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Short communication

Determination of bisbenzylisoquinoline alkaloids by high-performance liquid chromatography (II)

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Abstract

After the earlier analysis of nine bisbenzylisoquinoline alkaloids with ion pair chromatography, seven other bisbenzylisoquinoline alkaloids were analyzed using gradient elution with an acetonitrile–phosphate buffer (pH 8.0) mixture, and UV detection. Four alkaloids were detected in the stem woods of a Lauraceous plant, *Dehaasia triandra* Merr. and their contents determined. LC–MS suggested that a major unknown compound in the plant was also a bisbenzylisoquinoline alkaloid.

Keywords: Bisbenzylisoquinoline alkaloids; Alkaloids

1. Introduction

Bisbenzylisoquinoline (BBI) alkaloids, occurring primarily in Berberidaceae, Menispermaceae, Monimiaceae, Ranunculaceae [1] and lately found in Lauraceae [2,3], are reported to have many pharmacological activities such as antiarrhythmic, hypotensive, platelet-aggregation-inhibiting, histamine-antagonizing and anti-flagellated protozoa activities [4–9].

Recently some BBIs were determined in our laboratory by column liquid chromatography (HPLC) and capillary electrophoresis [10,11]. Nine BBI alkaloids, aromoline, obamegine, berbamine, homoaromoline, colorflammine, thalrugosine, norobaberine, tetrandrine and isotetrandrine, were analyzed by reversed-phase ion-pair chromatography [10]. Some BBI alkaloids were left untested at that time due to their much minor quantities isolated from the plant. In the present work, seven other BBIs, namely, daphnoline, norobamegine, dehatridine, 3′,4′-dihydrostaphasubine, norobaberine, norisotetrandrine and obaberine, were studied to complete the analysis of all BBIs available in our laboratory.

2. Experimental

2.1. Apparatus

2.1.1. HPLC–DAD

The equipment consisted of a pair of Shimadzu LC-10 AT pumps (Kyoto, Japan), a Rheodyne 7725i 5-μl manual injector (Cotati, CA, USA) and a Shimadzu SPD-M10A diode array detector. Separations were carried out on a Luna C_{18} column, 250×4.6 mm, 5 μm (Phenomenex, Torrance, CA, USA).

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2.1.2. HPLC–ESI-MS

ESI-MS was carried out with a Perkin-Elmer Sciex API 165 single quadrupole mass spectrometer (Sciex, Toronto, Canada) equipped with an ionspray interface and a Macintosh data system. The LC system included a Shimadzu LC-10 AD vp pump (Kyoto, Japan), a Rheodyne 8125 20-μl manual injector (Cotati) and a Luna C_{18} column, 150×2 mm, 5 μm (Phenomenex).

2.2. Chemicals and reagents

All the tested seven BBI alkaloids, daphnoline, dehatridine, 3′4′-dihydrostaphasubine, norisotetrandrine, norobaberine, norobamegine and obaberine, were isolated from the leaves or twigs of a Lauraceous plant *Dehaasia triandra* Merr. [3]. The identities of the substances were verified by UV, IR, ^{1}H NMR, ^{13}C NMR, and EI mass spectrometry. Papaverine hydrochloride was obtained from the Narcotic Bureau of the Government. Phosphoric acid (85%), ammonia solution (25%), methanol and acetonitrile were of analytic or chromatographic grade (Merck and Mallinckrodt). Ethanol (95%) and chloroform used in extraction were obtained locally. Water from a Barnstead water purification system (Dubuque, IA, USA) was used. A working standard solution containing the seven BBI alkaloids, each at 100 μl ml^{-1} in methanol, was prepared for the use of separation optimization. Papaverine hydrochloride was added as internal standard (100 μl ml^{-1}) for quantitative use.

2.3. Sample preparation [12]

The ground powders (300 g) of the stem woods of the plant *Dehaasia triandra* Merr. (collected in August 1992 in Lanyu, Taitung County, Taiwan) were extracted with 95% ethanol (21×5). After concentration the extract gave a residue (15 g) from which the alkaloids were extracted with 0.1 *M* hydrochloric acid (100 ml×4+50 ml×6). The aqueous layer, after being extracted with chloroform (50 ml×6), was alkalinified with ammonia water to pH 9.0 and again extracted with chloroform (50 ml×6). The two chloroform extracts were concentrated to give fractions A and B of 0.28 and 3.01 g, respectively. Fraction A contains only non-phenolic alkaloids, while Fraction B holds both phenolic and non-phenolic alkaloids.

