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Reversed-phase high-performance liquid chromatographic separation of epimeric alkaloid N-oxides from *Thalictrum simplex* L.

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Abstract

A simple and precise method was developed for the analytical and preparative reversed-phase HPLC separation of a mixture of epimeric pavine N-oxides containing 49.1% of (−)-thalimonine N-oxide A and 50.1% of (−)-thalimonine N-oxide B isolated from *Thalictrum simplex* L. (Ranunculaceae). A reversed-phase system with Nucleosil C_{18} analytical and preparative columns and ethanol–1.5% aqueous orthophosphoric acid (15:85) as the mobile phase was used. The epimeric pavine N-oxides were completely separated within 50 min.

Keywords: *Thalictrum simplex* L.; Alkaloid N-oxides; Thalimonine N-oxides; Pavine N-oxides

1. Introduction

The genus *Thalictrum* (Ranunulaceae) is a rich source of isoquinoline alkaloids. The alkaloid content of *Thalictrum* species used in traditional medicines in Tibet and Mongolia [1,2] has been systematically studied during the last few years [3–7]. Several known isoquinoline alkaloids together with three new pavine alkaloids and one new phenanthrene alkaloid were isolated from *Thalictrum simplex* L. of Mongolian origin [5–7]. The biological experiments performed with the new pavine alkaloid (−)-thalimonine isolated from *T. simplex* showed that the alkaloid possesses in vitro antiviral activity against Herpes simplex virus type 1 (HSV-1) and also in vitro and in vivo immunological activity. These results suggest further investigations of its potency in influencing HSV-1 infections and immune response [8].

A sample containing a mixture of pavine epimeric N-oxides was recently isolated from *T. simplex* [9]. Using well known classical chromatographic methods such as column chromatography (CC) and thin-layer chromatography (TLC), no separation of the mixture of epimers was achieved. HPLC methods for the separation and determination of free isoquinoline bases such as eschscholtzine and protopine [10], the chromatographic evaluation of free aporphine bases [11,12] and the separation of tertiary and quaternary alkaloids [13,14] have been reported. However, there is no published HPLC method for the analytical or preparative separation of

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epimeric N-oxides. A simple and precise method for the separation of epimeric pavine N-oxides by reversed-phase HPLC was developed and is described here.

2. Experimental

2.1. Apparatus

A Hewlett-Packard (HP) Model 1090 liquid chromatograph, equipped with an HP Model DR5 solvent-delivery system and an HP 1090 Series L diode-array detector was used for the analytical separation. A Perkin-Elmer Series 1 liquid chromatograph connected to a Perkin-Elmer LC-55 B UV detector and with a W + W Model 1200 chart recorder was used for the preparative separation.

2.2. Chemicals

Methanol and ethanol (HPLC grade), orthophosphoric acid (BioChemica MicroSelect) and chloroform (UV grade) were obtained from Fluka (Buchs, Switzerland). Sephadex LH-20 was purchased from Pharmacia (Uppsala, Sweden). Water was doubly distilled prior to use.

2.3. Plant material

The aerial parts of *T. simplex* were collected near Ulan Bator (Mongolia) during the full flowering period at the end of July 1991. The species was identified by Professors Ch. Sanchill and E. Ganbold (Institute of Botany, Mongolian Academy of Sciences). A voucher specimen (No. 83) is deposited in the Herbarium of the Institute of Botany, Mongolian Academy of Sciences, Ulan Bator.

2.4. Extraction procedure

Dried and ground plant material (1.5 kg) was extracted successively with cold light petroleum and ethanol. The ethanol extracts were evaporated to dryness in vacuo. The residue was suspended in 10% hydrochloric acid and subsequently extracted with hexane and chloroform. The acidic layer was made alkaline with 25% ammonia solution (pH 9–10) and extracted with chloroform. The chloroform extracts were evaporated to dryness in vacuo to give crude alkaloids. The crude alkaloid mixture was separated by column chromatography.

2.5. Chromatographic procedures

The isoquinoline alkaloid N-oxide mixture was eluted with diethyl ether–methanol (1:1) from an Al_2O_3 90 (Brockmann II, 70–230 mesh; Merck) column (400 mm × 19 mm I.D.). The mixture of epimeric pavine N-oxides was separated from other alkaloid N-oxides by gel filtration on a Sephadex LH-20 column (300 mm × 12 mm I.D.), eluted with acetone at a flow-rate of 2.5 ml/h.

A stainless-steel analytical Nucleosil 100-7 C_{18} column (250 mm × 4 mm I.D., 5 μm particle size) and a preparative Nucleosil 100-7 C_{18} column (250 mm × 10 mm I.D., 7 μm particle size) were used. Isocratic elution was performed at 22°C at a flow-rate of 1 ml/min for analytical separation and 9 ml/min for preparative separation. The dead volume was measured by the deviation of the baseline on injection of pure methanol. Sample injections were performed at 20-min intervals in order to allow complete equilibration of the columns between runs. Between each injection of the sample, a run with 20 μl of organic solvent (methanol or ethanol) was performed in order to wash the injector and the column. Mobile phase 1 was methanol–1.5% aqueous orthophosphoric acid (35:65) (pH 2.3) and mobile phase 2 was ethanol–1.5% aqueous orthophosphoric acid (15:85) (pH 2.1). Detection was performed at 280 nm in both cases. Samples of 15–20 μl were injected for the analytical separations and of 150 μl for the preparative separations. The samples were dissolved in 1–2 drops of chloroform and diluted with ethanol. Sample solution concentrations of 0.1–0.3 mg/ml were used for the analytical separations and of 33 mg/ml for the preparative separations. A chart speed of 1 cm/min was used during the prepara-

tive separation and the fractions were collected following the UV absorption profile.

