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Separation of stereoisomeric oxindole alkaloids from *Uncaria tomentosa* by high performance liquid chromatography

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Abstract

Two HPLC methods are presented that allow the rapid separation and determination of the stereoisomeric oxindole alkaloids present in the South American liana *Uncaria tomentosa* (Willd.) DC (Rubiaceae). Peak purities were established using a dual-wavelength technique. The effect of temperature on resolution was evaluated. The alkaloid pattern of individual plants changes with time. As these alkaloids are readily interconverted, a mild extraction procedure had to be established in order to retain the actual alkaloid composition.

1. Introduction

Although the alkaloids of the pantropical genus *Uncaria* have been surveyed extensively [1], only a few accounts have dealt with the constituents of the South American species *U. tomentosa* (Willd.) DC. In an early note, *U. tomentosa* was reported to contain tetracyclic indole and oxindole alkaloids [2]. Later, only pentacyclic oxindoles were found in plant material from Central Peru. However, it was not established whether the plant belonged to the species *U. tomentosa* or *U. guianensis*, both of which grow in that region [3]. The caryology of these two species was investigated [4], but difficulties in the correct identification of *Uncaria* species remained. Interrogation of locals from the Peruvian rain forest, who use *Uncaria* roots in traditional medicine, revealed that there were actually three varieties of *U. tomentosa*, which when freshly cut exhibit white-grey, yellow-brown or dark red root bark, and only the yellow-brown variety was used [5]. A reinvestigation showed the tetracyclic oxindoles rhynchophylline and isorhynchophylline to be the major alkaloids present in each of the three varieties, while the pentacyclic oxindole isopteropodine was found in high concentrations especially in the yellow-brown variety [6,7]. Recently, six pentacyclic oxindoles, namely pteropodine, isopteropodine, speciophylline, uncarine F, mitraphylline and isomitraphylline, have been identified in the root of *U. tomentosa*, but it was not specified which variety was used [8]. These inconsistent findings prompted us to develop reliable methods for the determination of above oxindole alkaloids.

2. Experimental

2.1. Equipment

A Merck–Hitachi HPLC system consisting of

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a Model L-6200 A pump, a Model L-4250 UV–Vis or Model L-4500 diode-array detector, a Model D-6000 interface and an IBM-compatible personal computer was used. The HPLC Manager Software D-6000 was from Merck–Hitachi. A Rheodyne Model 9125 sample injector fitted with a 200-μl polyether ether ketone (PEEK) loop was employed. LiChroCART 125 mm × 4 mm I.D. columns packed with LiChrospher 100 RP-18 (5 μm) were purchased from Merck (Darmstadt, Germany). The mixing chambers, injector and column were thermostated by a Peltier thermostat from Offenbeck Industrieelektronik (Langenzersdorf, Austria). The system suitability was checked by chromatographing a standard sample of pteropodine and determining the number of theoretical plates. A minimum of 7000 plates was found to be necessary in order to obtain the results reported here.

2.2. Reagents and materials

All reagents were of analytical-reagent grade, except acetonitrile, which was of HPLC grade, from Merck. Water was purified by use of Herco (Freiberg, Germany) Model HP40W reverse osmosis equipment. Eluents were gassed with helium. TLC plastic sheets (20 × 20 cm) precoated with a 0.2-mm layer of silica gel 60 F_{254} and silica gel 60 (70–230 mesh) for column chromatography were obtained from Merck. Plant material was collected and identified by K. Keplinger and D. Keplinger in the Province of Chanchamayo, Region of Andres Avelino Caceres (the former Departments of Junin and Pasco), Peru. Climate data (temperature, humidity, exposure to sunlight and rainfall) were also recorded [9].

2.3. Identification of alkaloids

The milled root bark of the yellow-brown variety was extracted with supercritical carbon dioxide and, after acid–base work-up of the fatty extract, the pure alkaloids were isolated by silica gel chromatography using mixtures of hexane, ethyl acetate and methanol with increasing polarity as the eluent. They were identified by analysis of two-dimensional correlated ^{1}H and ^{13}C NMR spectra and by comparison of melting points, mass spectra, hR_F values and isomerization behaviour with literature data [3,6,10–16]. Their structures and stereochemical notations are given in Table I. Additionally, natural mitraphylline and isomitraphylline were shown to be identical with the synthetic compounds prepared from ajmalicine [17].

