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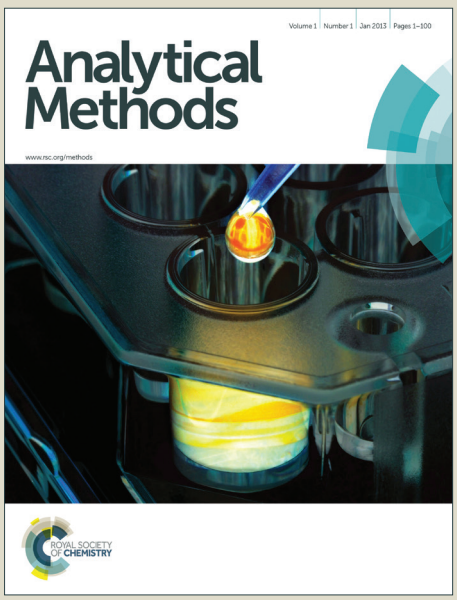

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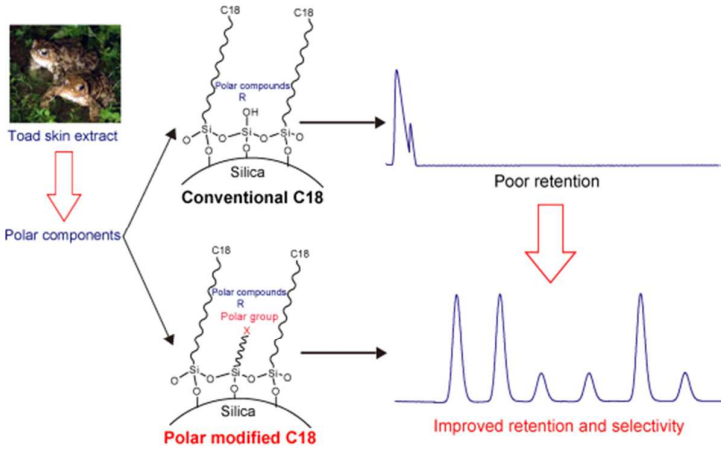

A novel method based on the polar-copolymerized C18 stationary phase was developed to separated polar compounds from toad skin.

Efficient purification of low molecular weight nitrogen polar compounds from skin of *Bufo bufo gargarizans* Cantor by reversed-phase high performance liquid chromatography with a polar-copolymerized C18 stationary phase

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Abstract

Cinobufacini (Huachansu) injections have been widely used in the treatment of various cancers in clinical application in China. As an aqueous extract of the skin of *Bufo bufo gargarizans* Cantor (toad skin), cinobufacini contains many polar compounds and the chemical profile of polar compounds was still unclear, which increase the risk of pharmacy safety. However, there are very few studies on the separation and purification of polar components from toad skin, especially in preparative scale. In this paper, a unique method based on a homemade polar-modified C18 stationary phase was developed and successfully applied to separate polar compounds from the toad skin. Compared with previous purification systems based on the C18 stationary phase, the poor retention problem of polar compounds was well resolved through use of the polar-modified C18 column and good resolutions were achieved as well. Seven compounds were purified and five of them (uracil, hypoxanthine, 3-hydroxy-4H-pyrazolo[4,5-d]pyridazin-7(1H)-one, thymine, bufothionine) were identified by MS, ^{1}H NMR and ^{13}C NMR. Additionally, 3-hydroxy-4H-pyrazolo[4,5-d]pyridazin-7(1H)-one was identified as a new compound and hypoxanthine was found from the skin of *Bufo bufo gargarizans* Cantor for the first time. These results indicate that the method show promise for the separation of polar compounds in toad skin.

1. Introduction

The skin of the *Bufo bufo gargarizans* Cantor (toad skin), a well-known traditional Chinese medicine (TCM), has been used in the treatment of various health problems, such as swelling, pain, heart failure, and various cancers in China[1-3]. Several methods have been developed mainly for the separation and purification of weak polar compounds (bufadienolides) from toad skin, including thin layer chromatography (TLC)[4-5], liquid chromatography (LC)[6-8], and preparative high performance liquid chromatography (prep-HPLC)[9-10]. More than 100 bufadienolides have been found from toad skin and its preparations[4-10]. However, the purification of

polar compounds remains lacking.

The water-soluble extract of toad skin, named cinobufacini (Huachansu) injection, has been widely used in the treatment several cancers in China for many years[11-13]. Some studies have suggested that, as an aqueous extract, alkaloids should be the major type of components in cinobufacini injection. But, till now, the chemical profile of polar compounds in this injection was still unclear, which increase the risk of pharmacy safety[14]. Recently, several analytical methods have been developed to obtain qualitative and quantitative information of cinobufacini injection[14-15]. However, the polar compounds of cinobufacini have been mainly investigated by high-performance liquid chromatography (HPLC)-diode array detection and HPLC-triple quadrupole mass spectrometry in analytical scale. To the best of our knowledge, there are very few reports[16] that have focused on the purification of polar compounds in the preparative scale, and only about ten polar compounds have been purified from toad skin due to the lack of separation materials and scarcity of proper methods. Therefore, it is urgently needed to develop new separation materials and proper separation methods for the purification of polar compounds of toad skin.

