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Preparative separation of naphthyltetrahydroisoquinoline alkaloids from *Ancistrocladus korupensis* by centrifugal partition chromatography☆

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Abstract

Crude extracts of *Ancistrocladus korupensis* contain a complex mixture of naphthyltetrahydroisoquinoline alkaloids, including the human immunodeficiency virus-inhibitory dimeric alkaloids michellamines A and B and the antimalarial monomeric korupensamines A–D. The efficient separation of michellamines A and B from these extracts has been accomplished by centrifugal partition chromatography. The chromatographic conditions used on a multi-channel cartridge unit (Sanki LLN) have been successfully scaled up with a newly developed, stacked-disk type centrifugal partition chromatography unit (Sanki NMF) for separating larger amounts of alkaloid mixtures with similar resolution. A refined, three-step process (solvent–solvent partitioning, centrifugal partition chromatography and HPLC) has been developed and applied to the scaled-up production of michellamine B for preclinical drug development.

1. Introduction

Recently, we disclosed [1,2] the isolation and identification of the unprecedented, human immunodeficiency virus (HIV)-inhibitory dimeric naphthyltetrahydroisoquinoline alkaloids, michellamines A and B (Fig. 1), from the tropical liana *Ancistrocladus korupensis*. We employed centrifugal partition chromatography as a key step in the initial analytical scale separation and purification of the michellamines. The selection of michellamine B by the US National Cancer Institute (NCI) for preclinical drug development has necessitated the development of suitable methods to provide much larger quan-

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☆ Part XIX in the series HIV-Inhibitory natural products. For Part XVIII, see J.B. McMahon, M.J. Currens, R.J. Gulakowski, R.W. Buckheit, Jr., C. Lackman-Smith, Y.F. Hallock and M.R. Boyd, *Antimicrob. Agents Chemother.*, submitted for publication.

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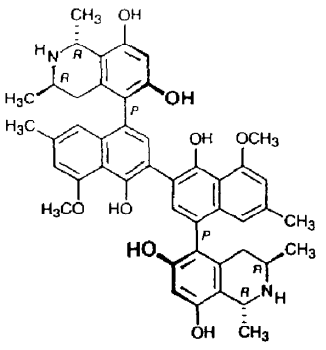
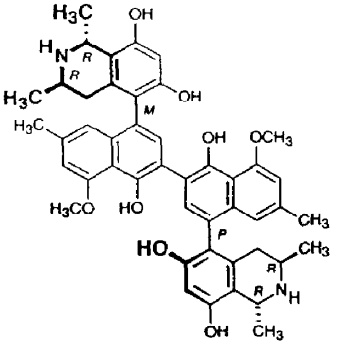
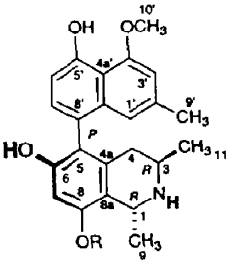


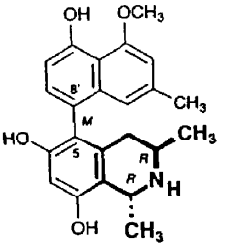
michellamine A



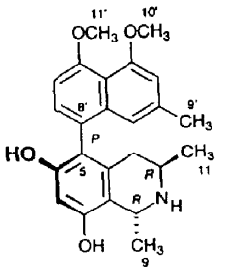
michellamine B



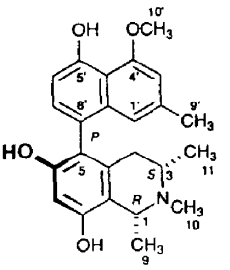
korupensamine A



korupensamine B



korupensamine C



korupensamine D

Fig. 1. Structures of michellamines A and B and korupensamines A–D. The designators P (plus) and M (minus) refer to absolute configuration about the chiral biaryl axis [6].

tities (e.g., hundreds of grams) of michellamine B for investigational new drug application (IND A)-directed formulation, toxicology and pharmacokinetics studies. In this paper, we report results of our efforts to optimize and scale up the counter-current separation of these alkaloids.

