Arch. Pharm. Res. Vol.21, No.5, pp. 565-569, 1998

Enantiomeric Ratio of Shikonin Derivatives as a Possible Key for the Determination of the Origin of Lithospermi Radix

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(Received April 27, 1998)

An HPLC method was developed to resolve the enantiomers of shikonin derivatives of the Lithospermi Radix. The optimum mobile phase on a Chiracel AD column was 5% isopropanol in *n*-hexane with flow rate of 1 ml/min. Establishment of this method made possible to determine the ratios of shikonin/acetylshikonin or alkanin/acetylalkanin in the same root. The correlation of the ratios of these substance pairs appeared characteristic for the country where they were originated from. All of the Korean species showed significantly higher ratios of shikonin/acetylshikonin and alkanin/acetylalkanin than the Chinese ones. This method would be useful to determine the origin of Lithospermi Radix.

Key words : Lithospermi Radix, Determination of the origin, Shikonin derivatives, Enantiomers, HPLC resolution

INTRODUCTION

The roots of *Lithospermum erythrorhizon* were used from ancient times for dyeing in Far East. The chemical components of the roots are isohexenylnaphthazarin, commonly known as shikonin, and its acetyl-, isobutyl-, dimethylacryl-, hydroxyisovaleryl-, isovaleryl-, methyl-*n*-butyl, anhydro- and deoxy-derivatives. The isohexenylnaphthazarin derivatives separated from this root are racemic mixtures. The configuration of shikonin was determined as R-form and that of alkannin as S-form. Alkannin occurs in *Alkanna tinctoria*, which grows in Europe. The use of alkannin as dyes was known to ancient Greeks and Romans who employed the plant for this purpose.

The root of *Lithospermum erythrorhizon* and *Alkanna tinctoria* exhibits antimicrobial (Tabata *et al.*, 1982; Papageorgiou *et al.*, 1979), anti-inflammatory (Tanaka *et al.*, 1986), immunostimulating (Wagner *et al.*, 1988), antitumor (Ahn *et al.*, 1996; Konoshima *et al.*, 1989; Sankawa *et al.*, 1977) and wound-healing (Papageorgiou *et al.*, 1980) activities. In Japan and China the Lithospermi Radix has been used as a major ingredient of an ointment for the treatment of wounds, skin disease and burns. The Lithospermi Radix is listed in Korean, Japanese and Chinese Pharmacopoeia. Alkannin and shikonin in the crude drugs or in preparations were quantitatively analyzed as total amount of enantiomers (Chen *et al.*, 1996; Chaisuksant *et al.*, 1993) and also as single enantiomer after chiral separation (Ikeda *et al.*, 1991; Yesilada *et al.*, 1996). To determine the total amount of alkannin and shikonin in the root of *Lithospermum erythrorhizon* or *Alkanna tinctoria*, it was necessary to hydrolyze the components because the isohexenylnaphthazarin derivatives occur in the acylated forms.

It is sometimes necessary to know the enantiomeric ratio of the isohexenylnaphthazarins in the natural state. It will be useful not only to understand the chemotaxonomy of the species and the dependence on the environment they grow up, but also to establish the method of quality control for the herbal drugs. For this purpose, an HPLC method is developed to analyze shikonin, alkannin and the respective acetyl derivatives in the Lithospermi Radix originated from different regions.

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MATERIALS AND METHODS

Preparation of samples

Seven samples of the Lithospermi Radix were purchased from herbal stores in Taejon City, Korea. Three of them were collected from different regions in Korea, three were imported from China and one was of unknown origin. Air dried root was ground to fine powder. Eight hundred milligrams of the powder was extracted with 10 ml $CHCl_3$ at room temperature for 12 hr. After filtration, the residue was extracted again under the

same conditions. Combined filtrate was divided in two parts and evaporated *in vacuo*. One part was dissolved in 2.0 ml isopropanol, filtered through 0.2 μm membrane filter and used as unhydrolyzed sample. Another part of residue was dissolved in 0.5 ml isopropanol and hydrolysed with 3 ml of 1 M NaOH at room temperature for 6 hr. The aqueous phase was adjusted to pH 3 with 1 M HCl and extracted with 5 ml $CHCl_3$ twice. The organic layer was washed with H_2O and evaporated to dryness *in vacuo*. The residue was dissolved in 1.0 ml isopropanol, filtered through 0.2 μm membrane filter and used as hydrolyzed sample.