Reversed-phase liquid chromatography (RPLC) has been widely utilized in many fields for the separation of medium and weak polar compounds due to its high separation efficiency[17-18]. But, in traditional RPLC, it is still challenging to separate polar compounds (e.g., highly polar natural products, contained in many drug components and biological samples) due to their weaker retention on the C18 stationary phase[19-20]. Also, normal-phase liquid chromatography (NPLC) has historically been utilized for the separations of polar compounds from TCMs using nonaqueous mobile phases with silica. Nevertheless, the application of NPLC for the separation of polar compounds has been very limited because of the drawbacks associated with the poor solubility, irreversible adsorption, and poor reproducibility[19]. Additionally, hydrophilic interaction liquid chromatography (HILIC) has been proved to be a useful technique for separation of polar compounds[19-24]. However, HILIC might not be suitable for the purification of polar compounds from complex sample matrices mainly due to their insufficient solubility in highly organic solvents and their

limited separation resolution in the preparative scale.

Besides the above mentioned methods, development of polar-modified C18 stationary phase (such as polar embedded or polar end-capped C18 stationary phase) is an alternative approach to separate polar compounds[25]. With the introduction of polar group, the polar-modified C18 stationary phase display more symmetrical peaks, alternative selectivity and stable retention in highly aqueous which also enhance the retention of polar compounds[26-27] Recently, based on the approach of horizontal polymerization on silica surface a polar-modified C18 stationary phase (C18HCE) was prepared in our laboratory[28]. As demonstration, the C18HCE column exhibited 100% aqueous mobile phase compatibility, suggesting that this material may have a great potential for the separation of polar compounds.

In the present study, based on this polar-modified C18 column, the poor retention problem of polar compounds on conventional C18 was successfully resolved and good resolution was obtained. Thus, seven polar compounds were successfully purified with high purity on the semi-preparative C18HCE column by preparative HPLC. Additionally, 3-hydroxy-4H-pyrazolo[4,5-d]pyridazin-7(1H)-one was identified as a new compound and hypoxanthine was found from the skin of *Bufo bufo gargarizans* Cantor for the first time. These results indicate the homemade polar-modified C18 show promise for the separation and purification of polar compounds in complex samples.

2. Materials and methods

2.1. Apparatus

An industrial HPLC system was assembled for the preparation of polar fraction from the crude sample in our laboratory. This system consists of a binary gradient module (P6000), a UV detector (UV6000) and a sample injector (A1359). Data were collected using a CXTH-3000 chromatography workstation.

A Waters Purification Factory was used for further purification and consisted of two 2525 binary gradient module (Waters, Milford, MA), an autosampler (Leap

Technologies, Carrboro, NC), and a 2498 UV detector (Waters). Data were recorded by MassLynx software (Waters, V. 4.1).

The analytical experiments were performed on an Alliance HPLC system consisting of a Waters 2695 HPLC pump and a Waters 2489 UV detector. The temperature was maintained at 30 ^{o}C for analytical separation. Data were collected by Waters Empower software (Milford, MA, USA).

The HPLC system was interfaced with a ZQ single quadrupole mass spectrometer from Waters Corporation. The main parameters of MS were as follows: capillary voltage was 3.0 kV; cone voltage was 25.00 V; source temperature was 120 ^{o}C; desolvation temperature was 320 ^{o}C; desolvation gas flow rate was 700 L/h; and cone gas flow rate was 50 L/h. The mass spectrometer was equipped with an ESI ion source and controlled by MassLynx version 4.0 (Holliston, MA, USA).

Identification of pure compounds was carried out using NMR. ^{1}H NMR spectra and ^{13}C NMR spectra were measured on a Bruker Ar III-600 spectrometer (^{1}H NMR at 600 MHz; ^{13}C NMR at 150 MHz) with DMSO-d_6 as solvent.

2.2. Reagents

Industrial grade ethanol and analytical grade methanol were purchased from Yuwang (Shandong, China). HPLC grade acetonitrile was purchased from Merck (Darmstadt, Germany). Trifluoroacetic acid (HPLC grade) was obtained from Acros (Cambridge, USA). Water for Alliance HPLC system was prepared by a Milli-Q system (Billerica, MA, USA). All reagents used in analysis were HPLC grade. The XUnion C18 column (250 mm × 4.6 mm, I. D. , 10 μm), XUnion C18 column (150 mm × 4.6 mm, I. D. , 5 μm), XCharge C18PN column (150 mm × 4.6 mm, I. D. , 5 μm) and XAqua C18 column (150 mm × 4.6 mm, I. D. , 5 μm), were purchased from Acchrom Techeologies Co. , Ltd. (Beijing, China). The Xterra MS C18 column (100 mm × 4.6 mm, I. D. , 5 μm), Symmetry C18 column (150 mm × 4.6 mm, I. D. , 5 μm) were purchased from Waters (Massachusetts, USA). C18HCE column (150 mm × 4.6

mm, I. D. , 5 μm) and C18HCE column (250 mm × 20 mm, I. D. , 5 μm) were prepared by our laboratory.

2.3. Preparation of the sample

Dried skin of the toad *Bufo bufo gargarizans* Cantor was collected from Shandong Province, and authenticated by the Institute of Medication, Xiyuan Hospital of China Academy of Traditional Chinese Medicine. The dried skin of *Bufo bufo gargarizans* Cantor (2 kg) was cut up into pieces, and then extracted with water/ethanol (v/v 5:95, 3 × 20 L, 2 h each) at 78 ^{o}C. The combined decoctions were dried by rotary evaporation at 60 ^{o}C in vacuum. The extract (70 g) was dissolved in 1.6 L of water/ methanol (v/v 30:70), and then extracted by n-heptane (3 × 1.6 L each). The methanol fraction was combined and concentrated to dryness in vacuo, which yielded 40 g of residue, and 20 g of residue was dissolved in 45 mL methanol at a concentration of 440 mg/mL and filtered through 0.45 μm membrane filter as the sample for preparation.