2. Experimental

2.1. Reagents

All solvents, either analytical or HPLC grade, were purchased from Baxter Scientific. Mineral

and acetic acids were obtained from Aldrich. Precoated amino TLC plates (10 × 10 cm, 250 μm layer) were obtained from EM Science. McIlvaine buffer (0.025 *M* citrate and 0.05 *M* phosphate) [3,4] was adjusted to pH 4.5 with phosphoric acid. The two-phase solvent systems used for separation of the alkaloid mixture were prepared by mixing $CHCl_3$–MeOH–0.5% mineral acid or McIlvaine buffer (5:5:3, v/v/v) in a separatory funnel; the two phases were then allowed to equilibrate overnight prior to separation and use.

2.2. Crude extracts of *Ancistrocladus korupensis*

Samples of leaves and twigs of the tropical liana *Ancistrocladus korupensis* were collected in Cameroon under NCI contract by D. Thomas and J. Jato. Fresh plant material was air-dried, stored in the freezer for at least three days prior to processing, and then ground in a Wiley mill to a coarse powder. That powder was successively extracted with CH_2Cl_2, CH_2Cl_2–MeOH (1:1, 2 ×) and MeOH (2–3 ×). The CH_2Cl_2–MeOH and MeOH extracts thus obtained were enriched in alkaloid content by solvent–solvent partitioning; they were suspended in MeOH–water (9:1, 500 ml) and defatted by extraction with hexane (10 × 250 ml). The aqueous phase was concentrated under reduced pressure to give the alkaloid-rich extracts.

2.3. Centrifugal partition chromatography

Analytical-scale separations were performed at 24°C with a Sanki (Kyoto, Japan) cartridge CPC, Model LLN, which contained 12 (type 250W) interconnected multi-channel partition cartridges. Each cartridge has 400 channels or microcells with a net volume of 21.3 ml. The 12-cartridge setup gives a total of 4800 partition channels and a volume of 256 ml. Solvent and sample delivery was achieved using a Sanki FCU valve module fitted with a 3 ml sample loop and a constant flow pump (Sanki LBP-V). An ISCO V^4 absorbance detector (254 nm) and Foxy fraction collector were connected to the main Sanki CPC unit. The unit was operated at 400 rpm with a flow-rate of 2.8 ml/min.

Preparative-scale separations were carried out on a newly developed stacked-disk type Sanki CPC Model NMF instrument, which measures 177.8 mm in diameter and 144.5 mm in height, with a total volume of about 1.5 l. A Rainin SD-1 HPLC pump was used for solvent delivery and the direction of flow was controlled by a Sanki FCU valve module fitted with a 12-ml sample loop. The outlet of the CPC was connected to a Linear multi-wavelength UV detector (254 nm), chart recorder (Linear Instruments, NV, USA) and an ISCO Foxy fraction collector. The unit was operated at 300 rpm with a flow-rate of 16 ml/min.

2.4. High-performance liquid chromatography

A Rainin SD-1 pump and UV-1 detector were used. Separations were accomplished with a Rainin Dynamax-NH_2 column (25 × 4.1 cm), eluting with CH_2Cl_2–0.1% $(NH_4)_2CO_3$ in MeOH (22:3) at 60 ml/min and monitoring at 255 nm.

3. Results

The initial collections of *Ancistrocladus korupensis* were extracted by our standard protocol (sequential steeping in 1:1 CH_2Cl_2–MeOH and MeOH, combined to provide a crude organic extract). This material was then subjected to a multi-step, acid–base partitioning scheme. First, the extract was distributed between $CHCl_3$ and 5% HCl. The aqueous phase was then made basic with NH_4OH and extracted successively with $CHCl_3$–MeOH (3:1, then 1:1) to recover the alkaloids. We have developed a simpler method to obtain a comparable, alkaloid-rich fraction.

The plant material is first extracted with CH_2Cl_2, which effectively removes both fat and other lipids and most pigments. Subsequent extraction with CH_2Cl_2–MeOH (1:1) and MeOH then yields alkaloid-containing extracts. These extracts are further enriched by solvent

partitioning between hexane and MeOH–water (9:1). The aqueous methanol fraction is roughly equivalent to the alkaloid (basic) fractions obtained in the original approach. This method offers two important advantages when viewed from the perspective of preparative-scale production. First, the extraction step has become part of the separation process, considerably simplifying the overall task. Second, solvent can be recycled at both the extraction (CH_2Cl_2) and partition (hexane) steps, resulting in substantial cost savings.

