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Simultaneous Determination of Bromhexine HCl and Baicalin in Chinese Compound Medicine by a Reversed-Phase Ion-Pair HPLC

SHUJUAN LIU, LIANG ZHAO, XIA LIU, FENG ZHOU AND SHENGXIANG JIANG*

Key Laboratory for Natural Medicine of Gansu Province, Lanzhou Institute of Chemical Physics, CAS, Lanzhou 730000, P. R. China

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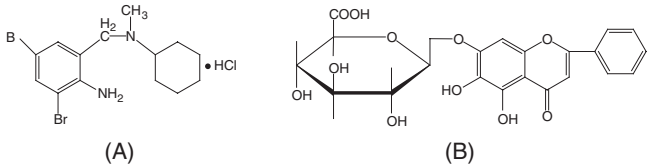
ABSTRACT

In this article, a reversed-phase ion-pair high-performance liquid chromatography (HPLC) method was established for simultaneous separation and quantitative analysis of bromhexine HCl (BHH) and baicalin in Chinese Ke-Chuan-Ling compound capsule. The influence of the mobile phase composition on the retention of the analytes was investigated. The optimal determination conditions consisted of acetonitrile/water/phosphoric acid/triethylamine (22/78/0.1/0.1, v/v/v/v) mobile phase with the addition of tetra-*n*-butylammonium bromide (TBA) (0.7 mmol L^{-1}, terminal concentration) as pairing ion, and a detection wavelength at 225 nm. In this case, baicalin and bromhexine HCl were eluted at 21 min and 27 min respectively and were separated successfully from other ingredients. Excellent linear relationship between the peak area and concentration was obtained in the range of 0.2~15.0 μg mL^{-1} for bromhexine HCl and 0.1~12.0 μg mL^{-1} for baicalin. The limit of detection of BHH was 0.1 μg mL^{-1} and it was 0.05 μg mL^{-1} for baicalin. The average recoveries of bromhexine HCl and baicalin were 97.7% and 99.1%, respectively. From the results it can be concluded that this method is simple, versatile, and reliable for the quality control of Chinese traditional medicines containing baicalin and/or bromhexine HCl.

Key words: pharmaceutical analysis, HPLC, bromhexine HCl, baicalin

INTRODUCTION

Ke-Chuan-Ling capsule is a traditional Chinese compound medicine for the treatment of cough, chronic bronchitis, pneumonia and asthma. This capsule consists of bromhexine HCl (BHH, chemical structure showed in Figure 1-A), *Scutellaria baicalensis* Georgi (Huangqin) and other Chinese medicinal herbs. Huangqin is an important Chinese medicinal herb widely used for the treatment of various inflammatory diseases[1], hepatitis, pyrexia[2] and tumors[1,3] in East Asian countries. Baicalin (chemical structure showed in Figure 1-B) is the main active component in Huangqin and has similar functions as Huangqin. BHH, an important mucolytic and expectorant drug used to cater for the therapeutic needs of cough and common cold[4], cooperates with other active components in this medicine. Owing to the complexity of this medicine, baicalin and BHH are chosen as the criterions for quality control. Therefore, it is essential to establish a method for the determination of these analytes. So far, baicalin and BHH have been investigated and determined separately using different analytical methods such as spectrophotometry[5-6], gas chromatography[7], atomic absorption spectrometry[8], extraction-flow injection method[9], potentiometric titration[10], thin-layer chromatography[5,11], liquid chromatography (LC)[12] and capillary electrophoresis[4,13-15], etc. Because of serious interference, most of these methods require many tedious clean up procedures such as distillation, liquid-liquid extraction and solid phase extraction as needed for the drugs. HPLC method is one of the simplest approaches to assay and monitor the drugs due to its accuracy and it does not require prior conversion of the drugs to the base form. However, it is difficult to get appropriate retention of both analytes on one column using ordinary mobile phase, because BHH is a methanol-soluble basic compound which cannot dissolve in water. On the other hand, baicalin is an acidic compound that is water-soluble. Moreover, interference by other components in the drug during the separation of analytes is another important factor that should be considered. Therefore, it is necessary to employ a reversed-phase ion-pair chromatography (RP-IPC) that is very suitable for the separation of ionic compounds. In this article, we optimize the separation by studying the influence of the various factors that affect the separation by a reversed-phase ion-pair chromatography. Under the optimal condition, BHH and baicalin get baseline separation without any interference of other components.



(A) (B)

Figure 1. Chemical structures of bromhexine HCl (A) and baicalin (B).