2.4. Separation of oxindole alkaloids

We developed two elution programmes for the separation of oxindole alkaloids by HPLC. For system I, acetonitrile–*tert.*-butyl methyl ether–10 m*M* aqueous phosphate buffer (pH 6.7)

Table 1
Retention times, hR_F values, absorbance ratios and stereochemistry of oxindole alkaloids

Alkaloid	t_R (min)		hR_F	A_{245}/A_{230}	A_{245}/A_{260}	Chiral centre[a]					
	System I	System II				C-3	N-4	C-7	C-15	C-19	C-20
Pteropodine (**1**)	12.7	2.9	65	1.62	1.45	*S*	*R*	*R*	*S*	*S*	*S*
Isopteropodine (**2**)	24.1	4.4	73	1.57	1.44	*S*	*R*	*S*	*S*	*S*	*S*
Speciophylline (**3**)	6.7	2.0	7	1.44	1.45	*R*	*S*	*S*	*S*	*S*	*S*
Uncarine F (**4**)	7.9	2.6	48	1.36	1.53	*R*	*S*	*R*	*S*	*S*	*S*
Mitraphylline (**5**)	8.8	2.3	18	1.40	1.65	*S*	*R*	*R*	*S*	*S*	*R*
Isomitraphylline (46)	11.8	3.2	60	1.33	1.69	*S*	*R*	*S*	*S*	*S*	*R*
Rhynchophylline (**7**)	13.7	4.1	4	1.53	1.61	*S*	*R*	*R*	*S*	–	*R*
Isorhynchophylline (**8**)	15.6	4.3	67	1.52	1.61	*S*	*R*	*S*	*S*	–	*R*

[a] This notation of stereochemistry was proposed by Poisson and Pousset [18].

(34:1:65) was used as the eluent at a flow rate of 1.0 ml/min. The column temperature was 12°C and detection was carried out at 245 nm. The typical pressure build-up was 110 bar. For system II, acetonitrile–10 m*M* aqueous phosphate buffer (pH 7.0) (45:55)was used as the eluent at a flow-rate of 1.3 ml/min. The column temperature was 80°C and detection was carried out at 245 nm. The typical pressure build-up was 55 bar.

Chromatograms were developed with ethyl acetate–hexane (9:1) and the spots were detected with UV light at 254 nm.

The absorbance ratios $A_{245\ nm}/A_{230\ nm}$ and $A_{245\ nm}/A_{260\ nm}$ were determined using diode-array detection.

2.5. Sample preparation

The buffers were chosen according to the eluent system used. Stock standard solutions of the standard alkaloids in acetonitrile were prepared and diluted appropriately with 10 m*M* aqueous phosphate buffer. The root bark (1.000 g) was finely milled and leached with 20 ml of methanol–water–1.2 *M* hydrochloric acid (50:50:1) for 1 h at 20°C and the extract was decanted and filtered; this procedure was repeated four times, and the combined extracts were diluted to a final volume of 100 ml. An aliquot of this solution was diluted 1:5 with aqueous phosphate buffer. For validation purposes the root bark (1.000 g) was also exhaustively extracted in a Soxhlet apparatus with methanol (150 ml). All samples were filtered through solvent-resistant 0.45-μm filters from Sartorius (Göttingen, Germany) and degassed by ultrasonification just prior to injection.

2.6. Standard preparation

The alkaloid mixture was suspended in boiling *tert.*-butyl methyl ether, filtered and recrystallized twice to yield pteropodine (**1**) with a purity higher than 98% (HPLC and microanalysis). Equilibration of pure pteropodine by heating in aqueous methanol at pH 9 gave a mixture containing 70% isopteropodine (**2**) and at pH 2 a mixture containing 40% speciophylline (**3**). From these enriched mixtures, the pure isomers were obtained by column chromatography as described above and used as standards.

3. Results and discussion

3.1. Quantification

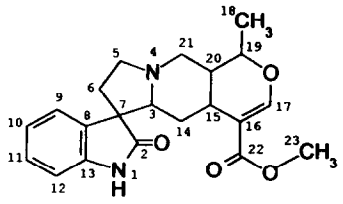
The purified alkaloids pteropodine (**1**), isopteropodine (**2**) and speciophylline (**3**) and synthetic mitraphylline (**5**) were used for calibration, giving identical plots within statistical error. A linear relationship between peak area and concentration of pteropodine was established in the ranges 0.05–20 μg/ml [system I; ten data points; correlation coefficient $R^2 = 0.998$; relative standard deviation (R.S.D.) = 1.07% for the five higher and 2.10% for the five lower concentrations] and 0.005–20 μg/ml (system II; twelve data points; $R^2 = 0.999$; R.S.D. was 0.65% for the six higher and 1.05% for the six lower concentrations). The detection limits were 5 ng/ml (system I) and 0.5 ng/ml (system II).