Sample solutions were made by dissolving separately a suitable amount of extracts A and B (0.5 and 1.5 mg ml^{-1}, respectively) in methanol. Papaverine (100 mg ml^{-1}) was added.

2.4. Chromatographic conditions

2.4.1. HPLC–DAD

The composition of the mobile phase was linearly changed from solvent mixture A [acetonitrile–0.2%–phosphoric acid (85%), 50:50, v/v, adjusted with ammonia water (5%) to pH 8.0] to solvent mixture B [acetonitrile–0.5%–phosphoric acid (85%) 80:20, v/v, adjusted with ammonia water (5%) to pH 8.0] with a gradient profile of 100% A–100% B/0–15 min followed by 100% B for 5 min. Flow rate, 1.0 ml min^{-1}; injection volume, 5 μl; ambient temperature, 23±2°C. The detection was at 215 nm with a bandwidth of 4 nm. UV spectra were recorded in the 200–400-nm range.

2.4.2. HPLC–ESI-MS

The mobile phase was an ammonium acetate buffer–acetonitrile (42:58) mixture. The buffer was prepared with 0.2% acetic acid adjusted to pH 8.0 with ammonia water. The flow-rate was 0.1 ml min^{-1}, with 20 μl min^{-1} being directed to the ESI interface via a splitter tee and restrictive tubing. The following conditions were used in ESI-MS with the positive ion mode: needle voltage, 4800 V; orifice plate voltage, 51 V; ring voltage, 210 V; nebulizer gas at position 8 and curtain gas at position 11.

3. Results and discussion

3.1. Method development

According to the number and nature of the diphenyl ether bonds which link two benzylisoquinoline (BI) monomers, bisbenzylisoquinoline (BBI) alkaloids are divided into many structural types [13]. The seven analytes tested in this work (Fig. 1) cover two types of BBI alkaloids, in which daphnoline, 3′4′-dihydrostaphasubine, norobaberine and obaberine are type VI,

