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QUALITATIVE AND QUANTITATIVE EVALUATION OF BISINDOLE USAMBARANE ALKALOIDS IN *STRYCHNOS USAMBARENSIS* ROOTS BY HIGH PERFORMANCE LIQUID CHROMATOGRAPHY-DIODE-ARRAY

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Abstract. A high performance liquid chromatographic method for the simultaneous determination of bisindole usambarane alkaloids from the roots of *Strychnos usambarensis* using a diode-array detector is proposed. The system, consisting of a RP-8 Select B column and a linear gradient elution with acetonitrile and acetate buffer allowed the separation of all the alkaloids in 20 min. Usambarensine was found to be the major tertiary alkaloid present in the roots. In order to demonstrate the possibility of quantifying the bisindole alkaloids in crude roots extracts, the quantitative evaluation of 3',4'-dihydrousambarensine, usambarensine and N_b-methylusambarensine was carried out.

Keywords: High performance liquid chromatography; diode-array detector; *Strychnos usambarensis*; bisindole usambarane alkaloids.

INTRODUCTION

Strychnos usambarensis Gilg (Loganiaceae) is a tree used traditionally by people of the Banyambo tribe who live along the Akagera river on the border between Rwanda and Tanzania. The leaves and roots are used as the main ingredient of a curarizing arrow poison (Angenot, 1971). This curarizing activity is mainly due to minor quaternary (dimeric) alkaloids such as curarine, calebassine, dihydrotoxiferine

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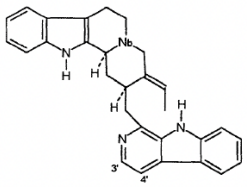
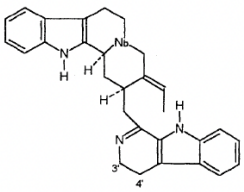
and afrocurarine (Angenot *et al.,* 1975b). The main quaternary alkaloids are monomers such as malindine and isomalindine **(5)** (Caprasse *et al.,* 1984). Tertiary bases such as usambarensine **(1)**, harman and akagerine **(4)** are also found in the root bark (Angenot and Bisset, 1971; Angenot *et al.,* 1975a; Massiot and Delaude, 1988). A number of these alkaloids form the usambarane group (usambarensine **(1),** 3',4'-dihydrousambarensine **(2)** and their N_b-methylated derivatives), some of which have been tested on protozoa such as *Plasmodium falciparum, Entamoeba histolytica,* and *Giardia intestinalis*. The results (Wright *et al.,* 1991, 1994) showed antiplasmodial properties for 3',4'-dihydrousambarensine **(2)**, and antigiardial and antiamoebic properties for usambarensine **(1)**. Antimitotic activities have also been described for these alkaloids (Leclercq *et al.,* 1986). Furthermore, the cytotoxicity and the selective antiprotozoal activity of usambarensine prompted the investigation and comparison of its effects with emetine by using mouse B16 melanoma cells cultivated *in vitro* (Bonjean *et al.,* 1996).

Although in recent years an high performance liquid chromatographic (HPLC) assay of monomeric indole alkaloids in *S. nux vomica* has been described (Gadi Biala *et al.,* 1996), no report of an HPLC method allowing the separation, identification and quantitation of bisindolic (usambarane) alkaloids has been published. The present paper describes a reversed-phase HPLC procedure which enables the qualitative and quantitative evaluation of these alkaloids.



usambarensine (1)



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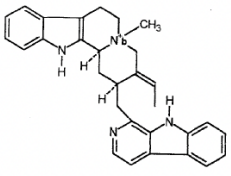
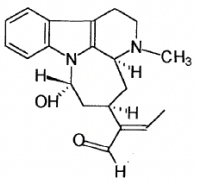
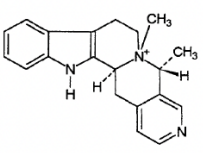


N_b-methylusambarensine (3)



akagerine (4)



isomalindine (5)

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EXPERIMENTAL

Materials. All the alkaloids used in this study were isolated from *S. usambarensis* in our laboratory and their structures were established by spectroscopic methods (Angenot and Bisset, 1971; Angenot *et al.,* 1975a). The roots were collected in March 1970 by one of the authors (LA) in Akagera National Park, Rwanda. They were immediately air-dried in Rwanda before being stored in Belgium at a temperature of 15°C. A voucher specimen of the plant was deposited in the herbarium of the National Botanical Garden of Belgium at Meise.

