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Development and optimization of a method for the analysis of Brazilein by HPLC with electrochemical detection

Xiaoling Yan, Wei Wang, Dongming Xing, Yunan Zhao, Lijun Du *

Laboratory of Pharmaceutical Sciences, Department of Biological Sciences and Biotechnology, Tsinghua University, Beijing 100084, China

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Abstract

The aim of this study was to establish an easy and accurate method for the determination of Brazilein in plant samples due to its potential pharmacological activities. High-performance liquid chromatography (HPLC) with electrochemical detection (ED) was used for the assay of Brazilein in this study for the first time. Crucial influence parameters including concentration of dodecane-1-sulfonic acid sodium salt (DSASS), inorganic modifier, tetrabutyl-ammonium hydroxide solution (TBAOH), and applied potential of proposed method were investigated. The proposed method is simple, rapid (analysis time: ∼10 min), sensitive [(detection limit: 0.6 ng per injection (20 μl) at a signal-noise ratio 3:1)], highly selective and precise (intra- and inter-day precisions were within 5%, $n = 7$). The calibration graph of Brazilein was linear in the range 0.6–150 ng per injection 20 μl. Recovery of Brazilein was over 92% by standard addition method.

Keywords: Brazilein; *Caesalpinia sappan* L.; Leguminosae; HPLC; Electrochemical detection

1. Introduction

Sappan Lignum, the dried heartwood of *Caesalpinia sappan* L. (Leguminosae), has been used in Chinese medicine as both an analgesic and an anti-inflammatory agent. The extract has been reported to have some pharmacological activities such as anti-atherogenic, inhibitors of iNOS activity, anti-complementary activity, antioxidant activity, anticonvulsant activity, antimicrobial activity [1–7], etc. Moreover Brazilein, one compound of the ethanolic extracts from Sappan Lignum, showed anticomplementary activity on the immune system in vitro [3]. All of the utmost importance, it was found in our lab recently that Brazilein exhibited a positive inotropic action in isolated cardiac tissues and inhibited Na^+, K^+-ATPase activity in vitro, and showed antioxidant activity both in vitro and in vivo. These indicated that Brazilein might become a potential natural compound in medicine in laboratory research.

However, no method has been reported for the analysis of Brazilein so far. It is becoming increasingly important to develop a suitable method to analysis of Brazilein for its further research. HPLC has been widely applied in detection and isolation of flavonoids from Leguminosae families [8–15]. The purpose of this paper is to develop and optimize a reversed-phase high-performance liquid chromatography (RP-HPLC) method for the determination of Brazilein, the potent flavonol compound from Sappan Lignum extracts.

Brazilein was isolated as reddish brown crystals whose structural formula (Fig. 1) [16] contains hydroxy of phenol and hydroxy functional groups. So it can be detected as other polyphenols by an UV detector [17–21] or by an electrochemical detector [22–25]. The limit detection of Brazilein by an UV detector has been tested in our lab. However, lower analytical sensitity restricted its further application in real biological samples where the detection sensitivity is a major concern. So we centered our focus on electrochemical detection in this paper.

High-performance liquid chromatography (HPLC) with electrochemical detection (ED) is valuable for the highly

* Corresponding author. Tel.: +86 10 62773630; fax: +86 10 62773630.
E-mail address: pharm@mail.tsinghua.edu.cn (L. Du).

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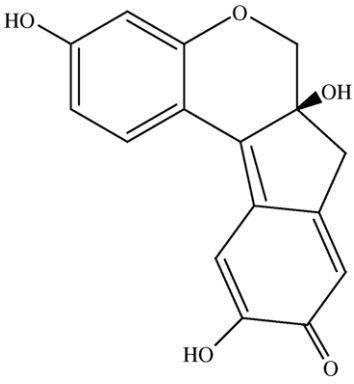

Fig. 1. Structural formula of Brazilein.

selective and sensitive analysis of trace amounts of compounds in complex matrices. Usually when choosing a mobile phase for use with electrochemical detectors, buffered systems to provide ionic strength for good electron transfer are used in mobile phase [26–29]. In this study, phosphate buffers were chosen as mobile phase in HPLC-ED system. In order to provide appropriate ionic strength, the concentration of phosphate buffer was focused on 75 mM for later studies.

However, such mobile phase composition mentioned above gave no promising electrochemical signal response of Brazilein even at high electrode potential. It was found through our experiments that dodecane-1-sulfonic acid sodium salt (DSASS) could remarkably improve the analytical sensitivity of Brazilein in HPLC-ED system. Meanwhile, the retention time of Brazilein was sharply shortened, which is not reasonable for subsequent analysis of Brazilein. And the worst thing was that peak width of Brazilein became too broad (∼4 min) which was not reliable for routine work. Therefore, TBAOH as an ion-pair reagent and sodium carbonate anhydrous (Na_2CO_3) as a general inorganic modifier were examined in order to obtain rational retention time and excellent peak width of Brazilein.