* Author for correspondence. Tel: 0931-4968205;
Fax: 0931-8277088; E-mail: lsjzf76@yahoo.com.cn

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MATERIALS AND METHODS

I. *Chemicals*

Standard BHH and baicalin are of AR grade and provided by Lanzhou Hesheng-Tang Medicine Factory; acetonitrile is of HPLC grade; tetra-*n*-butylammonium bromide (TBA), tetra-*n*-pentylammonium bromide (TPA), sodium 1-heptanesulfonate (SHS) (Tokyo Kasei, Kyogo, Japan), phosphoric acid and triethylamine are of AR grade. De-ionized water was used. The mobile phases were filtered through a 0.45-μm filter prior to use.

II. *Apparatus*

A Model 441 HPLC (Waters, USA) with a Rheodyne Model 7125 manual injection system fitted with a 20-μL sample loop, and a SPD-6AV type of UV-visible spectrophotometer (Shimadzu, Japan) as the detector were coupled to an HW chemical station (Nanjing, China). All separations were achieved on a C18 column (250 × 4.6 mm i.d., 5 μm, IBM instruments Inc.).

III. *Standard Preparation*

A solution containing 200 μg mL^{-1} BHH and 200 μg mL^{-1} baicalin in methanol was prepared and used as the standard mixture for HPLC analysis.

IV. *Sample Preparation*

Contents of the capsule (0.4 g) were ground into powder and mixed with 30 mL of methanol. After 40 min of ultrasonic vibrations, the mixture was filtrated through a glass filter. The residue was rinsed twice with 10 mL of methanol and the filtrates were mixed into the volumetric flask and diluted to 50 mL with methanol for HPLC analysis. Ethanol, water and acetonitrile were also used as the extraction solvents for comparison. The extracts were directly injected into the HPLC system.

V. *Chromatographic Conditions*

The selected mobile phase consisted of 22% (v/v) acetonitrile, 0.7 mmol L^{-1} TBA, 0.1% (v/v) phosphoric acid and 0.1% (v/v) triethylamine, with a pH of about 2.50. Owing to the different maximum absorbance (BHH is at 249 nm, and baicalin is at 278 nm), a wavelength of 225 nm was selected for reasonable sensitivity. The flow rate was 1.0 mL min^{-1} and the injection volume was 20 μL.

RESULTS AND DISCUSSION

I. *Effect of the Mobile Phase Composition*

The experimental variables optimized to accomplish adequate separation in eluting the analytes were the composition of the mobile phase. Among the different mobile phases tried, methanol/water (50/50, v/v) with the addition of 0.2% (v/v) H_3PO_4 was found to elute baicalin well, but eluted BHH as a distorted peak. Contrarily, BHH was eluted as a sharp peak with desired retention time in methanol/water (85/15, v/v) mobile phase containing 0.1% of triethylamine. However, in this mobile phase, baicalin was eluted very quickly within 5 min and thus was disturbed by the matrix. Therefore, the problem was how to prolong the retention time of baicalin and ensure appropriate resolution of both analytes. It was shown that the addition of positively charged ion-pair agent, i.e. TBA, to the mobile phase consisting of water/methanol /H_3PO_4/triethylamine (50/50/0.1/0.1, v/v/v/v) could significantly improve the separation with good peak shape and desired retention of both analytes. Furthermore, it was convenient to adjust the retentions of the analytes to avoid the disturbance of excipients, which would be further improved by replacing methanol with acetonitrile in the mobile phase.

II. *Effect of the Concentration of TBA*

TBA at different concentrations from 0.5 mmol L^{-1} to 25 mmol L^{-1} ensured the elution of the two analytes (Figure 2). The pairing ion, TBA^+, has great interaction with acidic compounds, such as residual silanol groups on the surface of silica column and baicalin. TBA^+ was adsorbed on the column through the hydrophobic alkyl chain and made the column a positively charged surface. Furthermore, more TBA resulted in more positive charges assembled on the column. In this case, baicalin was strongly adsorbed on the column via electrostatic interaction with adsorbed TBA^+, resulting in longer retention time of baicalin. On the other hand, if there was no TBA in the mobile phase, BHH was strongly adsorbed on the column due to the non-specific interaction with the residual silanol groups on the column. Due to the electrostatic repulsion with positively charged column and effective shielding of free silanol by the pairing ion, BHH exhibited a dramatic loss in retention time. It can be seen that the increase in TBA concentration up to 2.5 mmol L^{-1} weakened the retention of baicalin and at this point a maximal retention time occurred as shown in Figure 2. In that case, a saturated adsorption of TBA^+ on the column was achieved and resulted in equilibrium between adsorbed TBA^+ and baicalin, as well as between TBA^+ and BHH. Increasing in the ion-pair concentration to over 2.5 mmol L^{-1} would cause a little change in retention of BHH, but decreased retention time for baicalin due to the competitive adsorption on the column and the increased counterions (that was Br^- in this experiment).

