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Journal of Pharmaceutical and Biomedical Analysis 41 (2006) 124–128
JOURNAL OF PHARMACEUTICAL AND BIOMEDICAL ANALYSIS
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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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Received 12 July 2005; received in revised form 26 October 2005; accepted 27 October 2005
Available online 28 November 2005

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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doi:10.1016/j.jpba.2005.10.046

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

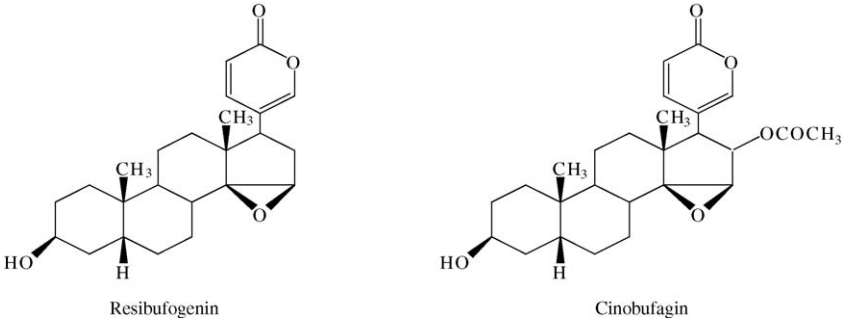

Fig. 1. Molecular structures of resibufogenin and cinobufagin.

to the micelle; therefore, they were difficult to be separated under normal MEKC conditions. It has been reported that the use of CD in MEKC is effective for the separation of lipophilic and closely related compounds [20,21], so our attention was focused on the use of β-CD as modifier.

3.2. Separation by CD-MEKC

The cyclodextrin-modified micellar electrokinetic chromatography (CD-MEKC) was firstly proposed by Terabe et al. [22]. In CD-MEKC, the solutes are distributed among three phases: the aqueous, the micellar and the CD phase. The solutes form inclusion complexes with CDs based on their size, geometry and physicochemical properties, while interactions with micelles are based on solute hydrophobicity. The inclusion of the solutes into the cavity of CD reduces the capacity factor of solute/micelle allowing for highly selective separations on condition that the distribution of the solutes towards either micelle or CD does not be completed. Therefore, the main parameters affecting the resolutions will be the nature and concentration of the CD and pH of the running buffer [22–25]. The main advantage of using a neutral CD is that the addition of an uncharged species to the background electrolyte (BGE) will not increase the Joule heat of the system. Subsequently, high separation voltages can still be utilized [26]. β-CD itself is electrically neutral and its outside surface is hydrophilic; accordingly, it could not penetrated into the SDS micelle, and migrate at an identical velocity with the bulk solution [27].

The optimization of the separation was performed by considering the importance of obtaining baseline resolutions in short analysis time. The effect of the β-CD concentration in the range 0–14 mM was investigated with using 10 mM borate buffer (pH 8.5), 40 mM SDS and 10% (v/v) of methanol. The resolution of the two active components became better as the concentration of β-CD increased (Fig. 2). The results indicate that resibufogenin formed a more stable complex with β-CD than did cinobufagin.

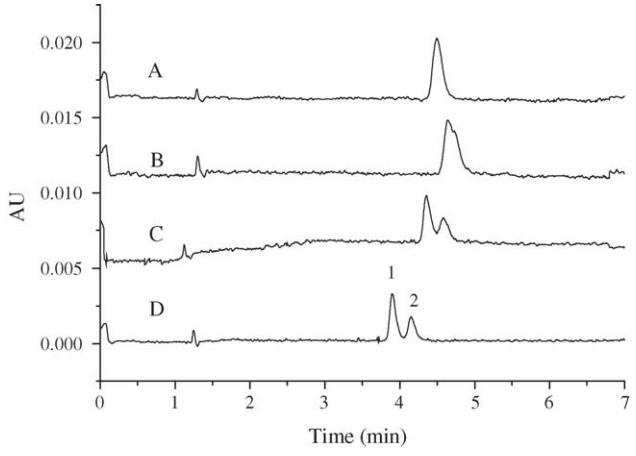

Fig. 2. Impact of β-CD concentration on separation of resibufogenin and cinobufagin. Operating conditions: 10 mM borate buffer (pH 8.5) with 40 mM SDS and 10% (v/v) of methanol. Injections: 0.3 psi for 3 s. Applied voltage: 20 kV, detection at 295 nm. (A) β-CD = 2 mM, (B) β-CD =4 mM, (C) β-CD = 6 mM and (D) β-CD = 12 mM. Peaks: 1 = resibufogenin, 2 = cinobufagin.