Using standard Brazilein as a target analyte, four critical parameters including concentration of DSASS, Na_2CO_3, TBAOH and applied potential were discussed in this study. Meanwhile, the linearity, LOD, RSD and recovery of the proposed HPLC-ED method were provided. Moreover, the validity of this HPLC-ED method was demonstrated by analysis of Brazilein in real plant samples.

2. Experimental

2.1. Apparatus

The HPLC-ED system, which consisted of a waters 600 controller, a waters^{TM} 600 pump, a 77251 injector with an effective volume of 20 μl (Waters, Milford, USA), and an esa coulochem II detector (ESA, Chelmsford, USA), was used. The chromatograms were recorded and integrated by ChromStation^{TM} Chromatography Data System (American TN Technologies, Walnut Creek, CA 94598, USA). All experiments were performed on a Kromasil-C_{18} HPLC column (5 μm, 150 mm × 4.6 mm, Rainbow, Beijing). The flow rate was 0.6 ml/min at room temperature. A pH meter (Model 828, ORION, China) with ±0.01pH resolution was used to measure the pH of run buffers throughout the experiment.

2.2. Chemicals and solutions

Standard Brazilein was kindly presented by Dr. Wei Wang (minimum content: 98%; Batch No:031006); sodium dihydrogen phosphate, and dodecane-1-sulfonic acid sodium salt (DSASS) were purchased from Beijing Reagents (China); sodium carbonate anhydrous, phosphoric acid, and tetrabutyl-ammonium hydroxide solution (TBAOH) were obtained from Shanghai Reagent Factory (China). All these reagents above were of analytical grade. Acetonitrile and methanol of HPLC grade (J.T. Baker, USA) were purchased from Beijing Reagent (China). Water for preparation of samples and buffered solutions was deionized by a Milli-Q purification system with a 0.2 μm fiber filter (Barnstead, CA, USA).

Aqueous solutions were prepared with deionized water (Barnstead, CA, USA). Standard stock solution of Brazilein was prepared in methanol at a concentration of 1.0 mg/ml. Prior to analysis, fresh working solutions were prepared by serial dilution from the stock solution with methanol. For the study of DSASS concentration, DSASS–NaH_2PO_4 (0.67 mM–2.7 mM/75 mM) run buffers were prepared by dissolving the appropriate amount of DSASS in NaH_2PO_4 (75 mM) buffer and adjusting pH to pH 3.0 with phosphoric acid. For the study of Na_2CO_3 concentration, Na_2CO_3–DSASS–NaH_2PO_4 run buffers (0.5 mM–5.0 mM/1.35 mM/75 mM) were prepared by serial dilution of a Na_2CO_3–DSASS –NaH_2PO_4 (5 mM/1.35 mM/75 mM) run buffer with DSASS–NaH_2PO_4 (1.35 mM/75 mM, pH 3.0). For the study of TBAOH concentration, TBAOH–Na_2CO_3–DSASS–NaH_2PO_4 run buffers (0.5%–2.5%/1 mM/1.35 mM/75 mM) were prepared by adding the appropriate volume of TBAOH into Na_2CO_3–DSASS–NaH_2PO_4 run buffer (1 mM/1.35 mM/75 mM). In this work, all buffers were filtered through 0.2 μm cellulose acetate membrane filters before use.

2.3. Sample preparation

The heartwood of *C. sappan* L. (1 kg, purchased from Drugstore of Songlan) was refluxed with 95% EtOH for three times and the extract was concentrated to residue (the ethanolic extracts) under reduced pressure. The residue was prepared in methanol at a concentration of 5.0 mg/ml. Prior to analysis, the sample was filtered through a 0.2 μm syringe filter.

3. Results and discussion

3.1. Concentration of dodecane-1-sulfonic acid sodium salt (DSASS)

At the beginning of the work, the effect of DSASS concentration on the limit of detection (LOD) of Brazilein (obtained at a 3:1 signal-to-noise ratio) was examined for the suitable mobile phase concentration. It was not until DSASS was added in the run buffer that the target electrochemical signal response was detected. This phenomenon indicated that DSASS might influence the degree of dissociation of Brazilein, which improved oxidation–reduction reaction of Brazilein. All experiments were carried out in duplicate ($n = 7$). The relationship between DSASS concentration and peak area of Brazilein was examined in this paper. Fig. 2 shows that peak area of Brazilein increased as DSASS concentration increased and reached maximum at the concentration of 1.35 mM. When DSASS concentration exceeded 1.35 mM, lower peak area of Brazilein was obtained by HPLC-ED. According to the experimental results, we can see that the retention time of Brazilein was shortened step by step (from 8.5 min, to 7.2, 5.5, 4.8, 4.5 min). Moreover, peak width of Brazilein became too broad (~4 min) for routine work. This observation may be related to that DSASS changed the D_m (diffusion modulus) of Brazilein, which accelerated the diffusion of Brazilein. Meanwhile, DSASS as an anion surfactant took part in the secondary chemical equilibrium (SCE) and changed the retention time of Brazilein. Despite of the irrational retention time of Brazilein, 1.35 mM DSASS was chosen for further studies.

