Journal of Chromatography B, 1026 (2016) 67–74

Contents lists available at ScienceDirect

Journal of Chromatography B

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

# Separation and characterization of bufadienolides in toad skin using two-dimensional normal-phase liquid chromatography × reversed-phase liquid chromatography coupled with mass spectrometry

Yun Zhang[a,1], Hongli Jin[a,1], Xiaolong Li[a], Jianqiang Zhao[b], Xiujie Guo[a], Jixia Wang[a], Zhimou Guo[a], Xiuli Zhang[a], Yanduo Tao[b], Yanfang Liu[a,*], Deliang Chen[c,**], Xinmiao Liang[a]

[a] Key Lab of Separation Science for Analytical Chemistry, Dalian Institute of Chemical Physics, Chinese Academy of Sciences, Dalian 116023, China
[b] Key Laboratory of Tibetan Medicine Research, Northwest Institute of Plateau Biology, Chinese Academy of Sciences, Xining 810001, PR China
[c] Wuhan Univ., Zhongnan Hosp., Dept. Internal Med., Div. Cardiol., Wuhan 430071, Hubei Province, PR China

ARTICLE INFO

*Article history:*
Received 20 July 2015
Received in revised form 3 November 2015
Accepted 10 November 2015
Available online 18 November 2015

*Keywords:*
Bufadienolides
Normal-phase liquid chromatography
Two-dimensional liquid chromatography
MS/MS
Toad skin

ABSTRACT

Bufadienolides possess various bioactivities especially antitumor. Due to the high structural diversity, the separation of bufadienolides often suffers from coelution problem on conventional RP columns. In this work, an off-line two-dimensional normal-phase liquid chromatography × reversed-phase liquid chromatography (2D-NPLC × RPLC) method was developed to separate and characterize bufadienolides in toad skin. Several RP and NP columns were evaluated with five reference bufadienlides. The XUnion C18 and XAmide columns exhibited superior chromatographic performances for bufadienlide separation, and were selected in RPLC and NPLC, respectively. RPLC was used in the second-dimension for the good compatibility with MS, while NPLC was adopted in the first-dimension. The orthogonality of the 2D-NPLC × RPLC system was investigated by the geometric approach using fifteen bufadienolide mixtures. The result was 49.6%, demonstrating reasonable orthogonality of this 2D-LC system. By combining the 2D-LC system with MS, 64 bufadienlides including 33 minor ones and 11 pairs of isomers in toad skin were identified. This off-line 2D-NPLC × RPLC allowed to solve the coelution problem of bufadienlides in one-dimension RPLC, and thus facilitated the identification significantly.



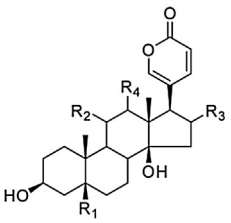
## 1. Introduction

The skin of *Bufo bufo gargarizans* Cantor, also known as toad skin, is one of the most famous traditional Chinese Medicine (TCMs). It has been used to treat numerous diseases in China for a long time, such as antitumor [1–3], anti-arrhythmia [4], and prevention of heart disease [5]. In recent years, increasing evidences have demonstrated that bufadienolides, a kind of polyhydroxy C-24 steroids with 2H-pyrone-2-one ring in the molecule, are main active components in toad skin. These compounds possess various bioactivities, including cardiotonic, renal sodium excretion, blood pressure stimulating [6,7], immunoregulatory [8], and antimicrobial [9]. Most notably, bufadienolides exhibit remarkable anticancer activities and a broad spectrum to many cancer cell lines [10,11]. The investigation of bufadienolides in toad skin has been a hotspot in the natural product research.

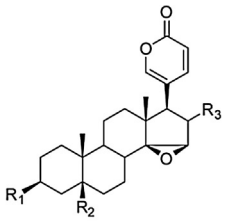
Up to now, about 60 bufadienolides in total have been reported in toad skin [12–16]. Due to the high structure diversity, the comprehensive separation and analysis of bufadienolides remains challenging. Reversed-phase liquid chromatography (RPLC) has been widely used for the separation of bufadienolides [17–19]. And coupled with mass spectrometry (MS) or tandem mass spectrometry (MS/MS), convenient identification of bufadienolides is achieved [20–24]. Hu et al. [21] have characterized bufadienolides in Chinese medicine-Venenum Bufonis by RPLC-ESI-MS/MS. A total of 30 bufadienolides were identified. Nonetheless, it was found that the separation of bufadienolides in toad skin was rather difficult, owing to the similar retention behaviors in RPLC. Besides, the char-

* Corresponding author. Fax: +86 411 84379539.
** Corresponding author.
*E-mail addresses:* liuyanfang@dicp.ac.cn (Y. Liu), deliangchen@sohu.com (D. Chen).
[1] These authors contributed equally to this work.

