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ANALYSIS OF LUPINE ALKALOIDS IN PLANTS BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY*

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SUMMARY

A high-performance liquid chromatographic method was devised for the qualitative and quantitative analysis of lupine alkaloids in plants. The separation of 22 naturally occurring lupine alkaloids was performed by adsorption chromatography (silica gel) with three solvent systems consisting of diethyl ether, methanol and ammonia solution and reversed-phase chromatography (octadecylsilica) with a buffered aqueous solution containing acetonitrile. The elution of alkaloids was monitored by UV absorption at 220 and 310 nm. Lupine alkaloids containing a 2-pyridone ring were detected by UV absorption at both wavelengths, while the alkaloids that do not possess this unsaturared heterocyclic ring lack UV absorption at 310 nm. The determination of lupine alkaloids was carried out by an external standard method using (−)-cytisine as the standard. The peak area for 1 μg of each alkaloid detected at 220 nm was measured and normalized for that of (−)-cytisine, and these relative factors were used for the determination of the alkaloids. The qualitative and quantitative analysis of lupine alkaloids in plants of the genus *Thermopsis* was performed by this method.

INTRODUCTION

Lupine alkaloids occur particularly in several Leguminosae genera and are also found sporadically in other plant families[1,2]. All contain one or two quinolizidine ring systems and are therefore called quinolizidine alkaloids. Their carbon skeleton is built up from two or three C_5 units of cadaverine derived from lysine[3,4]. Lupine

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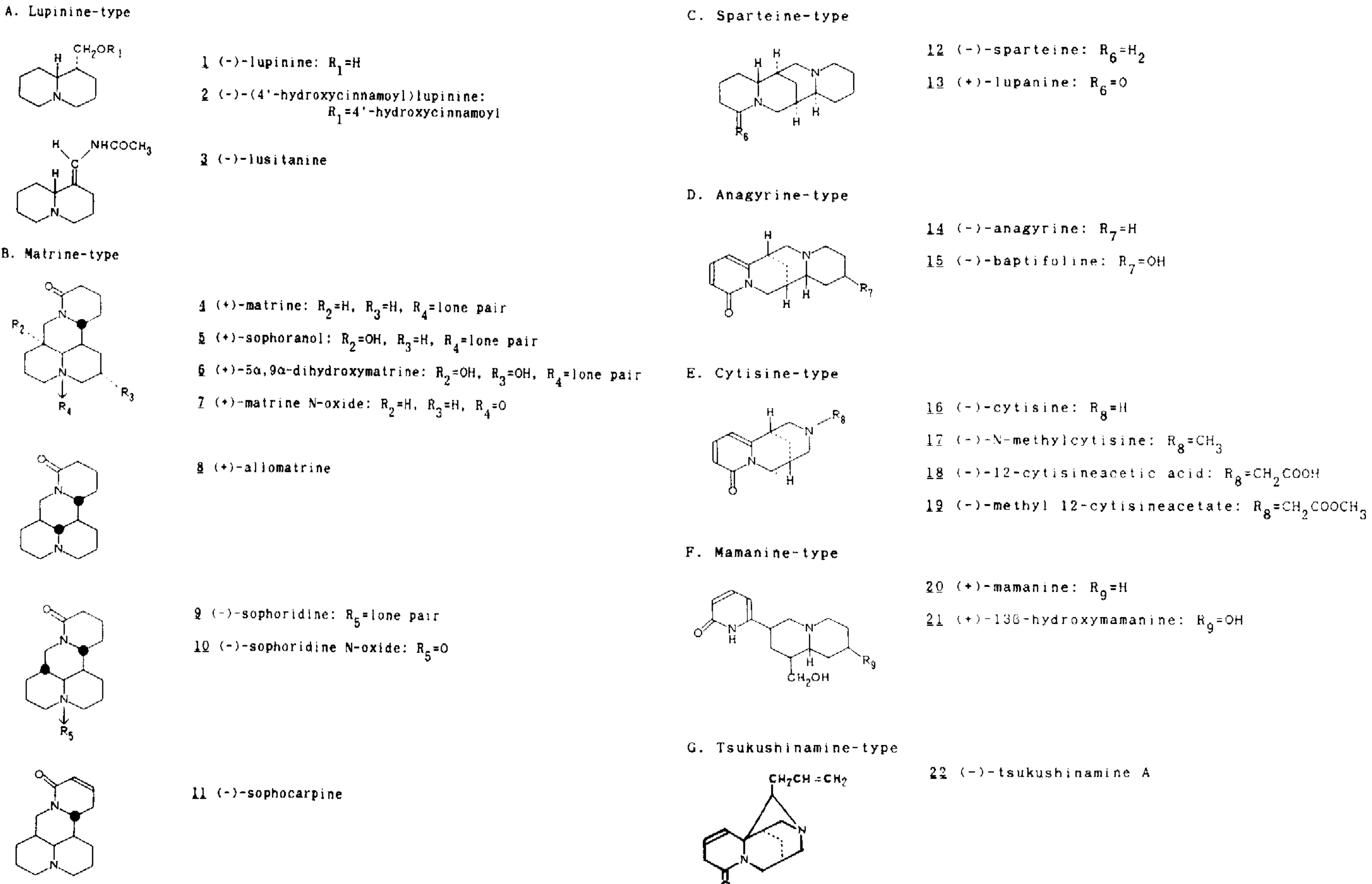

