A High-Performance Liquid Chromatography with UV–vis Detection Method for the Determination of Brazilein in Plant Extract

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

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

Abstract

A previously established mobile phase composition for a high-performance liquid chromatography (HPLC)–electrochemical detection (ED) method for the determination of Brazilein is further examined by HPLC–UV. Each component in the mobile phase is evaluated by the HPLC–UV system, and a convenient HPLC–UV method for the determination of Brazilein is developed. The mobile phase of this HPLC–UV method consists of sodium dihydrogen phosphate (NaH_2PO_4). The detection limit of Brazilein (signal-to-noise = 3) is 1 ng, and the calibration graph of Brazilein ranges from 1–120 ng per 20-µL injection. The validity of the proposed HPLC–UV method is demonstrated by the analysis of Brazilein in real plant samples. The differences between the HPLC–ED method and the proposed HPLC–UV method are given.

Introduction

Sappan Lignum, the dried heartwood of *Caesalpinia sappan* L. (Leguminosae), has been long used in Chinese medicine as both an analgesic and an anti-inflammatory agent, and its extracts have been reported to have some pharmacological activities (1–7). Based on the reported papers and our laboratory research, Brazilein, one of the ethanolic extracts, also exhibited many important pharmacological activities both in vitro and in vivo (3, 8–10).

Because of the potential use of Brazilein in laboratory, an accurate and sensitive high-performance liquid chromatography (HPLC) with electrochemical detection (ED) method was developed for its determination and was described along with an efficient mobile phase composition, including dodecane-1-sulfonic acid sodium salt (DSASS), tetrabutyl-ammonium hydroxide solution (TBAOH), sodium dihydrogen phosphate (NaH_2PO_4), and sodium carbonate anhydrous (Na_2CO_3) (8). However, this HPLC–ED method has a disadvantage in that it is time-consuming and requires complicated operation.

* Author to whom correspondence should be addressed: email pharm@mail.tsinghua.edu.cn.

Brazilein was isolated as reddish brown crystals, and its UV–vis spectrum showed prominent maximum absorptions at 445 and 556 nm (11). However, the method could only reach a limit of detection (LOD) of 150 ng per 20-µL injection for Brazilein, when pH 3.0 water was used as the mobile phase. In addition, this analytical sensitivity could not meet the requirements of biological analysis. When the mobile phase composition established in the HPLC–ED system was employed in the HPLC–UV system, the results showed that an LOD of 1 ng per 20-µL injection of Brazilein could also be reached, which was similar with that in the HPLC–ED system. This indicated that the mobile phase composition established in the HPLC–ED system might also play an important role in the HPLC–UV system. In order to explain this phenomenon, each component in the HPLC–ED system was examined by HPLC–UV. After investigating the influences of DSASS, TBAOH, NaH_2PO_4, and Na_2CO_3 by HPLC–UV, a much more convenient HPLC–UV method was developed and is described in this study. Also, the linearity, LOD, relative standard deviation (RSD), and recovery of the proposed HPLC–UV method are provided. Moreover, the validity of this HPLC–UV method is demonstrated by the analysis of Brazilein in real plant samples. In the end, the major differences between these two HPLC methods are given.

Experimental

Apparatus

An HPLC–UV system, consisting of a Waters pump control module, two Waters 515 HPLC pumps, a 7725 injector with an effective volume of 20 µL, and a Waters 996 photodiode array detector (Waters, Milford, MA), was used. The chromatograms were recorded and integrated by the Millennium^{32} Total Assurance Plan software (Waters). All experiments were performed on a Kromasil-C_{18} HPLC column (5 µm, 150 × 4.6 mm, Rainbow, Beijing, China). UV detection was performed at a single wavelength of 445 nm. The flow rate was 1.0 mL/min at room temperature. A pH meter (Model 828, Orion, Shanghai, China)

with ± 0.01 pH resolution was used to measure the pH of all buffers throughout the experiment.

