield nine standards (**1**–**9**, yield 0.017–0.078%). The semi-preparation HPLC separation of compounds **1** and **2** was carried out using 42% acetonitrile at a flow rate of 3.0 mL/min. Purification of **3**–**6** was performed using 55% acetonitrile at a flow rate of 3.0 mL/min. Compounds **7**–**9** was separated at a flow rate of 2.0 mL/min eluting with 82% MeOH, 85% MeOH and 90% MeOH, respectively. All purified compounds were stored at low temperature, protected from light and humidity.

The purities of these polyacetylenes were determined to be more than 98% by normalization of the peak areas detected by HPLC-DAD and further confirmed by quantitative NMR analysis.

2.5. Standard solutions for quantification

The standard stock solution of the mixture of the nine reference compounds was prepared by dissolving accurately weighted portions of the standards in MeOH, transferring it to a 2 mL volumetric flask, and then adding MeOH to make up the volume. A series of working standard solutions with gradient concentration was obtained by diluting the mixed standard stock solution. All the solutions were stored in a refrigerator at −4 °C.

2.6. Sample preparation for HPLC analysis

Finely powdered dried plant materials (0.4 g) were sonicated in 10 mL of MeOH for 30 min followed by centrifugation for 10 min at 3000 rpm. The procedure was repeated twice. Respective supernatants were combined and concentrated in a rotary evaporator. MeOH was used to dilute the concentrated solution under sonication, and the volume was made up to exactly 2 mL. Prior to use all samples were filtered through a 0.22 μm nylon membrane filter.

2.7. HPLC-DAD–MS analysis

The polyacetylenes in the MeOH extracts of *Bupleurum* were characterized by HPLC-DAD–ESI/MS analysis. Chromatography was performed on a TSKgel ODS-100 V C18 column (3 μm, 150 mm × 4.6 mm i.d., Tosoh Co., Tokyo, Japan) at a column temperature of 25 °C and a flow rate of 1 mL/min using acetonitrile (solvent A) and water (solvent B) as mobile phase with a linear gradient: 0–40 min (45–70% A), 40–60 min (70–80% A), 60–65 min (80–100% A). DAD was set to monitor at 254 nm, and the on-line UV spectra were recorded in the range 190–400 nm. By solvent splitting, about 38% of eluent from DAD was introduced into the ion source of mass spectrometry. LC–MS detection was performed directly after DAD measurements. Analyses were performed using a LC/MSD Ion Trap XCT mass spectrometer equipped with an ESI source. The acquisition parameters were as follows: collision gas, ultrahigh-purity helium (He); nebulizer gas, high purity nitrogen (N_2); nebulizer gas (N_2), 35 psi; dry gas (N_2), 12 L/min; dry temperature, 350 °C; HV voltage, 3500 V; mass range recorded *m/z* 200–1600, target mass *m/z* 300; compound stability, 100%; trap drive level, 100%; collision energy (Ampl), 0.3–2 V. Data acquisition was performed by using a Chemstation software (Agilent Technologies, USA).

2.8. Investigation of acute toxicity of Bupleurum longiradiatum

Powder of roots of *B. longiradiatum* was extracted with 95% aqueous ethanol at room temperature to afford crude extract. This extract was then suspended in water and partitioned successively with dichloromethane (CH_2Cl_2) and aqua-saturated *n*-BuOH.

For toxic studies, stock solutions of the extract and its fractions were prepared in 0.5% CMCNa at concentrations of 100 mg/mL and diluted with phosphate-buffered saline (PBS). ICR mice, half males and half females, with body weights of 19.3–22.0 g, were treated the testing drug solution by intragastric injection 8 h after fasting, with a dosing volume of 40 mL/kg body weight. The starting dose was 5000 mg/kg which was decreased according to the results. The control animals ($n = 10$) received 0.5% CMCNa alone (75.0 μg/animal). The death status of animals was consecutively observed for 14 days after administration.

The experimental protocol satisfied the Guidelines for Animal Experimentation approved by the Animal Experimentation Committee of the Second Military Medical University.