3.2. Concentration of Na_2CO_3

Although DSASS significantly improved the analytical sensitivity of Brazilein, it also resulted in peak width much broader, which is difficult for further quantitative analysis of Brazilein. In this paper, Na_2CO_3 as a general inorganic modifier was added in the run buffer to improve peak width of Brazilein [30]. Na_2CO_3 improved the peak width through dynamic ion-exchange. The competition between CO_3^{2-} and anion of Brazilein changed the retention properties and corrected the peak width of Brazilein. Besides that peak width of Brazilein was remarkably improved, there were no apparent changes in LOD and retention time of Brazilein when Na_2CO_3 concentration varied (results not shown). Five different concentrations of Na_2CO_3 were investigated in this study (0.5, 1.0, 2.0, 3.0 and 5.0 mM). Our experimental results demonstrated that 1 mM Na_2CO_3 could successfully improved the peak width of Brazilein. Hence, 1 mM Na_2CO_3 was chosen as the employed concentration for subsequent studies.

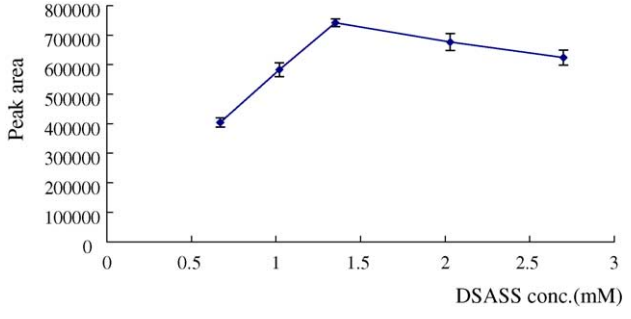

Fig. 2. Effect of DSASS concentration on peak area of Brazilein. Experimental conditions: a Kromasil-C_{18} HPLC column (5 μm, 150 mm × 4.6 mm); room temperature; applied potential 400 mV vs. Ag/AgCl reference electrode; flow rate, 0.6 ml/min; mobile phase, DSASS–NaH_2PO_4 run buffers (0.67 mM–2.7 mM/75 mM, pH 3.0): acetonitrile = 80:20 (v/v).

3.3. Concentration of TBAOH

In order to obtain proper retention time, TBAOH as an ion-pair chromatographic reagent was chosen to establish a more complex secondary chemical-equilibrium so as to change the retention time of Brazilein. In this study pH 7 was employed because that ion-pair combination needs suitable pH under which DSASS and TBAOH can form the ion-pairings. As expected, the retention time of Brazilein was prolonged while TBAOH concentration increased. And when TBAOH concentration reached 1% (v/v) from 0.5% (v/v), the retention time changed from about 8.5 min to 10 min, which is appropriate for subsequent studies. And when TBAOH concentration was higher than 1% (v/v) (higher concentration: 1.5%, 2.0%, and 2.5%), the retention time of Brazilein was still prolonged, but the extent was smaller than before. Consideration of routine work, 1% TBAOH was employed in this study.

3.4. Applied potential and background current

The relationships between applied potential and detection sensitivity of Brazilein and background current were investigated after mobile phase composition was completed. The relationships between peak height of Brazilein and background current and applied potential were illustrated in Table 1. Peak height of Brazilein increased significantly as the applied potential varied from 400 to 550 mV versus an Ag/AgCl reference electrode. Meanwhile the background current increased from 0.038 to 0.87 U accordingly. In order to maintain a stable baseline and constant signal response, 450 mV applied

Table 1
Relationships between applied potential and peak height and background current*

Applied potential (mV)	Peak height[a] (V)	Background current[a] (U)
400	62442	0.038
450	70381	0.072
500	77318	0.652
550	87368	0.87

* Flow rate: 0.6 ml/min.
[a] Calculation based on five duplicate injections of a standard sample.

potential versus Ag/AgCl reference electrode was employed to obtain the maximum LOD of Brazilein.