HPLC analysis

The high-performance liquid chromatograph equipped with a pump (HPLC Pump 64, Knauer), a Rheodyne injector and a diode array detector (L-3000 Multi Channel Photo Detector, Hitachi) controlled by a software (Diode Array Monitor software, Hitachi) was used. Stationary phase was Chiracel AD (4.6×250 mm, Daicel Chemical Industries, Ltd.). 5% isopropanol in *n*-hexane was used as mobile phase with flow rate of 1 ml/min. The injected volume was 20 μl and the absorbance was monitored at 480 nm.

Isolation of the enantiomeric mixture of shikonin and alkannin

One kilogram of root of *Lithospermum erythrorhizon* was powdered in 50~80 mesh and extracted overnight with 4 L of *n*-hexane in the absence of light at room temperature. The extract was evaporated *in vacuo* and 2 L of 7% NaOH solution was added. After stirring the solution under nitrogen gas, 10% H_2SO_4 solution was added in order to adjust pH 5. The precipitate and solution were extracted with ether twice. The organic layer was washed with water, dried over anhydrous Na_2SO_4 and evaporated to dryness *in vacuo*. The residue was recrystallized in *n*-hexane and 1 g of the enantiomeric mixture of shikonin was obtained. The melting point of the substance was 135~137°C and the chemical structure was determined as shikonin/alkannin by ^{1}H- and ^{13}C-NMR, MS, IR and UV spectroscopy.

Acetylation of the enantiomeric mixture

The enantiomeric mixture isolated (288 mg), dicyclohexylcarbodiimide (226 mg) and 4-dimethylaminopyridine (30 mg) were dissolved in 3 ml of dichloromethane. Acetic acid (60 mg) was added and the mixture was stirred under nitrogen gas for 30 min at 0°C then 3 hr at room temperature. Twenty milliliters of *n*-hexane were added and stirred for 10 min at room temperature. Precipitates were filtered and the solution was dried over anhydrous Na_2SO_4, evaporated to dryness *in vacuo*. The residue was dissolved in ethylacetate, chromatographed in silica gel column (2.5×20 cm) with the eluent of *n*-hexane: ethylacetate (4:1) and 240 mg of mixture of acetylshikonin and acetylalkannin were obtained. The melting point of the substance was 96~97.5°C and the chemical structure was determined as acetylshikonin/acetylalkannin by ^{1}H-NMR, MS, IR and UV spectroscopy.

Enantiomeric resolution of the mixture

The mixture of shikonin and alkannin was chromatographed on Chiralpak AD (4.6×250 mm, Daicel Chemical Industries, Ltd.) with 5% isopropanol in *n*-hexane and the effluents from 21.0 to 21.4 min and from 22.0 to 22.7 min were collected. The solution was evaporated to dryness *in vacuo* and the residue was dissolved in isopropanol. The mass spectra with electron spray ionization showed that the chemical structures of two substances were identical. The optical rotation was measured at 546 nm (JASCO Model J-700) because the value at 589 nm was unstable. The specific rotations of the substances with retention time 21.2 and 22.3 min were +233 and -242, which were not too far from the reported values (Toribara *et al.*, 1949) of shikonin and alkannin, respectively.

Acetylated mixture was chromatographed as described above and the effluents from 7.5 to 7.8 min and 8.2 to 8.5 min were collected. Mass spectrometry indicates that two substances are structurally identical. From the specific rotation, the former and the latter substances were identified as acetylalkannin and acetylshikonin, respectively.

shikonin: $[\alpha]_{546}^{25}$=+233 (c=0.03, isopropanol);
alkannin: $[\alpha]_{546}^{25}$=-242 (c=0.03, isopropanol);
acetylshikonin: $[\alpha]_{546}^{25}$=+296 (c=0.03, isopropanol);
acetylalkannin: $[\alpha]_{546}^{25}$=-278 (c=0.03, isopropanol).

