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Separation of the Ephedra Alkaloids by RPLC

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ABSTRACT

High-performance liquid chromatography with ODS columns has been the most popular technique in analyzing the Chinese herbal drugs. (–)-Ephedrine and (+)-pseudo-ephedrine are the major index components in Ephedrae Herba. In order to separate these two alkaloids, three ODS columns were evaluated. The relationships between SDS concentrations, brands, organic solvent ratios and pH values and retention times of the ephedrine and pseudoephedrine were also studied.

Key words: high-performance liquid chromatography, ephedrine, pseudoephedrine

INTRODUCTION

Ma-hung (Ephedra), a dry chamaephyte derived from Ephedrae Herba, is a traditional Chinese medicinal herb used to induce perspiration, reduce fever, treat coughing, and manage asthma[(1)]. It contains the following bioactive components: (–)-ephedrine **(E)**, (+)-pseudo-ephedrine **(PE)**, (–)-methylephedrine, (+)-methylpseudoephedrine, (–)-norephedrine, and (+)-norpseudoephedrine[(2, 3)].

(–)-Ephedrine and (+)-pseudo-ephedrine are the two major medicinal components in ephedra. Their chemical structures are shown in Figure 1. The methods for analysis of ephedra alkaloids are well documented. These include thin-layer chromatography (TLC)[(4, 5)], gas chromatography (GC)[(6)], carbon 13-NMR[(7)], isotachophoresis (ITP)[(8)], high performance liquid chromatography (HPLC)[(9-14)], and capillary electrophoresis (CE)[(15)]. Separation of the above six bioactive components is unable to be achieved by using TLC, GC, NMR, and ITP. Using HPLC is capable of separating 3~6 alkaloids; however, some improvements still need to be accomplished. Although the above 6 alkaloids can be efficiently separated by CE, HPLC has by now been used most often for ephedra alkaloids analysis. Unsatisfactory results in theoretical plate and resolution are a problem that needs to be overcome through the use of HPLC for ephedra alkaloids analysis. The reproducibility on routine analysis is also a problem. In 1996, Sagara *et. al.*[(14)] performed a HPLC method capable of quantitative determination of ephedrine and pseudo-ephedrine by using a reverse phase column and a mobile phase of acetonitrile and sodium dodecyl sulfate (SDS). In our preliminary study of developing the HPLC methodology for Ma-hung herbal analysis, we found that the brand and concentration of SDS significantly affects the separation of ephedra alkaloids. The purpose of this study was to research the effect of some analytical parameters on HPLC separation of ephedra alkaloids.

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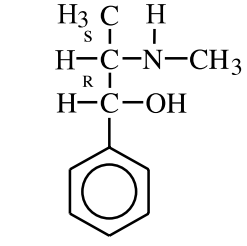
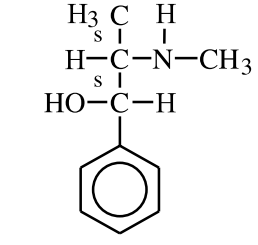
MATERIALS AND METHODS

I. *Reagents*

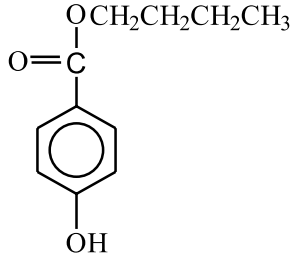
Ephedrine hydrochloride and pseudo-ephedrine hydrochloride were purchased from Aldrich (Milwaukee,



l-ephedrine **(E)**



d-pseudoephedrine **(PE)**



butyl p-hydroxybenzoate **(BPB)**

Figure 1. Structures of the two marker substances and the internal standard.

WI, USA). Phosphoric acid was obtained from Merck (Dermstadt, Germany). Butyl p-hydroxybenzoate (BPB) is the product of Nacalai Tesque (Kyoto, Japan). Sodium hydroxide was purchased from Sigma (St. Louis, MO, USA). SDS was purchased from either Merck or Sigma. A LC grade methanol and acetonitrile were from Fison (Loughborough, England). Deionized water was produced through a Milli-Q system (Millipore, Bedford, MA, USA). Ma-hung medicinal herb was purchased from a vendor on Di-Hua Street, Taipei.

