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Short communication

# Determination of bisbenzylisoquinoline alkaloids by high-performance liquid chromatography

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**Abstract**

Reversed-phase ion pair chromatography with UV detection was used to determine nine type VI and VIII bisbenzylisoquinoline alkaloids: aromoline, obamegine, berbamine, homoaromoline, colorflammine, thalrugosine, norobaberine, tetrandrine and isotetrandrine. The eluent was acetonitrile—0.05 *M* dihydrogen phosphate buffer (pH 3.0) (27:73, v/v) containing 0.1% diethylamine and 2 m*M* sodium heptanesulphonate. Five alkaloids were determined in the stem woods of a Lauraceous plant, *Dehaasia triandra* Merr. 

*Keywords:* Alkaloids; Aromoline; Obamegine; Berbamine; Homoaromoline; Colorflammine; Thalrugosine; Norobaberine; Tetrandrine; Isotetrandrine; Bisbenzylisoquinolines

## 1. Introduction

Bisbenzylisoquinoline alkaloids, occurring primarily in menispermaceae, berberidaceae, monimiaceae and ranunculaceae [1], exhibit many cardiovascular pharmacological activities; for example, tetrandrine was reported to show verapamil-like calcium antagonistic, antiarrhythmic and platelet aggregation-inhibiting actions [2–4], berbamine was suggested to show calcium channel blocking, isoproterenol and histamine antagonizing actions [5,6], and obamegine possessed α-adrenoreceptor-blocking and hypotensive activities [7].

Recent research on the alkaloidal constituents from the leaves of *Dehaasia triandra* Merr., a Lauraceous plant indigenous to Taiwan, revealed that the plant contained 11 bisbenzylisoquinoline alkaloids [8]. This study shows that the Lauraceous plants, especially the *Dehaasia* genus, might also become a source of these pharmacologically interesting molecules.

In view of the large number of species of Lauraceous plants (for example, about 10 species in the *Dehaasia* genus), an efficient analytical method was required to facilitate the study of the bisbenzylisoquinoline alkaloidal constituents in these plants. Up to now, however, no separation of such compounds using high-performance liquid chromatography (HPLC) has been reported. This work was undertaken to develop a simple and rapid HPLC method for the separation of nine type VI and VIII bisbenzylisoquinoline alkaloids. The method was used to determine the content of such alkaloids present in the stem woods of *Dehaasia triandra* Merr. Preceded by the same extraction procedure [9],

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this method also could be applied to the other plants mentioned above.

## 2. Experimental

### 2.1. Apparatus

The chromatographic equipment consisted of a Shimadzu LC-10 AT pump (Kyoto, Japan), a Rheodyne 7725i 20-μl manual injector (Cotati, CA, USA) and a Shimadzu SPD-M10A diode array detector. The chromatograms were recorded, analyzed using an 80486 DX2 66 PC, and printed on an HP DeskJet 660C printer.

Separations were carried out on a Microsorb-MV $C_{18}$ column, 250×4.6 mm, 5 μm (Rainin, Woburn, MA, USA).

### 2.2. Chemicals and reagents

Tetrandrine was obtained from First Medical University of Shanghai (China) and the other eight alkaloids, aromoline, obamegine, berbamine, homoaromoline, colorflammine, thalrugosine, norobaberine and isotetrandrine, were isolated from the leaves of the Lauraceous plant *Dehaasia triandra* Merr. [8]. The identities and purities of the substances were verified by TLC and HPLC and by UV, IR, $^1$H NMR and mass spectrometry. Codeine phosphate was supplied by the Narcotic Bureau of the Government (Taiwan). Potassium dihydrogen phosphate, diethylamine from Merck (Darmstadt, Germany), sodium *n*-heptanesulphonate from Acros (Geel, Belgium), hydrochloric acid from Carlo Erba (Milan, Italy) and acetonitrile of chromatographic grade from Mallinckrodt (Paris, KY, USA) were used. Ethanol (95%) and chloroform used in extraction process for sample preparations were supplied locally and distilled before use. Water was purified on a Barnstead water purification system (Dubuque, IA, USA).