Thus, crucial factors that affect optimal separation and detection sensitivity of Brazilein have been thoroughly studied. The chromatogram of standard Brazilein is shown in Fig. 3. This indicated the validity of the mobile phase composition established in this study.

3.5. Linearity, precision, detection limit of Brazilein

The calibration graph for Brazilein was constructed by plotting the peak area of Brazilein against the amount of standard Brazilein. Satisfactory linearity was obtained in the range 0.6–150 ng ($y = 3.03 \times 10^6 x - 5.70 \times 10^5$, $r = 0.999582$, y = peak area, x = amount of standard Brazilein in ng). Intra- and inter-day precisions (expressed in terms of RSD) for peak area were typically less than 5% ($n = 7$). The limit detection of Brazilein was 0.6 ng per injection (20 μl) at a signal-to-noise ratio of 3:1. The high precise and low LOD indicated that the proposed method was reliable for analyzing Brazilein.

3.6. Chromatography

The next effort was focused on the chromatogram of Brazilein in real plant samples. The chromatogram of Brazilein in real plant samples was shown in Fig. 4. Brazilein can be well separated without the interference of other compounds in the samples. Peak identification was carried out by the standard addition method. This chromatogram demonstrated that the application of the HPLC-ED method to the determination of Brazilein in plant samples was possible. The Brazilein content of this plant sample calculated by the calibration formula was 31.2 mg/g. A known amount of standard Brazilein was added to the plant extract and overall recoveries were estimated by the standard addition method. Brazilein recovery was over 92% by the standard addition method (Table 2). This indicated the validity of the calibration established in this study.

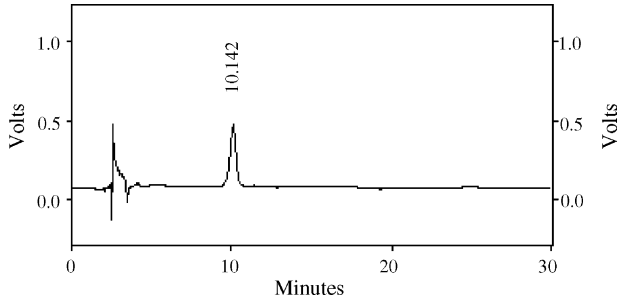

Fig. 3. Chromatogram of standard Brazilein. Experimental conditions: a Kromasil-C_{18} HPLC column (5 μm, 150 mm × 4.6 mm); room temperature; applied potential 450 mV vs. Ag/AgCl reference electrode; flow rate, 0.6 ml/min; mobile phase, TBAOH–Na_2CO_3–DSASS–NaH_2PO_4 run buffers (1%/1 mM/1.35 mM/75 mM, pH 7.0): acetonitrile = 80:20 (v/v).

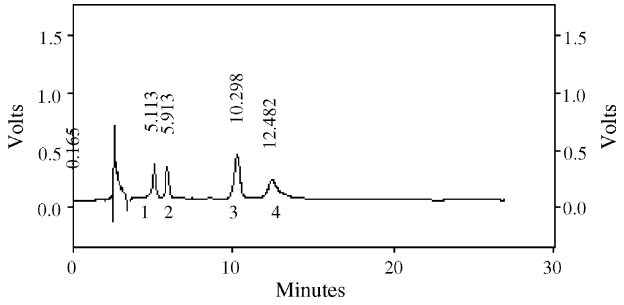

Fig. 4. Chromatogram of Brazilein in the extract of *Caesalpinia sappan* L. heartwood. Peak 3 = Brazilein. Experimental conditions: a Kromasil-C_{18} HPLC column (5 μm, 150 mm × 4.6 mm); room temperature; applied potential 450 mV vs. Ag/AgCl reference electrode; flow rate, 0.6 ml/min; mobile phase, TBAOH–Na_2CO_3–DSASS–NaH_2PO_4 run buffers (1%/1 mM/1.35 mM/75 mM, pH 7.0): acetonitrile = 80:20 (v/v).

Table 2
Recovery of Brazilein added in real plant sample

Added (mg/g)	Found (mg/g)	Recovery	
		mg/g	Percent
0	31.2[a]	–	–
5	35.8[a]	4.6	92
10	40.48[a]	9.28	92.8
20	49.84[a]	18.64	93.2

[a] RSD < 5% ($n = 7$).

4. Conclusions

This paper has reported a method for analysis of Brazilein in real plant samples. The conditions for optimal separation and detection sensitivity of Brazilein have been thoroughly studied. It has been applied to the identification and quantitation of Brazilein in real plant samples. Although peak identifications of every peak in Fig. 4 have not been carried out yet, this preliminary study for the determination of Brazilein established an interesting study for further analysis of Brazilein as well as other similar compounds of Sappan Lignum extracts in laboratories.

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