2.6. Isolation of the alkaloids after preparative reversed-phase HPLC separation

The collected fractions were basified to pH 9–10 with 25% ammonia solution and the alkaloids were extracted with chloroform, dried over anhydrous Na_2SO_4 and the solvent was evaporated in vacuo.

3. Results and discussion

The alkaloid N-oxides are easily separated from the free bases by classical chromatographic methods such as CC and TLC if the sample contains only one alkaloid N-oxide [15] or if the N-oxides are present in not too small amounts [16]. The separation is more complex if more than one N-oxide is present in the crude alkaloid mixture or if the N-oxides are present in low concentrations in the sample. A sample containing a mixture of pavine and aporphine N-oxides of *T. simplex* was isolated as a polar fraction by classical CC on neutral alumina [7,9]. Gel filtration performed on Sephadex LH-20 columns at a low flow-rate of the eluent allowed the separation of the pavine N-oxides (20 mg) from aporphine N-oxide (1.5 mg) according to the increasing size of the molecules. The ^1H NMR spectrum of the pavine N-oxide showed that the fraction contains a mixture of pavine N-oxide epimers with the structures shown in Fig. 1. All attempts to separate the mixture of

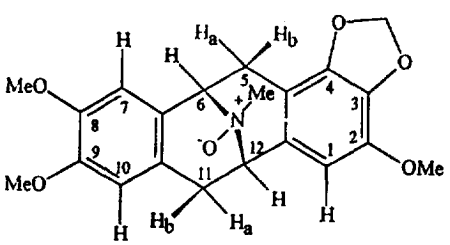

Fig. 1. Structure of (−)-thalimonine N-oxide A. The structure of (−)-thalimonine N-oxide B is similar, with the Me and O^- interchanged.

epimers by classical chromatographic methods were unsuccessful.

Reversed-phase high-performance liquid chromatography with a Nucleosil C_{18} column was finally successful in separating the mixture. A mixture of acetonitrile with different proportions of aqueous orthophosphoric acid gave no separation. The replacement of acetonitrile with methanol (mobile phase 1) showed better results. Optimum separation between the epimers with phase 1 was achieved when the proportions of methanol and 1.5% aqueous orthophosphoric acid were 35:65 under isocratic conditions, R_s = 1.20 (Fig. 2a). An increase or decrease in methanol content or of the percentage of orthophosphoric acid in the mobile phase decreased the separation. All experiments with gradient elution were unsuccessful.

Replacement of methanol with ethanol (mobile phase 2) in the same proportions showed poor resolution of the epimers. Optimum separation was achieved in 50 min with mobile phase 2 when the proportions of ethanol and 1.5% aqueous orthophosphoric acid in the mobile phase were 15:85 (Fig. 2b).

The influence of the variations in the mobile phase on the selectivity α and the resolution R_s is shown in Table 1. The preparative separation was performed at a flow-rate of 9 ml/min, and the retention times of the epimers decreased to 20 min. There was a small decrease in the resolution of the epimers, but changing the flow-rate to 6, 5, or 3 ml/min did not give better results. The proportions of the epimeric N-oxides in the mixture tested by analytical HPLC showed 49.1% of (−)-thalimonine N-oxide A and 50.9% of (−)-thalimonine N-oxide B.

Fractions with different purities of the epimeric N-oxides obtained during the preparative separation are shown in Table 2. The purity of each fraction was checked by analytical HPLC. Fraction 2, containing 3 mg of 83.4% pure (−)-thalimonine N-oxide A (Fig. 3a, Table 2) and fraction 9, containing 3 mg of 93.5% pure (−)-thalimonine N-oxide B (Fig. 3b, Table 2) were subjected to the physical measurements (NMR, MS, UV, IR, etc.) necessary for the structural elucidation of the epimers [9]. The developed