Fig. 1. Structures of lupine alkaloids. The absolute configurations (+)-mamanine (20) and (+)-13β-hydroxymamanine (21) have not been determined.

alkaloids are well known as constituents of poisonous plants because of their toxicity towards mammals. Some of them exhibit potentially useful pharmacological activities[5].

The most important methods for the determination of lupine alkaloids in biochemical and chemotaxonomic studies have hitherto been gas chromatography (GC), gas chromatography–mass spectrometry (GC–MS) and thin-layer chromatography (TLC)[5]. These methods, however, have some disadvantages with regard to resolution and involatile samples. High-performance liquid chromatography (HPLC) is expected to be superior for the determination of lupine alkaloids in plant extracts, but no systematic investigation of its application has been reported so far.

In this study, we devised a qualitative and quantitative HPLC method for lupine alkaloids.

EXPERIMENTAL

Chemicals

The 22 natural lupine alkaloids used as standard samples were isolated and identified in the course of our recent studies[6–14]. Their structures are shown in Fig. 1. Diethyl ether, methanol and water were used immediately after all-glass distillation. Ammonia solution and acetonitrile of the highest grade available were used without further purification.

HPLC

LiChrosorb Si 60 (5 μm) was purchased from Kanto Chemicals (Tokyo, Japan) and Inertsil ODS (5 μm) from Gaschro-Kogyo (Tokyo, Japan). HPLC was carried out with a Senshu HPLC system constiting of an SSC-3100 pump, an SSC-3110 pump controller and SSC-E1E005 manual injector, equipped with a 4- or 20-μ sample loop, an SASC-3510 column oven, an SSC Y-1000 variable UV detector and a Sekonic SS-250F recorder. As highly volatile diethyl ether is used as a component of the eluent, the temperature of the column oven was maintained at 20°C. The eluents were sonicated for 1 min before use. The sample was dissolved in methanol and filtered through a Shodex DT filter (ED-03, 0.45 μm) prior to injection.

Plant extracts

The aerial parts of *Thermopsis lupinoides* Link and *T. chinensis* were collected in the Medicinal Plant Gardens of Chiba University in May 1986. Fresh plant tissues were homogenized and extracted with 75% ethanol. The neutral and acidic materials were removed by extraction with dichloromethane (twice) after acidification (pH 1) with hydrochloric acid. The basic fraction was extracted with dichloromethane (twice) from the saturated solution made alkaline with potassium carbonate. The basic fraction were analysed by HPLC after filtration.

RESULTS AND DISCUSSION

The HPLC conditions for the separation of lupine alkaloids are summarized in Table I. The separation of the alkaloids was performed satisfactorily in both the normal-phase (silica gel) and reversed-phase (octadecylsilica) modes.

TABLE I

HPLC CONDITIONS FOR THE ANALYSIS OF LUPINE ALKALOIDS

Mode	*Column*	*Solvent*	*Flow-rate (ml/min)*	*Detection (nm)*
Normal phase	LiChrosorb Si 60 (5 μm), 250 × 4.6 mm I.D.	(A) 15% Methanol in diethyl ether–5% ammonia solution (25:1, v/v) (B) 25% Methanol in diethyl ether–5% ammonia solution (25:1, v/v) (C) 50% Methanol in diethyl ether–5% ammonia solution (25:2, v/v)	1.5	220 and 310
Reversed phase	Inertsil ODS (5 μm), 150 × 4.6 mm I.D.	(D) Acetonitrile–5 m*M* potassium phosphate buffer (pH 5.5) (1:9, v/v)	1.0	220 and 310