3. Results and discussion

3.1. Structural characterization

Chemical investigation on the CH_2Cl_2 extract of *B. longiradiatum* led to the isolation of two new polyacetylenes (**1**, **3**), along with seven known polyacetylenes, namely bupleurotoxin (**2**) [12,18], acetylbupleurotoxin (**4**) [12,18], (2*Z*,9*Z*)-pentadecadiene-4,6-diyn-1-ol (**5**) [26], (2*Z*,8*E*,10*E*)-pentadecatriene-4,6-diyn-1-ol (**6**) [12], bupleurynol (**7**) [18,27], (2*E*,4*E*,9*Z*)-octadecatriene-6-yne-1,18-diyl diacetate (**8**) [12], and (2*E*,4*E*,9*Z*)-heptadecatriene-6-yn-1-yl acetate (**9**) [12] (Fig. 1). In this work, for the first time, the structures of compounds **1** and **3** were determined on the basis of UV, IR, MS and NMR data (see Supplementary material).

Compound **1** was assigned the molecular formula $C_{17}H_{22}O_2$, due to the molecular ion peak at *m/z* 258.1618 in HREIMS spectrum. Its UV, IR and ^1H and ^{13}C NMR spectra data were almost same as those of **2** [12,18], which led to the conclusion that they were stereo-isomers. The stereochemistry change was located about the double bond C-8/C-9, which had a *Z* stereochemistry ($J_{8,9} = 11.0$ Hz) in **1**. This stereochemistry was confirmed by the shielding experienced by C-7 and C-10 in the ^{13}C NMR spectrum. Thus, **1** was defined as (2*Z*,8*Z*,10*E*)-heptadecatriene-4,6-diyne-1,14-diol.

Compound **3** gave a molecular formula $C_{19}H_{24}O_3$, as shown by the HREIMS at *m/z* 300.1726 $[M]^+$, revealing the compound to be 42 amu more than that of **1**. The ^1H and ^{13}C NMR spectroscopic data of 3 were very similar to those of **1**, with the difference being the appearance of an acetate group [δ_H 2.05 (3H, s); δ_C 170.0 (s), 21.2 (q)] in **3**. Considering the significant downfield shift of the signal of H-14 from δ_H 3.63 in **1** to δ_H 4.90 in **3**, the acetate group was presumed to be placed at the C-14 position. This was further confirmed by the HMBC correlation from H-14 (δ_H 4.90) to the ester carbonyl (δ_C 170.0). Therefore, **3** was elucidated as (2*Z*,8*Z*,10*E*)-1-hydroxyheptadecatriene-4,6-diyn-14-yl acetate.

3.2. Optimization of extraction and chromatographic conditions

In order to obtain quantitative extraction, variables involved in the procedure, including extraction solvent, extraction repetitions and extraction time were optimized. In this study, four different types of extraction organic solvent, including MeOH, MeOH–CH_2Cl_2 1:1 (v/v), CH_2Cl_2 and *n*-hexane were evaluated for their extraction efficiency using ultrasonication method. The best solvent was found to be MeOH which allowed a complete extraction of the tested polyacetylenes in relatively high amounts. In addition, extraction repetitions (1–3 times) and extraction time (30, 60, 90 min) were also evaluated. The results suggested that ultrasonication with MeOH for 30 min twice was simple and effective for extraction.

To quantify the investigated compounds, full separation of major polyacetylenes is necessary. However, some peaks of the analytes were usually overlapped due to the extremely similar structures between different polyacetyelenes, especially for those stereo-isomers (compound **1**/**2**; compound **3**/**4**) (Fig. 1). Thus, slowly gradient elution with different elution systems, including acetonitrile–water, MeOH–water, acetonitrile–acetic acid and MeOH–acetic acid in various proportions, were investigated, respectively. The results suggested that a linear gradient elution of acetonitrile–water system gave the best resolution and the majority of tested constituents could be efficiently separated within 50 min (Fig. 2). DAD detection was employed at wavelength range of 200–400 nm to investigate the UV spectra of the nine polyacetylenes (Fig. 3). It was found that 254 nm was the best