RESULTS AND DISCUSSION

Effects of isopropanol concentration on resolution of enantiomers

The concentration of isopropanol in mobile phase plays an important role on capacity factors and resolutions of enantiomers. It was found that the resolution of shikonin-alkannin (Fig. 2) and acetylshikonin-acetylalkannin mixtures (Fig. 3) increased by decreas-

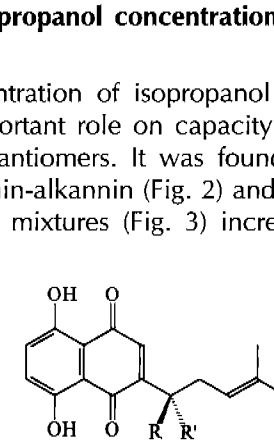

Fig. 1. The chemical structures of shikonin, alkannin and their acetyl derivatives. Shikonin (SK): R=OH, R'=H; Alkannin (AK): R=H, R'=OH; Acetylshikonin (ASK): R=$OCOCH_3$, R'=H; Acetylalkannin (AAK): R=H, R'=$OCOCH_3$.

ing the isopropanol concentration. Thus, if isopropanol concentration was decreased from 5% to 3%, there was only a slight increase of resolution but the capacity factor increased drastically. Hence, 5% isopropanol in *n*-hexane was selected as an appropriate eluent for the analysis of the mixtures. The chromatograms of the standard mixture (Fig. 4) and hydrolyzed and unhydrolyzed samples prepared from the Lithospermi Radix (Fig. 5) show that the components are base line separated. Alkannin and shikonin were detected only from hydrolyzed samples and acetylalkannin and acetylshikonin were detected mostly from unhydrolyzed samples.

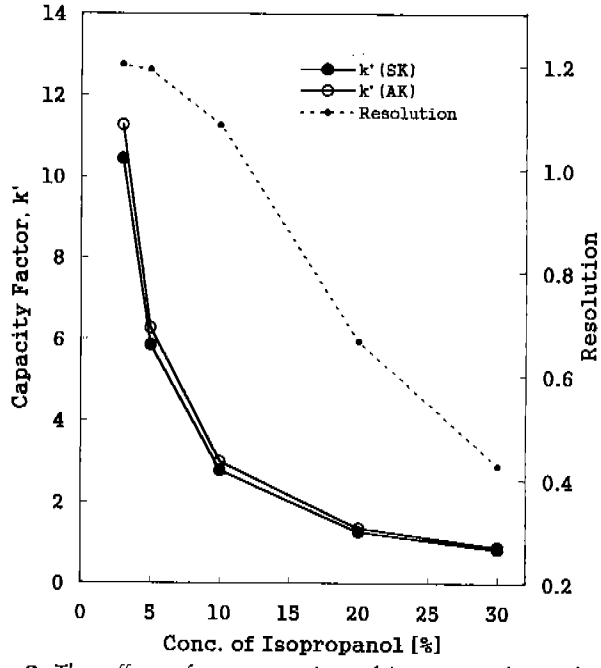

Fig. 2. The effect of concentration of isopropanol in n-hexane on capacity factors and resolutions of shikonin and alkannin. Column: Chiracel AD (4.6×250 mm); flow rate: 1 ml/min.