In this CD-MEKC system investigated, there existed hydrophobic interactions, hydrogen bonding effects between the analytes and the micelles, and inclusion-complex interactions between the analytes and β-CD. It was assumed that the inclusion of the analytes into the cavity of β-CD reduces the capacity factor of solute/micelle, and inclusion-complex stability of analytes with β-CD seemed to be predominant interaction contrasted to those of analytes with micelles in the course of separation. A more stable inclusion complex of the analyte with β-CD is formed at β-CD concentrations higher than 10 mM. β-CD tended to be easily precipitated when it was used at high concentration; therefore, we employed 12 mM β-CD for the analysis in our study.

To verify the effect of running buffer pH on migration behavior, experiments were performed with pH ranging from 8.0 to 10.0. A decrease in resolution was observed upon increasing pH. As the pH value increased, the velocity of EOF increased slightly and the migration times of the analytes decreased. The effect of the analytes on SDS micelles and β-CD was gradually moderated. Although the use of a pH buffer lower than 8.5 could improve the resolution to some point, the migration time is inadequate because of prolonging analysis time. The effect of buffer concentration on resolution and migration time was also investigated by varying the concentration of borate from 10 to 50 mM, at pH 8.5. From this experiment, we observed that increasing buffer concentration lengthened the migration time and enhanced the separation slightly, however, band tailing and broadening was observed and the detection became insensitive with increasing buffer concentration. Hence, 10 mM borate (pH 8.5) was selected.

The influence of the concentration of SDS in the range 20–100 mM on the separation of the two active components was studied. The resolution increased with increasing SDS concentration of the BGE up to 40 mM and then decreased with longer migration times. This result is not attributable to an increase in ionic strength, because the EOF was not changed significantly over the whole SDS concentration range. The distribution of the analyte between the micelle and the nonmicellar aqueous phase including β-CD directly affects the resolution. SDS monomers can have their hydrophobic tails included in the CD cavity along with the analyte. This could change the nature of the analyte and CD interaction and consequently, the resolution. Since the two compounds are highly hydrophobic, neutral species, the solubilization into the micelle or interaction with the micelle will be probably increased with an increase of SDS concentration and this lead to increasing partition of analytes into the micellar phase and decreasing the interaction with CD, resulting in degradation of the quality of the separation as reflected by poor resolution and longer migration times. In this study, it was also found the peak symmetry deteriorated with increasing SDS concentration due to the increasing mobility mismatch between analytes and buffer constituent. Therefore, 40 mM SDS was chosen. The resolution in CD-MEKC can be improved by modifying the buffer by adding organic solvents [28]. It has been considered that the organic modifier can have two roles: (1) improving the solubility of the solutes and (2) decreasing the affinity of the solute for the hydrophobic cavity of CD.

The addition of methanol affected resolution: resibufogenin and cinobufagin were completely resolved from each other with the addition of 10% (v/v) methanol, whereas less than 10% (v/v) addition brought incomplete resolution. But the addition of 15% deteriorates the separation.

The effect of the applied voltage from 10 to 25 kV was investigated. It was found that the separation efficiency of the two compounds was not improved with the voltage increasing. A voltage of 20 kV yielded the best compromise in terms of run time, separation current and was used for all experiments. The total separation time takes less than 5 min.

Therefore, the optimum BGE conditions, i.e., the conditions giving both high resolution and short migration time, were found to be 12 mM β-CD, 40 mM SDS and 10% (v/v) of methanol in 10 mM borate buffer (pH 8.5) with an effective voltage of 20 kV.

3.3. Validation of the method

The linear relationship between the concentrations of two compounds and the corresponding peak areas were found in the concentration range 12.3–800 μg ml^{-1} for resibufogenin and 13.8–810 μg ml^{-1} for cinobufagin, respectively. The regression equations of these curves and their correlation coefficients were calculated as follows: resibufogenin, $y = 60.33x + 331.14$ ($r = 0.9982$), cinobufagin $y = 49.06x - 200.17$ ($r = 0.9995$), where y and x were the peak area and the concentration (μg ml^{-1}) of the two active components, respectively. The limits of detection (LOD, S/N = 3) of the analytes were 1.90 μg ml^{-1} for resibufogenin and 2.26 μg ml^{-1} for cinobufagin, respectively.