### 2.3. Standard preparation

A reference solution containing nine bisbenzylisoquinoline standards, aromoline, obamegine, berbamine, homoaromoline, colorflammine, thalrugosine, norobaberine, tetrandrine and isotetrandrine, each at 100 μg ml$^{-1}$ in methanol was prepared. Codeine phosphate was added as internal standard (100 μg ml$^{-1}$) for quantitative use.

### 2.4. Sample preparation [9]

The ground powders (300 g) of stem woods of the plant *Dehaasia triandra* Merr. (collected in August 1992 in Lanyu, Taitung County, Taiwan) were extracted with 95% ethanol (2 l×5). Concentration of the ethanolic extract afforded a residue (15 g) from which the alkaloids were extracted with 0.1 *M* hydrochloric acid (100 ml×4+50 ml×1). The acidic aqueous layer, after being extracted with chloroform (50 ml×6), was alkalinified with ammonia water to pH 9 and again extracted with chloroform (50 ml×6). These two chloroform extractions were concentrated to give fractions A and B of 0.28 and 3.01 g, respectively.

### 2.5. Chromatographic conditions

The mobile phase was prepared by adding acetonitrile to a 0.05 *M* potassium dihydrogen phosphate buffer (27:73, v/v) containing 2 m*M* sodium heptanesulphonate and 0.1% diethylamine (v/v). The pH of the buffer was adjusted with 10% HCl to 3.0 before mixing with acetonitrile.

The mobile phases were filtered (0.45 μm, Millipore) and degassed (ultrasonic) before use. The flow-rate was 1.0 ml min$^{-1}$ and the column was operated at room temperature.

The detection wavelength was set at 207 nm with a bandwidth of 4 nm. The UV absorption spectra were recorded in the 200–400 nm range.

## 3. Results and discussion

### 3.1. Method development

Bisbenzylisoquinoline (BBI) alkaloids are built up of two benzylisoquinoline (BI) units linked mostly by ether bridges. A variety of structural patterns arises in the BBI molecules due to differences in the number and nature of the diphenyl ether linkages and

the sites on the two BI units at which these ether bonds originate [10]. The nine compounds selected for the analysis (Fig. 1) cover two types of BBI alkaloids, in which aromoline, homoaromoline, colorflammine and norobaberine are type VI BBI alkaloids, while obamegine, berbamine, thalrugosine, tetrandrine and isotetrandrine are type VIII ones. Both types have two diphenyl ether linkages, with one (the $C_{11}$–O–$C_{12'}$ linkage) being identical, whereas the other has $C_7$–O–$C_{8'}$ and $C_8$–O–$C_{7'}$ linkages for type VI and type VIII BBIs, respectively.

Due to the higher stability in acidic than in alkaline solutions of the phenolic groups present in some of the test alkaloids, the separation was performed in acidic buffers. Pietta et al. [11] also stated that acidic mobile phases or acidic amine–phosphate buffers can be useful for improving the peak shapes and separations for the analysis of basic substances such as alkaloids.

Simple alkylamines, such as diethylamine and triethylamine, are the most commonly used additives as a competing base in the analysis of various alkaloids [12]. In this work, following some preliminary studies, mobile phases of 0.05 *M* dihydrogen phosphate buffer (containing 0.1% of diethylamine (v/v), the pH being adjusted to acidic)–acetonitrile mixtures with various proportions were tested, and it was found that berbamine–homoaromoline and thalrugosine–norobaberine peaks were always partially coalesced even at the optimum ratio of the eluent mixture (73:27, v/v). In the analysis of alkaloids, the technique of ion-pair

