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Rapid separation and determination of resibufogenin and cinobufagin in toad venom and Liushen tablet by β-cyclodextrin modified micellar electrokinetic chromatography

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Abstract

A rapid cyclodextrin modified micellar electrokinetic chromatography (CD-MEKC) method was proposed for the determination of resibufogenin and cinobufagin in the Chinese herbal extracts from toad venom and its medicinal preparation (Liushen tablet). The two components have the close structural similarity and similar hydrophobicity, which result in poor resolution in normal MEKC. The addition of neutral β-CD to the MEKC system was found to improve the separation of the studied compounds. The effects of several CD-MEKC parameters on the resolutions were evaluated systematically. Based on the investigation, a background electrolyte solution consisting of 10 mM borate buffer adjusted to pH 8.5, 40 mM sodium dodecyl sulfate (SDS), 12 mM β-CD and 10% (v/v) of methanol was found to be optimal conditions for the fast separation. The contents of resibufogenin and cinobufagin were successfully determined within 5 min, with satisfactory repeatability and recovery.

Keywords: Micellar electrokinetic chromatography; β-Cyclodextrin; Resibufogenin; Cinobufagin; Toad venom

1. Introduction

Dried toad venom is called "Chan su" or "toad cake" in China and "Senso" in Japan obtained from the postauricular and skin glands of toad. It is often found in traditional Chinese medicine ingredients, such as Liushen tablet [1] and Niuhuangxiaoyan tablet [2]. These Chinese medications have been widely used in China, Japan and other Asian countries for a long time, and over the last decade, have gained considerable favor in the United States and other places of the world. Toad venom is used as a topical anesthetic and cardiac medication [3,4]. It can eliminate toxic material; relieve carbuncle, furuncle, cellulites and multiple abscesses [5]. Recently, toad venom has been utilized in the treatment of cancer [6–9]. However, overdose may cause nausea, vomiting, diarrhea, and even general paralysis [10]. Recent reports indicate that toad venom toxicity carries a high mortality rate in the United States [2,11,12]. Resibufogenin and cinobufagin are two major components isolated from toad venom. So, identification and determination of resibufogenin and cinobufagin will play an important role in the safety, efficacy and therapeutic reproducibility of toad venom and its medical preparations.

Several methods such as thin layer chromatography (TLC) [13], high performance liquid chromatography (HPLC) [14–17] have been reported for the analysis of resibufogenin and cinobufagin. The literature shows that HPLC is the most common method for determination these active components in Chinese herbal medicines or biological matrices. In most cases, the analytical times of these HPLC methods reported were usually above 15 min, even more than 50 min. In order to further confirm the structural identification of compositions of Chinese herbs, LC/MS/MS method [18] was also performed in determination the content of resibufogenin and cinobufagin, but the operation procedure was more complicated comparing with that of HPLC. The obvious disadvantage of most HPLC methods in the analysis of resibufogenin and cinobufagin is the high cost of magnitude

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of mobile phase and time-consuming, which would not meet the needs of high throughput analysis. On the other hand, capillary electrophoresis (CE) has been proved to be a complementary and attractive alternative to the more established methods. CE offers the advantages of excellent separation efficiency, high resolution and rapid analysis. In addition, the amount of the sample and solvent usage of CE is minimum. However, to the best of our knowledge, there are no reports on the separation and determination of the two active components by CE. The aim of this work is to develop a fast CE method for the assay of resibufogenin and cinobufagin in toad venom and its medical preparations.

2. Experimental

2.1. Instruments

All separations were performed on MDQ CE instrument (Fullerton, CA, USA) equipped with on-column didode-array detection (DAD) system. Beckman Coulter MDQ 32 Karat software was used for instrumental control and data analysis. A 31.2 cm × 50 μm i.d. uncoated fused silica capillary (Yong-Nian Optical Fiber Factory, Hebei, China) was utilized with an effective length of 21 cm. Separation were carried out using an electrical voltage of 20 kV, and the temperature of the capillary was maintained at 25 °C, while 295 nm was selected as the detection wavelength. Samples were introduced into the capillary via hydrodynamic injection by applying 0.3 psi for 3 s (1 psi = 6894.76 Pa).