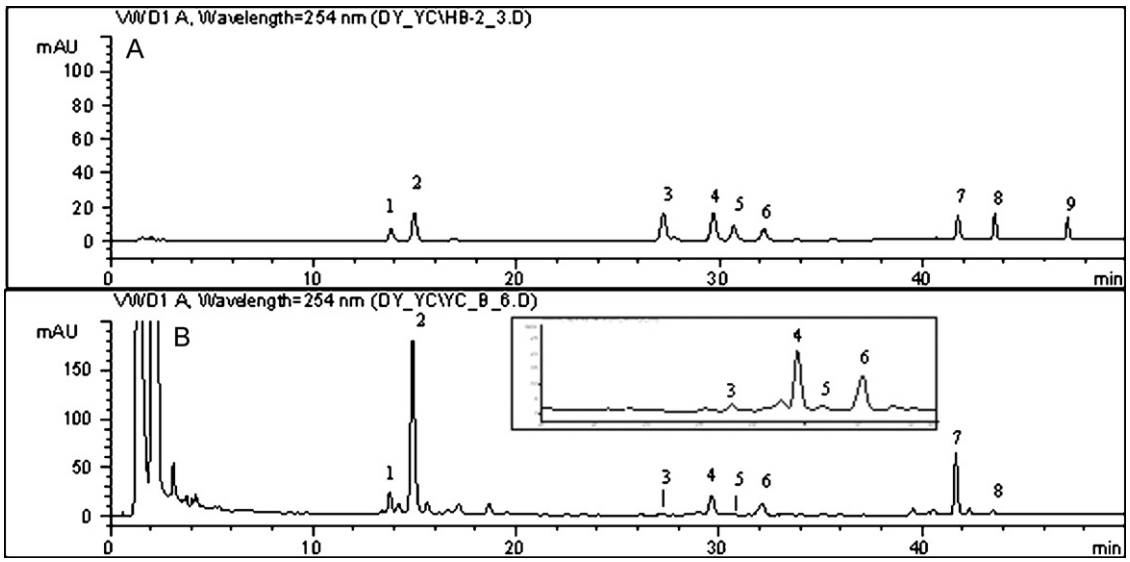

Fig. 2. Representative HPLC chromatograms of (A) mixed standards and (B) methanol extract of *Bupleurum longiradiatum*. For peak identification, see Fig. 1. Experimental conditions as in Section 2.8.