Chemicals and solutions

Standard Brazilein was kindly provided by Dr. Wei Wang (minimum content: 98%, Batch No. 031006), and NaH_2PO_4 and DSASS were purchased from Beijing Reagents (Beijing China), and Na_2CO_3, phosphoric acid (H_3PO_4), chloroform ($CHCL_3$). and TBAOH were obtained from Shanghai Reagent Factory (Shanghai, China). All these reagents were of analytical grade. HPLC-grade acetonitrile (ACN) and methanol (MeOH) (J.T. Baker, Phillipsburg, NJ) were purchased from a local distributor (Beijing Reagent). Water for the preparation of samples and buffered solutions was deionized by a Milli-Q purification system with a 0.2-µm fiber filter (Millipore, Barnstead, CA).

Aqueous solutions were prepared with deionized water (Millipore). Prior to use, a standard stock solution of Brazilein was freshly prepared in methanol at a concentration of 1.0 mg/mL and stored in a brown volumetric flask. Fresh working solutions were prepared by serial dilution from the stock solution with methanol. For the evaluation of DSASS and TBAOH concentration, two types of run buffers were required. DSASS–TBAOH–NaH_2PO_4–Na_2CO_3 run buffers (1.0, 1.35, 2.0mM:1%:75mM:1mM, pH 3.0) were prepared by dissolving the appropriate amount of DSASS in TBAOH–NaH_2PO_4–Na_2CO_3 solution (1%/75mM/1mM) and adjusting to pH 3.0 with H_3PO_4. NaH_2PO_4–Na_2CO_3 run buffers (75mM:1mM, pH 3.0) were prepared by dissolving the appropriate amount of NaH_2PO_4 and Na_2CO_3 in deionized water and adjusting to pH 3.0 with H_3PO_4. For the assessment of NaH_2PO_4 concentration, NaH_2PO_4 run buffers (10–80mM) were prepared by serial dilution of a NaH_2PO_4 run buffer (80mM, pH 3.0) with deionized water. For the evaluation of Na_2CO_3 concentration, Na_2CO_3–NaH_2PO_4 run buffers (0, 0.5, 1.0, and 2.0mM/20mM) were prepared by dissolving the appropriate amount of Na_2CO_3 in 20mM NaH_2PO_4 buffer and adjusting the pH to 3.0 with H_3PO_4. For the evaluation of the organic modifier present in the mobile phase, the ACN to inorganic run buffer ratio was varied by adjusting the flow-rate of two HPLC pumps. In this work, all run buffers were filtered through 0.45-µm cellulose acetate membrane filters before use.

Table I. Effect of Sodium NaH_2PO_4 Concentration on the Retention Time and LOD of Brazilein*

	Brazilein	
NaH_2PO_4 conc. (mM)	Retention time (min)†	Brazilein LOD (ng/20 µL)‡
0	7.8 ± 0.07	150
10	8.3 ± 0.06	6.75
20	8.2 ± 0.07	1.18
40	9.1 ± 0.08	1.18
80	8.7 ± 0.06	1.18

* Flow rate = 1.0 mL/min.
† Mean ± standard deviation (n = 5).
‡ LOD was calculated based on five duplicate injections of a standard sample.

Samples preparation

The heartwood of *Caesalpinia sappan* L. (1 kg, purchased from a local drugstore in Songlan) was refluxed with 95% EtOH for three times, and the extract was concentrated to residue (the ethanolic extracts) under reduced pressure, thus obtaining sample 1. The second plant sample was obtained by silica gel separation using sample 1. The methanol–chloroform elution was also concentrated to residue under reduced pressure. The two residues were dissolved in methanol at a concentration of 5.0 mg/mL. Prior to analysis, these two samples were filtered through a 0.45-µm syringe filter.

Results and Discussion

Effect of DSASS and TBAOH

Because DSASS had a significant effect on the LOD of Brazilein in the HPLC–ED system, its effect in the HPLC–UV system was also examined. Three concentrations of DSASS (1.0, 1.35, and 2.0mM), at which Brazilein reached an LOD around the maximum by the HPLC–ED method, were investigated in this study.