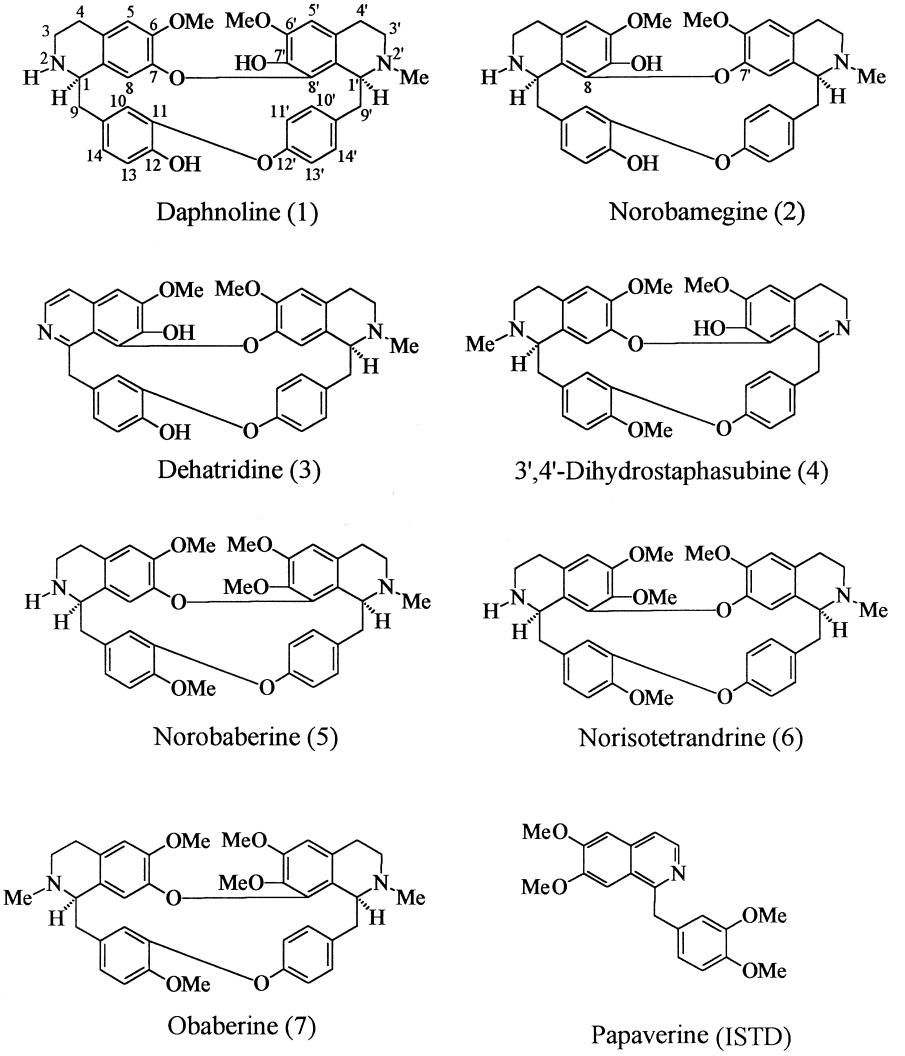

Fig. 1. Structures of the bisbenzylisoquinoline alkaloids analyzed.

while norobamegine, dehatridine and norisotetrandrine belong to type VIII. Both types have two diphenyl–ether linkages, with one (the C_{11}–O–$C_{12'}$ linkage) being identical, the other being C_7–O–$C_{8'}$ and C_8–O–$C_{7'}$ linkages for type VI and type VIII BBIs, respectively.

The relative polarities of these BBIs are related to their phenol and amine functions. The pK_a of the phenols is about 10.0. The pK_a values of the amines and isoquinoline are about 7.0 and 6.4, respectively (by analogy with apomorphine [14] and papaverine [15]).

The ion-pair chromatographic method used previously [10] was tested first. The mobile phase was a mixture of acetonitrile and phosphate buffer (pH 3.0) containing a small amount of diethylamine and sodium heptanesulfonate. Under such acidic conditions, peaks of norobaberine and obaberine appeared as broad humps, which was not suitable for the analysis of real samples.

Some researchers suggested working at high pH (e.g., >7) for the separation of basic compounds [16]. Weak bases such as alkaloids are less ionized at higher pH values, thereby the retention caused by the

silanol–sample interaction can be diminished [17]. A column stable at alkaline pH [18] was accordingly used. The buffer was 0.2% phosphoric acid adjusted with 5% ammonia water to pH 8.0 and the mobile phase was acetonitrile–buffer (50:50, v/v). At this pH the amine functions of the BBI solutes are largely unprotonated, making the ion-pairing agents useless. The peak shapes of norobaberine and obaberine turned out normal. All alkaloids were baseline separated and no tailing occurred. However, to accelerate the elution of the last peak, a linear gradient was applied. The original acetonitrile–buffer (50:50) solvent giving an appropriate retention time (4.5 min) to the earliest eluting BBI peak was taken as the initial solvent (solvent A), and an acetonitrile–buffer mixture (80:20, v/v) as the final solvent (solvent B).

Through the adjustment of buffer concentration, resolutions between peaks of the BBIs and their concomitant trace impurities could be improved slightly. Buffer pH was found to have great influence on separation. The retention time increased with solution pH along the tested range 7.0–9.0 for all compounds. The differing extent of increase in retention times among these compounds allowed us to locate an optimum pH for separation.

The three-dimensional spectrochromatogram obtained with the optimum conditions is shown in Fig. 2.

As found before [10], the elution orders of the BBI analytes were related to their structural features as follows: (1) phenol functions, (2) amine functions (secondary amine, tertiary amine, or an isoquinoline nucleus), and (3) type VI or type VIII configuration. The weighing order of their contribution to solute hydrophilicity and thereby its elution is: 1>2>3. For example, 3′,4′-dihydrostaphasubine and norisotetrandrine were eluting ahead of norobaberine and obaberine, respectively.

