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On-line identification of the constituents of Buyang Huanwu decoction in pig serum using combined HPLC–DAD–MS techniques

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Abstract

Buyang Huanwu decoction (BYHWD) is a widely used Chinese traditional compound medicine that has proved effective in treating cerebrovascular illnesses; however, its active substances have remained unknown. In this paper, serum chemistry and combined high-performance liquid chromatography (HPLC), photodiode-array detection and mass-spectrometry techniques were used to study the constituents of BYHWD from pig serum after oral administration. A total of 45 characteristic HPLC peaks were detected from serum containing drug. The chemical structures of nine of the peaks were tentatively elucidated as 7,3′-dihydroxy-4′-methoxyisoflavone-7-*O*-glucuronide (**P1**), 7-hydroxy-4′-methoxyisoflavone-7-*O*-glucuronide (**P2**), 7,2′,4′-trihydroxy-3′-methoxyisoflavane-7-*O*-sulphate (**P3**), 3-hydroxy-9,10-dimethoxypterocarpan-3-*O*-glucuronide (**P4**), 7,2′-dihydroxy-3′,4′-dimethoxyisoflavane-7-*O*-glucuronide (**P5**), 3-hydroxy-9,10-dimethoxypterocarpane-3-*O*-sulphate (**P6**), 4(1H)-quinolinone (**P7** or **P8**), 4-hydroxyquinoline (**P8** or **P7**) and oleic acid (**P9**). All of the identified peaks, with the exception of **P9**, were metabolites of the constituents of BYHWD in vivo.

Keywords: Buyang Huanwu decoction; Serum containing drug; HPLC–DAD–MS; Metabolite

1. Introduction

Chinese traditional compound medicines (CTCMs) represent a valuable medicinal legacy. CTCMs have made essential contributions to the growth and prosperity of the Chinese nation for centuries, as well as exerting important international influences. Unlike Western medicines, CTCMs are made up of various Chinese drugs, and their chemical constituents are multiplicity. At present, CTCMs are facing difficulties because their curative effects are largely based on the prescribing doctor's experiences, and their effective constituents and mechanisms of action are not clear, which seriously restricts their development in the international market.

Buyang Huanwu decoction (BYHWD) is a famous CTCM that was first mentioned in "*Yilin Gaicuo*" (*Correction on Errors in Medical Classics*), which was written during the Qing Dynasty (1830). It is widely used in China to invigorate the body, promote blood flow and activate meridians. In clinical applications, BYHWD has proved effective in treating cerebrovascular diseases, such as blood deficiencies and sequelae of stroke [1–3]. BYHWD is composed of Huangqi [Radix Astragali, the dried roots of *Astragalus membranaceus* (Fisch.) Bge. var. *mongholicus* (Bge.) Hsiao], Danggui (Guiwei) [Radix Angelicae Sinensis, the dried lateral roots of *Angelica sinensis* (Oliv.) Diels], Chishao [Radix Paeoniae Rubra, the dried roots of *Paeonia lactiflora* Pall.], Chuanxiong [Rhizoma Chuanxiong, the dried rhizomes of *Ligusticum chuanxiong* Hort.], Honghua [Flos Carthami, the dried flowers of *Carthamus tinctorius* L.], Taoren [Semen Persicae, the dried seeds of *Amygdalus persica* L.] and Dilong [Pheretima, the dried bodies of *Pheretima aspergillum* (E. Perrier)], all of which are recorded in the Chinese Pharmacopoeia (2005) [4–10].

The constituents of BYHWD are numerous and diverse, including flavonoids, alkaloids and saponins, and it remains unclear which constituents are responsible for the actions of the drug. The present paper examined the constituents of pig serum after oral administration of BYHWD using combined

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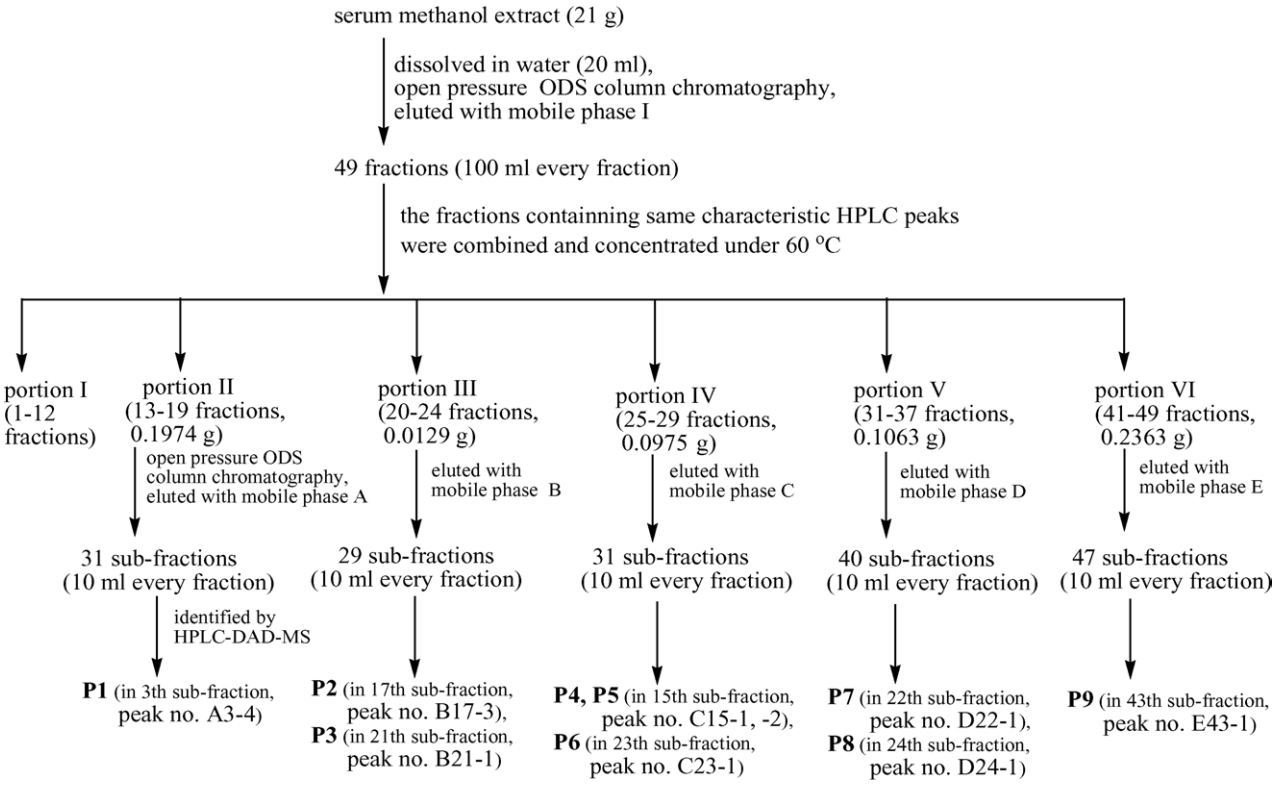

Fig. 1. Flow chart for separating the serum of pigs administrated BYHWD orally. Mobile phase I: from water to methanol gradually; mobile phase A: 5% methanol to 15% methanol; mobile phase B: 8% methanol to 20% methanol; mobile phase C: 10% methanol to 30% methanol; mobile phase D: 20% methanol to 50% methanol; and mobile phase E: 30% methanol to 70% methanol.

high-performance liquid chromatography (HPLC), photodiode-array detection (DAD) and mass spectrometry (MS) techniques.

2. Experimental

2.1. Instrumentation and chromatographic materials

The HPLC system consisted of the HP1100 series LC apparatus (Hewlett-Packard Inc., USA), including a DAD, a high-pressure binary-gradient solvent-delivery pump, an autosampler and a thermostatted column compartment. A Zorbax SB C_{18} column (5 μm; 250 × 4.6 mm) was used to analyze all of the samples; the flow rate was 1.0 ml/min, the injection volume was 20 μl and the temperature of the column oven was 20 °C. The results of HPLC of serum containing drug and its fractions eluted from the ODS column were compared with those of control serum, using 0.0025% phosphoric acid–acetonitrile

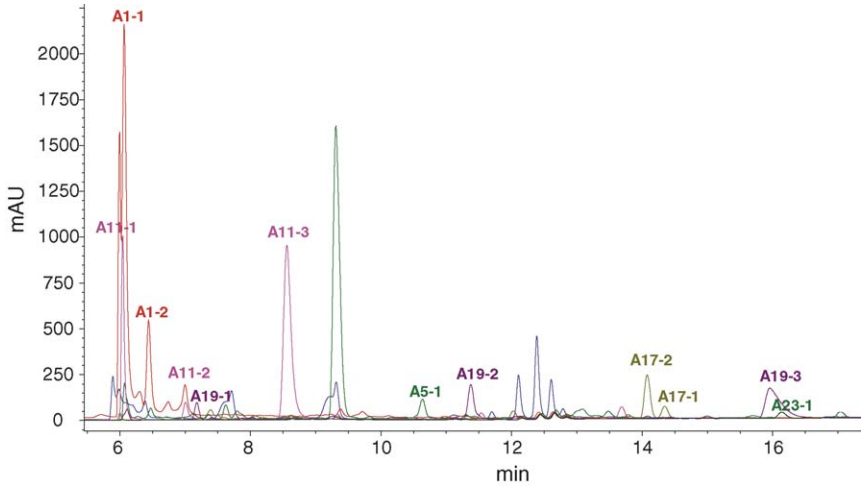

Fig. 2. Overlapping of HPLC signals containing subfractions A1, -A5, -A11, -A17, -A19, -A23 and control blank sample (210 nm); mobile phase, 0.0025% phosphoric acid–acetonitrile (100:0–0:100 in a linear gradient over 30 min); flow rate, 1 ml/min; (—) control blank sample; (—) A1; (—) A5; (—) A11; (—) A17; (—) A19; and (—) A23.