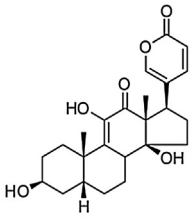
http://dx.doi.org/10.1016/j.jchromb.2015.11.015
1570-0232/



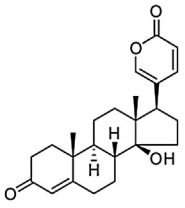
* 1. Bufarenogin: $R_1$ = H $R_2$ = =O $R_3$ = H $R_4$ = β-OH
3. Desacetylbufotalin: $R_1$ = H $R_2$ =H $R_3$ = β-OH $R_4$ = H
* 5. Telocinobufagin: $R_1$ = OH $R_2$= H $R_3$ = H $R_4$ = H
* 8. Bufotalin: $R_1$ = H $R_2$ =H $R_3$ = β-OAc $R_4$ = H
11. Bufalin: $R_1$ = H $R_2$ =H $R_3$ = H $R_4$ = H



2. Desacetylcinobufotalin: $R_1$ = OH $R_2$ = OH $R_3$ = β-OH
* 6. Desacetylcinobufagin: $R_1$ = OH $R_2$ = H $R_3$ = β-OH
10. Marinobufagin: $R_1$ = OH $R_2$ = OH $R_3$ = H
12. 3-keto-Deacetylcinobufagin: $R_1$= =O $R_2$ = H $R_3$ = β-OH
14. Cinobufagin: $R_1$ = OH $R_2$ = H $R_3$ = β-OAc
15. Resibufogenin: $R_1$ = OH $R_2$ = H $R_3$ = H



4. Argentinogenin



* 9. 3-Dehydrobufagin

**Fig. 1.** The chemical structures of the reference bufadienolides. * used for the development of 2D-LC system.

acterization of minor bufadienolides or isomers may suffer from co-elution problems in one-dimensional RPLC. Hence, improved chromatographic separation of bufadienolides is of great significance for the identification.

Two-dimensional liquid chromatography (2D-LC) has become a potent tool for the separation of complex mixtures [25–28]. Liu et al. [29] has developed a two-dimensional reversed-phase liquid chromatography/hydrophilic interaction chromatography (2D-RPLC/HILIC) method for the purification of bufadienolides from toad skin. In addition, RPLC coupled with positively charged C18 column was used to efficiently separate the bufadienolides in toad skin [30]. These works indicated that 2D-LC is capable of providing significantly improved separation for bufadienolides comparing to one-dimension LC, mainly ascribed to the increased peak capacity and selectivity.

In the case of constructing a successful 2D-LC system, Orthogonality is an important issue. Commonly, a pair of phases with different separation mechanisms would minimize the retention correlation between dimensions [31,32], and thus good orthogonality could be obtained. Normal-phase liquid chromatography (NPLC) has been adopted as an alternative mode to RPLC for the distinct chromatographic mechanisms [33,34]. And the combination of NPLC and RPLC offers a high degree of orthogonality in the analysis of various compounds [35,36]. To our best knowledge, two-dimensional NPLC × RPLC (2D-NPLC × RPLC) method has not been applied in the bufadienolide separation.

In this work, we aimed to develop an off-line 2D-NPLC × RPLC method for the separation and identification of bufadienolides in toad skin. RP and NP columns were assessed to establish the 2D-LC system. Its orthogonality was evaluated by the geometric approach. Using this 2D-LC method coupled with mass spectrum, bufadienolides in toad skin were characterized systematically.

## 2. Materials and methods

### 2.1. Apparatus and reagents

Most HPLC separation was performed on an Alliance HPLC system consisting of a waters 2695HPLC pump and a waters 2996 photodiode array detector. Data acquisition and processing were conducted by Waters Empower software. The second dimensional RPLC-MS/MS was performed on an Agilent 1290 Infinity LC instrument (Aglient, USA) coupled to Agilent 6540 series QTOF-MS (Agilent, USA), which equipped with electrospray ionization (ESI) source, diode-array detector (DAD), automatic sample injector and column thermostat.

Water was prepared by a Milli-Q system (Billerica, MA, USA). Methanol, isopropanol and *n*-hexane were of chromatographic grade (Yuwang, China). Acetonitrile was purchased from Tedia (Fairfield, USA) and formic acid was obtained from Acros (Cambridge, USA). Fifteen reference bufadienolides were listed as follows: bufarenogin (1), desacetylcinobufotalin (2), desacetylbufotalin (3), argentinogenin (4), telocinobufagin (5), desacetylcinobufagin (6), bufadienolide (7), bufotalin (8), 3-dehydrobufagin (9), marinobufagin (10), bufalin (11), 3-keto-deacetylcinobufagin (12), bufadienolide (13) cinobufagin (14) and resibufogenin (15). Compounds 7 and 13 belonged to bufadienolides, according to UV and MS data. The chemical structures required further identification. Other reference bufadienolides were isolated from toad skin in our previous work [30]. The structures (shown in Fig. 1) were identified by UV, MS and NMR.

All the columns used in this work were purchased from Acchrom (Beijing, China) and listed as follows: XAmide (150 mm × 4.6 mm, I. D., 5 μm), Unitary Diol (150 mm × 4.6 mm, I. D., 5 μm), Click XIon (150 mm × 4.6 mm, I. D., 5 μm) and XUnion C18 (150 mm × 4.6 mm, I. D., 5 μm).

### 2.2. Sample preparation

Toad skin was collected from Shandong Province and authenticated by the Institute of Medication, Xiyuan Hospital of China Academy of Traditional Chinese Medicine. 100 g of dried toad skin was cut up into pieces and decocted with 700 mL of methanol for three hours. The extraction procedure was repeated twice. The decoctions were combined and dried with rotary evaporation at 60 °C in vacuum. 3.5 g of the extract was redissolved in 50 mL of 70% methanol, and then extracted with *n*-heptane (3 × 50 mL each). The methanol fraction was dried in vacuum and yielded