2.4. Polar fraction preparation

In this study, a preparative XUnion C18 column (250 mm × 100 mm, 10 μm) was adopted in the preparative HPLC system at a flow rate of 330 mL/min and the detection wavelength was 300 nm. The mobile phases used were water (A) and methanol (B_1) and gradient condition was: 0-7 min, 25-40 B_1%; 7-60 min, 40-65 B_1%; 60-65 min, 65-100 B_1%; 70-75 min, 100-100 B_1%. In this system, about 18 g extract of toad skin was fractionated in 1 injection and 22 fractions (labeled as F1–F22) were collected. The fraction 1 (F1) was eluted near at the dead time and was selected as polar compounds for further separation.

2.5. Chromatographic conditions in analysis of F1

Initially, the F1 was injected on the XUnion C18 (250 mm × 4.6 mm, I. D., 10

μm) column using an analytical HPLC system with the same gradient condition in Section 2.4 (0-7 min, 25-40 B_1%; 7-60 min, 40-65 B_1%; 60-65 min, 65-100 B_1%; 70-75 min, 100-100 B_1%.) and the flow rate was 0.7 mL/min (shown in Fig. 1).

Secondly, three conventional C18 columns, XUnion C18 column (150 mm × 4.6 mm, I. D. , 5 μm), XTerra MS C18 column (150 mm × 4.6 mm, I. D. , 5 μm), Symmetry C18 column (150 mm × 4.6 mm, I. D. , 5 μm) with a weaker elution condition were used. The mobile phases were 0.1% trifluoroacetic acid aqueous solution (A) and 0.1% trifluoroacetic acid acetonitrile (B_2); the flow rate was 1.0 mL/min; the gradient condition was 0-30 min, 5-10 B_2%; the column temperature was maintained at 30 ^{o}C; the detective wavelength was 230 nm; the flow rate was 1.0 mL/min.

Finally, another three polar-modified columns: C18HCE column (150 mm × 4.6 mm, I. D. , 5 μm), XAqua C18 column (150 mm × 4.6 mm, I. D. , 5 μm) and XCharge C18PN column (150 mm × 4.6 mm, I. D. , 5 μm) were tried. The mobile phases were 0.1% trifluoroacetic acid aqueous solution (A) and 0.1% trifluoroacetic acid acetonitrile (B_2). The gradient condition was 0-30 min, 0-10 B_2%. The column temperature was maintained at 30 ^{o}C, the detective wavelength was 230 nm and the flow rate was 1.0 mL/min.

2.6. Polar compounds isolation procedure

The isolation of polar compounds was performed on the semi-preparative HPLC system with the C18HCE column (250 mm × 20 mm, I. D., 5 μm). The mobile phases were 0.1% trifluoroacetic acid aqueous solution (A) and 0.1% trifluoroacetic acid acetonitrile (B_2). The gradient condition was 0-30 min, 0-10 B_2%; 30-40 min, 10-10 B_2%; the detective wavelength was 230 nm and the flow rate was 18 mL/min. F1 (500 mg) was eluted with the above gradient program for 3 times to afford compounds **P1**-**P7**.

3. Results and discussion

3.1. Preparation of polar components

As a biological sample, toad skin contains large numbers of compounds covering a wide range of polarity and molecular weight. To simplify the crude sample, a conventional C18 column was used for the rough separation mainly according to their differences in hydrophobicity. Although the C18 is not suitable for the separation of polar compounds due to their poor retention, we can take advantage of the hydrophobic property of C18 to cluster the polar compounds. In present study, the fraction 1 (F1, as shown in Fig. 1A, corresponding time range was 2.7 min to 5.8 min), which was eluted nearly at dead time on the C18 column, was selected as the polar components for further purification. In addition, to further test the reliability of the preparation of F1, with the same gradient condition in Section 2.4, the F1 was injected on an analytical XUnion C18 (250 mm × 4.6 mm, I. D., 10 μm) column using an analytical HPLC system (shown in Fig. 1B). By compared with Fig. 1A and Fig. 1B, the consistency in retention times between the analytical scale and the preparative scale indicated that the pre-fractionation strategy by prep-HPLC is reproducible and reliable. According to the above results, we may preliminarily deduce that the F1 is rich in polar components.

3.2. Optimizing the purification method for polar fraction using C18 columns

As displayed in Fig. 1B, although only one main peak was observed, it may still contain many polar compounds which were co-eluted near the void volume of the column. In this elution condition, purification of polar compounds may be impossible due to their poor retentions on the C18 column. Thus, with three conventional C18 columns, mild elution conditions were tried to enhance the retentions of polar components. As displayed in Fig. 2A, Fig. 2B and Fig. 2C, the retentions of polar compounds were enhanced, and more peaks were separated from the cluster peaks (Fig. 1). Unfortunately, the poor retentions and the inadequate separation resolutions of polar compounds also suggested that the conventional C18 stationary phase is not

suitable for the separation of polar compounds even when using a mild elution condition.