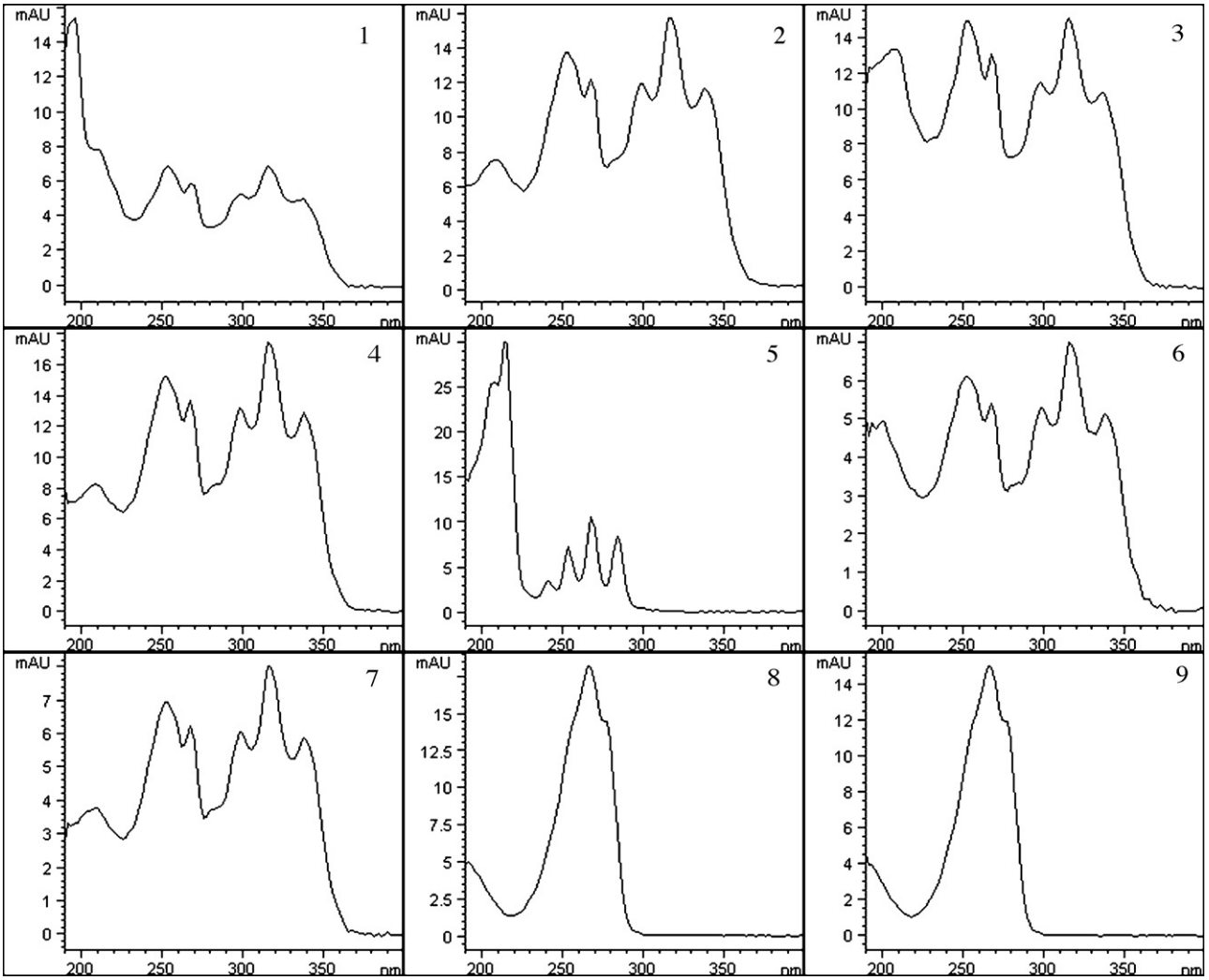

Fig. 3. Characteristic UV absorption spectra of the nine standard compounds. For peak identification, see Fig. 1.

Table 2
Statistical analysis of the linear regression equation employed in the determination of the nine polyacetylenes.

Compound	Calibration curve[a]	r^2	Test range (μg/mL)	LOD[b] (ng)	LOQ[c] (ng)
1	$y = 12120.1x - 17.042$	0.9998	2.50–250	0.41	1.0
2	$y = 35883x + 10.667$	0.9999	0.63–630	0.16	0.42
3	$y = 32582x - 34.717$	0.9999	0.35–175	0.18	0.70
4	$y = 27604.1x - 43.215$	1.0000	1.83–915	0.23	0.61
5	$y = 6090.5x + 0.5432$	0.9999	0.4–200	0.67	2.0
6	$y = 42115.5x - 73.645$	0.9994	0.34–170	0.23	0.57
7	$y = 46582x + 64.21$	0.9998	0.52–520	0.52	1.3
8	$y = 2933.3x - 0.268$	1.0000	0.53–132.5	0.88	2.65
9	$y = 24121.3x - 4.6214$	1.0000	0.235–235	0.31	0.78

[a] The calibration curves were constructed by plotting the peak areas versus the concentration of each analyte. Each calibration curve included six data points.
[b] Limits of detection.
[c] Limits of quantification.

wavelength for the detection because almost all the investigated constituents had higher absorbance, better separation and steady baseline there. Fig. 2 shows typical HPLC profiles of a mixed standards and a MeOH extract of whole plants of *B. longiradiatum*.

3.3. Method validation

The linearity of each analyte was determined by using a series of working standard solutions, and each standard solution was measured in triplicate. Good linear relationships were gained, and the correlation coefficients of all the calibration curves were found to be higher than 0.9994 (Table 2). Limits of detection (LOD) and quantification (LOQ) under the present chromatographic conditions were determined on the basis of response and slope of each regression equation at a signal-to-noise ratio (S/N) of 3 and 10, respectively. The LOD and LOQ ranged in 0.16–0.88 ng and 0.42–2.65 ng, respectively (Table 2).

Intra- and inter-day variations were chosen to determine the precision of the developed method. The intra-day variation was determined by analyzing the same mixed standard solution for six times within one day. While for inter-day variability test, the solution was examined in triplicate for consecutive 3 days. The R.S.Ds of retention times and peak areas were taken as a measure tool, and were less than 1.88% for all nine compounds (Table 3).