3.2. Precision and recovery

The precision (R.S.D.) was determined by analysis of six preparations of a root extract and was 0.90% (system I) and 1.03% (system II). The recovery was determined by spiking two preparations of the extract at two different levels of pteropodine before analysis. Extracts containing approximately 10 μg/ml of total alkaloids were spiked at levels of 15 and 20 μg/ml. The recovery ranged from 99 to 101% (system I) and from 99 to 103% (system II).

3.3. Chromatography

The constituents of *U. tomentosa* are of interest because this plant is used in traditional medicine in South America for the treatment of diseases that in our interpretation can be attributed to immune system disorders. We have isolated and identified eight oxindole alkaloids from *U. tomentosa* so far. A superior separation of



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pteropodine (**1**), isopteropodine (**2**), speciophylline (**3**), uncarine F (**4**), mitraphylline (**5**) and isomitraphylline (**6**) was achieved by isocratic elution using HPLC system I. A typical chromatogram is given in Fig. 1a. In the course of validation, peak purity is an essential criterion. As the stereoisomeric compounds **1–6** exhibit virtually identical UV spectra, absorbance ratios at suitably selected wavelengths were calculated. Any impurities that co-eluted or overlapped with the peak of interest would be indicated by a distortion of the flat-topped peak

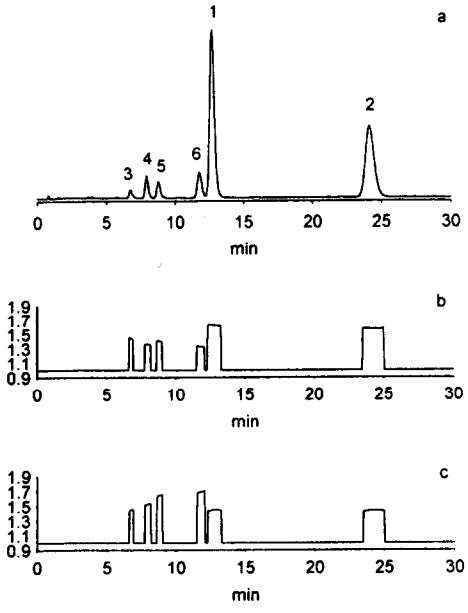


Fig. 1. (a) Chromatogram of the alkaloid mixture obtained by extraction of *Uncaria tomentosa* root bark with supercritical CO_2. Separation of pteropodine (**1**), isopteropodine (**2**), speciophylline (**3**), uncarine F (**4**), mitraphylline (**5**) and isomitraphylline (**6**) was carried out using HPLC system I (see text). Absorbance ratio plots using (b) 245/230 nm and (c) 245/260 nm, threshold set at 2% of highest peak.

in the ratiogram. We propose the use of 245 nm as the numerator wavelength because the absorption maxima of the alkaloids are between 243 and 247 nm. The denominator wavelength should be selected where the absorption is small, such that the resulting ratio exhibits a large value. We used 230 and 260 nm, respectively. It can be clearly seen in Fig. 1b and c that by this method the alkaloids can be distinguished and that the shapes of the peaks indicate homogeneity.

In contrast to a recently published method [8], the use of pure acetonitrile as the organic solvent instead of acetonitrile–methanol (1:1) led to better resolution. The admixture of a small amount of *tert.*-butyl methyl ether as a second modifier gave a further improvement in resolution. The effect of temperature on resolution has been reported in the range 9–30°C [8]. We found a different temperature dependance for our system I (Fig. 2a). It seemed of interest to evaluate the effect of higher temperatures also. These experiments led to the development of eluent system II. The temperature dependance of resolution using this system in the range 50–80°C is depicted in Fig. 2b. Complete separation of the alkaloids was achieved at 80°C within 5 min, as can be seen in Fig. 3. It is noteworthy that the sequence of elution of two pairs of alkaloids is reversed, namely that of **5–4** and **6–1**. The elution behaviour of these isomers, especially the fact that **2** is eluted so much later than the others, is not fully understood [19]. Retention times for both HPLC systems, hR_F values for TLC and absorbance ratio values are given in Table 1. As both methods use isocratic elution, no additional equilibration time is required. Thus, especially system II provides excellent resolution with a minimum turnaround time.