NMR and TLC analyses of the alkaloid fraction from the *Ancistrocladus korupensis* extracts obtained from the acid–base partition from the initial protocol, or from the aqueous methanol fraction from the revised protocol, indicated a complex mixture of naphthylisoquinoline alkaloids. Michellamines A and B were initially separated from the alkaloid complex by centrifugal partition chromatography (Sanki LLN), using $CHCl_3$–MeOH–0.5% HBr (5:5:3) in the descending mode [1]. As shown in Fig. 2A, the dimeric michellamines were strongly retained in the stationary phase and partially resolved, while the accompanying monomeric alkaloids (korupensamines A–D, Fig. 1) eluted earlier.

The influence of the acid component on the separation was investigated by replacing HBr with different acids. $HClO_4$ and HI gave separations similar to that obtained with HBr, but with somewhat decreased resolution and retention (Fig. 2B and C). Substitution of HOAc (at 0.5, 2.5 or 5%) for HBr resulted in a dramatic loss of resolution and significant increase in retention. Only small amounts of non-polar constituents were eluted in the descending mode. These results might be related to acid strengths and the facility of ion pairing between the alkaloids and the acid modifiers.

The use of an aqueous buffer in place of an acid modifier for the counter-current separation of alkaloids has been reported [3,4]. We studied the separation with $CHCl_3$–MeOH–pH 4.5 McIlvaine buffer (5:5:3). Overall retention of alkaloids was increased relative to the HBr-containing solvent system (Fig. 2D); indeed, the dimeric michellamines were selectively retained

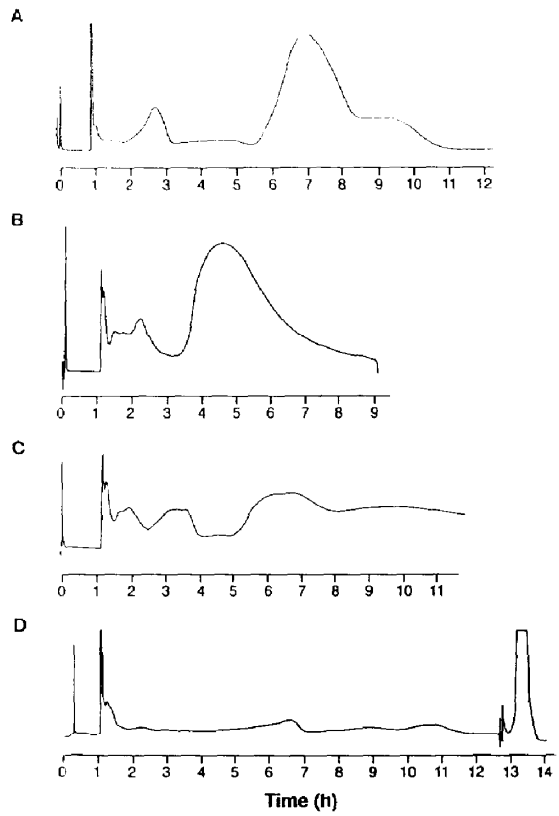


Fig. 2. Comparison of different centrifugal partition chromatography solvent combinations. All analyses performed on a Sanki CPC, model LLN with 12 analytical cartridges, with flow-rates of 2.8 ml/min at 400 rpm. Injections were 350–400 mg crude alkaloid mixture; descending mode was used in each case; detection by UV at 254 nm. (A) $CHCl_3$–MeOH–0.5% HBr (5:5:3); (B) $CHCl_3$–MeOH–0.5% $HClO_4$ (5:5:3); (C) $CHCl_3$–MeOH–0.5% HI (5:5:3); (D) $CHCl_3$–MeOH–pH 4.5 McIlvaine buffer (5:5:3).

in the stationary phase. Switching to the ascending elution mode resulted in coelution of michellamines A and B. Another disadvantage of the buffer system was the amount of salt (citrate and phosphate) in the fractions of interest; additional manipulations would be required prior to further purification (HPLC) of michellamines A and B. While it was likely that the retention and resolution of the michellamines could be modified by step gradient addition of counterions to the buffer [4], this would further complicate the separation by introducing even more salt to the fractions of interest.