II. *Instruments and Analytical Conditions*

HPLC pump: Waters 510 × 2
Injection valve: Waters U6K
Mobile phase controller: waters 680 automated gradient controller
Detector: Shimadzu SPD-M10AVP photodiode array detector
Data processor: Shimadzu CLASS-LC10
Guard column: μ-bondapak^{TM}C$_{18}$ (Millipore, Milford, MA, USA)
Analytical column: Cosmosil 5C$_{18}$-MS, 5 μm, 25 cm x 4.6 mm (Nacalai Tesque, Kyoto, Japan)
Mobile phase: 25 mM SDS (adjusted to pH 4.0 by 0.1% phosphoric acid): acetonitrile (65/35, v/v)
Flow rate: 0.8 mL/min
Detection wavelength: 210 nm

III. *Methods*

(I) *Selection of Analytical Column*

Three C$_{18}$ analytical columns with different types of packing materials (Table 1) were selected. The above mobile phase was used and the injection was performed in duplicate to study the effect of different types of packing materials of analytical column on separation efficiency.

(II) *Study on the Brands and Concentrations of SDS in Mobile Phase*

A suitable analytical column was selected and the mobile phases containing 0, 5, 7.5, 10, 12.5, 15, 17.5, 20, 25, 30, 40, 50, 60, 75, 90, 100, 110, and 125 mM SDS (Merck) were tested. The injection for each concentration was performed in duplicate.

A Sigma brand SDS with the concentrations of 5, 10, 15, 20, and 25 mM in mobile phase was tested. The analytical method and duplicate injections were performed as mentioned above.

(III) *Study on Acetonitrile Ratio in Mobile Phase*

Because of salt, the mobile phase should contain a proper amount of water to prevent salt precipitate and to obtain a more stable baseline. The following ratios of SDS solution/acetonitrile in mobile phase were tested: 70/30, 65/35, 60/40, 55/45, 50/50, 45/55, 40/60, 35/65, and 30/70. The analytical method and duplicate injections were performed as mentioned above.

(IV) *Study on the pH Effect of Mobile Phase*

A suitable concentration of SDS was selected and the mobile phases with pHs in the range of 2.0~8.0 were adjusted using phosphoric acid and ammonia water. The analytical method and duplicate injections were performed as mentioned above.

IV. *Suitability Evaluation of Analytical Conditions*

An internal standard solution (IS) was prepared by dissolving 100 mg BPB in 100 mL of 50% methanol and a standard stock solution was prepared by dissolving 50 mg ephedrine and 50 mg pseudo-ephedrine in 100 mL of 50% methanol.

(I) *Reproducibility*

The standard stock solution (2 mL) and IS (1 mL) were spiked into a 10-mL volumetric flask and the 50% methanol was then added to the volume. The test solution was thus prepared. The intraday and interday analyses were performed separately 6 times using the optimum HPLC condition.

(II) *Detection Limit*

A proper series of dilutions were injected to HPLC and the detection limit was determined based on the signal to noise ratio (S/N ratio) of 3.

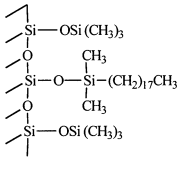
Table 1. Different packing material and type of column

Name[a]	Pore size (Å)	Surface area (m2/g)	Ratio of carbon (%)	Bonding type[b]
Cosmosil 5C$_{18}$	110	330	20	mono
Cosmosil 5C$_{18}$-MS	120	300	16	mono
Cosmosil 5C$_{18}$-AR	120	300	16	polymer

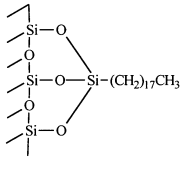
[a] Three columns were from Nacalai Tesque (Kyoto, Japan).
Particle size, 5 μm; Column length, 25cm; Column diameter, 4.6 mm