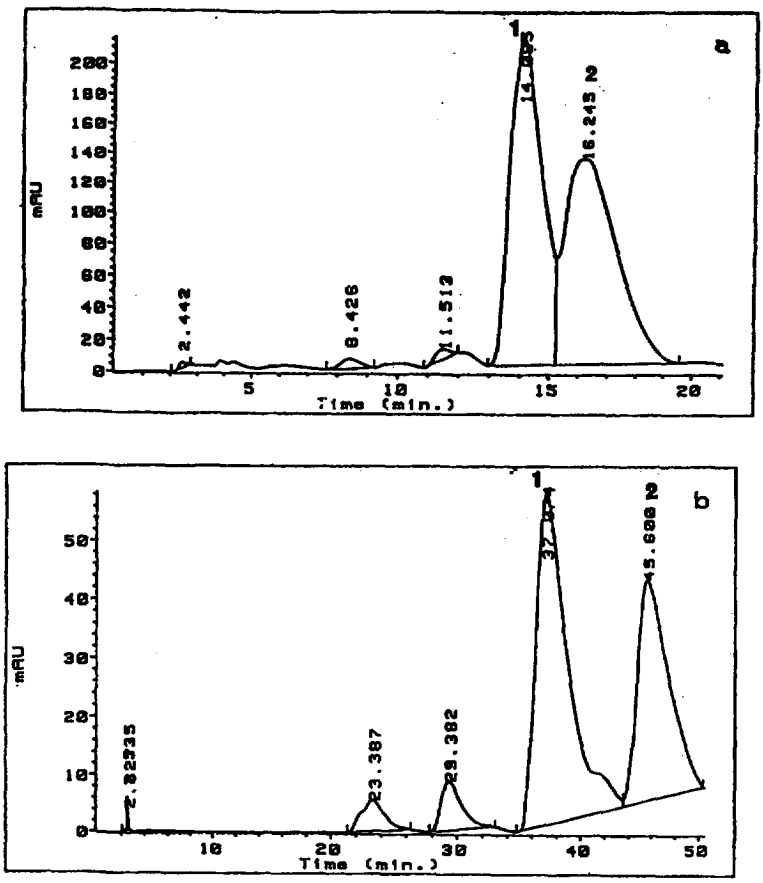

Fig. 2. Chromatograms of epimeric pavine N-oxides from *Thalictrum simplex*: (−)-thalimonine N-oxide A (**1**) and (−)-thalimonine N-oxide B (**2**) eluted with different mobile phases: (a) methanol–1.5% aqueous orthophosphoric acid (35:65); and (b) ethanol–1.5% aqueous orthophosphoric acid (15:85).

Table 1
Influence of variations of the mobile phase on the selectivity α and the resolution R_s

Parameter[a]	Mobile phase[b]			
	1	2	2a	2b
k'_1	4.78	13.15	5.84	5.68
k'_2	5.66	16.40	7.14	6.94
α	1.18	1.25	1.22	1.22
R_s	1.20	2.80	1.86	1.59

[a] k'_1 and k'_2 = capacity factors of (−)-thalimonine N-oxide A (**1**) and (−)-thalimonine N-odixe B (**2**), respectively.
[b] Mobile phases: 1 = methanol–1.5% aqueous orthophosphoric acid (35:65); 2 = ethanol–1.5% aqueous orthophosphoric acid (15:85); 2a = ethanol–1.5% aqueous orthophosphoric acid (25:75); 2b = ethanol–1.5% aqueous orthophosphoric acid (35:65).

Table 2
Retention times (t_R) and contents of (−)-thalimonine N-oxide A (**1**) and (−)-thalimonine N-oxide B (**2**) in different fractions after reversed-phase HPLC separation

Fraction	t_R (min)	Amount (mg)	Content of (−)-thalimonine N-oxide (rel. %)	
			1	**2**
1	9	1.0	73.9	26.5
2	10	3.0	83.4	16.6
3	11	2.5	74.5	25.5
4	12	2.0	65.3	34.7
5	13	3.0	42.0	56.0
6	14	1.6	21.8	78.2
7	16	1.8	11.6	88.4
8	17	1.3	9.2	90.8
9	19	3.0	6.5	93.5

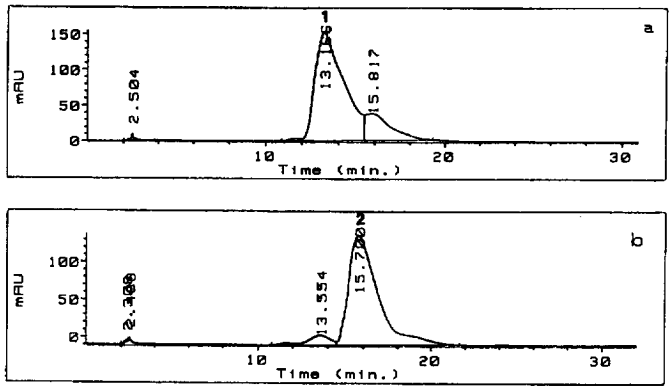

Fig. 3. Chromatograms of separated epimeric pavine N-oxides from *Thalictrum simplex*. (a) (−)-Thalimonine N-oxide A (**1**); and (b) (−)-thalimonine N-oxide B (**2**).

method is a simple reversed-phase HPLC method for the separation of epimeric pavine N-oxides of equal polarity.

The new secoisoquinoline alkaloid (−)-hyperectine, recently isolated from Mongolian *Hypecoum erectum*, was found to be present in the N-ammine–imino equilibrium state by ^{1}H NMR and HPLC analyses. The HPLC separation was performed with phase 2 in the above-described method with gradient elution, $R_s = 3.40$. The last result shows that the method can be applied not only for the separation of epimeric N-oxide mixtures [17].

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