2.2. Reagents

Resibufogenin and cinobufagin (the structures were shown in Fig. 1) were purchased from the National Institute for Control of Pharmaceutics and Biological Products, Beijing, China. Samples of toad venom and Liushen tablet were purchased from a local herbal store. All chemicals used in the analysis were of analytical reagent grade. Sodium tetraborate decahydrate, sodium dodecyl sulfate (SDS), methanol, ethanol and acetonitrile were purchased from Beijing Chemical Reagents Plant. β-Cyclodextrin (β-CD) was purchased from the Development Center of Special Chemical Reagents (Tianjin, China). Deionized water was used throughout this study.

2.3. Preparation of standards and buffer solution

Stock solutions (1 mg/ml) of resibufogenin and cinobufagin were prepared in methanol, and diluted by methanol to obtain the desired concentration before use. The running buffer consisted of 10 mM sodium tetraborate decahydrate, 40 mM SDS and 12 mM β-CD was prepared in deionized water. Prior to the analysis, the pH of the buffer solution was adjusted with 0.1 M HCl or NaOH and added 10% (v/v) of methanol. All solutions were filtered through 0.45 μm syringe filter before analysis.

2.4. Sample preparation

0.20 g of fine powder of toad venom and Liushen tablet were accurately weighed respectively and extracted with 20 ml chloroform by refluxing for 5 h. The extracts were filtered through a filter paper. The extraction procedure was repeated three times, and the extracts were combined and concentrated to dryness. The residue was diluted to 5 ml with methanol, which was then passed through a 0.45 μm membrane filter before analysis.

3. Results and discussion

3.1. Separation by normal MEKC

Resibufogenin and cinobufagin are uncharged molecules under neutral, acid and weak alkaline conditions indicating that simple capillary zone electrophoresis (CZE) cannot be suitable for analysis. Thus, for simultaneous separation and determination of both the two components, a micellar electrokinetic chromatographic (MEKC) system was adopted. The separation of neutral solutes in MEKC is mainly due to their partitioning between an aqueous phase and a micellar phase. In preliminary experiments, normal MEKC conditions where there were no modifiers added to the buffer were tested. In the range of the concentration of SDS within 10–120 mM, borate buffer 10–100 mM and pH 7.0–11.0, no separation of the two compounds was observed. The resolution in MEKC can be improved by modifying the buffer by adding organic solvents. Organic solvents can decrease the EOF and affinity of the hydrophobic solute for the micellar phase [19]. But successful separation was not obtained with addition of methanol, ethanol or acetonitrile. The two analytes have close structural similarity and similar hydrophobicity

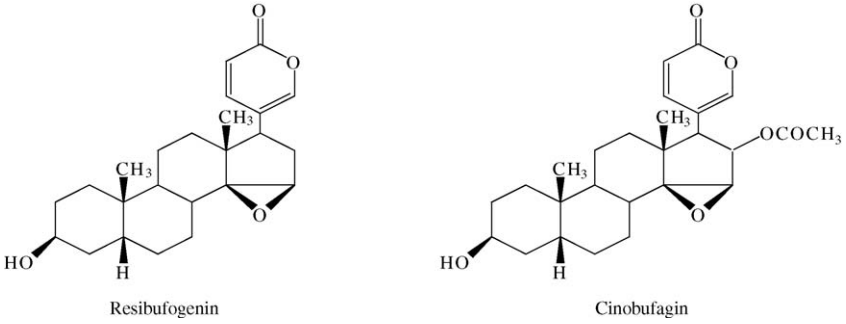

Fig. 1. Molecular structures of resibufogenin and cinobufagin.