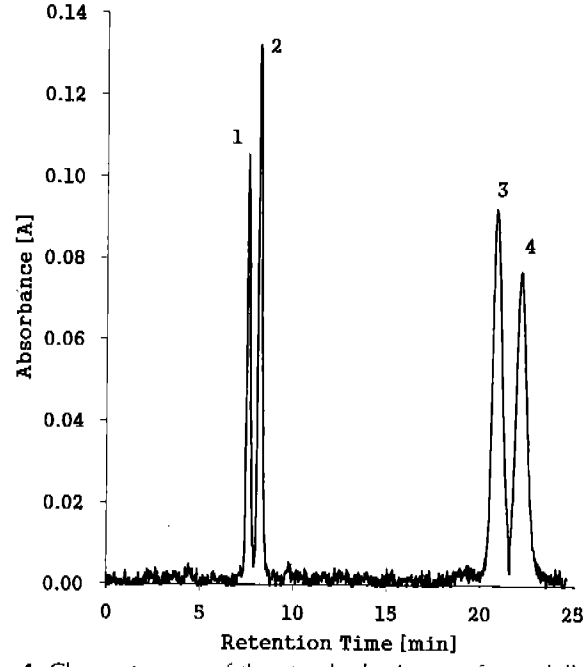

Fig. 4. Chromatogram of the standard mixture of acetylalkannin (1), acetylshikonin (2), shikonin (3) and alkannin (4). Column: Chiracel AD (4.6×250 mm); eluent: 5% isopropanol/*n*-hexane; flow rate: 1 ml/min.

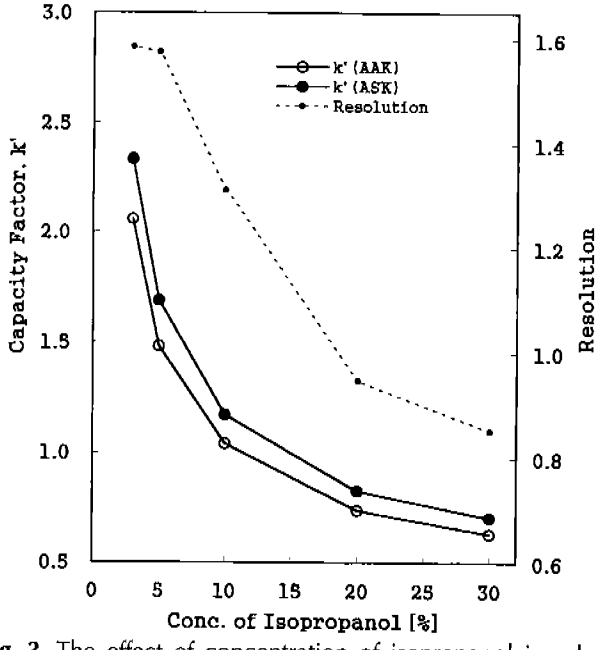

Fig. 3. The effect of concentration of isopropanol in *n*-hexane on capacity factors and resolutions of acetylshikonin and acetylalkannin. HPLC conditions, same as Fig. 2.

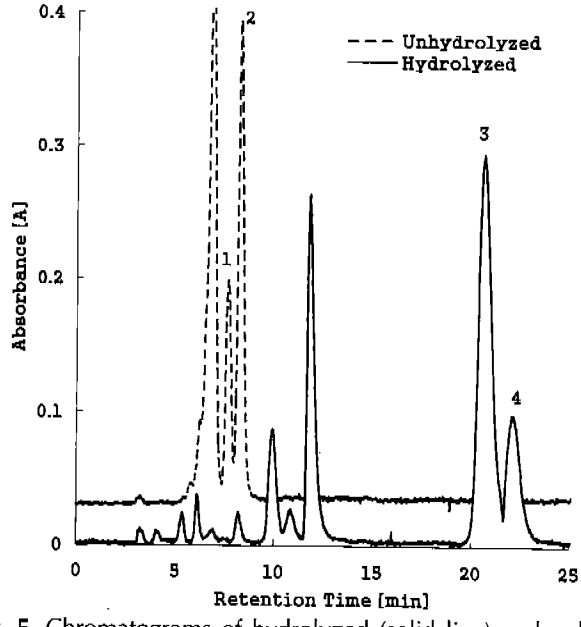

Fig. 5. Chromatograms of hydrolyzed (solid line) and unhydrolyzed (dashed line) samples prepared from the Lithospermi Radix. HPLC conditions and peak identification: see Fig. 4.