The repeatability was evaluated by performing six successive injections and expressed as relative standard deviation (R.S.D.) for both migration time and peak area. The R.S.D. of the migration time and the peak area of each peak were 1.51, 3.63% for resibufogenin and 1.22, 2.58% for cinobufagin (intra-day), and 2.63, 4.25% for resibufogenin and 2.89, 3.97% for cinobufagin (inter-day, for a 5-day period), respectively.

The accuracy was evaluated by means of recovery studies. It was determined by adding suitable amounts of the analytes of known concentration to plant extracts. The recoveries of the resibufogenin and cinobufagin were 96.8 and 105.3%, 93.1 and 94.5% ($n = 5$) for the extract from toad venom and Liushen tablet, respectively.

3.4. Determination of the analytes in toad venom and its medicinal preparations

The extracts of toad venom and Liushen tablet were injected directly and separated under the optimum conditions described above. Fig. 3 shows the electropherograms of the extracts of toad venom and Liushen tablet, respectively. Peaks were identified with comparing the migration times and the spectra of the separated compounds and standards, and spiking standards to the sample solutions. The analytical results were summarized in Table 1.

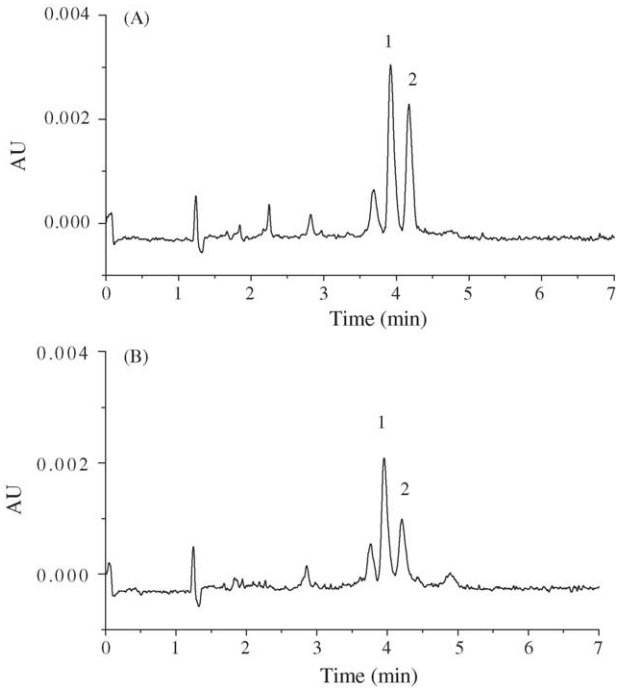

Fig. 3. Electropherograms of real samples Peaks: 1 = resibufogenin, 2 = cinobufagin. Operating conditions: 10 mM borate buffer (pH 8.5) with 40 mM SDS, 12 mM β-CD and 10% (v/v) of methanol. Injections: 0.3 psi for 3 s. Applied voltage: 20 kV, detection at 295 nm. (A) Toad Venom and (B) Liushen tablet.

Table 1
Results for the determination of resibufogenin and cinobufagin in real samples ($n = 5$)

Sample	Analyte	Content (g/100 g)	R.S.D. (%)
Toad venom	Cinobufagin	0.90	2.39
	Resibufogenin	1.56	1.96
Liushen tablet	Cinobufagin	0.45	3.93
	Resibufogenin	1.04	2.57

4. Conclusions

The baseline separation of resibufogenin and cinobufagin was achieved in less than 5 min by β-CD modified MEKC. The influence of the concentration and pH of the BGE has been evaluated with the goal of developing a CD-MEKC method suitable for the simultaneous analysis of both the two components. This method also promises to be applicable to the quality control of the two compounds in the complex extract of toad venom and its medicinal preparations. Compared with HPLC separation, this CD-MEKC method is faster, cost-effective and more suitable for high throughput analysis.

Acknowledgements

This project is financially supported by the Foundation for "Bairen Jihua" of CAS in 2001 and National Natural Science Foundation of China (no. 20375045).

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