In order to examine the recovery of the developed method, mixed standard solutions with three different concentration levels (high, middle and low) were added to the known amounts of *B. longiradiatum* samples. Then the resultant samples were extracted and analyzed with the proposed method and triplicate experiments were performed at each level. The percentage recoveries were calculated according to the following equation: (detected amount − original amount)/spiked amount × 100. As shown in Table 3, the developed analytical method was reproducible with good accuracy in the range of 96.5–104.8%.

3.4. Qualitative analysis of polyacetylenes

Peaks were identified by comparison of their chromatographic characteristics and UV absorption spectra with the standards compounds and confirmed by mass analysis. Table 4 shows the retention times of the standards, as well as their maximal UV absorption wavelengths and MS data.

The UV spectra were useful in the detection of the polyacetylenes, because these compounds usually have typical chromophoric groups that exhibited characteristic UV profiles [20]. Due to the characteristic UV absorption pattern of a diene-diyne-ene structure, polyacetylenes **1**–**4**, **6**, **7** were rapidly confirmed by their UV profiles, with maximal UV wavelengths at 334, 313, 294, 277, 264 and 249 nm. Polyacetylene containing an ene-diyne structure (**5**) was also confirmed by maximal absorption wavelength at 280, 264, 254, 238 and 210 nm, while compounds **8** and **9** with a diene-yne-diene moiety were observed to have maximal absorption peaks at 267 and 280 nm. Based on the characteristic UV profile (Fig. 3, Table 4), it is easy to detect polyacetylene in crude extracts, though they usually have similar retention times to other types of compounds on RP-HPLC column.

Besides the diagnostic UV spectra, their MS data were also compared with those of standards. The MS spectra of polyacetylenes were acquired in positive ion mode, which showed $[M+H]^+$ and $[M+Na]^+$ ions, as well as some other fragment ions, such as $[M+H-H_2O]^+$ and $[M+H-CH_3COOH]^+$. Therefore, HPLC-DAD–MS combines the efficient separation capabilities of HPLC and the great power in structural characterization of UV and MS, and provides a new powerful approach to identify the constituents in plant extracts rapidly and accurately (Table 4).

3.5. Analysis of polyacetylenes in samples

The newly developed HPLC-DAD–MS method was applied to quantify nine polyacetylenes (compounds **1**–**9**) in a total of 27

Table 3
Precision and recoveries of the analytical method for the nine polyacetylenes.

Compound	Intra-day precision ($n = 6$)		Inter-day precision ($n = 3$)		Recovery (%) (mean, $n = 9$)
	R.S.D. of t_R[a] (%)	R.S.D. of PA[b] (%)	R.S.D. of t_R (%)	R.S.D. of PA (%)	
1	0.13	0.22	0.16	0.77	98.3
2	0.07	0.24	0.13	0.77	104.8
3	0.08	0.30	0.21	0.38	97.4
4	0.03	0.37	0.27	1.88	107.9
5	0.01	0.25	0.09	1.24	99.7
6	0.04	0.55	0.16	1.17	101.2
7	0.03	0.18	0.03	1.07	96.6
8	0.01	0.12	0.04	0.53	102.4
9	0.09	0.30	0.11	0.76	96.5

[a] Retention time.
[b] Peak area.

Table 4
Chromatographic, UV and mass spectral data of the polyacetylenes analyzed by HPLC-DAD–MS.

Identification	t_R (min)	(+)ESI-MS (*m/z*)	λ_{max} (nm)
(2*Z*,8*Z*,10*E*)-Heptadecatriene-4,6-diyne-1,14-diol (**1**)	13.81	259.2 $[M+H]^+$	334, 313, 294, 277, 264, 249
Bupleurotoxin (**2**)	14.95	259.2 $[M+H]^+$	334, 313, 294, 277, 264, 249
(2*Z*,8*Z*,10*E*)-1-Hydroxyheptadecatriene-4,6-diyn-14-yl acetate (**3**)	27.22	241.3 $[M+H-CH_3COOH]^+$ 301.2 $[M+H]^+$ 323.2 $[M+Na]^+$	334, 313, 294, 277, 264, 249
Acetylbupleurotoxin (**4**)	29.69	241.3 $[M+H-CH_3COOH]^+$ 301.3 $[M+H]^+$ 323.2 $[M+Na]^+$	334, 313, 294, 277, 264, 249
(2*Z*,9*Z*)-Pentadecadiene-4,6-diyn-1-ol (**5**)	30.69	217.6 $[M+H]^+$	280, 264, 251, 238, 210
(2*Z*,8*E*,10*E*)-Pentadecatriene-4,6-diyn-1-ol (**6**)	32.19	215.6 $[M+H]^+$	334, 313, 294, 277, 264, 249
Bupleurynol (**7**)	41.74	243.2 $[M+H]^+$	334, 313, 294, 277, 264, 249
(2*E*,4*E*,9*Z*)-Octadecatriene-6-yne-1,18-diyl diacetate (**8**)	43.56	383.3 $[M+Na]^+$ 361.4 $[M+H]^+$	280, 267
(2*E*,4*E*,9*Z*)-Heptadecatriene-6-yn-1-yl acetate (**9**)	47.15	226.3 $[M+H-CH_3COOH]^+$	280, 267