3.4. Sample preparation

Often, too little attention is paid to the extraction process in phytochemical research. It is known that oxindole alkaloids which are spiro structures undergo isomerization easily when heated with acids or bases [10,11]. We investigated the behaviour of these alkaloids in solu-

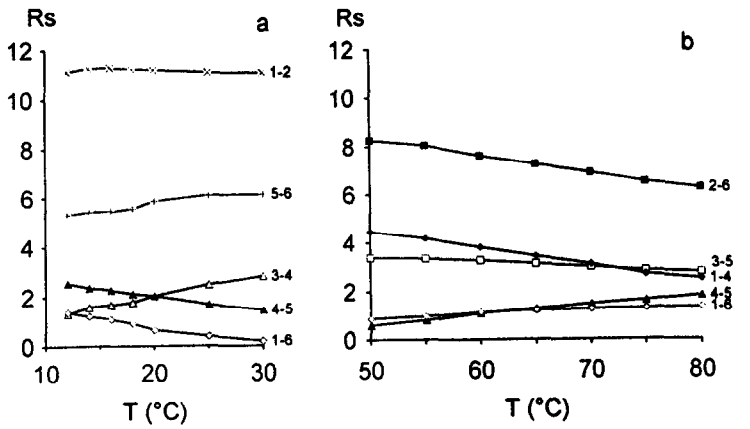


Fig. 2. Effect of temperature on resolution of the alkaloid pairs (a) **1–2**, **5–6**, **3–4**, **4–5** and **1–6** using HPLC system I (see text) in the range 12–30°C, and (b) **2–6**, **3–5**, **1–4**, **4–5** and **1–6** using HPLC system II (see text) in the range 50–80°C.

tions at different pH values and at different temperatures. We found that equilibration in neutral or basic solutions took place even at room temperature and proceeded rapidly under reflux conditions. However, in acidic solutions the isomerization was slower in general and almost ceased at room temperature. Hence, the usual continuous extraction technique in a Soxhlet apparatus, where the extracted material is in contact with a boiling solvent (methanol) during a prolonged period, could not be applied. The change of alkaloid distribution in a model mixture of pteropodine isomers **1–4** effected by boiling in methanol is shown in Fig. 4. The same applies for mitraphyllines and rhynchophyllines. Of course, these conversions also take place in the plant, but not after it has been dried. The mixture obtained by carbon dioxide extraction (Fig. 1a) has clearly undergone isomerization. It is evident that extracts obtained in this manner would not reflect the actual proportions of alkaloids as they occur in the plant. In contrast, when the root was extracted repeatedly with acid at room temperature, no isomerization was observed. Validation of the extraction techniques showed that the acid method yielded a 92.4% recovery (S.D. 3.7%) in authentic proportions compared with the Soxhlet method, which was assumed to give 100% (S.D. 2.8%) but showing artifacts.

The results of determinations using the different extraction methods are given in Table 2. All the following results were obtained by the acid extraction method.

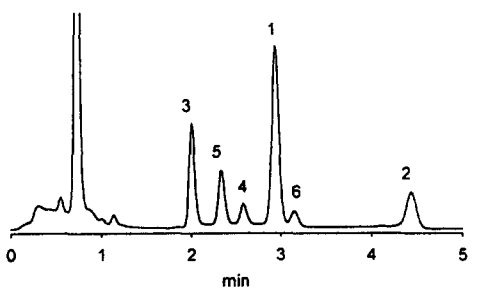


Fig. 3. Chromatogram of an acid extract of *Uncaria tomentosa* root bark containing the alkaloids **1–6** in authentic proportions. The separation was carried out using HPLC system II (see text).

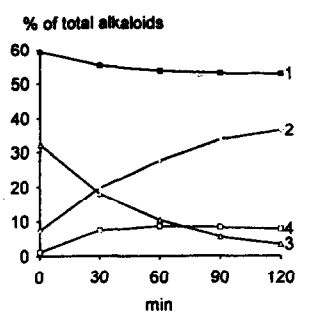


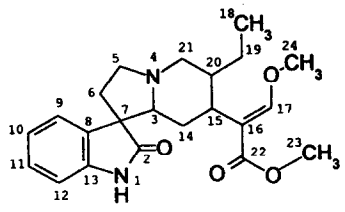
Fig. 4. Generation of artifacts by an unsuitable extraction procedure, *i.e.*, by boiling a model mixture of pteropodine isomers **1–4** in methanol.