It also can be seen that at the intersection of these two curves (about 1.0 mmol L^{-1} TBA, Figure 2), BHH and baicalin could not be separated from each other. Meanwhile, at over 1.5 mmol L^{-1} TBA, BHH would be interfered by the excipient matrices or other analytes. In

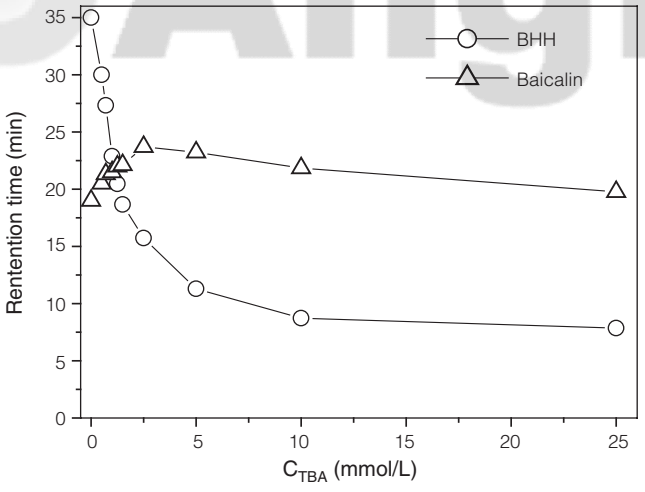

Figure 2. Effect of TBA concentration on the retention. Mobile phase at different concentration of TBA in acetonitrile/water/H_3PO_4/triethylamine (22/78/0.1/0.1, v/v/v/v); flow rate of 1.0 mL min^{-1}; detection at 225 nm on an UV/vis detector.

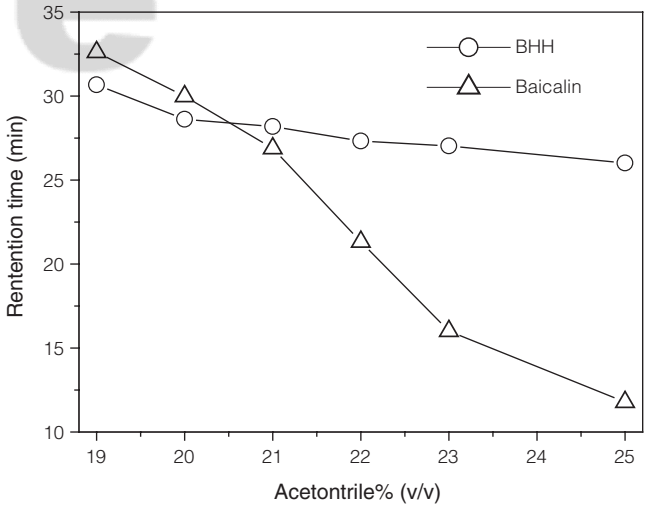

Figure 3. Dependence of retention time with acetonitrile content in mobile phase. Mobile phase, different content of acetonitrile in water with 0.1% H_3PO_4, 0.1% triethylamine and 0.7 mmol L^{-1} TBA as the additives. See Figure 2 for other effects.

this study, 0.7 mmol L^{-1} TBA in the mobile phase was chosen for the efficient separation.

III. *Effect of Acetonitrile Content*

Figure 3 shows the dependence of retention time of the analytes on acetonitrile content in the mobile phase. It can be seen that the increase of acetonitrile content caused rapid decrease in the retention time of baicalin but slight downtrend in BHH. More organic solvent in the mobile phase could weaken the adsorption of TBA^+ on the column, resulting in selectively sharp decrease in retention of the oppositely charged solute, i.e. baicalin. Therefore, the organic solvent content altered the selectivity as well as the resolution of the two analytes. Acetonitrile content below 21% would deteriorate the resolution. It can be seen that the peak symmetry decreased considerably with decreasing acetonitrile content. In addition, a tendency for tailing to occur was observed for both analytes and gave asymmetric peaks below an acetonitrile content of 20%. Apparently, the control of retention of baicalin could be improved by altering the acetonitrile content in the eluent. In conclusion, a concentration of 22% acetonitrile was selected for the best separation, under the condition that a relatively high resolution and no disturbance in the separation would occur.