3.2. Method validation

The standard and sample solutions were prepared in methanol for practical reasons. The BBI compounds were readily soluble in methanol and their recovery (for re-use) from methanol were much more easy. No degradation of the BBI compounds were found for the standard solution stored in a re-

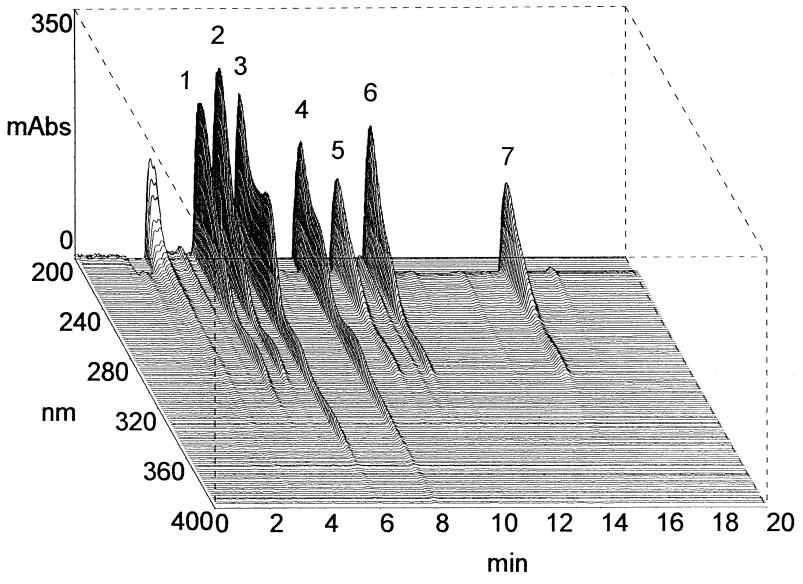

Fig. 2. Spectrochromatogram of the seven BBI alkaloids mixture. Luna C_{18} column, 25×0.46 cm, 5 μm; linear gradient from 100% A to 100%/0–15 min, followed by 100% B/5 min. (A) ACN–0.2% H_3PO_4, 50:50, v/v, pH 8.0 (adjusted with 5% NH_4OH); (B) ACN–0.5% H_3PO_4, 80:20, v/v, pH 8.0 (adjusted with 5% NH_4OH). Ambient; 1 ml min^{-1}; 215 nm; 5 μl sample. Compound identities are indicated in Fig. 1.

frigerator for 20 days. The sample solution was also stable for at least 2 days when stored alike.

The within-day repeatability ($n=7$) and between-day precision ($n=3$) of retention times were within 0.5 and 1.1% relative standard deviation (RSD), respectively. The precisions of peak area ratios of analyte to internal standard were tested at 10 μl level. The repeatability ($n=7$) and between day precision ($n=3$) were all within 2.0% RSD.

Six standard solutions of the alkaloids (10–200 μg ml^{-1}) were run for the calibration curve, with papaverine (100 μg ml^{-1}) as internal standard. The linear relationships between peak area ratios and concentrations injected were verified by the coefficients of correlation (r) for the seven BBIs (between 0.997 and 0.999). The limits of detection (LOD, $S/N=3$) were 225–535 pg (in amount) or 45–107 ng ml^{-1} (in concentration). The common wavelength of maximum absorption for the analytes was 207 nm. The LODs were taken at 215 nm because the baseline drifted at 207 nm on using the gradient elution.

The accuracy of the method was tested by analyzing the mixture prepared by adding suitable amounts of four alkaloids (3′,4′-dihydrostaphasubine, norobaberine norisotetrandrine and obaberine) to the Fraction B preparations with known contents of these alkaloids. The recoveries of the alkaloids were between 82 and 114%.

3.3. Determination of the BBI compounds in the plant

With analyte-spiking and spectrum-matching techniques, four tested alkaloids, 3′4′-dihydrostaphasubine, norobaberine, norisotetrandrine and obaberine, were identified in the samples prepared from the stem woods of the plant *Dehaasia triandra* Merr. The similarity indexes of spectra for the comparing peaks were all greater than 0.9980 (1.0000 for complete overlap). The above four BBIs were found in Fraction B (Fig. 3), while only obaberine was detected in Fraction A.