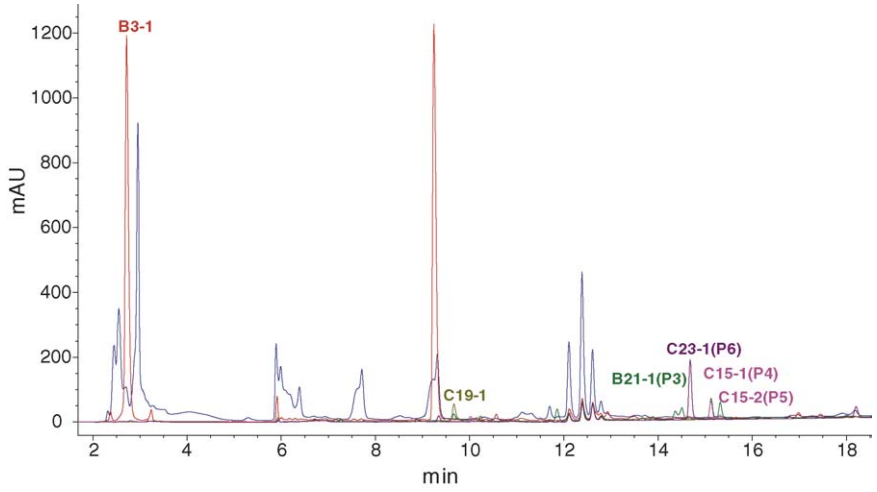

Fig. 3. Overlapping of HPLC signals containing subfractions B3, -B21, -C15, -C19, -C23 and control blank sample (210 nm); mobile phase, 0.0025% phosphoric acid–acetonitrile (100:0–0:100 in a linear gradient over 30 min); flow rate, 1 ml/min; ( ) control blank sample; ( ) B3; ( ) B21; ( ) C15; ( ) C19; and ( ) C23.

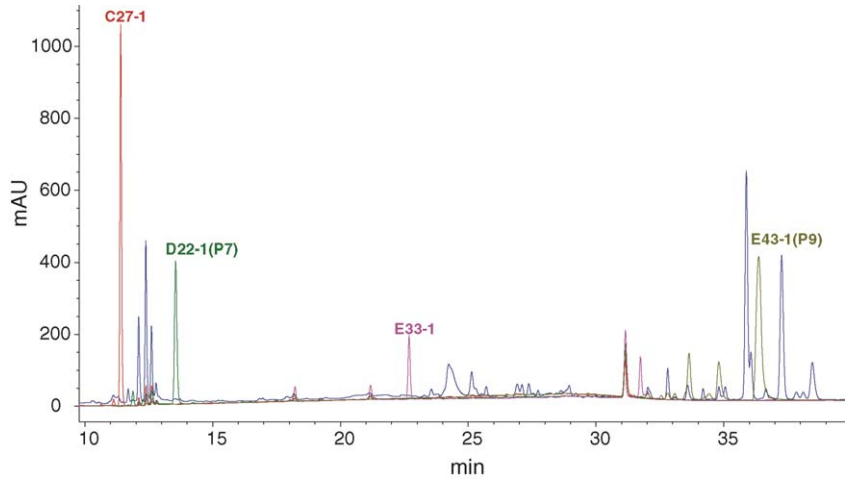

Fig. 4. Overlapping of HPLC signals containing subfraction C27, -D22, -E33, -E43 and control blank sample (210 nm); mobile phase, 0.0025% phosphoric acid–acetonitrile (100:0–0:100 in a linear gradient over 30 min); flow rate, 1 ml/min; ( ) control blank sample; ( ) C27; ( ) D22; ( ) E33; and ( ) E43.

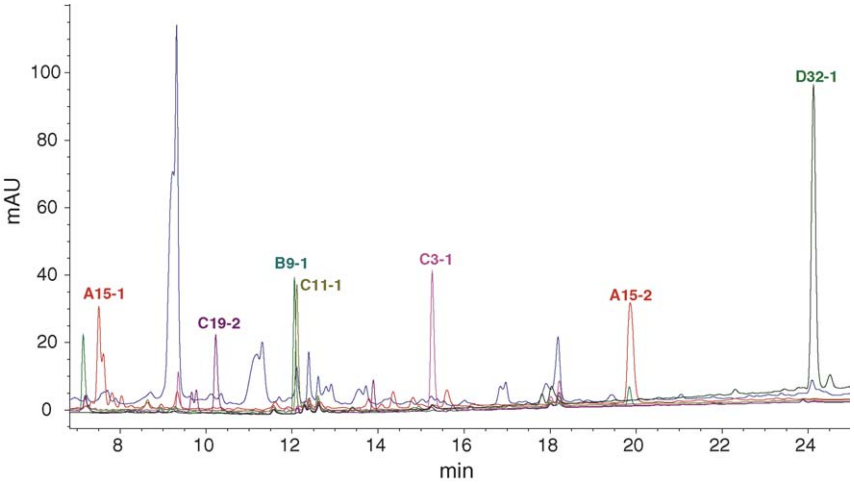

Fig. 5. Overlapping of HPLC signals containing of subfractions A15, -B9, -C3, -C11, -C19, -D32 and control blank sample (230 nm); mobile phase, 0.0025% phosphoric acid–acetonitrile (100:0–0:100 in a linear gradient over 30 min); flow rate, 1 ml/min; ( ) control blank sample; ( ) A15; ( ) B9; ( ) C3; ( ) C11; ( ) C19; and ( ) D32.

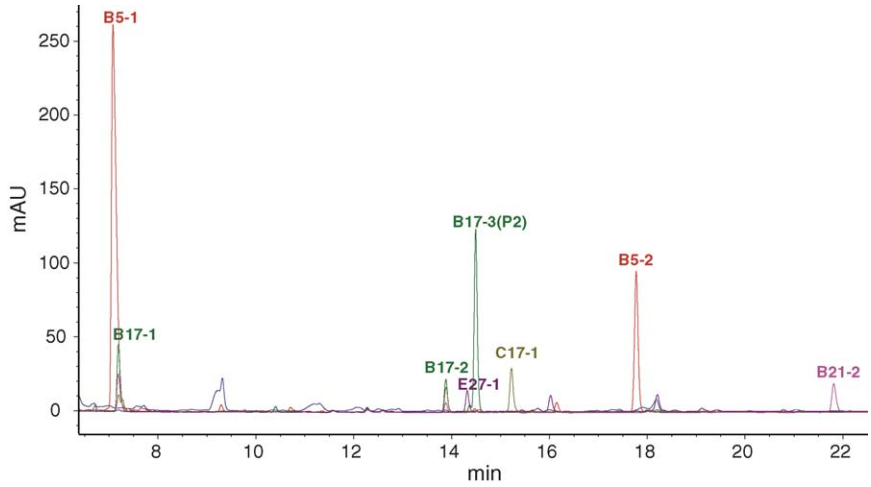

Fig. 6. Overlapping of HPLC signals containing subfractions B5, -B17, -B21, -C17, -E27 and control blank sample (254 nm); mobile phase, 0.0025% phosphoric acid–acetonitrile (100:0–0:100 in a linear gradient over 30 min); flow rate, 1 ml/min; ( ) control blank sample; ( ) B5; ( ) B17; ( ) B21; ( ) C17; and ( ) E27.

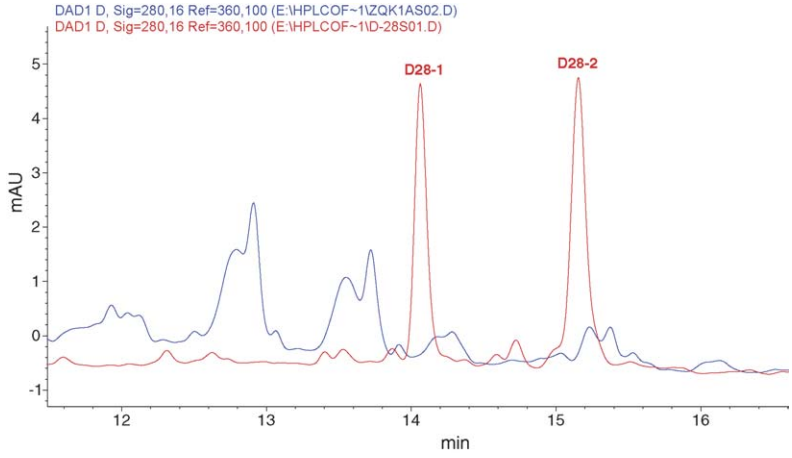

Fig. 7. Overlapping of HPLC signals containing subfractions D28 and control blank sample (280 nm); mobile phase, 0.0025% phosphoric acid–acetonitrile (100:0–0:100 in a linear gradient over 30 min); flow rate, 1ml/min; ( ) control blank sample and ( ) D28.

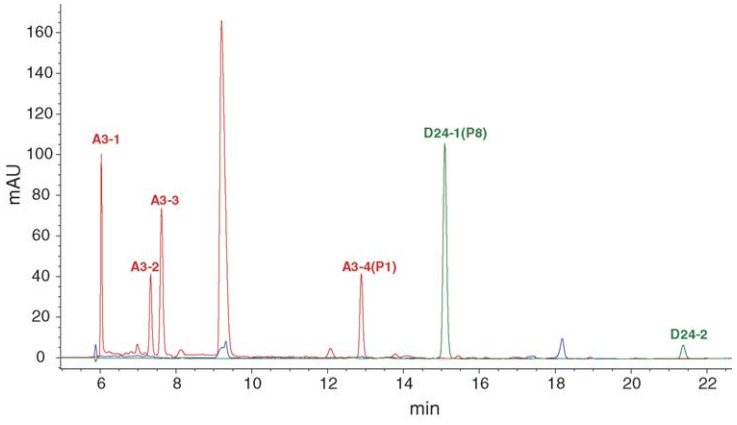

Fig. 8. Overlapping of HPLC signals containing subfractions A3, -D24 and control blank sample (300 nm); mobile phase, 0.0025% phosphoric acid–acetonitrile (100:0–0:100 in a linear gradient over 30 min); flow rate, 1 ml/min; ( ) control blank sample; ( ) A3; and ( ) D24.