As demonstrated, the polar-modified C18 stationary phase is an alternative approach to separate polar compounds. On the one hand, the introduced polar group increases the polar interaction between the solutes and absorbents; on the other hand, the polar-modified C18 stationary phase exhibits 100% aqueous compatibility, which allows use of all the aqueous mobile phase. To enhance the retentions of polar compounds, with milder elution conditions, three polar-modified C18 stationary phases were introduced for the separation of polar compounds. Compared to the conventional C18 stationary phases, the retentions of polar compounds were enhanced significantly on the columns by introduction of polar groups, which increased polar interactions between the solutes and absorbents. Additionally, the polar-modified C18 columns (Fig. 2D, Fig. 2E and Fig. 2F) have higher resolution and better selectivity than those of conventional C18 columns (Fig. 2A, Fig. 2B and Fig. 2C). Among the polar-modified C18 columns, the XAqua C18 column (Fig. 2E) and the C18HCE column (Fig. 2F) showed better resolution than the XCharge C18PN column (Fig. 2D). Moreover, the peaks were more evenly distributed on the C18HCE column, which could benefit separation, especially in preparative scale. In addition, when the loading amount was increased ten-fold (injection volume: from 10 μL to 100 μL, 2 mg), we found that the resolutions (Fig. 3B) were as good as those of Fig. 2F on the C18HCE column, while on the XAqua C18 column, the last two peaks overlapped each other (Fig. 3A). Based on the discussion mentioned above, the separation of polar components on homemade C18HCE showed better retentions and resolutions. These better phenomena on the column could be ascribed to the characterization of polar-modified alkyl stationary phase. As demonstrated in our previous study[28], the C18HCE stationary phase (C18-C3Cl) exhibited 100% aqueous mobile phase compatibility and low silanol activity. Moreover, by compared with conventional C18, with the introduction of 3-chloropropyl trichlorosilane, C18HCE showed different selectivity in the separation of polar components. Therefore, the preparative C18HCE column was employed for separation of polar compounds in the following purification

process.

3.3. Purification of polar fraction

To further validate the practicality of this method, the preparative separation of F1 was performed. A semi-preparative C18HCE column (250 mm × 20 mm, 5 μm) and a suitable HPLC condition (Section 2.6) were employed to increase the preparative resolution. As shown in Fig. 3, good separation of most compounds from fraction F1 was achieved. Three injections of F1 (500 mg) were separated (Fig. 4A, Fig. 4B and Fig. 4C) and satisfactory reproducibility of different injections was obtained in the preparative chromatography. Peaks eluted in the same retention time for different injections were combined and dried by rotary evaporation at 40 ^{o}C in vacuum. Ultimately, seven compounds with high-purity were obtained.

3.4. Identification of polar compounds

As demonstrated in Section 3.3, seven compounds were purified and five of them were identified by ^{1}H NMR, ^{13}C NMR, DEPT-135 and 2D-NMR. As shown in Fig. 5, seven purified compounds of F1 were tested on the analytical C18HCE column with the same HPLC condition (see Section 2.5). The five compounds (shown in Fig. 6) are identified as uracil (**P1**, 15.2 mg)[16, 29], hypoxanthine (**P2**, 30.8 mg)[30], 3-hydroxy-4H-pyrazolo[4,5-d]pyridazin-7(1H)-one (**P3**, 6.5 mg), thymine (**P4**, 22.0 mg)[16, 29], and bufothionine (**P7**, 60.0 mg)[16, 31] respectively. Till now, the structure of **P5** (8.3 mg) and **P6** (35.8 mg) were not identified. However, **P5** was isolated as a white amorphous powder, positive ESI-MS m/z: 160.0 $[M+H]^{+}$, which suggested that it might be a low molecular weight nitrogen polar compound according to the nitrogen rule. **P6** was also isolated as a white amorphous powder, positive ESI-MS m/z: 202.9 $[M+H]^{+}$, which indicated that it could be a nitrogen compound with even number of nitrogen atoms according to previous report[14]. Additionally, 3-hydroxy-4H-pyrazolo[4,5-d]pyridazin-7(1H)-one was identified as a new compound and hypoxanthine was isolated for the first time from *Bufo bufo gargarizans* Cantor.

Identifications of five compounds were listed as follows:

Compound **1** (**P1**), the molecular formula: $C_4H_4N_2O_2$, positive ESI-MS m/z: 112.9 $[M+H]^+$; ^{1}H-NMR (600 MHz, DMSO-d_6): δ, 5.46 (1H, d, J = 7.6 Hz, H-1), 7.40 (1H, dd, J = 7.6 Hz, H-2), 10.8 (1H, s, H-3), 11.0 (1H, s, H-5); ^{13}C-NMR (150 MHz, DMSO-d_6): δ, 100.7 (C-5), 142.7 (C-6), 152.0 (C-2), 164.8 (C-4). The ^{1}H-NMR and ^{13}C-NMR spectral data were in agreement with those of uracil[16, 29].

Compound **2** (**P2**), the molecular formula: $C_5H_4N_4O$, positive ESI-MS m/z: 137.0 $[M+H]^+$; ^{1}H-NMR (600 MHz, DMSO-d_6): δ, 8.21 (1H, s, H-2), 8.97 (1H, s, H-8); ^{13}C-NMR (150 MHz, DMSO-d_6): δ, 147.8 (C-2), 150.2 (C-4), 117.2 (C-5) , 154.4 (C-6), 139.9 (C-8). The ^{1}H-NMR and ^{13}C-NMR spectral data were in agreement with those of hypoxanthine[30].