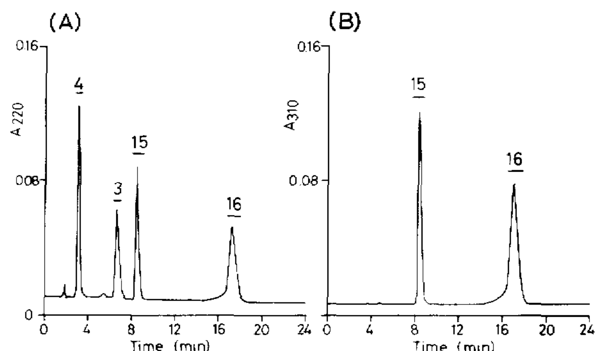

Fig. 2. HPLC profile of standard alkaloids with solvent system B. HPLC was carried out according to Table I. The sample consisted of (−)-lusitanine (3) (0.4 μg), (+)-matrine (4) (2.0 μg), (−)-baptifoline (15) (2.0 μg), (−)-cytisine (16) (2.0 μg) and (+)-matrine N-oxide (2.0 μg). UV detection: (A) 220 nm; (B) 310 nm.

The chromatographic patterns of standard alkaloids are shown in Figs. 2 and 3 (normal-phase mode) and in Fig. 4 (reversed-phase mode). Table II summarizes the retention times of lupine alkaloids in four chromatographic systems and the cytisine constants (see below). The N-oxides of lupine alkaloids, such as matrine N-oxide (7) and sophoridine N-oxide (10), were not eluted from the LiChrosorb Si 60 column by solvents A and B, but they were eluted by solvent C. The N-oxides were also chromatographed on the ODS column with slight tailing.

For the qualitative analysis of lupine alkaloids, their elution was monitored by

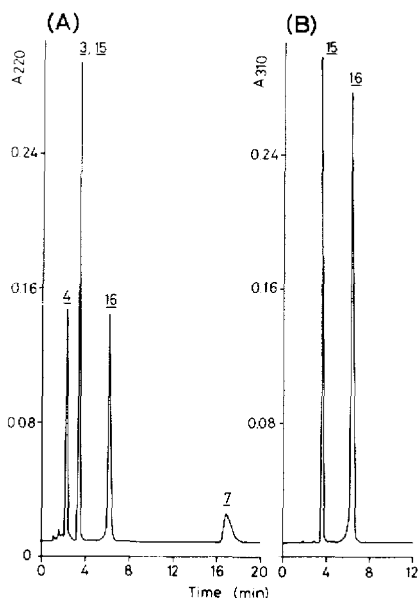

Fig. 3. HPLC profile of standard alkaloids with solvent system C. Conditions and samples as in Table I and Fig. 2.

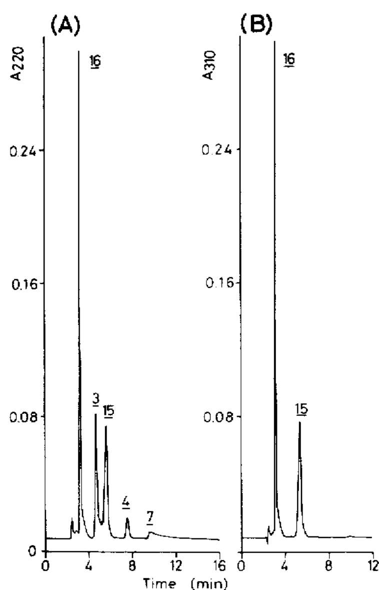

Fig. 4. HPLC profile of standard alkaloids solvent with system D. Conditions and samples as in Table I and Fig. 2.

UV absorption at 220 and 310 nm. The alkaloids that contain a 2-pyridone ring, such as (−)-cytisine, (−)-baptifoline and (+)-mamanine, were detected by their UV absorption at both wavelengths[15]. On the other hand, the alkaloids that do not possess this unsaturated heterocyclic ring, such as (+)-matrine and its N-oxide (+)-lupanine, (−)-lusitanine and (−)-sparteine, lack UV absorption at 310 nm. From these characteristic UV properties and the retention times in four chromatographic systems, we were able to identify the alkaloids in plant extracts with the known lupine alkaloids.