[b]

mono



polymer



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RESULTS AND DISCUSSION

Three ODS analytical columns with different types of packing material from the same producer were tested in this study. As shown in Figure 2, the peaks of interest appeared in the same order and the tendency of peak mobility was identical but a slight difference in retention times was observed using the above 3 different columns. Of these, the C$_{18}$ column

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contains more carbons so as to be able to retain peaks of interest much longer than the others. The shorter retention times, but with lower and wider peaks were observed using the C_{18}-AR, which also showed a lower theoretical plate than the C_{18}-MS column. Therefore, the C_{18}-MS column was selected to be the analytical column for further study.

The effect of Merck and Sigma brands SDS on retention times is shown in Figure 3. SDS containing a negative ion is able to react with ephedra alkaloids to form neutral ion pair complexes, which can be well retained and separated in the C_{18} column. Nevertheless, the retention times of ephedra alkaloids could vary using different brands of SDS reagent. This could be due to the impurities in SDS reagents; however, further study needs to be carried out to explain this result. Figure 4 demonstrates how the concentrations of SDS change the orders of internal standard (BPB) and ephedra alkaloid peaks. The peaks of ephedrine, pseudo-ephedrine, and BPB accompanied with a solvent peak appeared at around 5 min as using the mobile phase of 35% acetonitrile without the addition of SDS. The BPB peak appeared after ephedra alkaloid peaks when an SDS concentration of less than 12.5 mM was used; while the BPB peak came before ephedra alkaloid peaks when more than 15 mM SDS was added. SDS could enhance the ion strength of the mobile phase resulting in extending the retention times of peaks. Continuing the increase of SDS concentration could allow an ion pair complex between SDS (an anion) and ephedra alkaloids (base compounds) to be formed and therefore increase the retention times of the ephedra alkaloids. However, a physical bonding by Van der Waals forces between SDS and the carbon of packing material in the reverse phase column could occur as increasing the concentration of SDS. The excess SDS covers the functional groups of packing material and the hydrophilic group ($-OSO_3^-$) of SDS is exposed enabling a normal phase-like property to be performed. This mechanism explains why the BPB peak appeared before the ephedra alkaloid peaks when SDS concentration increased, and its retention time decreased as there was a continuing increase in SDS concentration. The following formula can elucidate the above phenomenon:

$$CH_3(CH_2)_{11}\text{-}OSO_3^- + \text{Si-}C_{18} \rightarrow \text{Si-}C_{18} /\!/ CH_3(CH_2)_{11}\text{-}OSO_3^-$$

Instead of a C_{18} group which exhibits a hydrophobic

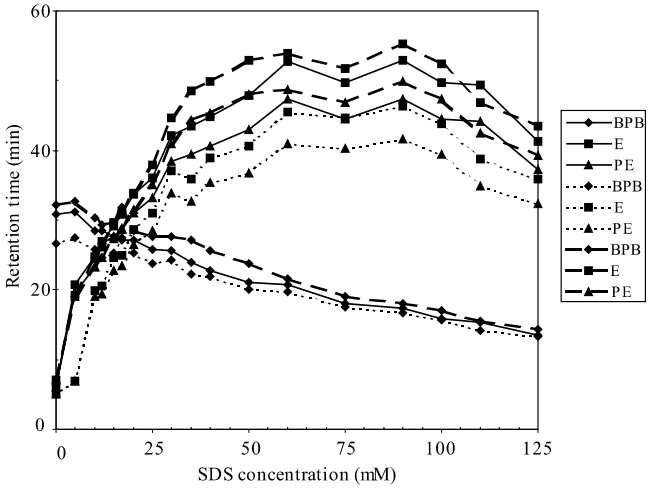

Figure 2. Effect of SDS concentrations on retention times with different columns.
(—: C_{18}-MS; ····· : C_{18}-AR; ——: C_{18})