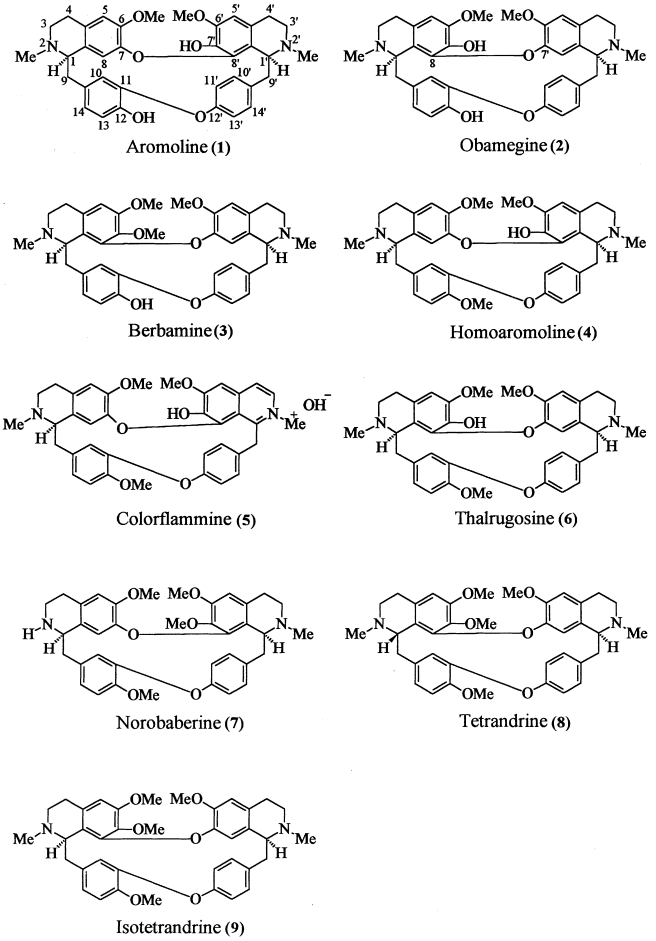


Fig. 1. Bisbenzylisoquinoline alkaloids used for the analysis.

chromatography in reversed-phase HPLC are considered to be of great importance with regard to separation selectivity [13]. With the addition of sodium heptanesulphonate to the buffer, the above two-peak pairs were separated and the retention time (as well as the capacity factor) of each compound was increased in proportion to the amount of the ion-pairing agent added (Fig. 2). To maintain an acceptable resolution within a short analysis time, 2 m*M* of sodium heptanesulphonate was selected for use.

To facilitate the ion pair formation, the BBI alkaloids should exist in the form of protonated amines. The acidity of the buffer system was therefore investigated to find an appropriate pH for separation. Below pH 3.5 the retention times remained nearly constant; however, when the pH was raised above 4.0, all the retention times increased, with the peak of tetrandrine being gradually merged with isotetrandrine and completely overlapped at pH 4.5. The increase of retention times with increasing pH was due to the formation of the free (non-protonated) form of the alkaloids, which were more retained by the stationary phase. A pH of 3.0 was selected for the analytical use.

In these alkaloids, aromoline and obamegine have

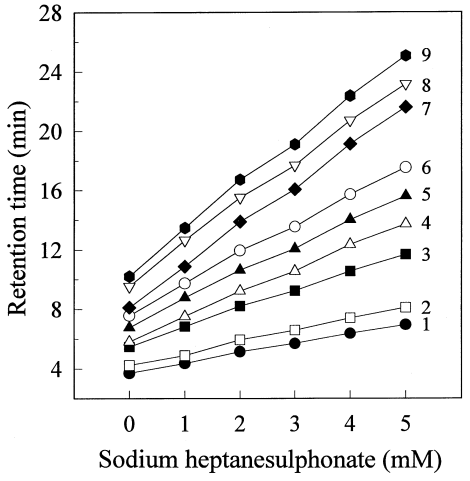


Fig. 2. Retention times of the bisbenzylisoquinoline alkaloids versus concentration of sodium heptanesulphonate. Eluent, 0.05 *M* sodium dihydrogen phosphate (containing 0.1% diethylamine, 0–5 m*M* sodium heptanesulphonate, pH 3.0)–acetonitrile (73:27, v/v); flow-rate, 1.0 ml $min^{-1}$; UV detection at 207 nm. Compound identities as indicated in Fig. 1.