Overall, the $CHCl_3$–MeOH–0.5% HBr (5:5:3) system evolved as the most advantageous of those examined, providing acceptable resolution of michellamine B and the other alkaloids as well as offering ease of workup, since no desalting step was necessary. A further advantage was the acquisition of the target compound as a hydrobromide salt, which proved to be a more stable form for storage than the free base.

In view of the requirement for multi-gram quantities of michellamine B for preclinical development, the analytical/semi-preparative-scale centrifugal partition chromatograph (Sanki LLN) was not a viable option for scale-up production of the compound. The maximum injection of crude alkaloid mixture which could be effectively resolved on this instrument was 350–400 mg.

A preparative-scale instrument (Sanki NMF) of 1.5 l capacity was expected to give a five- to six-fold increase in loading capacity with comparable resolution. Under similar operating conditions ($CHCl_3$–MeOH–0.5% HBr, 5:5:3, descending; 400 rpm; 16 ml/min flow-rate), virtually identical separations have been achieved (Fig. 3A). Reducing the spin rate to 300 rpm and the flow-rate to 15 ml/min reduced detector noise and improved resolution and, therefore, capacity

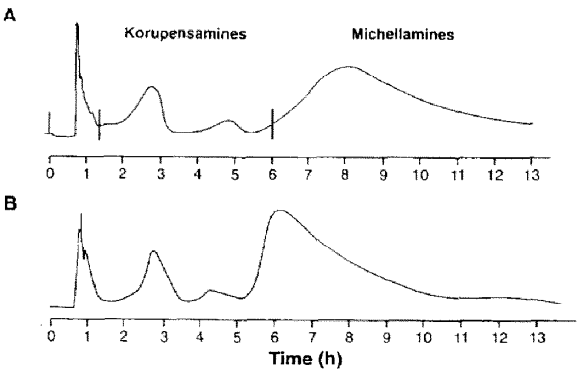


Fig. 3. Effect of spin rate on resolution in preparative-scale centrifugal partition chromatography. Analyses performed on a Sanki NMF (1.5 l capacity) with $CHCl_3$–MeOH–0.5% HBr (5:5:3) in the descending mode; detection by UV at 254 nm; (A) 2.1-g injection at 400 rpm, 16 ml/min; (B) 9.0-g injection at 300 rpm, 15 ml/min.

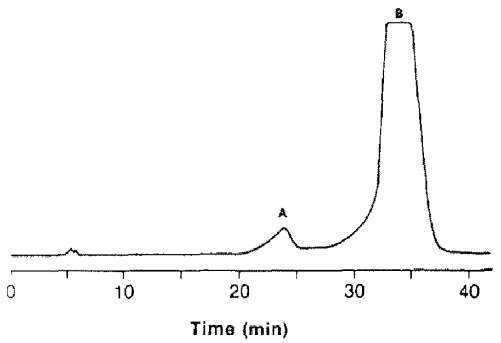


Fig. 4. Preparative HPLC purification of michellamine B. Conditions: Rainin Dynamax-Amino column (25 × 4.1 cm, 8 μm) eluted with CH_2Cl_2–0.1% $(NH_4)_2CO_3$ in MeOH (22:3) at 60 ml/min. Injection 100 mg, UV detection at 255 nm. Peaks: A = michellamine A; B = michellamine B.

(Fig. 3B). Currently, 8–9-g aliquots of crude alkaloid mixture are loaded and separated five times per week in our laboratory.

Final purification of michellamine B utilized normal-phase HPLC on an aminopropyl-bonded phase column (Rainin Dynamax-Amino, 25 × 4.1 cm). In the interests of cost reduction, environmental concern and health safety, we have been able to replace the $CHCl_3$ originally used at this stage [1] with CH_2Cl_2. Elution with CH_2Cl_2–0.1% $(NH_4)_2CO_3$ in MeOH (22:3) provides excellent separation (Fig. 4) of michellamines A and B with added loading capacity. This rapid and efficient, three-step process is currently providing 2.5 g per week of pure michellamine B for further preclinical investigation and drug development. In addition, this protocol has also facilitated access to the monomeric alkaloids (korupensamines), recently identified as antimalarial constituents of *A. korupensis* [5].

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