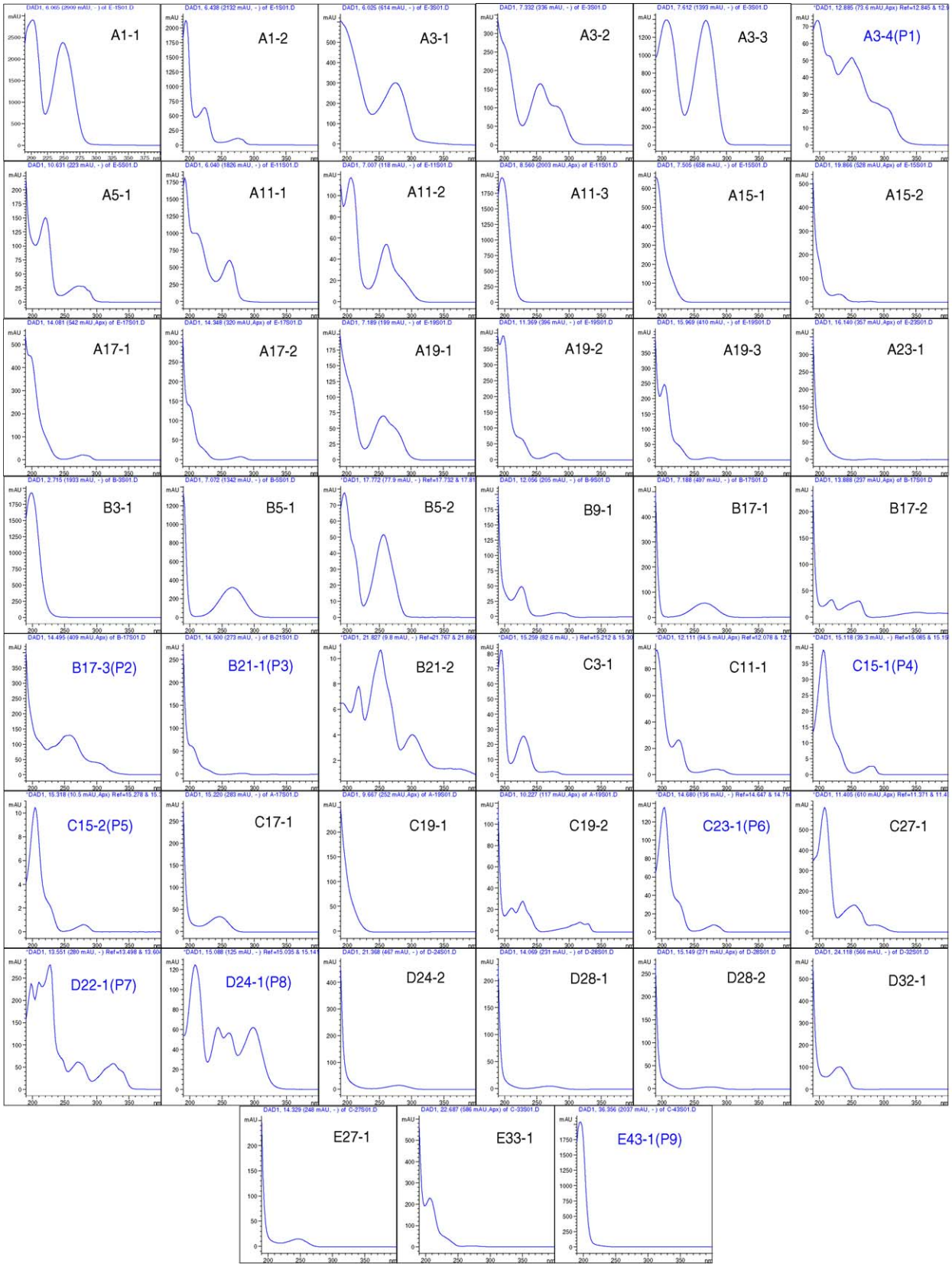

Fig. 9. UV spectra of 45 HPLC peaks, which were detected in the serum of pigs administrated BYHWD orally.

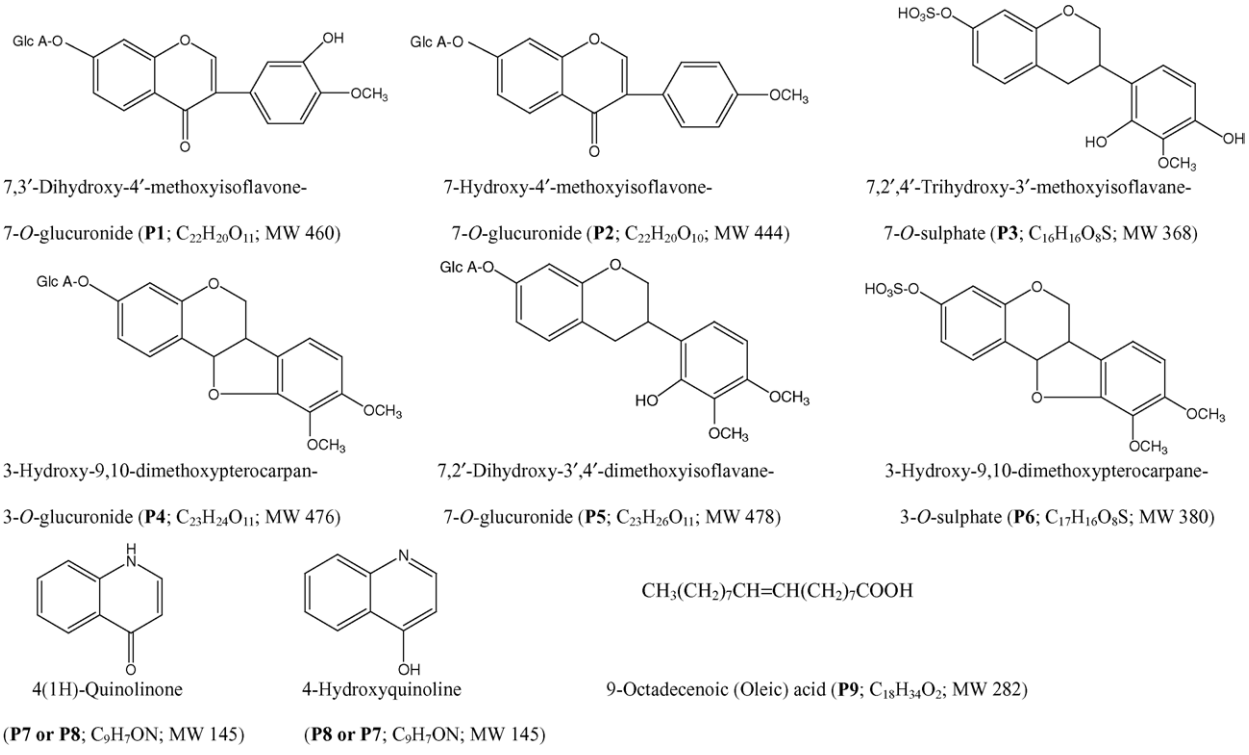

Fig. 10. Chemical structures of **P1**–**P9**, which were detected in the serum of pigs administrated BYHWD orally.

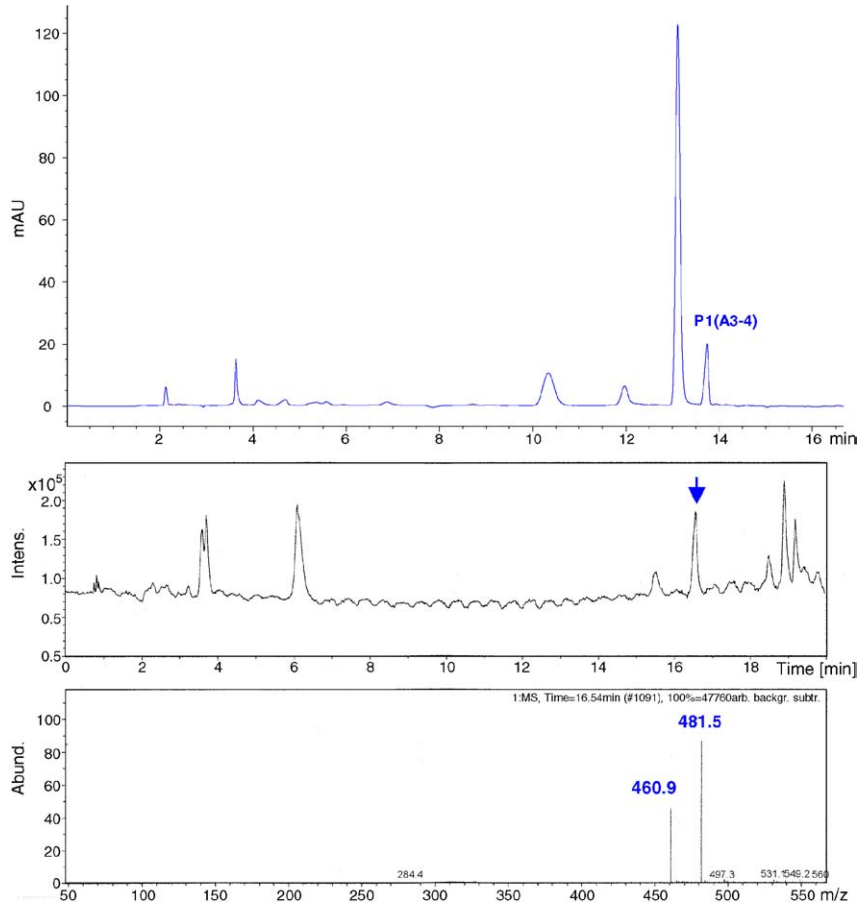

Fig. 11. HPLC (upper) and on-line total ion chromatogram (middle) of subfraction A3, the spectrum of $[M-H]^-$ ion at m/z 460.9 and $[M+Na-H]^-$ ion at m/z 481.5 (lower) of **P1** by LC–MS. Mobile phase, 5% acetonitrile (0–7 min) and then to 50% acetonitrile in a linear gradient (7–20 min); flow rate, 1 ml/min; detection wavelength, 300 nm.