two phenolic substitutions, berbamine, homoaromoline, colorflammine and thalrugosine have one such, while norobaberine, tetrandrine and isotetrandrine are non-phenolics. The gross elution trend is determined by the number of phenolic substitutions each compound possesses. It could be seen that with identical molecular mass the type VI compounds, namely, aromoline and homoaromoline, were eluted ahead of their type VIII positional isomers, obamegine and thalrugosine, respectively. This leads to the speculation that the type VIII BBIs are more lipophilic compared with the type VI isomers. Berbamine being less retained than thalrugosine could be attributed to the higher hydrophilicity of the molecule incurred by the hydroxyl substitution at the $C_{12}$ position than at the $C_7$ position. The elution of colorflammine after homoaromoline was due to the higher lipophilicity of the quaternary isoquinolinium moiety possessed by the former than the protonated tetrahydroisoquinoline part of the latter. Norobaberine, tetrandrine and isotetrandrine were non-phenolic, but norobaberine was eluted before the latter two. This was because one of the nitrogens in norobaberine is demethylated, which rendered the molecule more hydrophilic. Tetrandrine is the diastereomer of isotetrandrine. The (*S*) configuration of $C_1$ in tetrandrine caused the molecule to be less retained by the stationary phase. The three-dimensional spectrochromatogram for the nine BBI alkaloids with codeine phosphate as internal standard is shown in Fig. 3.

### *3.2. Method validation*

The reproducibility of the retention times of the method, for seven replicate injections on 3 days, was found to be within 0.2% relative standard deviation for each BBI alkaloid. The reproducibilities of the peak height ratios of the BBIs relative to the internal standard (codeine phosphate at 100 μg $ml^{-1}$) were tested on two concentration levels (10 and 200 μg/ml) ($n=7$, for 3 days) and the relative standard deviations were less than 1.0% for both levels. The detection limits ($S/N=3$) were found to range between 200 and 1630 pg (Table 1). For the calibration curves, six standard solutions of the alkaloids covering the range 10–200 μg $ml^{-1}$ were run, with codeine phosphate as internal standard (100 μg

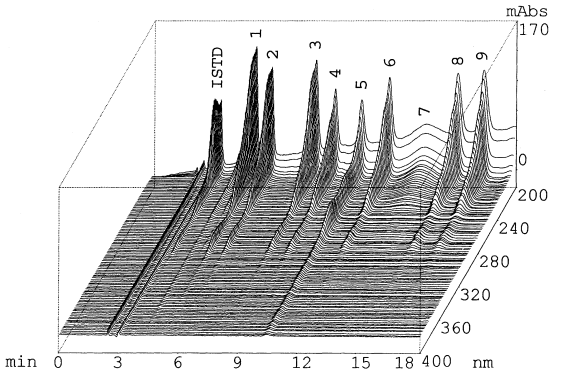


Fig. 3. Spectrochromatogram of the standard mixture of the bisbenzylisoquinoline alkaloids and codeine phosphate (internal standard). Eluent, 0.05 *M* sodium dihydrogen phosphate (containing 0.1% diethylamine, 2 m*M* sodium heptanesulphonate, pH 3.0)–acetonitrile (73:27, v/v); flow-rate, 1.0 ml $min^{-1}$; UV detection at 207 nm. Compound identities as indicated in Fig. 1.

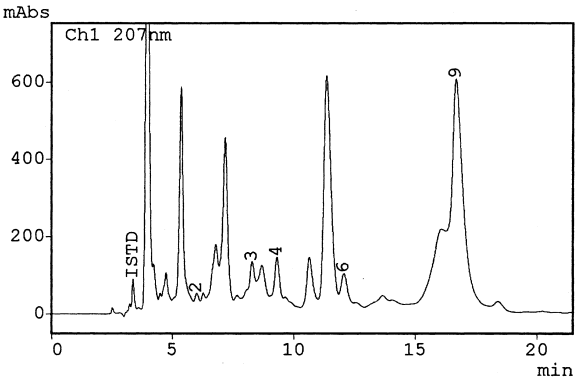


Fig. 4. Chromatogram of the Fraction B sample preparation from the stem wood of *Dehaasia triandra* Merr. Chromatographic conditions as described in Fig. 3. Compound identities as indicated in Fig. 1.