Table 2
Comparison of extraction methods

Alkaloid	Soxhlet extraction		Acid extraction	
	mg/g root bark	% of total alkaloids	mg/g root bark	% of total alkaloids
Pteropodine (1)	6.51	51.62	5.75	49.01
Isopteropodine (2)	3.76	29.80	2.28	19.44
Speciophylline (3)	0.38	3.03	1.81	15.41
Uncarine F (4)	0.64	5.09	0.55	4.70
Mitraphylline (5)	1.03	8.15	1.13	9.65
Isomitraphylline (6)	0.29	2.30	0.21	1.79
Total alkaloids	12.61		11.73	

3.5. Plant analysis

It was found that the three varieties can be related to the climate, the dark red variety preferring a warm and moist environment, the white-grey variety preferring cool and dry conditions and the yellow-brown variety being in between. However, no correlation of alkaloid contents and the colour of the root bark could be established, as can be seen in Table 3, which gives the alkaloid content of three samples collected at the same time (July 1990). Only small stripes of the root bark were taken and the root was returned to the ground each time in order not to destroy the plant. Interestingly, samples from several individual plants harvested in different years displayed completely different alkaloid patterns. Only two major alkaloids were detected in these samples, rhynchophylline (**7**) and isorhynchophylline (**8**). Individual plants



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switched from one alkaloid pattern to the other over the years. In all instances either the pteropodine and mitraphylline isomers **1–6** or the rhynchophyllines **7** and **8** were found as major constituents (Table 4). The total alkaloid content varied from 0.036 to 3.83% (w/w) of dried root bark. The plant material was always collected from June to October, but there are reports of the Asian species *Mitragyna parvifolia* changing its oxindole alkaloids within several

Table 3
Alkaloid content of three differently coloured root bark samples of *Uncaria tomentosa*

Alkaloid	Alkaloid content (mg/g root bark)		
	White-grey root bark	Yellow-brown root bark	Dark red root bark
Pteropodine (1)	7.54	5.20	10.53
Isopteropodine (2)	3.94	2.49	2.99
Speciophylline (3)	8.16	2.80	4.06
Uncarine F (4)	3.00	0.79	0.86
Mitraphylline (5)	3.03	4.70	1.83
Isomitraphylline (6)	0.52	1.89	0.04
Total alkaloids	26.20	17.88	20.30

Table 4
Variation of alkaloid content in the root bark of *Uncaria tomentosa* collected at different times

Alkaloid	Alkaloid content (mg/g root bark)										
	Plant 1[a]				Plant 2[b]			Plant 3[c]			
	October 1985	July 1987	July 1990	July 1992	June 1983	October 1985	July 1987	August 1981	June 1983	October 1985	July 1987
Pteropodine (1)	3.14	2.65	6.18	0.04	1.29	3.83	0.04	0.48	0.04	0.48	0.43
Isopteropodine (2)	1.01	1.53	2.72	0.01	0.40	1.66	0.01	0.16	0.01	0.15	0.12
Speciophylline (3)	1.65	2.32	3.61	0.03	1.72	2.49	0.03	0.28	0.02	0.35	0.24
Uncarine F (4)	0.35	0.61	0.86	0.17	0.39	0.66	0.00	0.02	0.00	0.03	0.01
Mitraphylline (5)	1.91	3.59	5.48	0.11	1.07	3.62	0.07	0.76	0.04	1.04	0.60
Isomitrphylline (6)	0.80	1.63	2.17	0.04	0.66	1.52	0.08	0.26	0.05	0.38	0.24
Rhynchophylline (7)	0.01	0.08	0.05	13.82	20.33	0.09	12.53	3.36	0.08	7.12	4.52
Isorhynchophylline (8)	0.02	0.03	0.02	14.23	12.39	0.03	8.63	8.86	0.12	7.17	5.94
Total alkaloids	8.89	12.44	21.09	28.45	38.25	13.90	21.39	14.18	0.36	16.72	12.10

[a] Plant 1 is of the white-grey variety.
[b] Plant 2 is of the white-grey variety.
[c] Plant 3 is of the yellow-brown variety.

months [20,21]. Climate data also did not offer an explanation for this remarkable fact [9]. So far it cannot be deduced from our results whether seasonal or long-term fluctuations occur. At least it can be stated that the alkaloid distribution of the species *U. tomentosa* is subject to change, thus explaining the inconsistent findings of earlier investigations.

4. Acknowledgement

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