Bupleurum samples from different species or locations. All the contents were calculated with external standard method, and data of the quantitative analyses were expressed as mean ± deviation (Table 5).

It was found that compounds **1**–**8** were detected from all the three samples of *B. longiradiatum*, while their contents differed greatly from each other. Among the tested polyacetylenes, bupleurotoxin (**2**), acetylbupleurotoxin (**4**), and bupleurynol (**7**) were determined as the main compounds at concentrations of about 2.25–0.18 mg/g, 3.91–0.02 mg/g, and 1.00–0.03 mg/g, respectively. Comparatively, relatively smaller amounts of compounds **1**, **3**, **5**, **6**, and **8** were observed in the analyzed *B. longiradiatum*, while no detectable amount of compound **9** was determined in this species. Since the analysis was performed using fresh plant materials, we suggested that compound **9** might be artifacts that form during storage or extraction. From the above results, *B. longiradiatum* represents a rich source of polyacetylenes, which is in accordance with the results reported previously [12,18]. Pharmacological studies on the polyacetylenes suggested that these compounds possess strong toxicity, and might be responsible for the toxicity of *B. longiradiatum* [11,18,28].

For other species of *Bupleurum* genus, *B. smithii*, *B. bicaule* and *B. angustissimum* contained compounds **6**–**8**, and *B. smithii* var. *parvifolium* also showed the presence of compounds **5**–**8**. However, no polyacetylenes (**1**–**9**) were detectable from the authentic *Chaihu* samples (*B. chinense*, *B. scorzonerifolium*), and from five other *Bupleurum* species (*B. marginatum* var. *stenophyllum*, *B. wenchuanense*, *B. komarovianum*, *B. rockii* and *B. candollei*). These results indicated that the distribution and the contents of the tested polyacetylenes in different *Bupleurum* species vary significantly, and they were much higher in two samples of *B. longiradiatum* (LO-2, LO-2A) than in the other samples, which suggested that these two batches might have stronger toxic effects than others. Thus, in order to ensure its safety clinical use, the commercially prepared crude drugs of *Chaihu* in herb markets should be labeled before circulation, and the qualitative and quantitative determination of polyacetylenes is useful for the assessment of the toxicity of crude herbal material.

It should be noted that polyacetylenes **1**–**4** are specific for *B. longiradiatum*, though the detection of compounds **5**–**8** was observed from *B. smithii*, *B. smithii* var. *parvifolium*, *B. bicaule* and *B. angustissimum*, and the isolation of various polyacetylenes has been reported from other *Bupleurum* species, including *B. falcatum* [26], *B. acutifolium* [27], *B. salicifolium* [29] and *B. spinosum* [30]. Therefore, the detection of these four characteristic polyacetylenes (**1**–**4**) could be used to reveal the identification of the toxic *B. longiradiatum*, and to differentiate the toxic plants from the medicinal *Chaihu* plants.

3.6. Acute toxicity of *Bupleurum longiradiatum*

There are cases of acute human poisoning caused by the ingestion of *B. longiradiatum*, after mistaking it for *Chaihu*. Previous investigation on the acute toxicity of this plant also showed its strong poisoning in mice, with LD_{50} value of 3000.0 mg/kg [11].