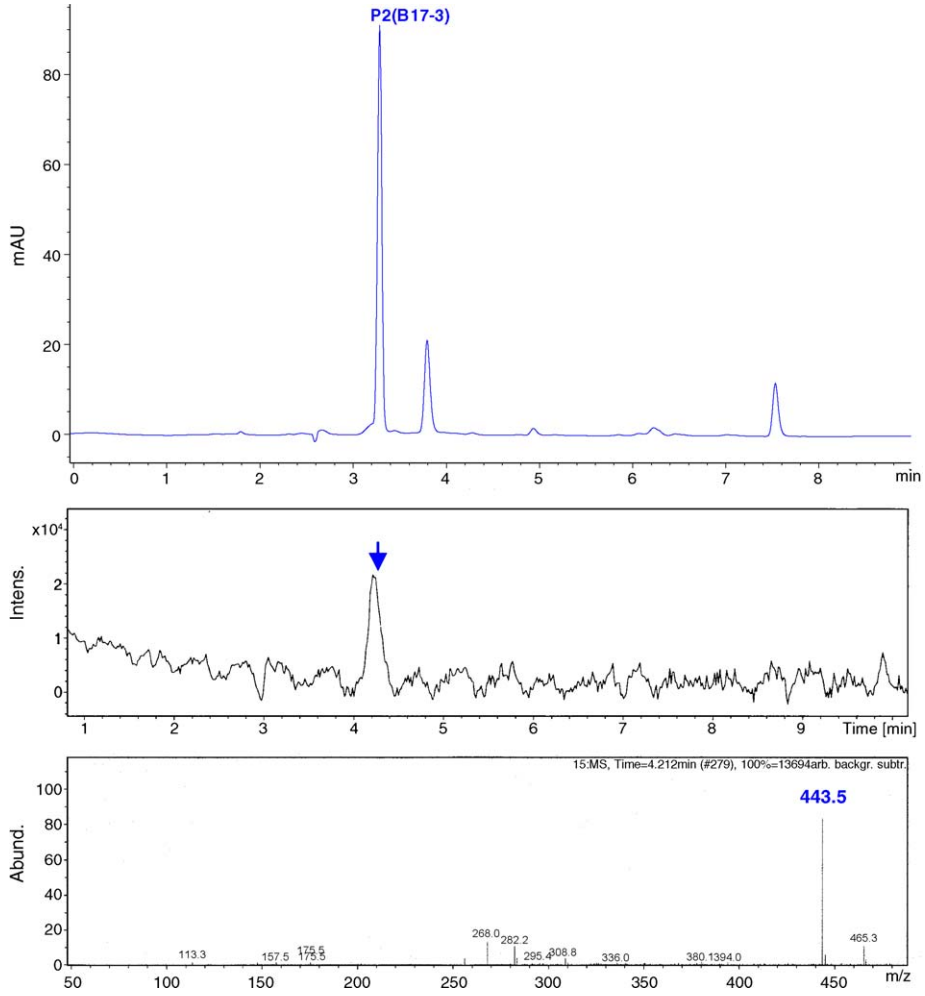

Fig. 12. HPLC (upper) and on-line total ion chromatogram (middle) of subfraction B17, the spectrum of $[M-H]^-$ ion at m/z 443.5 (lower) of **P2** by LC–MS. Mobile phase, 20% acetonitrile to 80% acetonitrile in a linear gradient (0–15 min); flow rate, 1 ml/min; detection wavelength, 254 nm.

(100:0–0:100 in a linear gradient over 30 min) as the mobile phase.

HPLC–electrospray ionization (ESI)–ion trap mass spectrometry (MS^n) of **P1**–**P9** was performed using a Varian 6000/ESQUIRE-LC system (Bruker/HP Inc., USA) in series with an ultraviolet (UV) detector and an ESI–MS^n detector. The ESI–MS^n working conditions were as follows: drying gas (N_2) temperature, 300 °C; drying gas, 12 l/min; capillary voltage, 4000 V; ions observed at mass range m/z 15–800; accumulation time, 50 ms; and nebulizer pressure, 40 psi. The water–acetonitrile mobile phases were as follows: 5% acetonitrile (0–7 min) to 50% acetonitrile (7–20 min) in a linear gradient for **P1** in subfraction A3; 20% acetonitrile to 80% acetonitrile (0–15 min) in a linear gradient for **P2** in subfraction B17; 30% acetonitrile to 60% acetonitrile (0–20 min) in a linear gradient for **P3** in subfraction B21; 15% acetonitrile for **P4** and **P5** in subfraction C15; 5% acetonitrile to 30% acetonitrile (0–15 min) in a linear gradient and then keep 30% acetonitrile for 15 min for **P6** in subfraction C23; 20% acetonitrile to 100% acetonitrile (0–20 min) in a linear gradient for **P7** in subfraction D22; 35% acetonitrile to 100% acetonitrile (0–15 min) in a linear gradient for **P8** in subfraction D24; 80% acetonitrile to 100% acetonitrile (0–8 min) in a linear gradient and then keep 100% acetonitrile for 15 min for **P9** in subfraction E43.

The acetonitrile (Fair Lawn, NJ, USA) and phosphoric acid (Fairfield, OH, USA) used were of HPLC grade. Wahaha purified water (Hangzhou, Zhejiang, China) was used for HPLC. The methanol used was of analytical grade (Beijing Chemical Factory, Beijing, China). Custom Bonded ODS (90–130 μm, Alltech, USA) was used to separate the serum containing drug.

2.2. Animals

Male miniature pigs (each weighing ∼25 kg) were obtained from the Chinese Academy of Agricultural Science (Beijing, China). The animals were maintained in an environmentally controlled breeding room (Animal Center for Peking University Health Science Center, Beijing, China) for 5 days before starting the experiments, and were fed a standard laboratory diet.

2.3. Crude drugs and preparation of BYHWD

Huangqi (No. 99001), Danggui (Guiwei; No. 99002), Chishao (No. 99003), Chuanxiong (No. 99004), Honghua (No.

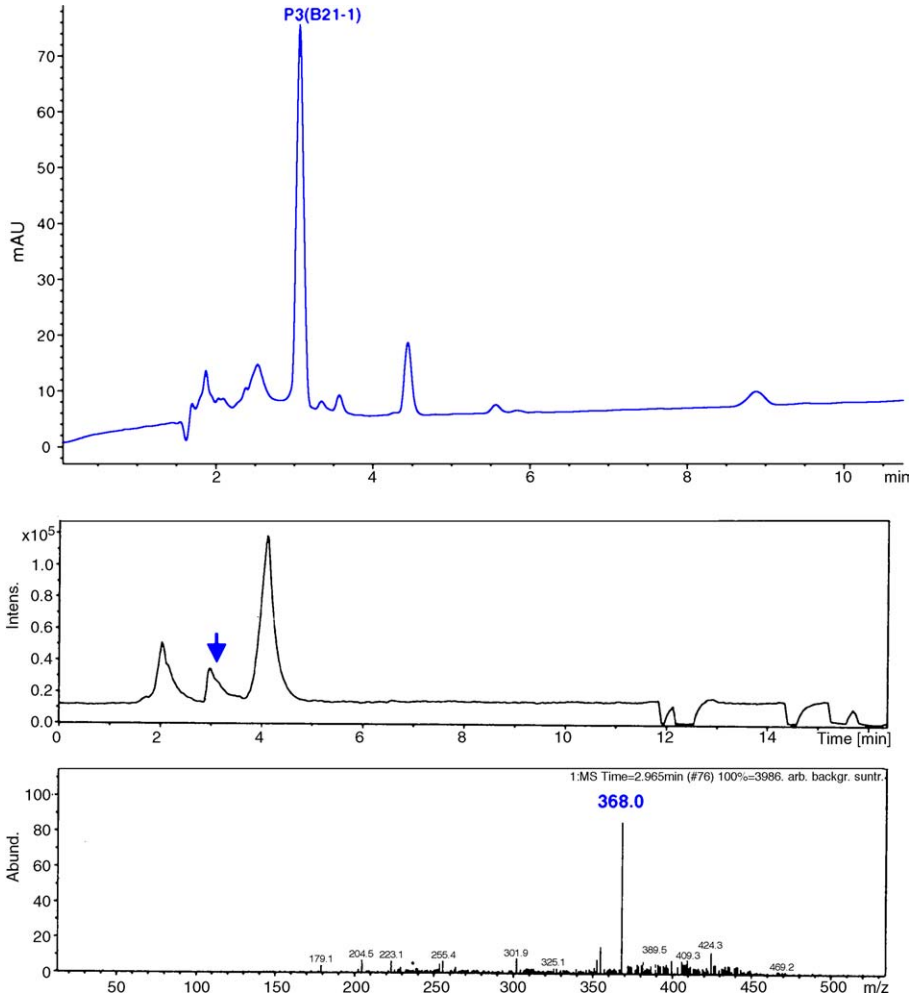

Fig. 13. HPLC (upper) and on-line total ion chromatogram (middle) of subfraction B21, the spectrum of $[M-H]^-$ ion at *m/z* 368.0 (lower) of **P3** by LC–MS. Mobile phase, 30% acetonitrile to 60% acetonitrile in a linear gradient (0–20 min); flow rate, 1 ml/min; detection wavelength, 210 nm.