Calibration and detection limits

The calibration functions of the enantiomers represent as a straight line in the range of 0.02~0.129 mg/ml for shikonin, 0.02~1.355 mg/ml for alkannin, 0.01~0.756 mg/ml for acetylshikonin and 0.01~0.2 mg/ml for acetylalkannin. The correlation coefficients of them are 1.000, 1.000, 0.999 and 0.998 respectively. The detection limits were 0.005 mg/ml (S/N>3) for acetylshikonin and acetylalkannin and 0.01 mg/ml (S/N>3) for shikonin and alkannin.

Evaluation of results

The ranges of contents of acetylalkannin, acetylshikonin, alkannin and shikonin in six Lithospermi Radix were 0.30~2.92, 0.78~6.50, 0.42~2.79 and 1.17~7.96 μg/g, respectively. The enantiomer compositions of acetylshikonin and shikonin were 71.6% and 72.9 %, respectively. As shown in Table I, the differences of contents of enantiomers between Korean and Chinese samples were not significant at p>0.01. Hence, it was not possible to distinguish the origin of samples by the contents of enantiomers. To determine the characteristic of the sample more accurately, the ratio of shikonin/acetylshikonin is plotted versus the ratio of alkannin/acetylalkannin. As shown in Fig. 6, the plotted points form two clusters, one cluster composed of Korean samples is appearing on right upper corner and the other composed of Chinese samples in the middle of plot area. The Korean samples can be distinguished from others by this method. An unknown sample was interpolated into the plot and appeared in the cluster of Chinese origin and identified as a sample with Chinese origin. This method was found to be useful to determine the origin of Lithospermi Radix.

Table I. The mean contents (μmol/g of root) of acetylalkannin, acetylshikonin, alkannin and shikonin in Lithospermi Radix with Korean and Chinese origin

	Korea	China
Acetylalkannin (A)	0.42±0.05#	1.46±1.34
Acetylshikonin (B)	1.08±0.30#	3.41±2.86
B/(A+B)×100	71.71±3.29#	71.48±2.46
Alkannin (C)	0.73±0.11#	1.50±1.20
Shikonin (D)	2.12±0.67#	4.01±3.53
D/(C+D)×100	73.57±5.65#	72.23±2.76

#The contents of enantiomers in Korean samples were not significantly different at p>0.01, when compared with that in Chinese samples. n=3.

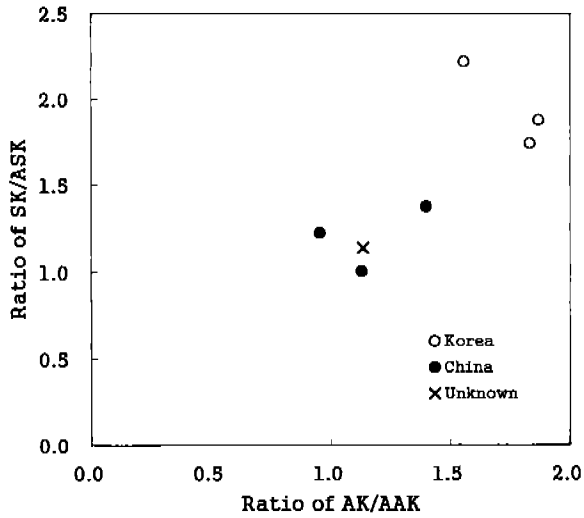

Fig. 6. Plot of shikonin/acetylshikonin ratio versus alkannin/acetylalkannin ratio in the Lithospermi Radix collected in Korea (opened circle), imported from China (closed circle) and of unknown origin (cross). The sample of unknown origin could be identified as a sample imported from China.

ACKNOWLEDGEMENTS

The authors thank Professor B. Chankvetadze, Tbilisi State University, Georgia for valuable advice. This work was supported by GRANT No. KOSEF 951-0711-056-2 from the Korea Science and Engineering Foundation.

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