To further investigate the toxicity of *B. longiradiatum* and its constituents, we examined the acute toxicity of the extract and its fractions. The LD_{50} value was determined based on the dosage and the mortality, and was calculated according to Bliss's probit method [31]. The results indicated that the CH_2Cl_2 fraction and the ethanol extract exhibited much higher toxicity, with LD_{50} values of 37.5 mg/kg (95% CI: 32.8–42.9 mg/kg), and 77.7 mg/kg (95% CI: 67.7–89.3 mg/kg), respectively. However, *n*-BuOH fraction showed no toxic reactions, with the maximum tolerated dose

Table 5
Contents of the nine polyacetylenes in samples of different *Bupleurum* (mean ± deviation, $n = 3$).

Sample	Content[a] (mg/g)							
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**
LO-1A	0.13 ± 0.01	0.18 ± 0.01	0.01[d]	0.02[d]	0.02[d]	0.02[d]	0.03[d]	0.08[d]
LO-2	0.30 ± 0.01	2.25 ± 0.04	0.16 ± 0.01	3.91 ± 0.12	0.45 ± 0.01	0.16 ± 0.01	1.00 ± 0.04	0.19 ± 0.01
LO-2A	0.43 ± 0.01	0.39 ± 0.01	0.02[d]	1.12 ± 0.05	0.09[d]	0.05[d]	0.31 ± 0.02	0.05[d]
SM-1	–[b]	–	–	–	–	0.33 ± 0.01	0.11 ± 0.01	0.27 ± 0.02
SM-2	–	–	–	–	–	0.31 ± 0.01	0.11 ± 0.02	0.51 ± 0.01
PA-1	–	–	–	–	0.23 ± 0.02	0.11 ± 0.02	0.04[d]	+[c]
PA-2	–	–	–	–	–	0.15 ± 0.01	0.05[d]	0.42 ± 0.02
BI-1	–	–	–	–	–	0.22 ± 0.02	0.09[d]	0.28 ± 0.01
AN-1	–	–	–	–	–	0.03[d]	+	+

[a] Average of triplicates.
[b] Under detection limit.
[c] Under quantification limit.
[d] Standard deviation (SD) <0.01.

(MTD) >15 g/kg. Chemical screening found that polyacetylenes were the main constituents in the toxic CH_2Cl_2 fraction, which were in accordance with the results reported by Zhao et al. [12,18]. Based on the above results and the literature data [11,18,28], we suggested that the polyacetylenes are likely to be toxic constituents that responsible for the toxicity of *B. longiradiatum*, though further toxicological investigation still need to be carried out to confirm the work.

4. Conclusion

In this work, a HPLC-DAD–MS method has been developed for simultaneous determination of nine polyacetylenes in *Bupleurum* for the first time. Among them, compounds **1** and **3** are two new polyacetylenes. Furthermore, the established method was applied for the qualitative and quantitative evaluation of 27 *Bupleurum* samples, which showed a great variety in the distribution and the contents of the polyacetylenes. It was found that the polyacetylenes (**1**–**8**) were abundant in *B. longiradiatum*, and were responsible for the toxicity of this plant. Polyacetylenes **5**–**8** were also identified in *B. smithii*, *B. smithii* var. *parvifolium, B. bicaule* and *B. angustissimum*. However, no polyacetylenes were detected from the authentic *Chaihu* samples and some other *Bupleurum* species, namely *B. marginatum* var. *stenophyllum*, *B. wenchuanense*, *B. komarovianum*, *B. rockii* and *B. candollei*. The results demonstrated that the toxic *B. longiradiatum* could readily be distinguished from the authentic *Chaihu* samples and other *Bupleurum* species based on the polyacetylene profiles. We also suggest that the determination of polyacetylenes, such as bupleurotoxin (**2**), acetylbupleurotoxin (**4**), and bupleurynol (**7**), should be recorded in some authorized publications and applied to the quality evaluation for *Chaihu* to ensure its safety clinical usage.

Acknowledgments

The work was supported by program NCET Foundation, NSFC (30725045), the Special Program for New Drug Innovation of the Ministry of Science and Technology, China (2009ZX09311-001, 2008ZX09308-005), China International Science and Technology Cooperation Project (2007DFC31030), Shanghai Leading Academic Discipline Project (B906) and in part by the Scientific Foundation of Shanghai China (07DZ19728, 09DZ1975700, 09DZ1971500).

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.chroma.2010.12.007.

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