IV. *Effect of Triethylamine Content*

It had been demonstrated that triethylamine could improve the peak shape of basic compounds on account of its ability to shield residual silanols. If no triethylamine was added, BHH was eluted as a distorted and broadening peak. When increasing the content of triethylamine, both BHH and baicalin exhibited slight change in retention (Figure 4) because slight increase in triethylamine content

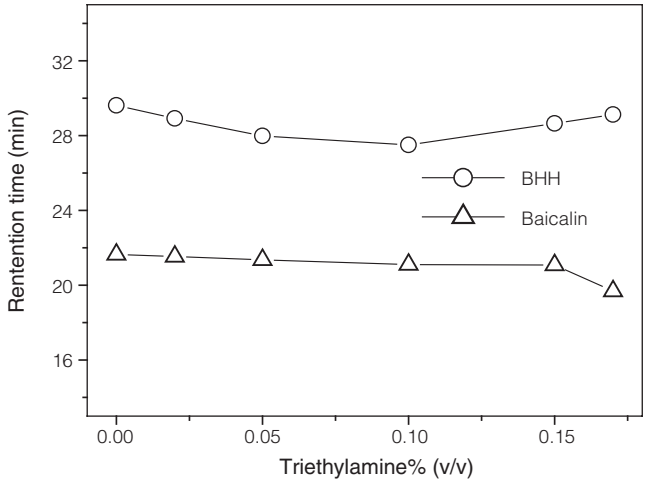

Figure 4. Effect of triethylamine content on the retention. Mobile phase, different percentage of triethylamine in acetonitrile/water/H_3PO_4 (22/78/0.1, v/v/v) with the addition of 0.7 mmol L^{-1} of TBA. See Figure 2 for others.

resulted in a small change in pH due to the presence of H_3PO_4. 0.1% triethylamine was selected because relative minimal retention time and better peak shape for BHH were achieved at this point. Under the above conditions, baicalin and BHH were eluted at 21 min and 27 min, respectively (See Figure 5-A).

V. *Influence of Different Ion Pair Reagents*

The nature (type and chain length of the hydrophobic group) of the pairing ion is an important parameter in the retention of ionic solutes in RP-IPC, so we chose two different ion-pair agents to investigate their influences. Appropriate amounts of TPA and SHS were separately added into the mobile phase without changing other condi-

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Table 1. Linearity and recovery of each substance and its corresponding equation

Comp.	Equation of calibration curve	Correlation coefficient	Concentration range (μg mL^{-1})	Detection limit (μg mL^{-1})	Mean content (μg per capsule)	Recovery (% ± SD, n = 5)	Reproducibility (RSD%)
BHH	Y = 24639.85 X - 695.58	R = 0.9996	0.2–15.0	0.1	776	97.7 ± 1.51	1.30
Baicalin	Y = 108738.81 X - 5167.6	R = 0.9997	0.1–12.0	0.05	1800	99.1 ± 0.53	0.72