Compound **3** (**P3**) was isolated as a white amorphous powder. Its molecular formula was deduced to be $C_5H_4N_4O_2$ by HRESIMS [*m/z* 153.0409$[M+H]^+$, calcd for $C_5H_5N_4O_2$, 153.0409]. ^{1}H-NMR (600 MHz, DMSO-d_6): δ_H, 11.40 (1H, s, H-1), 10.96 (1H, s, H-4), 7.88 (1H, s, H-6) and 12.40 (1H, s, OH); ^{13}C-NMR (150 MHz, DMSO-d_6): δ_C, 153.4 (C-3), 145.9 (C-3a), 145.1 (C-6), 109.3 (C-6a) and 151.1 (C-7). The ^{13}C NMR and DEPT spectra furnished four quaternary carbons, one methine and all signals of compound **3** were ascribed completely on the basis of HSQC and HMBC experiments. In Long-range correlations (HMBC), correlations H-1 (δ_H, 11.40) with C-3 (δ_C, 153.4) and C-6a (δ, 109.3); H-4 (δ_H, 10.96) with C-3, (δ_C, 153.4), C-3a (δ_C, 145.9) and C-6a (δ_C, 109.3); H-6 (δ_H, 7.88) with, C-3a (δ_C, 145.9), C-6a (δ_C, 109.3) and C-7 (δ_C, 151.1) confirmed the skeleton of compound **3**. On the basis of these observations and other HMBC correlations (Fig. 6 and Table 1), the structure of **P3** was established and named 3-hydroxy-4H-pyrazolo[4,5-d]pyridazin-7(1H)-one.

Compound **4** (**P4**), the molecular formula: $C_5H_6N_2O_2$, positive ESI-MS m/z: 127.1 $[M+H]^+$; δ, 7.25 (1H, s, H-3), 10.59 (1H, s, H-4), 11.00 (1H, s, H-5), 1.73 (3H, s, CH); ^{13}C-NMR (150 MHz, DMSO-d_6): δ, 108.1 (C-5), 138.2 (C-6), 152.0 (C-2), 165.4 (C-4), 12.3 (CH_3). The ^{1}H-NMR and ^{13}C-NMR spectral data were in agreement with those of thymine[16, 29].

Compound **5** (**P7**), the molecular formula: $C_{12}H_{14}N_2O_4S$, positive ESI-MS m/z:

282.7 $[M+H]^+$; ^{1}H-NMR (600 MHz, DMSO-d_6): δ, 11.36 (1H, s, NH), 7.33 (1H, d, J = 11.2, 2-H), 3.26 (2H, t, J = 5.8 Hz, H-3), 4.06 (2H, t, J = 5.8 Hz, H-4), 3.74 (6H, s, CH_3-N^+-CH_3), 7.35 (1H, d, J = 8.7 Hz, 7-H), 7.46 (1H, d, J = 8.7 Hz, 8-H); ^{13}C-NMR (150 MHz, DMSO-d_6): δ, 132.1 (C-1a), 120.7 (C-1b), 120.7 (C-2), 105.2 (C-2a), 18.7(C-3), 68.2 (C-4), 125.4 (C-5a), 137.4 (C-6), 119.2 (C-7), 105.2 (C-8), 54.5 (CH_3), 54.5 (CH_3). The ^{1}H-NMR and ^{13}C-NMR spectral data were in agreement with those of bufothionine[16, 31].

4. Conclusions

In this study, a new separation method based on a homemade polar-modified C18HCE was developed to purify the polar compounds from *Bufo bufo gargarizans* Cantor. With the application of a polar-modified C18 column, the poor retention problem was resolved and good resolutions were obtained as well. Therefore, seven high pure compounds were obtained via preparative HPLC. Among them, compound **2** (**P2**) was found to be hypoxanthine, which was isolated for the first time from skin of *Bufo bufo gargarizans* Cantor. Compound **3** (**P3**) was identified as a new compound (3-hydroxy-4H-pyrazolo[4,5-d]pyridazin-7(1H)-one). Totally, seven compounds were purified and five of them were identified by MS and NMR. As an alternative method used to separate polar compounds under RPLC mode, the established method was demonstrated to be feasible and potent for the separation and purification of polar compounds from toad skin. This methodology is anticipated to be extended to other complex mixtures, such as TCMs, pharmaceutical preparation and biological samples.

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Figure captions

Fig. 1. Preparative HPLC chromatogram of the methanol extract fraction on the XUnion C18 column (250 mm × 100 mm, I.D., 10 μm). The gradient elution condition was shown in Section 2.4; flow rate: 330 mL/min; UV detection at 230 nm (Fig. 1A); Analytical HPLC chromatogram of F1 on the XUnion C18 column (250 mm ×4.6 mm, I.D., 10 μm). The gradient elution condition was shown in Section 2.4; flow rate: 0.7 mL/min; column temperature: 30 ^{o}C; UV detection at 230 nm (Fig. 1B).

Fig. 2. HPLC chromatograms of F1 on XUnion C18 column (150 mm ×4.6 mm, I.D., 5 μm) (A), XTerra MS C18 column (150 mm ×4.6 mm, I.D., 5 μm) (B), Symmetry C18 column (150 mm ×4.6 mm, I.D., 5 μm) (C), XCharge C18PN column (150 mm ×4.6 mm, I.D., 5 μm) (D), XAqua C18 column (150 mm ×4.6 mm, I.D., 5 μm) (E) and C18HCE column (150 mm × 4.6 mm, I.D., 5 μm) (F). The gradient elution condition is shown in Section 2.5; injection volumn: 10 μL; l flow rate: 1 mL/min; column temperature: 30 ^{o}C; UV detection at 230 nm.

Fig. 3. HPLC chromatograms of F1 on XAqua C18 column (250 mm ×4.6 mm, I.D., 5 μm) (A) and C18HCE column (250 mm ×4.6 mm, I.D., 5 μm) (B). The gradient elution condition is shown in Section 2.5; injection volumn: 100 μL; flow rate: 1 mL/min; column temperature: 30 ^{o}C; UV detection at 230 nm.