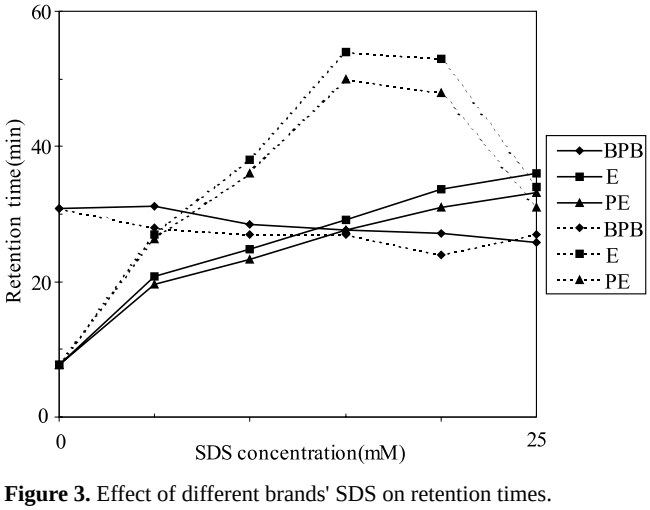

Figure 3. Effect of different brands' SDS on retention times.
(—: SDS from Merck; ····· : SDS from Sigma)

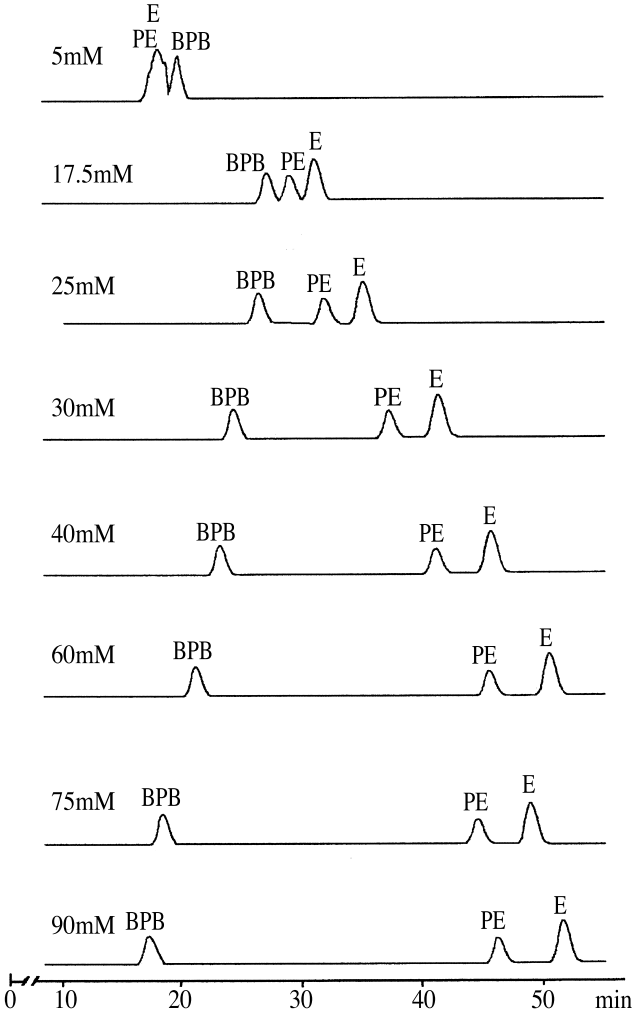

Figure 4. HPLC chromatograms with different SDS concentrations. (Merck)

property, a new active layer with negative ion or polar group was exposed. The ephedra alkaloids then bond to Si-C_{18} // $CH_3(CH_2)_{11}$-OSO_3^- via an ion pairing , ion exchange and/or ion interaction reactions. The ephedra alkaloids are thus separated based on the differences of the above bonding ability. Increasing the SDS concentration is more likely to increase the above bonding ability. However, the overload SDS in organic solvent could cause a salting out effect that could damage the analytical column resulting in decreasing the peak resolution. An optimal concentration at 25 mM was thus selected to perform a study on effects of organic solvent and pH value.