In order to determine the amount of each alkaloid in the plant extracts, the alkaloids were analysed by adsorption chromatography with the solvent systems B and C. The lupine alkaloids were determined by the external standard method, using (−)-cytisine as the standard. The peak area of 1 μg of each alkaloid at 220 nm was measured and normalized to that of (−)-cytisine as 1.00. Hence the "cytisine constant" is the relative absorbance at 220 nm of 1 μg of each alkaloid with respect to cytisine. The area of the cytisine peak varies linearly with its concentration. The cytisine constants of lupine alkaloids are summarized in Table II. From these data, the 22 lupine alkaloids can be determined. The limit of detection was 0.01–0.2 μg for each alkaloid.

We applied this HPLC method to the qualitative and quantitative analysis of lupine alkaloids in the aerial parts of plants of the genus *Thermopsis* growing in Japan. We were able to identify the five major alkaloids in the basic fraction prepared as described under Experimental. As shown in Table III, these plants accumulated alkaloids of the sparteine, anagyrine and cytisine types and not those of the lupinine and matrine types. The main base in *T. chinensis* is N-methylcytisine, as reported previously[16]. It is assumed to be biosynthesized from cytisine by a cytisine-specific N-methyltransferase[17,18]. The main alkaloid in *T. lupinoides*, in contrast, is lupanine,

TABLE II

RETENTION TIMES AND CYTISINE CONSTANTS OF LUPINE ALKALOIDS

The retention times were determined in four chromatographic systems. Cytisine constants were obtained by normalizing the peak area of 1 μg of each alkaloid to that of (−)-cytisine as 1.00. Determinations were carried out by adsorption chromatography.

*Alkaloid No.**	*Absorption at 310 nm*	*Retention time in four solvent systems** (min)*				*Cytisine constant*
		A	*B*	*C*	*D*	
1	−	8.03	7.90	5.83	9.50	0.10
2	−	2.56	2.70	1.93	−***	0.65
3	−	6.72	6.90	3.52	4.30	2.84
4	−	3.42	3.12	2.10	7.01	0.64
5	−	5.20	4.03	2.61	8.51	0.59
6	−	13.0	6.15	2.73	4.28	0.36
7	−	−	−	16.9	8.80	0.53
8	−	6.31	6.45	3.58	5.90	0.48
9	−	9.72	10.7	5.42	4.95	0.47
10	−	−	−	21.4	9.32	0.31
11	−	3.26	3.20	2.43	8.30	0.14
12	−	21.3	35.0	−	−	0.39
13	−	5.17	5.40	3.20	4.90	0.33
14	+	3.83	3.75	2.46	7.02	0.44
15	+	13.2	8.40	3.52	4.71	0.55
16	+	23.8	17.1	5.86	2.73	1.00
17	+	5.97	4.62	2.86	5.90	0.80
18	+	−	18.4	1.88	2.68	1.21
19	+	4.25	3.38	2.43	8.30	0.86
20	+	11.7	7.73	3.34	4.20	0.74
21	+	−	26.7	5.47	2.62	0.60
22	−	9.30	8.16	4.14	5.14	0.40

* See Fig. 1.
** See Table I.
*** The sample was not eluted from the column.

TABLE III

LUPINE ALKALOIDS IN PLANTS OF THE GENUS *THERMOPSIS*

The fresh aerial parts of *T. chinensis* and *T. lupinoides* were extracted and the basic fractions were analysed by HPLC as described under Experimental.

Alkaloid	*Concentration (mg/g fresh weight)*	
	T. chinensis	*T. lupinoides*
Anagyrine (14)	0.017	0.141
N-Methylcytisine (17)	0.212	0.164
Lupanine (13)	0.004	1.273
Baptifoline (15)	0.010	0.082
Cytisine (16)	0.048	0.003

which is regarded as the precursor of alkaloids of the anagyrine and cytisine types[19]. Hence it is suggested that the ability to oxidize lupanine to anagyrine and cytisine is low in the aerial parts of *T. lupinoides* compared with that in *T. chinensis*. Biosynthetic and stereochemical relationships of these alkaloids are further problems to be solved.

Tsukushinamine-type alkaloids are new cage-type lupine alkaloids isolated from *Sophora franchetina* Dunn, which is a native and very rare shrub in Japan[9]. As far as we know, alkaloids of this type have not so far been found in other plant species. Our HPLC method should be useful not only for screening lupine alkaloids in plants but also for biochemical, physiological, and chemotaxonomic studies of these alkaloids.

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