Fig. 4. Preparative chromatograms of F1 on C18HCE column (250 mm ×20 mm, I.D., 5 μm). The gradient elution condition was shown in Section 2.8; injection volumn: 800 μL; flow rate: 18 mL/min; column temperature: 30 ^{o}C; UV detection at 230 nm.

Fig. 5. The chromatograms of F1 and seven purified compounds from F1. The gradient elution condition was shown in Section 2.6; injection volumn: 10 μL; flow rate: 0.7 mL/min; column temperature: 30 ^{o}C; UV detection at 230 nm.

Fig. 6. Structures of polar compounds **P1-4**, **P7** and HMBC correlations of compound **P3**.

Figures

Fig. 1

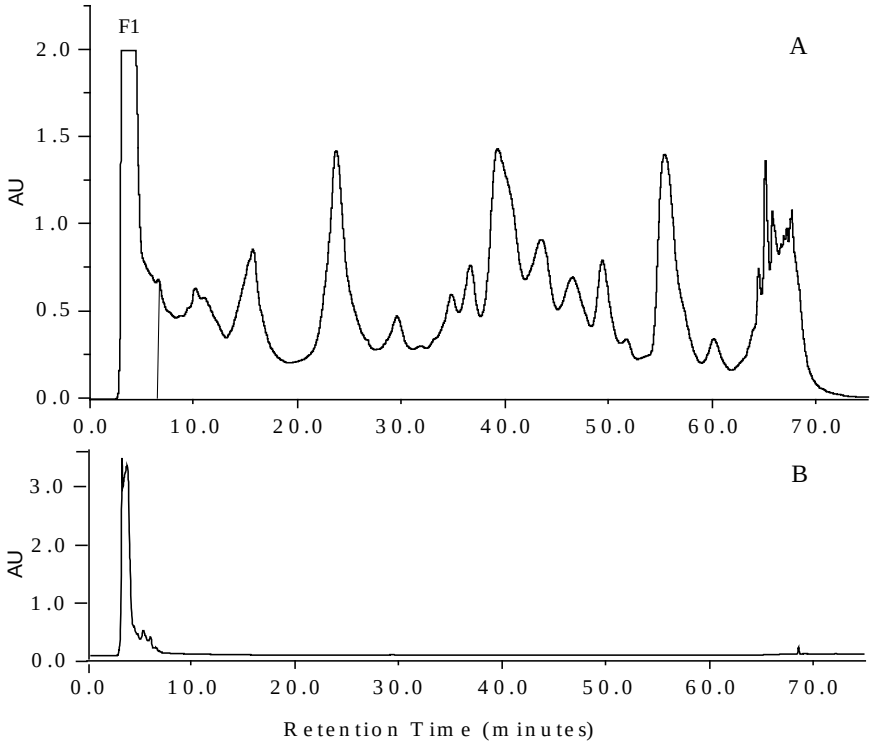

Fig. 2

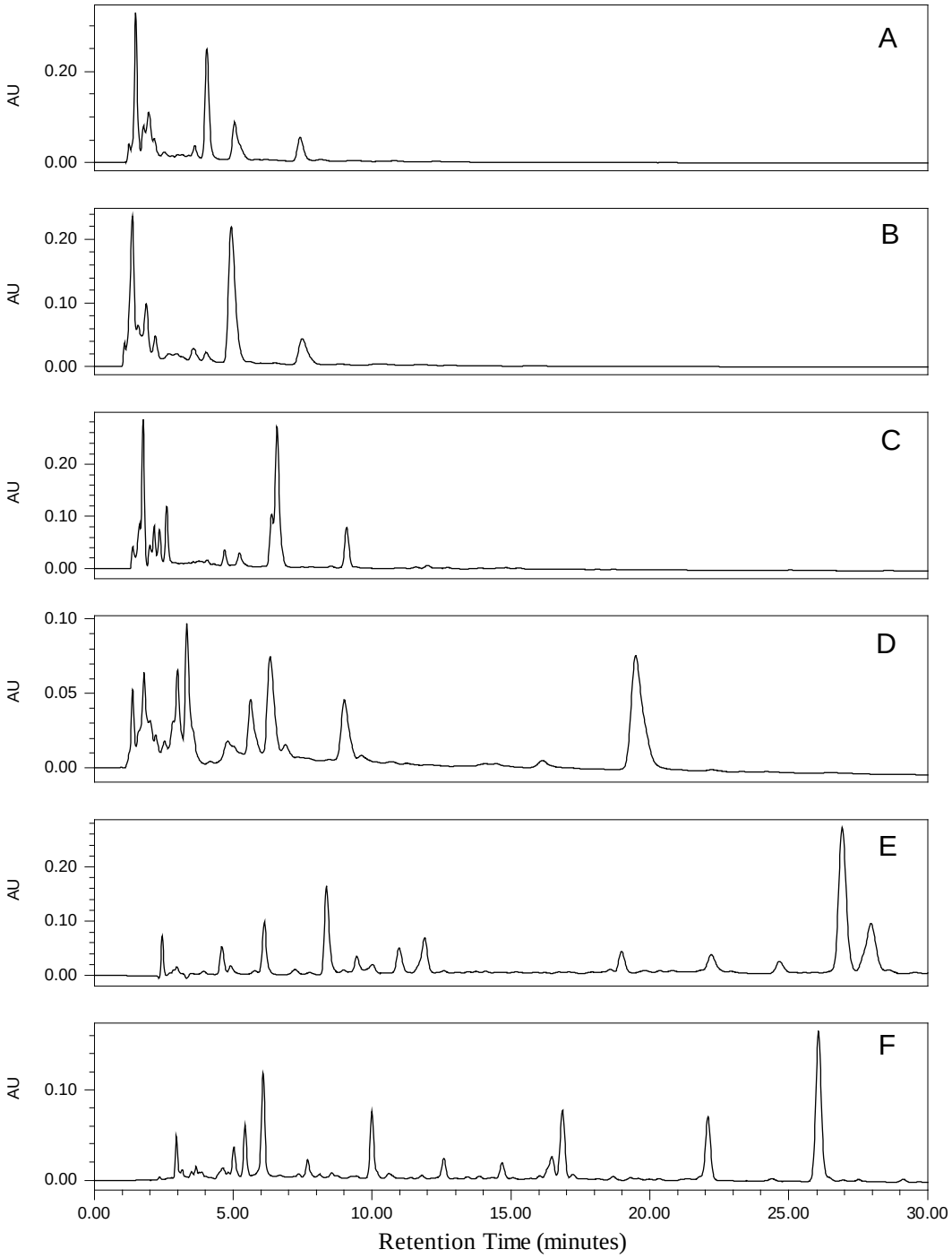

Fig. 3

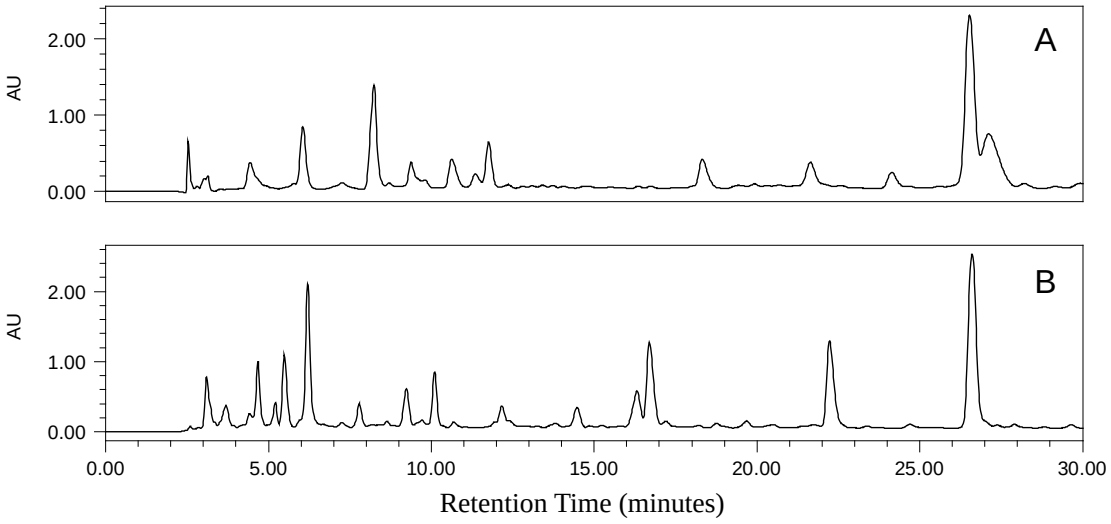

Fig. 4