Table 1
Retention time and detection wavelength of the 45 characteristic HPLC peaks detected in the serum of pigs administrated BYHWD orally

Peak no.	Retention time (min)	Detection wavelength (nm)
A1-1	6.07	210
A1-2	6.44	210
A3-1	6.03	300
A3-2	7.33	300
A3-3	7.62	300
A3-4 (**P1**)[a]	12.89	300
A5-1	10.64	210
A11-1	6.04	210
A11-2	7.01	210
A11-3	8.56	210
A15-1	7.51	230
A15-2	19.86	230
A17-1	14.08	210
A17-2	14.35	210
A19-1	7.18	210
A19-2	11.37	210
A19-3	15.96	210
A23-1	16.14	210
B3-1	2.72	210
B5-1	7.09	254
B5-2	17.77	254
B9-1	12.06	230
B17-1	7.30	254
B17-2	13.88	254
B17-3 (**P2**)[a]	14.49	254
B21-1 (**P3**)[a]	14.51	210
B21-2	21.81	254
C3-1	15.26	230
C11-1	12.11	230
C15-1 (**P4**)[a]	15.12	210
C15-2 (**P5**)[a]	15.32	210
C17-1	15.22	254
C19-1	9.67	210
C19-2	10.23	230
C23-1 (**P6**)[a]	14.68	210
C27-1	11.41	210
D22-1 (**P7**)[a]	13.55	210
D24-1 (**P8**)[a]	15.09	300
D24-2	21.37	300
D28-1	14.06	280
D28-2	15.15	280
D32-1	24.13	230
E27-1	14.32	254
E33-1	22.70	210
E43-1 (**P9**)[a]	36.36	210

Table 1 (*Continued*)

[a] Structure identified.

99005), Taoren (No. 99006) and Dilong (No. 99007) were purchased from herb companies in Hunyuan (Shanxi), Minxian (Gansu), Duolun (Inner Mongolia), Chengdu (Sichuan), Urumchi (Xinjiang), Anguo (Hebei) and Guangzhou (Guangdong), respectively, and were identified by Prof. Shaoqing Cai (Peking University, Beijing, China). All voucher specimens with the same sample number were deposited in the Herbarium of Pharmacognosy, School of Pharmaceutical Sciences, Peking University. The powdered medical component drugs of BYHWD (12.5 kg) at a ratio of 120:6:4.5:3:3:3:3 Huangqi:Danggui (Guiwei):Chishao:Chuanxiong:Honghua:Taoren:Dilong (dosage = 142.5 g/person/day dry weight), according to "*Yilin Gaicuo*", were extracted three times with boiling water (volume over weight values per extraction = 10-, 8- and 6-fold, respectively; duration = 30 min per extraction). The solution obtained was concentrated and lyophilized. The dried powdered BYHWD extract was stored at 4 °C and made up to a final concentration of 3 g/ml before use.

2.4. Preparation of serum samples

The pigs were divided into two groups: an experimental group (group A; $n = 8$) and a control group (group B; $n = 2$). Each animal in group A was administrated BYHWD orally at a dose of 4 ml/kg twice daily (7:00 a.m. and 7:00 p.m.) for a total of seven doses. These eight pigs were exsanguinated from the arteria carotis at 1 h after the last dosing, and a total of 9700 ml blood was obtained and centrifuged for 15 min at 3000 rpm. The serum (4320 ml) was separated, frozen to a dry powder (433 g) and denoted "serum containing drug". The two pigs in group B were administrated water at a dose of 4 ml/kg twice daily (7:00 a.m. and 7:00 p.m.) for a total of seven doses. Serum was obtained from the control group animals using the procedure described above, and was denoted "control serum".

2.5. Processing and analysis of serum sample extracts

The dry powder of the serum containing drug (433 g) was sonicated three times with 5000 ml methanol (30 min per sonication). The extract obtained was concentrated at 60 °C to produce concentrated extract (21 g), which was dissolved in 20 ml water before separation by ODS column chromatography. The flow chart for separating the serum extract is shown in Fig. 1. HPLC data for the 49 fractions eluted from the ODS column were compared with HPLC of the control serum respectively, using 0.0025% phosphoric acid–acetonitrile among 100:0–0:100 in a

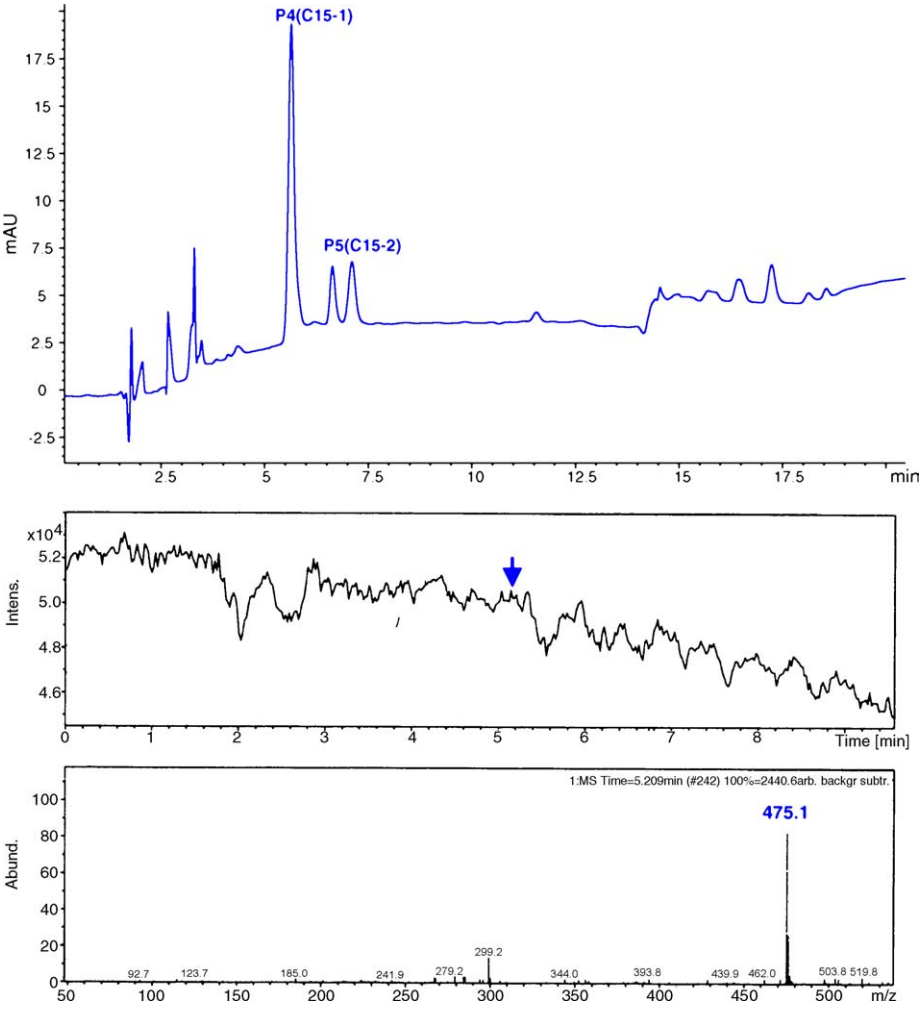

Fig. 14. HPLC (upper) and on-line total ion chromatogram (middle) of subfraction C15, the spectrum of $[M-H]^-$ ion at m/z 475.1 (lower) of **P4** by LC–MS. Mobile phase, 15% acetonitrile; flow rate, 1 ml/min; detection wavelength, 210 nm.

linear gradient over 30 min as the mobile phase. Based on the characteristic HPLC peaks that were only found in the serum containing drug, the 49 fractions were combined into six portions (I–VI). Portions I–VI were further separated into different subfractions by ODS column chromatography, and a total of 45 characteristic HPLC peaks were detected, which occurred in 27 different subfractions (Figs. 1–9, Table 1). Among these 45 peaks, the structures of nine peaks (**P1**–**P9**, Fig. 10) with abundant ion fragments were studied further using combined HPLC–DAD–MS technologies (Figs. 11–19, Table 2).

3. Structural identification of P1–P9

The chemical structures of **P1**–**P9** were identified as 7,3′-dihydroxy-4′-methoxyisoflavone-7-*O*-glucuronide (**P1**), 7-hydroxy-4′-methoxyisoflavone-7-*O*-glucuronide (**P2**), 7,2′,4′-trihydroxy-3′-methoxyisoflavane-7-*O*-sulphate (**P3**), 3-hydroxy-9,10-dimethoxypterocarpan-3-*O*-glucuronide (**P4**), 7,2′-dihydroxy-3′,4′-dimethoxyisoflavane-7-*O*-glucuronide (**P5**), 3-hydroxy-9,10-dimethoxypterocarpane-3-*O*-sulphate (**P6**), 4(1H)-quinolinone (**P7** or **P8**), 4-hydroxyquinoline (**P8** or **P7**) and oleic acid (**P9**).

3.1. Identification of the structures of *P1* and *P2*

P1 (subfraction A3, peak no. A3-4) and **P2** (subfraction B17, peak no. B17-3) had similar UV absorptions, with maxima at 200, 250 and 300 (sh) nm (Fig. 9), which were typical of flavonoids. **P1** showed $[M-H]^-$ and $[M+Na-H]^-$ ions at *m*/*z* 460.9 and 481.5, respectively (Fig. 11), and two one-level splitting fragment ions were detected at *m*/*z* 283.0 $[M-175-2H]^-$ and 174.7 $[Glc\ A-H_2O-H]^-$. Moreover, one product ion at *m*/*z* 267.8 $[283-CH_3]^-$ was detected by two-level splitting MS of the reactant ion at *m*/*z* 283. Based on these findings, the structure of **P1** was identified as 7,3′-dihydroxy-4′-methoxyisoflavone-7-*O*-glucuronide ($C_{22}H_{20}O_{11}$, molecular weight [MW] 460), which was inferred to originate from the glucuronic acid metabolite [11] of 7,3′-dihydroxy-4′-methoxyisoflavone (calycosin; MW 284; present in Huangqi [12]) or other compounds with related structures in vivo (for example, by glucuronidation in phase II metabolism).