$ml^{-1}$), and the relationships between peak height ratios ($y$) and concentrations injected ($\mu g\ ml^{-1}$) ($x$) are listed in Table 1.

### 3.3. Application

With the aid of peak-spiking and spectrum-matching techniques, five tested BBIs, obamegine, berbamine, thalrugosine, homoaromoline and isotetrandrine, were identified in the samples prepared from the stem woods of the plant *Dehaasia triandra* Merr. For the spectrum-matching procedure, the similarity indexes (value of 1.0000 for complete overlap) of spectra for the peaks to be compared were calculated, the values of which in this work were all greater than 0.9980. The above five BBIs were found in Fraction B (Fig. 4), while only isotetrandrine was detected in Fraction A. In Fig. 4 the peak appearing in the front part of isotetrandrine (compound 9) was identified as obaberine, the *N*-methylated derivative of norobaberine. This compound could be completely separated from isotetrandrine at the expense of a much longer retention time (more than 30 min) by decreasing the eluent strength.

The contents of the five BBI alkaloids in the Fraction A and B preparations are listed in Table 2. The total contents in the stem wood part of the plant calculated by combining the results from these two fractions were 0.7, 3.0, 2.7, 3.5 and 31.8 ppm for obamegine, berbamine, homoaromoline, thalrugosine and isotetrandrine, respectively. It can be seen that

Table 1
Detection limits ($S/N=3$) and linear relationship between peak-height ratios ($y$) and concentrations ($\mu g\ ml^{-1}$) ($x$) for the bisbenzylisoquinoline alkaloids

| Compound | Detection limit (pg) | Slope | Intercept | $r^a$ |
|---|---|---|---|---|
| Aromoline | 220 | 0.0126 | −0.0469 | 0.997 |
| Obamegine | 280 | 0.0116 | −0.0322 | 0.999 |
| Berbamine | 210 | 0.0122 | −0.0108 | 0.997 |
| Homoaromoline | 240 | 0.0076 | −0.0190 | 0.998 |
| Colorflammine | 480 | 0.0055 | −0.0207 | 0.999 |
| Thalrugosine | 270 | 0.0088 | −0.0213 | 0.999 |
| Norobaberine | 1630 | 0.0017 | −0.0022 | 0.997 |
| Tetrandrine | 290 | 0.0082 | −0.0035 | 0.999 |
| Isotetrandrine | 200 | 0.0082 | −0.0061 | 0.999 |

[a] $r$, correlation coefficient.

Table 2
Contents of the bisbenzylisoquinoline alkaloids in the sample preparations of the stem woods of *Dehaasia triandra* Merr. and the recoveries of these alkaloids

| | Contents (ppm)[a] | | Recovery (%)[a] |
|---|---|---|---|
| | Fraction A | Fraction B | Fraction B |
| Obamegine | — | 1813±25 | 87.8±4.9 |
| Berbamine | — | 7885±76 | 107.8±4.4 |
| Homoaromoline | — | 7122±60 | 88.0±2.7 |
| Thalrugosine | — | 14 320±93 | 93.7±4.5 |
| Isotetrandrine | 65 523±12 | 76 395±65 | 109.1±3.7 |

[a]Mean±S.D. ($n$=3).

isotetrandrine constituted the major part of these BBI alkaloids (about 73%).

The accuracy of the method was tested by analyzing the mixture prepared by adding suitable amounts of the five alkaloids to the Fraction B preparations with known contents of these alkaloids. The recoveries of the alkaloids listed in the Table 2 are good in view of the minor contents of some of these alkaloids and the interference by the other constituents in the mixture.

## 4. Conclusions

Notwithstanding the close similarities in the structures of these nine type VI and VIII BBI compounds, a base-line separation was achieved by the proposed ion pair chromatographic method. After a conventional treatment to the stem part of a Lauraceous plant *Dehaasia triandra* Merr., five such alkaloids were detected and their contents were quantitated. This method also could be applied to other Lauraceous plants and the plants containing these type VI and VIII BBI alkaloids mentioned at the beginning of this paper.

## Acknowledgements

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