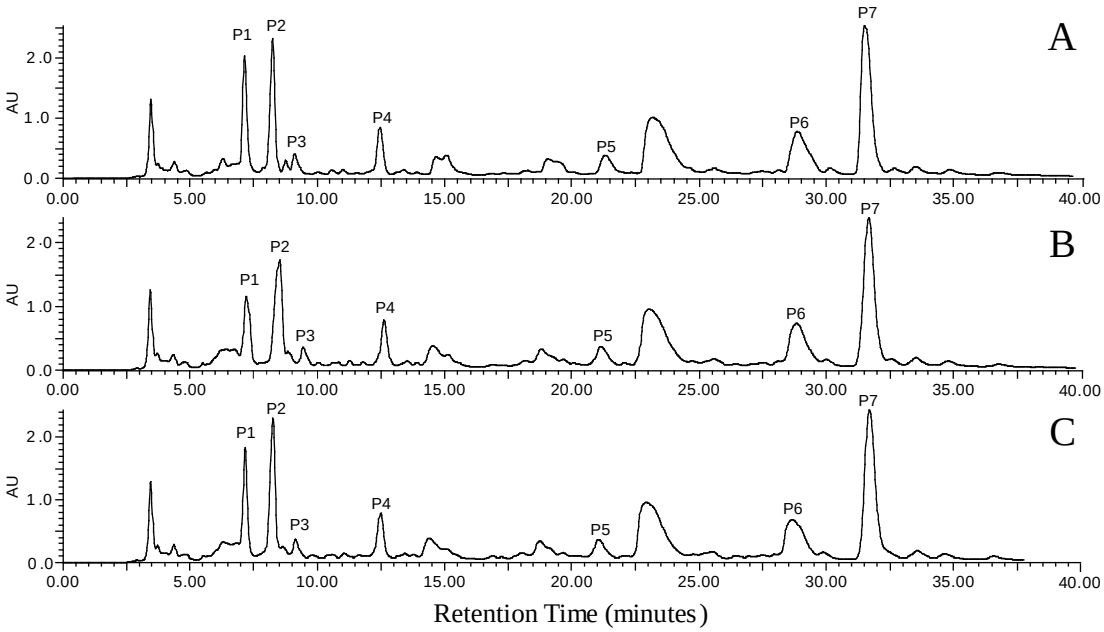

Fig. 5

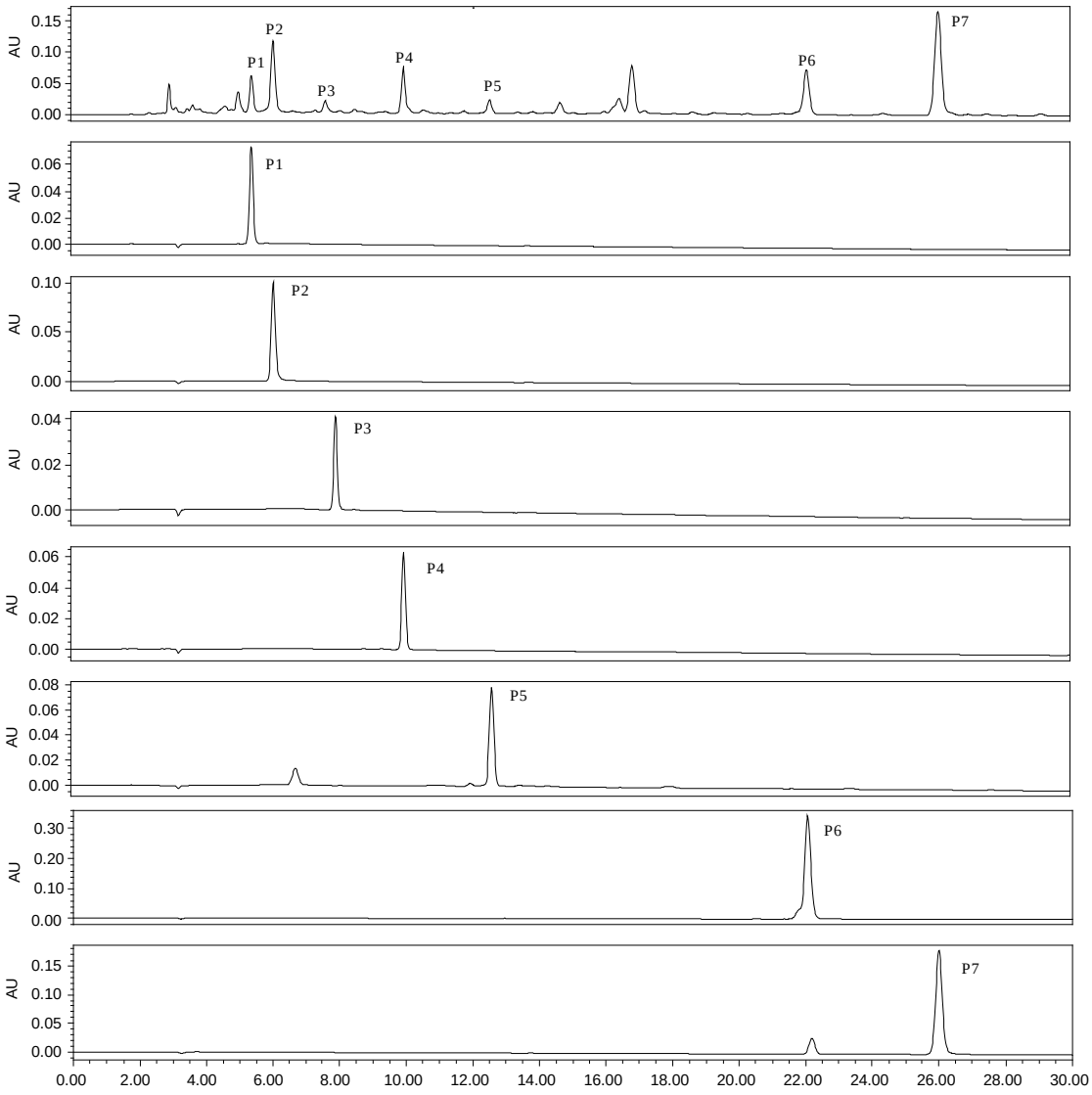

Fig. 6

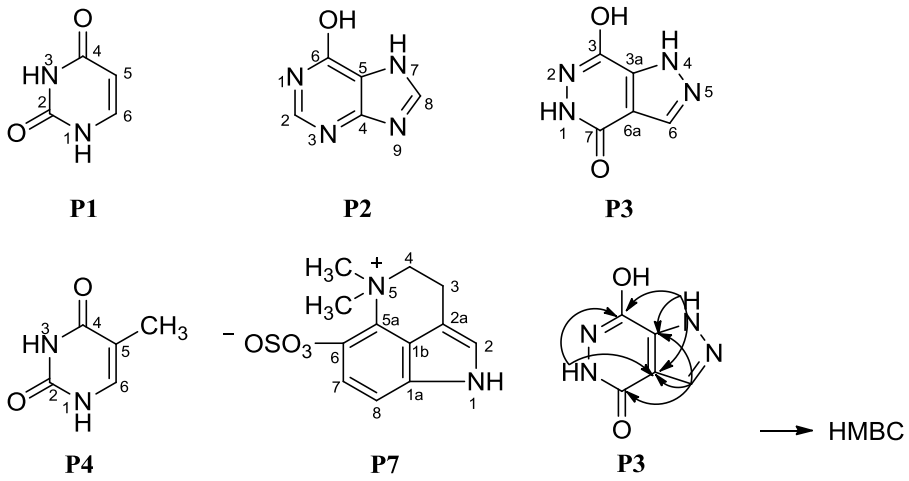

Table 1. NMR Spectral data of **P3** (DMSO-d_6, δ in ppm)

No.	δ_C	δ_H (J in Hz)	HMBC
1-NH		11.40	C-3, C-6a
2-N			
3-C	153.4		
3-OH		12.40	
3a-C	145.9		
4-NH		10.96	C-3, C-3a, C-6a
5-N			
6-C	145.1	7.88	C-3a, C-6a, C-7
6a-C	109.3		
7-C	151.1		