Sample preparation. *Extraction of the alkaloids:* the crude extracts were prepared by methanol and ethyl acetate extraction. The dried powdered root (500 mg) of *S. usambarensis* was subjected to extraction under agitation with 40 mL of solvent at room temperature for 2h. The solution was then filtered, and the residue was extracted twice again with 20 mL of the same solvent. The three extracts were bulked and the residue obtained after evaporating the solvent was dissolved in methanol and the volume adjusted to 20.0 mL with methanol.

Standard alkaloid solution: approximately 10 mg of each alkaloid was precisely weighed and dissolved in 100.0 mL of methanol and appropriate dilutions were made for the calibration curve. These solutions were then filtered through a 0.45 µm membrane HVLP filter and injected directly onto the HPLC system: the injection volume was 10 µL.

HPLC procedure. A Hewlett Packard (Palo Alto, CA, USA) model HP G1311A quaternary pump (with HP 1100 Series vacuum degasser) equipped with a diodearray detector model HP 1040 M-series 2, operating at 254 nm was employed. The column was a Lichrospher 60 RP Select B (250 x 4mm i.d.; particle size of 5 µm; Merck, Darmstadt, Germany). The mobile phases were: solvent **A**-acetonitrile; solvent **B**-an aqueous solution of sodium acetate (2.1 g in 800 mL) adjusted to pH 3.5 with phosphoric acid (0.5%): the final volume was brought to 1 L with HPLC quality water. Elution was with a linear gradient from 20% to 35% **A** in **B** over 20 min at a flow rate of 1.0 mL/min. All reagents were of analytical grade standards.

RESULTS AND DISCUSSION

The use of a linear gradient of the mobile phases described above was required to give a complete separation of the alkaloids of the roots of *S. usambarensis* in a single chromatographic run. Reversed-phase chromatography achieved good baseline stability and high resolution. By combining this HPLC method with a diode-array detector and advanced software, it was possible to check the purity of the eluted peaks. Alkaloids in the sample were identified by characteristic ultraviolet spectra (Angenot and Bisset, 1971; Angenot *et al.,* 1975a) given by the diode array detector and by relative retention times (Table 1).

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Table 1. Retention times of standard alkaloids using the HPLC system described in the Experimental section

Alkaloid	Retention time (min)[a]	Relative standard deviation (%)
Malindine	6.17	0.59
Isomalindine (**5**)	6.98	0.58
Harman	7.61	1.07
Akagerine (**4**)	11.84	0.53
3′,4′-Dihydrousambarensine (**2**)	12.54	0.45
N_b-Methyldihydrousambarensine	13.32	0.51
Usambarensine (**1**)	15.05	1.00
N_b-Methylusambarensine (**3**)	16.46	0.74

[a] Mean values ($n = 8$).

In order to find the optimum conditions for extracting the bisindole usambarane alkaloids from the roots of *S. usambarensis,* extracts were made using ethyl acetate and methanol: the chromatograms are shown, respectively, in Figs. 1a and 1b. HPLC analysis of the extracts revealed larger peak areas for usambarensine and particularly *N*b-methylusambarensine in the methanolic extracts. Furthermore, the chromatograms of the methanolic extracts contained additional peaks being the major quaternary alkaloids such as malindine and isomalindine **(5)**. These quaternary monomers were not present in the ethyl acetate extract, except for the N_b-methyl derivatives of usambarensine and dihydrousambarensine which have hybrid structures, possessing both tertiary amine and quaternary ammonium functional groups, which give these alkaloids some special solubilities (Angenot and Bisset, 1971).