The contents of these four BBI compounds in the stem wood of the plant were 495, 320, 7 and 1590 μg/g, respectively. Obaberine constituted the major part of the tested BBI alkaloids (about 66%).

In Fig. 3 two large peaks are seen in front of

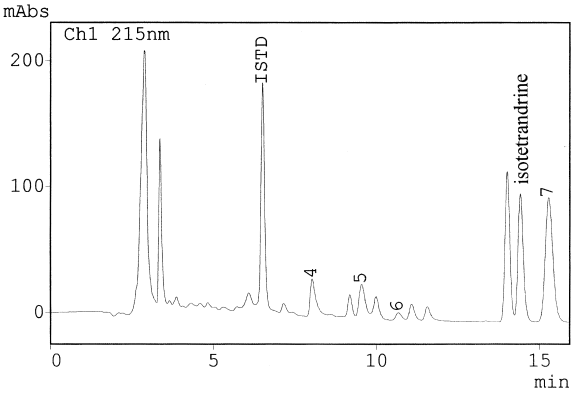

Fig. 3. Chromatogram of the Fraction B sample preparation from the stem woods of *Dehaasia triandra* Merr. Chromatographic conditions are as described for Fig. 2. Compound identities are indicated in Fig. 1.

obaberine. The one nearer to obaberine was identified as isotetrandrine which was included in the previous study [10]. The other was not found among the BBI compounds ever tested and LC–MS was used to investigate this unknown. A mass range of 500–700 amu was monitored to acquire the peaks. With the LC conditions (see Section 2.4.2) this unknown was baseline separated from the other compounds. The molecular weight of 606 (Fig. 4) and its UV spectrum (not shown) suggested that this compound was a BBI molecule. The late retention time might be caused by the absence of phenolic substitution.

4. Conclusions

This work provided an alternative approach to the analysis of BBI alkaloids. In contrast to the acidic condition used in the earlier separation [10], alkaline buffer was taken in this work, because norobaberine and obaberine peaks remained as humps with acidic mobile phases unless the buffers were changed to neutral or alkaline. Combined with the earlier study [10], the total of 15 BBI alkaloids available in our laboratory were analyzed.

As stated at the beginning of this article, many plants contain BBI alkaloids which are pharmacologically very interesting. The developed analytical methods will facilitate the studies of the BBI al-

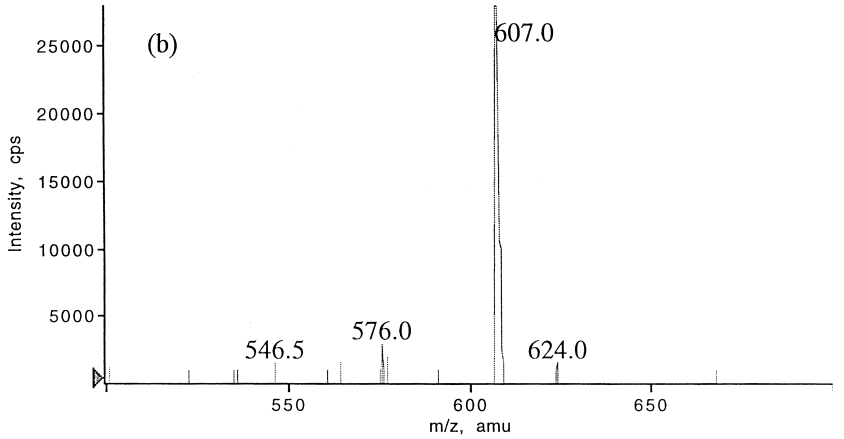

Fig. 4. Mass spectrum of the unknown compound eluting just before isotetrandrine in Fig. 3. LC conditions: ACN–0.2% HOAc, 42:58, v/v, pH 8.0 (adjusted with NH_4OH); Luna C_{18} column, 15×0.2 cm, 5 μm; flow-rate, 0.1 ml min^{-1} with splitting. ESI-MS (positive ion mode) conditions: needle voltage, 4800 V; orifice plate voltage, 51 V; ring voltage, 210 V; nebulizer gas position, 8 and curtain gas position, 11; detection mass range, 500.0–700.0 amu.

kaloids in these plants. Besides, these methods will contribute to the chemotaxomy of these and related plants.

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