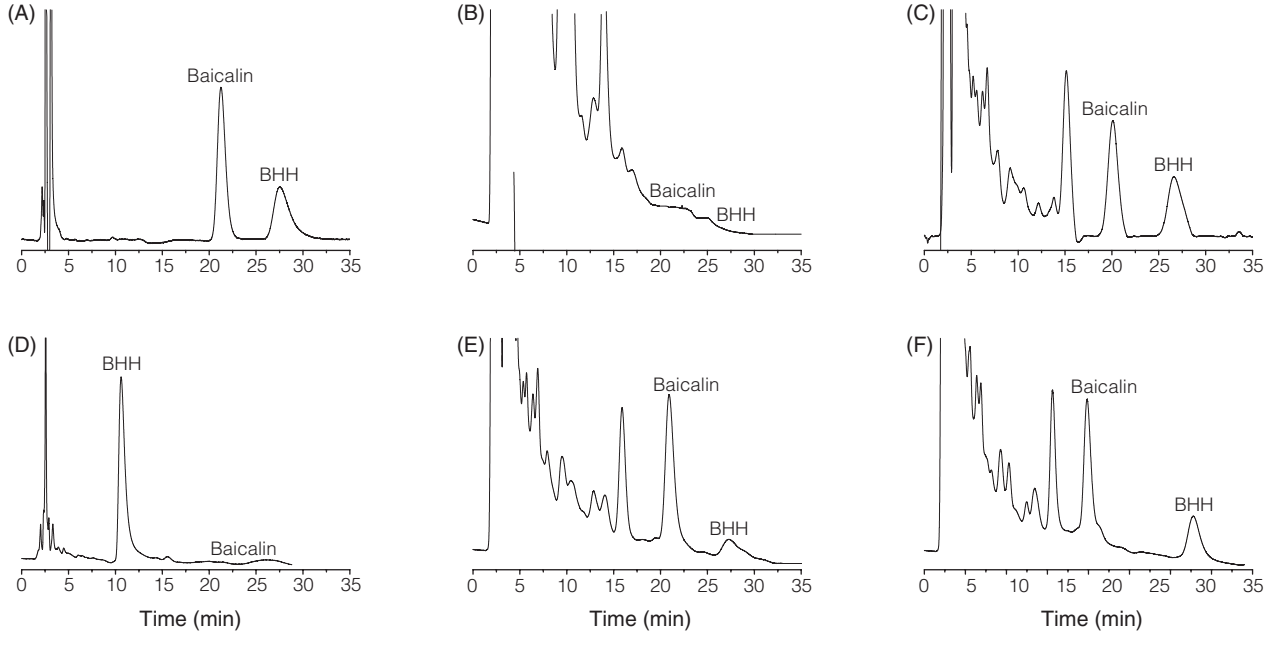

Figure 5. Representative chromatograms of baicalin and BHH. (A) standard analytes; (B)blank sample, using methanol as the extraction solvent; (C) sample, using methanol as the extraction solvent; (D) acetonitrile as the extractant for sample; (E) water as extractant for sample; (F) ethanol as extractant for sample. Chromatographic condition: (A), (B), (C) and (E) were under the optimal condition; (D) 5.0 mmol L^{-1} TBA was added in mobile phase other than 0.7 mmol L^{-1}. See Figure 2 for others; (F) 0.5 mmol L^{-1} TBA was used other than 0.7 mmol L^{-1}. See Figure 2 for others.

tions. The results indicated that SHS had contrary roles to TBA on the two analytes. For baicalin, its retention time decreased when the concentration of SHS increased due to the electrostatic repulsions. Meanwhile, SHS deferred the elution by adsorbing BHH. Such result would not be appropriate for the analysis and separation of the analytes.

It had been demonstrated that the retention of oppositely charged solute usually increases with increasing chain length of the pairing ion given identical condition. The same phenomenon was observed in this experiment. TPA had similar effect on the retentions and needed less concentration to attain the same result as that with TBA since TPA was more hydrophobic than TBA. To certain extent, TPA may be an alternative to TBA as they have similar effect on the selectivity and separation.

VI. *Selection of Extraction Solvent*

Figure 5 shows the representative chromatograms of different solvents as extractants. Using ethanol or water as the extraction solvent, much less BHH was extracted than that using methanol or acetonitrile, indicating that BHH was slightly soluble in ethanol and water. Contrarily, more baicalin was extracted using methanol, water or ethanol than that using acetonrtrile, demonstrating that acetonrtrile was not suitable for the extraction of baicalin. Comparing the four different extractants, methanol was the best extractant for both analytes.

VII. *Linearity*

Aliquots (2.5, 5, 10, 15 and 20 mL) of the standard solution were transferred into the volumes and diluted to 25 mL with methanol separately. The following results were obtained at the optimal separation conditions. The calibration curves were prepared by plotting the areas of BHH and baicalin versus their concentrations, respectively. The regression equations, correlation coefficients, linear response ranges, detection limits as well as the average content of BHH and baicalin in one capsule are shown in Table 1.

VIII. *Recoveries and Reproducibility*

Certain amounts of BHH and baicalin were added to the actual sample containing known quantities of BHH and baicalin and then the mixture was ground into powder. After 40 min of ultrasonic vibrations in methanol, the filtrates were then determined by HPLC directly. The standard deviations (n = 5) and the mean recoveries of

BHH and baicalin are presented in Table 1. It can be seen that the method has high recoveries for the analytes.

Five samples with same quantity derived from the same batch were prepared into solutions according to above sample preparation procedure. By injections of the solution of these samples, the relative standard deviations (RSD) of the chromatographic procedure were determined. The results are shown in Table 1 indicating high reproducibility of the method.

CONCLUSIONS

The proposed method for the chromatographic separation and determination of BHH and baicalin allows the determination and convenient control of this drug in pharmaceuticals. TBA as the pairing ion influenced the behavior of baicalin and BHH, making desirable elution and simultaneous determination possible. The reversed-phase ion-pair HPLC method described for the analysis of BHH and baicalin is simple, rapid and precise, with the following advantages: 1. it is an isocratic HPLC system; 2. no pretreatment is required except for extraction; 3. the simultaneous determination of both analytes is possible for the first time. Therefore, this method can easily be applied to other BHH and/or baicalin formulations, as well as separation of BHH and/or baicalin biological materials.

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