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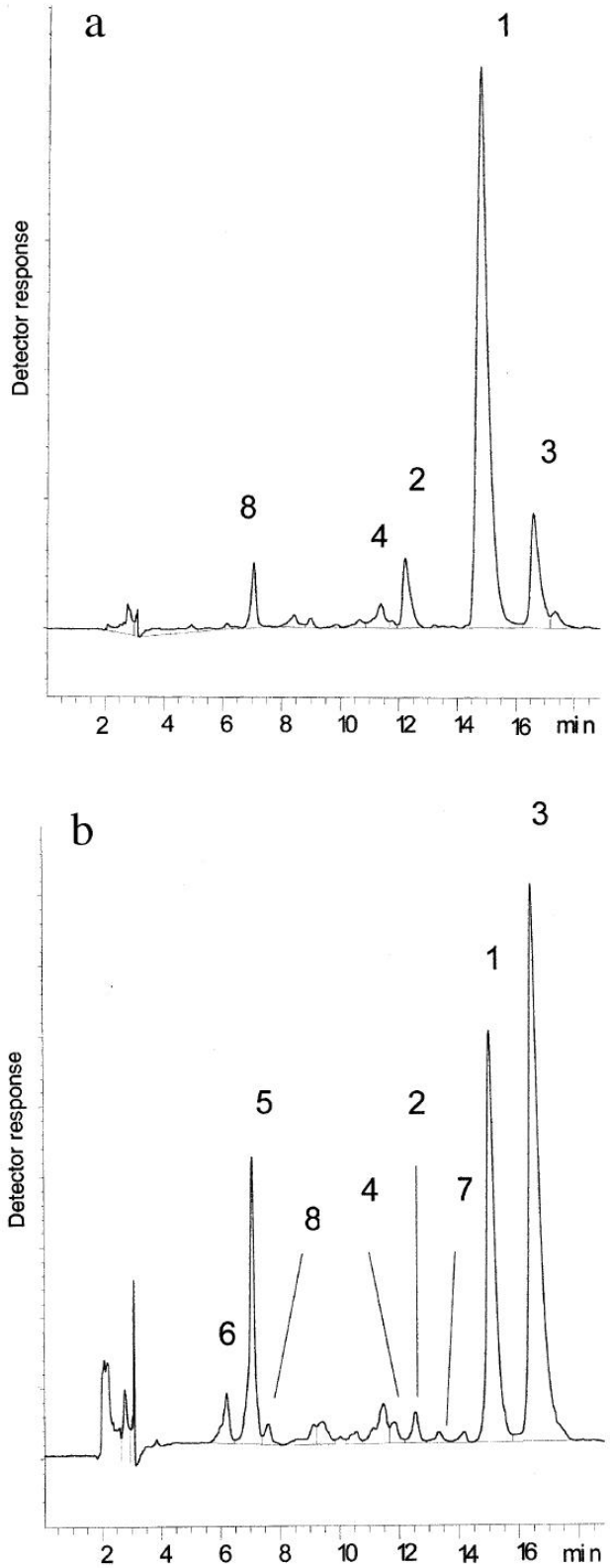

Figure 1. HPLC chromatograms of crude extracts from roots of *Strychnos usambarensis* obtained using (a) ethyl acetate, and (b) methanol as extracting solvent. (For chromatographic protocol see Experimental section). Key to peak identity: **1,** usambarensine; **2,** 3′,4′-dihydrousambarensine; **3,** N_b-methylusambarensine; **4,** akagerine; **5,** isomalindine; **6,** malindine; **7,** N_b-methyldihydrousambarensine; and **8,** harman.

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In order to demonstrate the repeatability of the HPLC method, the levels of the three major bisindolic alkaloids, usambarensine **(1)** 3',4'-dihydrousambarensine **(2),** and N_b-methylusambarensine **(3)** were determined in six methanolic extracts of the roots. The results are shown in Table 2, the major alkaloids found in the roots being **1** and **3**.

Table 2. Determination of the concentrations (% w/w) of bisindole alkaloids in six different extracts of *S. usambarensis* roots

Extract number[a]	Usambarensine (1)	3′,4′-Dihydrousambarensine (2)	N_b-Methylusambarensine (3)
1	0.223	0.068	0.501
2	0.234	0.073	0.495
3	0.231	0.072	0.513
4	0.224	0.071	0.502
5	0.223	0.072	0.508
6	0.230	0.069	0.497
Relative standard deviation	2.09%	2.73%	1.35%

[a] Extracts were prepared and analysed on six different days in order to determine repeatability and intermediate precision.

Three calibration graphs were prepared from peak area measurements obtained following injection of 10 µL of mixtures of usambarensine, 3',4'-dihydrousambarensine or N_b-methylusambarensine standards over the concentration range of 10–100 µg/mL. The resulting curves were linear over this range and the correlation coefficients were near 0.999 (see Table 3).

Table 3. Parameters determined for the calibration curves of standard compounds 1–3

	Usambarensine (1)	3′, 4′-Dihydrousambarensine (2)	N_b-Methylusambarensine (3)
Constants for the equation	$a = 16.134$	$a = 19.460$	$a = 29.033$
$y = ax + b$[a]	$b = +29.703$	$b = -5.814$	$b = -122.641$
Correlation coefficients	0.99935	0.99815	0.99944

[a] where x = amount of alkaloid (µg/mL) and y = peak area (arbitrary units).

In order to test the accuracy of the extraction assay, standard usambarensine, 3',4'-dihydrousambarensine and N_b-methylusambarensine were added before extraction to 500 mg of powdered samples. The peak area corresponding to each alkaloid was found to increase proportionally with the added concentration of standard.

In conclusion, the described HPLC method could be used as a rapid and sensitive method for identification, separation and dosage of different compounds of the usambarensine class.

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