P2 showed a $[M-H]^-$ ion at *m*/*z* 443.5 (Fig. 12), two one-level splitting product ions at *m*/*z* 267.0 $[M-175-2H]^-$ and 174.8 $[Glc\ A-H_2O-H]^-$, and one two-level splitting ion at *m*/*z* 251.8 $[267-CH_3]^-$. Based on these data, the structure

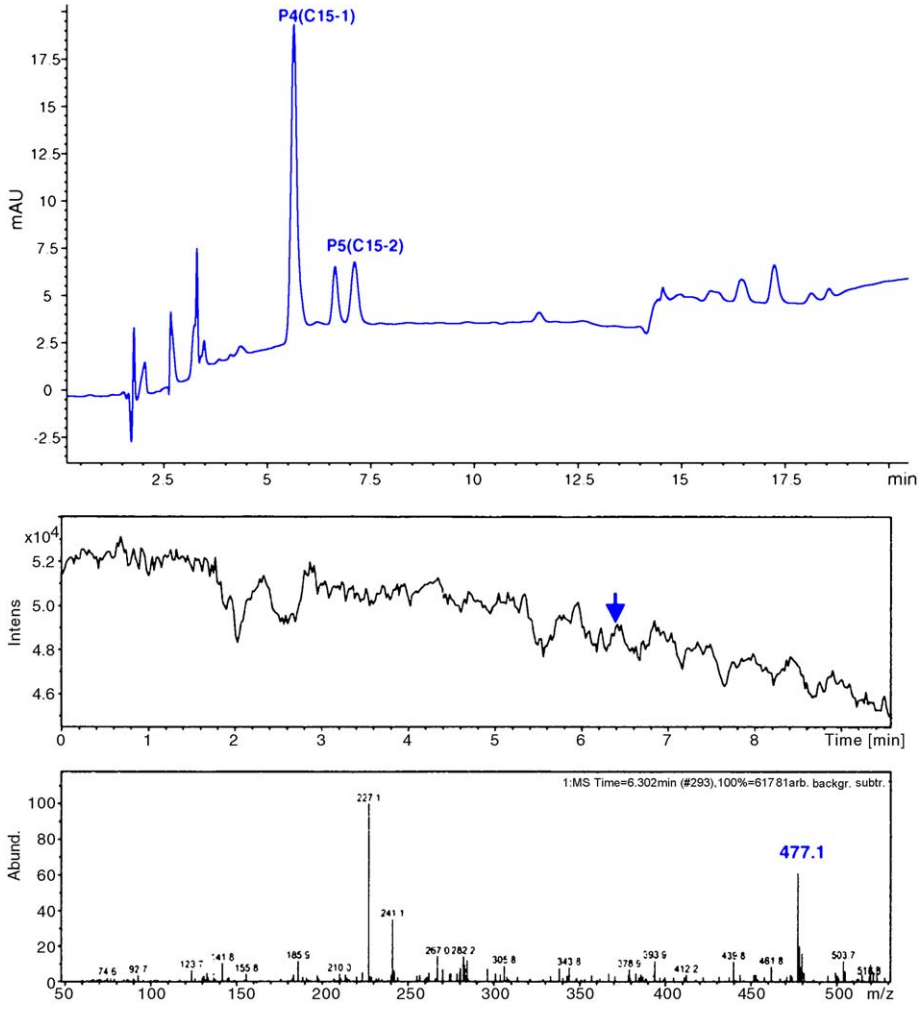

Fig. 15. HPLC (upper) and on-line total ion chromatogram (middle) of subfraction C15, the spectrum of $[M-H]^-$ ion at *m*/*z* 477.1 (lower) of **P5** by LC–MS. Mobile phase, 15% acetonitrile; flow rate, 1 ml/min; detection wavelength, 210 nm.

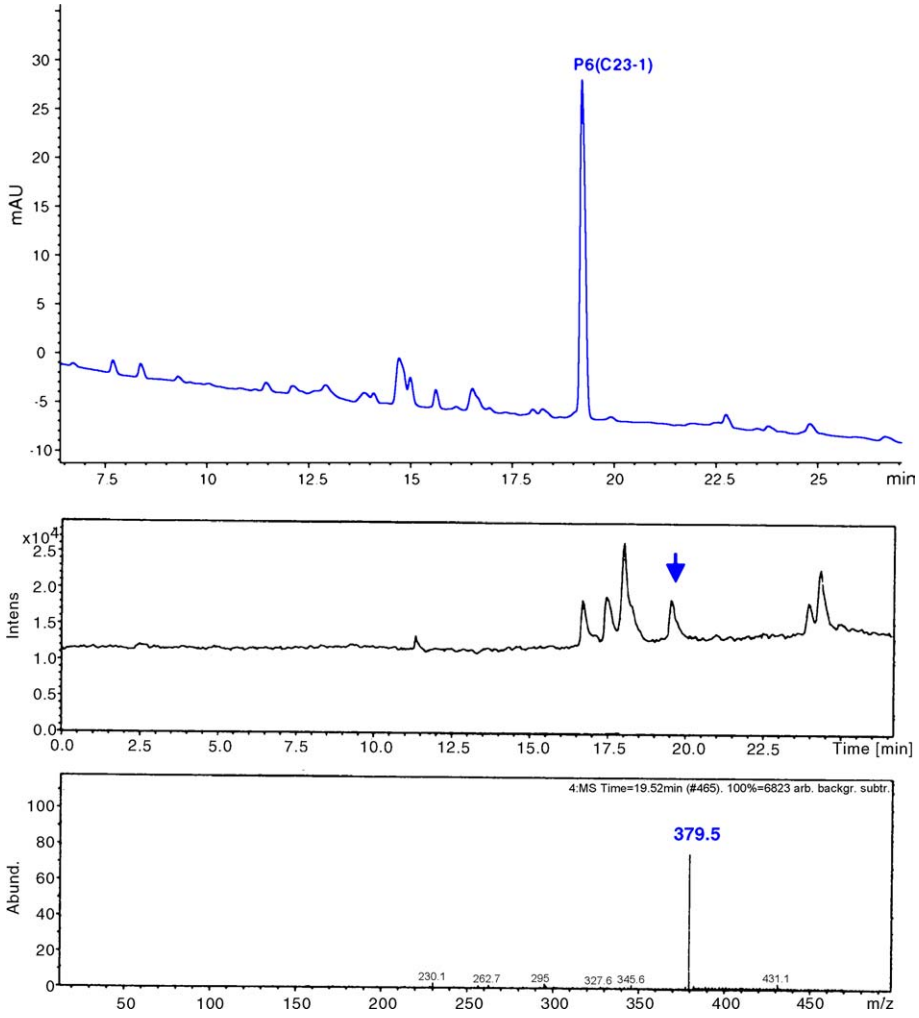

Fig. 16. HPLC (upper) and on-line total ion chromatogram (middle) of subfraction C23, the spectrum of $[M-H]^-$ ion at *m/z* 379.5 (lower) of **P6** by LC–MS. Mobile phase, 5% acetonitrile to 30% acetonitrile in a linear gradient (0–15 min) and then keep 30% acetonitrile for 15 min; flow rate, 1 ml/min; detection wavelength, 210 nm.

Table 2
ESI–MS^n values of **P1**–**P9** and characteristic HPLC peaks detected in the serum of pigs administrated BYHWD orally

Peak no.	$[M-H]^-/[M+H]^+$ (*m/z*)	Fragment ions (*m/z*)	Identified chemical structures
P1	481.5 $[M+Na-H]^-$ 460.9 $[M-H]^-$	460 → 283.0, 174.7 283 → 267.8	7,3′-Dihydroxy-4′-methoxyisoflavone-7-*O*-glucuronide
P2	443.5 $[M-H]^-$	443 → 267.0, 174.8 267 → 251.8	7-Hydroxy-4′-methoxyisoflavone-7-*O*-glucuronide
P3	368.0 $[M-H]^-$	367 → 349.1, 286.9 287 → 271.9, 254.9, 176.8, 164.8, 146.9, 134.9, 120.9, 108.9	7,2′,4′-Trihydroxy-3′-methoxyisoflavane-7-*O*-sulphate
P4	475.1 $[M-H]^-$	475 → 457.2, 298.9, 283.8, 174.6	3-Hydroxy-9,10-dimethoxypterocarpan-3-*O*-glucuronide
P5	477.1 $[M-H]^-$	477 → 300.9, 174.6 301 → 285.9, 270.8, 178.8, 146.8, 134.8, 108.9	7,2′-Dihydroxy-3′,4′-dimethoxyisoflavane-7-*O*-glucuronide
P6	379.5 $[M-H]^-$	379 → 299.0 299 → 283.9, 268.8 284 → 268.8 269 → 240.8	3-Hydroxy-9,10-dimethoxypterocarpane-3-*O*-sulphate
P7 or **P8**	146.0 $[M+H]^+$ 168.1 $[M+Na]^+$	146 → 129.0, 118.4, 109.0, 92.3, 73.9, 69.6	4-Hydroxyquinoline
P8 or **P7**	146.1 $[M+H]^+$ 167.9 $[M+Na]^+$	146 → 130.2, 117.2, 91.2, 84.0, 77.0, 73.0, 52.0	4 (1H)-quinolinone
P9	281.3 $[M-H]^-$	281 → 141.2, 127.2, 113.2, 111.0, 97.0, 95.2, 82.8, 81.0, 70.8	9-Octadecenoic acid (oleic acid)

of **P2** was identified as 7-hydroxy-4′-methoxyisoflavone-7-*O*-glucuronide ($C_{22}H_{20}O_{10}$; MW 444), which was inferred to originate from the glucuronic acid metabolite of 7-hydroxy-4′-methoxyisoflavone (formononetin; MW 268; present in Huangqi [12]) or related structures in vivo (for example, by glucuronidation in phase II metabolism).

3.2. Identification of the structures of *P3–P6*

P3–**P6** had similar UV absorptions, with maxima at 205, 220 (sh) and 280 nm (Fig. 9), which were typical of flavonoids.