The results showed that the LOD of Brazilein was stable at 1 ng when the DSASS concentration increased in the HPLC–UV system. This indicated that DSASS might not affect the analytical sensitivity of Brazilein in the HPLC–UV system. Considering the ion-pair relationship between DSASS and TBAOH, run buffers without DSASS and TBAOH (Na_2CO_3–NaH_2PO_4, 75mM:1mM, pH 3.0) were then examined. The results showed that the LOD of Brazilein could also reach 1 ng. This further confirmed that although DSASS was important in the HPLC–ED system, it might be unnecessary for achieving an acceptable LOD for Brazilein in the HPLC–UV system.

Concentration of NaH_2PO_4

Excluding the effects of DSASS and TBAOH, the effects of NaH_2PO_4 and Na_2CO_3 were then examined. NaH_2PO_4 was examined first. The effect of NaH_2PO_4 concentration on the retention time and LOD of Brazilein (obtained at a 3:1 signal-to-noise ratio) was examined to obtain a suitable mobile phase concentration. A pH meter was used to measure the pH of the mobile phase, which was maintained at pH 3.0 throughout the experiment. Table I shows the relationships between the retention time and LOD of Brazilein and NaH_2PO_4 concentration. As the NaH_2PO_4 concentration increased, the LOD of Brazilein increased and reached a maximum at 20mM NaH_2PO_4. Moreover, as the NaH_2PO_4 concentration changed, the UV–vis spectrum of Brazilein still showed prominent maximum absorptions at 445 and 556 nm. However, the retention time of Brazilein remained relatively constant when the NaH_2PO_4 concentration changed. Accordingly, 20mM NaH_2PO_4 was employed for later work in order to retain maximum detection sensitivity of Brazilein in the HPLC–UV system.

Concentration of Na_2CO_3

Na_2CO_3 was used as the inorganic modifier to improve the peak symmetry of Brazilein caused by DSASS in the HPLC–ED

system. Four concentrations of Na_2CO_3 (0, 0.5, 1.0, and 2.0mM) were examined by HPLC–UV. The results showed that the concentration of Na_2CO_3 had no effect on the LOD of Brazilein in the HPLC–UV system. Moreover, Na_2CO_3 exists as phosphate at pH 3.0 in solution.

It was concluded that in the HPLC–UV system, it was NaH_2PO_4, not DSASS, that influenced the analytical sensitivity to detect Brazilein. NaH_2PO_4 might affect the degree of dissociation of Brazilein, changing its UV signal response. By comparing the HPLC–ED system with the HPLC–UV system, the LOD of Brazilein was influenced by different chemicals in different HPLC systems. This was because the redox and UV–vis absorption of Brazilein required different functional groups, respectively. Meanwhile, DSASS and NaH_2PO_4 might influence different functional groups of Brazilein.

Table II. Comparisons of Mobile Phase Composition, RSD, Analytical Range, LOD, and Recovery of Brazilein Obtained by Proposed HPLC–UV Method and Published HPLC–ED Method

	HPLC–ED (8)	HPLC–UV
RSD	5%	2.5%
Analytical range	0.6~150 ng	1~120 ng
LOD	0.6 ng	1.1 ng
Recovery	over 92%	over 94%
Mobile phase composition	DSASS–TBAOH–Na_2CO_3–NaH_2PO_4	NaH_2PO_4