Under a reverse phase analytical condition, increasing the organic solvent portion in the mobile phase can decrease the capacity factor (K') so as to keep the less polar compounds from being retained in the analytical column. The maximum absorption of ephedra alkaloids was determined to be 210 nm, which was therefore used as the UV wavelength for detection. The mobile phase containing methanol could result in an unstable baseline, thus acetonitile was used to replace methanol. Results showed that the retention times of compounds decreased with increasing the acetonitile portion in the mobile phase. As more than 45% acetonitrile as the mobile phase was used, the six ephedra alkaloids were eluted in 6 min because the SDS ion-pairing effect was dismissed. When less than 30% acetonitrile was used, the ephedra alkaloids were retained in the column for more than 100 min. A mobile phase of 35% acetonitrile was therefore used for analysis in this study.

The effect of mobile phase pH at 2.0, 3.0, 4.0, 5.0, 6.0, 7.0 or 8.0 on the capacity factor is shown in Figure 5. As can be seen, the mobility of peaks is not significantly different between pH 2.0~5.0. At pH higher than 6.0, retention times increased significantly together with a drifting baseline. Therefore, a lower pH value was favorable for analysis.

The intraday and interday analyses were performed 6 times individually, to evaluate the reproducibility of the developed method using a mobile phase of 35% acetonitrile in 25 mM SDS solution adjusted to pH 4.0 by phosphoric acid. The relative standard deviation (RSD) of retention times and detection limit were calculated as shown in Table 2. The RSDs of ephedrine and pseduo-ephedrine in intraday analysis are 0.03 and 0.04%, respectively, and in interday analysis are 0.05 and 0.06%, respectively, indicating that a satisfactory reproducibility in retention times can be performed by using the developed method. The detection limits of ephedrine and pseudo-ephedrine were determined to be 0.35 and 0.39 μg/mL, respectively.

CONCLUSION

Ephedrine and pseudo-ephedrine are the two major ephedra alkaloids used as the index components for determining the quality of ephedra raw material and its products. Analysis of the above two compounds can be achieved by using a reverse phase analytical condition. The resolution of the peaks is more likely affected by the packing materials in analytical column, SDS brands and its concentration, and acetonitrile content in mobile phase, but less affected by the pH value of the mobile phase.

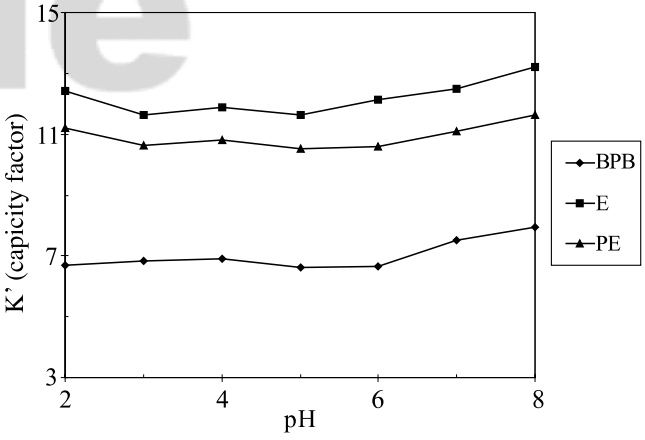

Figure 5. Effect of pH on capacity factor (K').

Table 2. Data for reproducibility of retention time and detection limit

	Reproducibility (n=6)		Detection limit
Compound	Intraday RSD(%)	Interday RSD(%)	(μg/mL)
E	0.03	0.05	0.35
PE	0.04	0.06	0.39

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麻黃生物鹼之逆相層析探討

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摘　　要

搭配ODS管柱的逆相高效液相層析(RPLC)是目前最常用來分析中藥材指標成分的技術。本文旨在以三不同ODS材質的逆相層析管柱，探討不同SDS(sodium dodecyl sulfate)濃度與品牌、有機溶劑含量及酸鹼值對麻黃指標成分麻黃素((—)-ephedrine)與偽麻黃素((+)-pseudoephedrine)之滯留時間的影響。

關鍵詞：高效液相層析，麻黃素，偽麻黃素