P3 (subfraction B21, peak no. B21-1) showed a $[M-H]^-$ ion at *m/z* 368.0 (Fig. 13), and two product ions at *m/z* 286.9 $[M-SO_3H-H]^-$ and 349.1 $[M-OH-H]^-$, which implied that substitutes of $-SO_3H$ and $-OH$ existed in the structure of **P3**. Furthermore, the two-level splitting product ions at *m/z* 271.9 $[287-CH_3]^-$, 254.9 $[287-OH]^-$, 176.8, 164.8, 146.9, 134.9, 120.9 and 108.9 were detected from the reactant ion at *m/z* 287, which was similar to that of 7,2′-dihydroxy-3′,4′-dimethoxyisoflavane-7-*O*-β-D-glucoside (MW 464) present in Huangqi [13]. Therefore, **P3** was predicted to have a similar aglycon unit to 7,2′-dihydroxy-3′,4′-dimethoxyisoflavane-7-*O*-β-D-glucoside. The MW of the aglycon of **P3** was 288 $[M-SO_3]^-$, which was 14u smaller than that of 7,2′-dihydroxy-3′,4′-dimethoxyisoflavane (MW 302). **P3** was thus predicted to have three hydroxyl and one methoxyl substitutes, which differed from 7,2′-dihydroxy-3′,4′-dimethoxyisoflavane (which has two hydroxyl and two methoxyl substitutes). Based on these data, the structure of **P3** was tentatively determined as 7,2′,4′-trihydroxy-3′-methoxyisoflavane-7-*O*-sulphate ($C_{16}H_{16}O_8S$; MW 368), which might have originated from the metabolite of 7,2′-dihydroxy-3′,4′-dimethoxyisoflavane or related structures conjugated with sulphate in vivo (for example, by sulphation in phase II metabolism).

P4 (subfraction C15, peak no. C15-1) showed a $[M-H]^-$ ion at *m/z* 475.1 (Fig. 14), four one-level splitting product ions at *m/z* 457.2 $[M-H_2O]^-$, 298.9 $[M-175-H]^-$, 283.8 $[M-175-CH_3-H]^-$ and 174.6 $[Glc\ A-H_2O-H]^-$. The structure of **P4** was therefore identified as 3-hydroxy-9,10-dimethoxy-pterocarpan-3-*O*-glucuronide ($C_{23}H_{24}O_{11}$; MW 476), which was the glucuronic acid metabolite of 3-hydroxyl-9,10-dimethoxy-pterocarpane-3-*O*-glucoside (MW 462; present

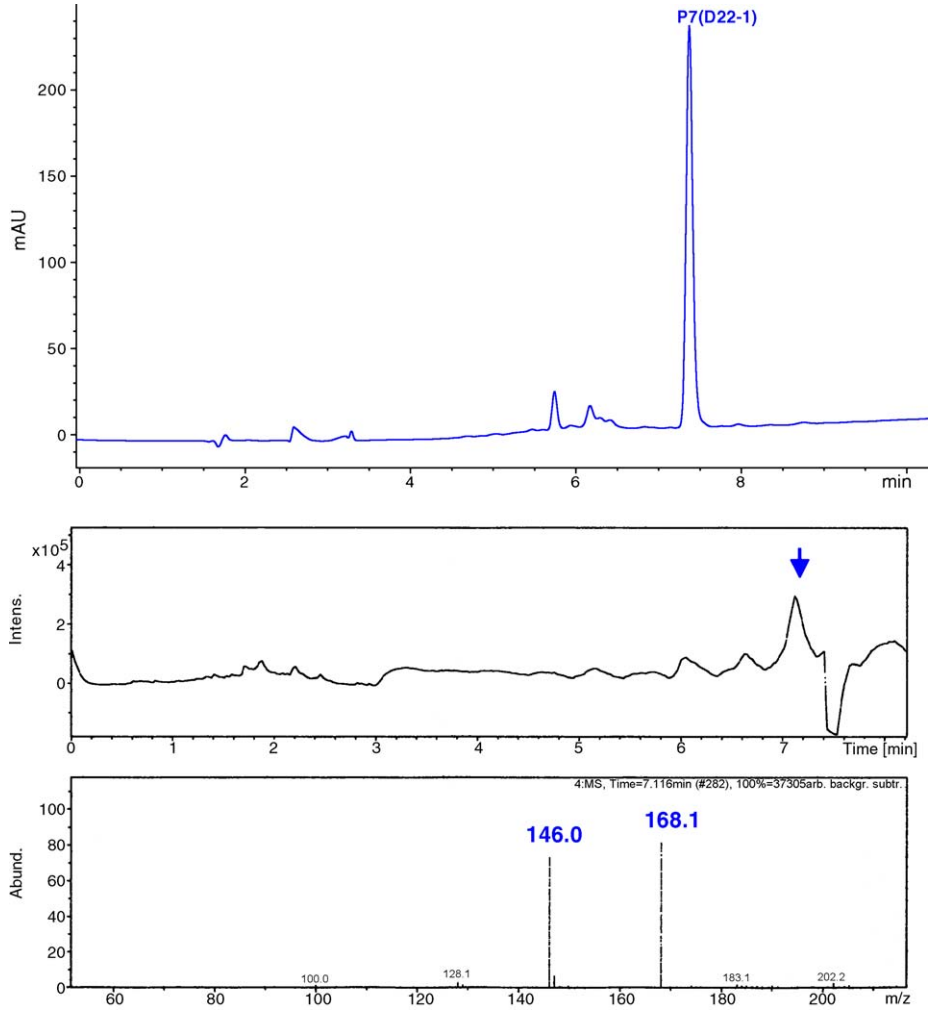

Fig. 17. HPLC (upper) and on-line total ion chromatogram (middle) of subfraction D22, the spectrum of $[M+H]^+$ ion at *m/z* 146.0 and $[M+Na]^+$ ion at *m/z* 168.1 (lower) of **P7** by LC–MS. Mobile phase, 20% acetonitrile to 100% acetonitrile in a linear gradient (0–20 min); flow rate, 1 ml/min; detection wavelength, 210 nm.

in Huangqi [14]) or related structures in vivo, both having the same aglycon unit (MW 299).

P5 (subfraction C15, peak no. C15-2) showed a $[M-H]^-$ ion at *m/z* 477.1 (Fig. 15), two one-level prominent product ions at *m/z* 300.9 $[M-175-2H]^-$ and 174.6 [Glc A $-H_2O-H]^-$, and six two-level splitting product ions at *m/z* 285.9 $[301-CH_3]^-$, 270.8 $[301-2CH_3]^-$, 178.8, 146.8, 134.8 and 108.9 from the reactant ion at *m/z* 301. The structure of **P5** was therefore identified as 7,2′-dihydroxy-3′,4′-dimethoxyisoflavane-7-*O*-glucuronide($C_{23}H_{26}O_{11}$; MW 478), which was the glucuronic acid metabolite of 7,2′-dihydroxy-3′,4′-dimethoxyisoflavane-7-*O*-glucoside (present in Huangqi [13]) or related structures in vivo, both having the same aglycon unit (MW 301).

P6 (subfraction C23, peak no. C23-1) showed a $[M-H]^-$ ion at *m/z* 379.5 (Fig. 16) and a product ion at *m/z* 299.0 $[M-SO_3H-H]^-$. Furthermore, two-level fragment ions at *m/z* 283.9 $[299-CH_3]^-$ and 268.8 $[299-2CH_3]^-$, three-level fragment ions at *m/z* 268.8 $[284-CH_3]^-$, and four-level fragment ions at *m/z* 240.8 $[269-H_2O]^-$ were also detected. It was thus easy to infer that **P6** originated from the metabolite of 3-hydroxy-9,10-dimethoxypterocarpane-3-*O*-β-D-glucoside present in Huangqi [14], both having same aglycon unit (MW 299), and the structure of **P6** was identified as 3-hydroxy-9,10-dimethoxypterocarpane-3-*O*-sulphate ($C_{17}H_{16}O_8S$; MW 380).

3.3. Identification of the structures of *P7 and P8*

P7 (subfraction D22, peak no. D22-1) and **P8** (subfraction D24, peak no. D24-1) had similar $[M+H]^+$ and $[M+Na]^+$ ions at *m/z* 146 and 168 (Figs. 17 and 18). The UV spectrum of **P7** showed maximum wavelength of absorption (λ_{max}) values (nm) of 200, 215, 230, 260 and 325 (Fig. 9). The UV spectrum of **P8** showed λ_{max} values (nm) of 210, 245, 255 and 300 (Fig. 9). Based on the results of time of flight (TOF)–MS, the precise ion $[M+H]^+$ mass of **P7** was 146.0635, and that of **P8** was 146.0615. Therefore, the protonated molecular formulae of **P7** and **P8** were predicted to be C_9H_8ON (MW 146.0600) or $C_3H_8O_2N_5$ (MW 146.0673). One-level splitting product ions of **P7** were observed at *m/z* 129.0, 118.4, 109.0, 92.3, 73.9 and 69.6, and one-level splitting product ions of **P8** were detected at *m/z* 130.2, 117.2, 91.2, 84.0, 77.0, 73.0 and 52.0. Based on these data, a phenyl substitute was predicted to exist in the structures of **P7** and **P8**. Therefore, only C_9H_8ON was suitable for the