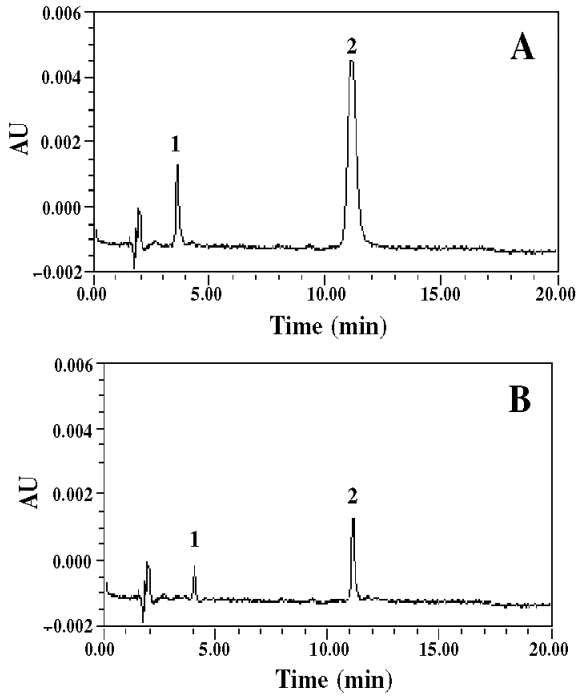

Figure 1. Chromatogram of Brazilein in the extract of *Caesalpinia sappan* L. heartwood. A and B are chromatograms for samples 1 and 2, respectively. Peak 2 represents Brazilein. The experimental conditions were: a Kromasil-C_{18} HPLC column (5 µm, 150 × 4.6 mm); room temperature; UV filter, 445 nm; flow rate, 1.0 mL/min; and mobile phase, NaH_2PO_4 (20mM, pH 3.0): ACN (80:20, v/v).

Influence of organic modifier present in mobile phase

The amount of ACN in the mobile phase significantly affected the retention time and LOD of Brazilein. When the ACN to inorganic run buffer ratio increased from 15:85 to 25:75, the retention time of Brazilein was shortened gradually, though the LOD of Brazilein remained constant as before. But when the ratio decreased to 10:90, no UV signal response of Brazilein could be detected. Although a high ACN to inorganic run buffer ratio can shorten the analysis time, the optimal ratio necessary to attain the highest level of resolution and shortest analysis time was concluded to be 20:80.

Precision, linearity, and LOD 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 1–120 ng ($y = 2452.901x - 26913.9$, $r = 0.999904$, where y is the peak area and x is the amount of standard Brazilein in ng). Intra- and inter-day precisions (expressed in terms of RSD) for peak area were typically less than 2.5% ($n = 7$). The LOD was 1 ng per injection (20 µL) at a signal-to-noise ratio of 3:1. Differences between both HPLC systems on mobile phase composition, RSD, analytical range, LOD, and recovery of Brazilein are summarized in Table II.

Plant sample analysis

The chromatograms of Brazilein in real plant samples were also examined (Figure 1). The chromatograms were much more concise than those obtained by the HPLC–ED system. This was because many components of the plant extract did not exhibit UV–vis signal response under the mobile phase composition at the wavelength of 445 nm. Peak identification was carried out by the standard addition method. The Brazilein content of two plant samples calculated by the calibration formula was 38.6 and 425.8 mg/g, respectively. A known amount of Brazilein was added to the plant extract, and overall recoveries were estimated by the standard addition method. Brazilein recovery was over 94% by the standard addition method (Table III). This demonstrated that the application of the proposed HPLC–UV method for the determination of Brazilein in plant samples was possible.

Table III. Recovery of Brazilein Added in Real Plant Sample

Added (mg/g)	Found (mg/g)	Recovery (mg/g)	Recovery %
0	38.6*	–	–
5	43.3*	4.70	94%
10	48.18*	9.58	95.8%
20	57.56*	18.96	94.8%

* RSD = 2.5% ($n = 7$)

Conclusion

The effect of each component in the mobile phase of the HPLC–ED system was further investigated by applying and evaluating the effect of each component with the HPLC–UV system. According to the experimental results, NaH_2PO_4 could improve the UV–vis absorption of Brazilein at the wavelength of 445 nm. Meanwhile, a much more convenient HPLC method was established for the analysis of Brazilein. This new method rectified the time-consuming disadvantage of the HPLC–ED method. Moreover, the high sensitivity of this HPLC–UV method makes it possible for its further application in biological analysis. Because the UV–vis detection does not destroy the analytical samples, this HPLC–UV system will be suitable for the isolation and purification of Brazilein on plant study.

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