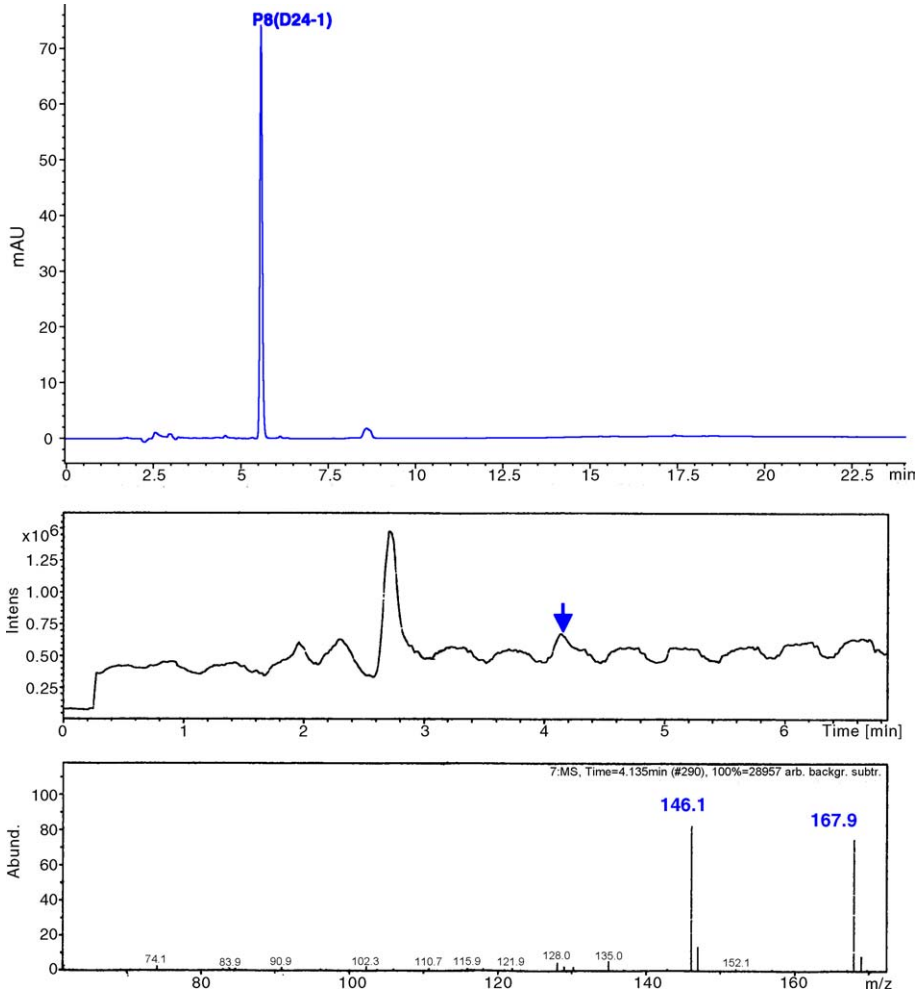

Fig. 18. HPLC (upper) and on-line total ion chromatogram (middle) of subfraction D24, the spectrum of $[M+H]^+$ ion at *m/z* 146.1 and $[M+Na]^+$ ion at *m/z* 167.9 (lower) of **P8** by LC–MS. Mobile phase, 35% acetonitrile to 100% acetonitrile in a linear gradient (0–15 min); flow rate, 1 ml/min; detection wavelength, 300 nm.

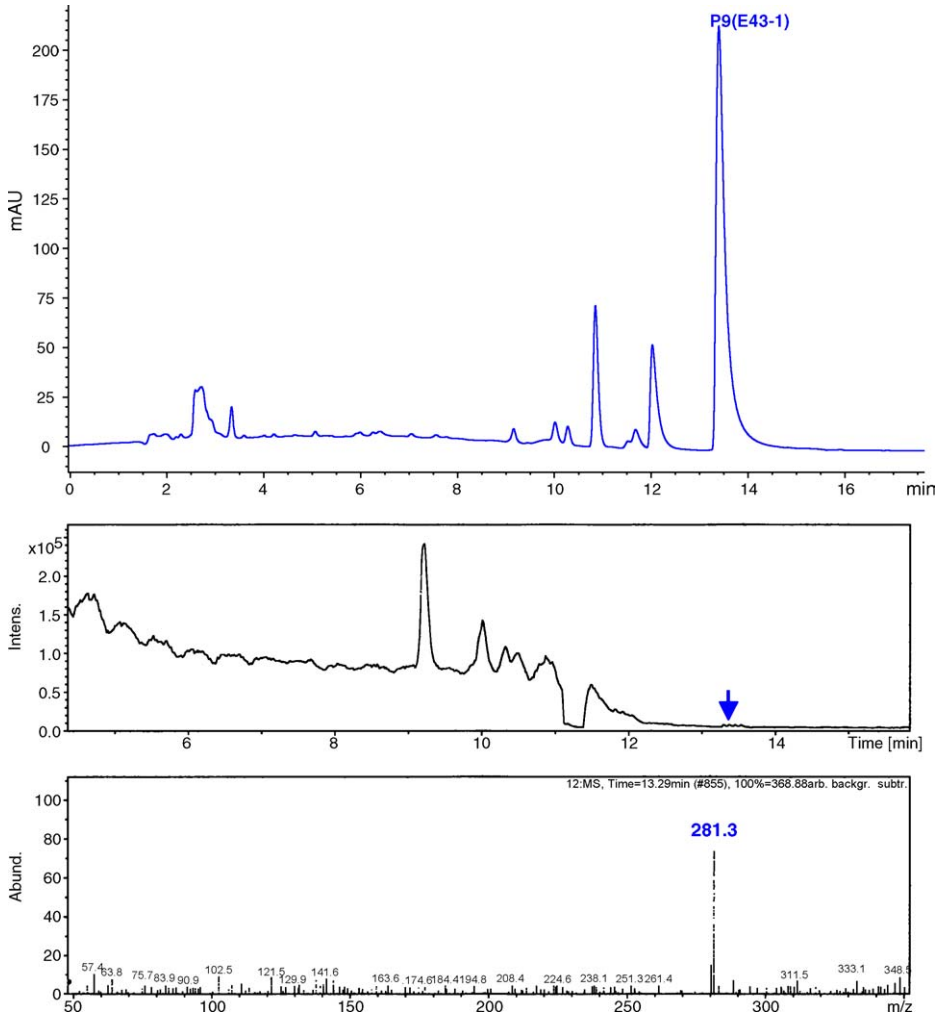

Fig. 19. HPLC (upper) and on-line total ion chromatogram (middle) of subfraction E43, the spectrum of $[M-H]^-$ ion at *m/z* 281.3 (lower) of **P9** by LC–MS. Mobile phase, 80% acetonitrile to 100% acetonitrile in a linear gradient (0–8 min) and then keep 100% acetonitrile for 15 min; flow rate, 1 ml/min; detection wavelength, 210 nm.

protonated molecular formulae of **P7** and **P8**, which were alkaloid containing phenyls. According to the published literature [15], 3-methoxy-1-methyl-4(1H)-quinolinone ($C_{11}H_{11}O_2N$) is present in Chuanxiong, and has more C_2H_4O unit than **P7** and **P8**. Therefore, the structures of **P7** and **P8** were tentatively predicted to be 4(1H)-quinolinone (**P7** or **P8**; C_9H_7ON; MW 145) and 4-hydroxyquinoline (**P8** or **P7**; C_9H_7ON; MW 145), which might originate from the metabolite of 3-methoxy-1-methyl-4(1H)-quinolinone in vivo.

*3.4. Identification of the structure of **P9***

P9 (subfraction E43, peak no. E43-1) showed a $[M-H]^-$ ion at *m/z* 281.3 (Fig. 19). The UV maximum absorption of **P9** was observed at 200 nm (Fig. 9). According to the published literature [16,17], 9-octadecenoic (oleic) acid ($C_{18}H_{34}O_2$; MW 282) is present in Danggui and Honghua. One-level splitting MS fragments of **P9** were observed at 141.2 $[CH_2=CH-(CH_2)_5-COO]^-$, 127.2 $[CH_2=CH-(CH_2)_4-COO]^-$, 113.2 $[CH_2=CH-(CH_2)_3-COO]^-$, 111.0 $[CH_3-(CH_2)_5-CH=CH]^-$, 97.0 $[CH_3-(CH_2)_4-CH=CH]^-$, 95.2 $[CH_2=CH-CH_2-CH=CH-CO]^-$, 82.8 $[CH_3-(CH_2)_3-CH=CH]^-$, 81.0 $[CH_2=CH-CH=CH-CO]^-$ and 70.8 $[CH_2=CH-COO]^-$, which were identical to those of $CH_3(CH_2)_7CH=CH(CH_2)_7COOH$. Therefore, the structure of **P9** was identified as 9-octadecenoic acid (oleic acid).

4. Discussion

Using combined HPLC–DAD–MS techniques, 45 characteristic HPLC peaks were detected in serum containing drug (that is, serum from pigs administrated BYHWD orally), and the structures of nine (**P1**–**P9**) of these peaks were identified. **P1**–**P8** were phase II metabolites in vivo. **P1**, **P2**, **P4** and **P5** were metabolites of isoflavonoids and isoflavanoids (present in Huangqi) conjugated with glucuronic acid. **P3** and **P6** were metabolites of isoflavanoids (present in Huangqi) conjugated with sulphate. **P7** and **P8** were alkaloids, which might have originated from metabolites of alkaloids present in Chuanxiong. **P9** was oleic acid, an original constituent present in Danggui and Honghua. In order to assign the substitution positions of methoxyl, hydroxyl, sulphate, glucuronide and others in **P1**–**P8**

correctly, additional information (for example, nuclear magnetic resonance spectra) will be required. Therefore, the chemical structures of **P1**–**P9** determined in this paper are only tentative.

We found that the majority of the compounds identified were isoflavonoids and isoflavanoids. There are two possible reasons for this result: first, the isoflavonoids and isoflavanoids in BYHWD were easily absorbed in vivo; and second, the structures of isoflavonoids and isoflavanoids were suitable for UV detection. Whether the other structural types present in BYHWD, such as saponins, were also absorbed in vivo warrants further investigation.

Pharmacological studies by our research group have shown that 7-hydroxy-9,10-dimethoxy-pterocarpane-7-*O*-β-D-glucoside, calycosin, formononetin and calycosin-7-*O*-β-D-glucoside are the active constituents of BYHWD in treating cerebrovascular illnesses. Moreover, all of these constituents can increase the fluidity of the brain cell membrane in ischemia-reperfusing rats to achieve normal or nearly normal levels [18].

To clarify the mechanisms of BYHWD in treating cerebrovascular illnesses, it is first necessary to study the active constituents. According to our results, we believe that a valid approach is to study the main active chemical constituents of CTCMs from serum containing drug using modern analytical technologies.

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