ELSEVIER

Journal of Chromatography A, 685 (1994) 259–262

JOURNAL OF CHROMATOGRAPHY A

Separation of alkaloids by pH-zone-refining counter-current chromatography

Ying Ma, Yoichiro Ito*, Edward Sokolosky, Henry M. Fales

Laboratory of Biophysical Chemistry, National Heart, Lung, and Blood Institute, National Institutes of Health, Bethesda, MD 20892, USA

First received 16 May 1994

Abstract

pH-Zone-refining counter-current chromatography was applied to the separation of alkaloids from a crude extract of *Crinum moorei* using a multilayer coil planet centrifuge. After methyl *tert.*-butyl ether and water were equilibrated, triethylamine (5–10 m*M*) was added to the organic phase and hydrochloric acid (5–10 m*M*) to the aqueous phase. The separation was performed by eluting the column with either the organic phase (displacement mode) or the aqueous phase (reverse-displacement mode) while the other phase was used as the stationary phase. From 3 g of the extract, crinine, powelline and crinamidine were separated in 2.5–7 h with minimum overlapping

1. Introduction

Counter-current chromatography (CCC) [1–3] is a liquid–liquid partition chromatography not requiring solid support in the separation column and offering various advantages including high sample recovery, high purity of fractions and large sample loading capacity. During the past decade, high-speed CCC has been widely used for preparative separations of natural products [3,4].

pH-Zone-refining CCC [5–8] is a recently developed preparative method which yields characteristic rectangular peaks of analytes comparable to those observed in displacement chromatography [9]. The method has been successfully applied to separations of ionizable compounds including various amino acid derivatives [5–7,10,11], hydroxyxanthene dyes [5,12,13], indole auxins [7], etc.

The technique operates in two different modes: reverse-displacement mode [5] and displacement mode [10]. In the reverse-displacement mode, the aqueous mobile phase elutes the analyte retained in the organic stationary phase by the action of a retainer. In the displacement mode, the displacer in the organic mobile phase transfers the analyte from the aqueous stationary phase to the organic mobile phase in a manner analogous to that observed in displacement chromatography. Both modes result in similar elution patterns of analytes except that the order of elution is reversed.

In this paper, pH-zone-refining CCC was applied to the separation of three basic alkaloids from a crude extract of *Crinum moorei* using both reverse-displacement and displacement modes. Each analyte was identified by both MS

* Corresponding author.

0021-9673/94/$07.00
SSDI 0021-9673(94)00667-9

and NMR. The advantages of each operation mode are discussed.

2. Experimental

2.1. CCC apparatus

A commercial model (Ito Multilayer Coil Separator/Extractor, Potomac, MD, USA) of the high-speed CCC centrifuge was used throughout the present studies. The basic design of the apparatus was given elsewhere [4].

The separation column was prepared in our laboratory by winding a single piece of 160 m × 1.6 mm I.D. polytetrafluoroethylene (PTFE) tubing around the column holder hub with 16 layers and 325 ml capacity.

The revolution speed of the apparatus was regulated with a speed controller (Bodine Electric Co., North Chicago, IL, USA). An optimum speed of 600–800 rpm was used in the present studies.

2.2. Reagents

Methyl *tert.*-butyl ether (HPLC grade), triethylamine (reagent grade) and hydrochloric acid (reagent grade) were purchased from Fisher Scientific, Fair Lawn, NJ, USA.

The crude alkaloid extract from *Crinum moorei Hook f.* was extracted as described by Boit [14] and stored at room temperature for ca. 35 years.

2.3. Preparation of solvent phases and sample solutions

The solvent pairs were prepared as follows: about equal volumes of methyl *tert.*-butyl ether and distilled water were thoroughly equilibrated in a separatory funnel at room temperature and the two phases separated. To the upper organic phase triethylamine was added to make the solution in that base 5–10 m*M* (pH 9.2–9.6) while the lower aqueous phase was acidified with hydrochloric acid to 5–10 m*M* (pH 2.3–1.7).

The sample solution was prepared by dissolving 3 g of crude alkaloids extract in 60 ml of a phase mixture consisting of equal volumes of each phase. The pH of the sample solution was adjusted to about 6.0 with HCl.

2.4. Separation procedures

Two different operation modes were employed. In the reverse-displacement mode the organic phase was used as the stationary phase and the lower aqueous phase as the mobile phase. In the displacement mode, the above relationship was reversed, i.e. the lower aqueous phase became the stationary phase and the upper organic phase the mobile phase.

In the reverse-displacement operation, the column was first entirely filled with the organic phase containing 5 m*M* triethylamine (retainer base) followed by sample injection through the sample port. Then, the acidified aqueous phase containing 5 m*M* HCl (eluent acid) was pumped into the inlet of the column in the head to tail elution mode while the column was rotated at the initial speed of 600 rpm. The revolution speed was raised to 800 rpm after 20 fractions had been collected. The above change of the column rotation minimized the carryover of the stationary phase. The effluent from the outlet of the column was continuously monitored with a UV monitor (Uvicord S; LKB Instruments, Bromma/Stockholm, Sweden) at 206 nm and collected into test tubes at 1-min intervals (3.3 ml/tube) with a fraction collector (Ultrorac, LKB Instruments).

In the displacement operation, the column was filled with the aqueous phase containing 10 m*M* HCl (retainer acid) followed by sample injection through the sample port. Then the organic phase containing 10 m*M* triethylamine (displacer) was eluted through the column at a flow-rate of 3.3 ml/min in the tail-to-head elution mode while the apparatus was rotated at 600 rpm. In this elution mode, the two phases establish a reversed pressure gradient through the column where the pressure at the column inlet often plunges into a negative range causing suction of extra solvent from the reservoir through one-way check valves of the metering pump. In order to

prevent this complication, a piece of long narrow PTFE tubing (3 m × 0.4 mm I.D.) was placed at the outlet of the monitor to raise the column pressure. This device also prevented formation of gas bubbles inside the flow cell of the UV monitor which would disturb recording of the elution curve.

In both operation modes, after the desired peaks were eluted, the apparatus was stopped and the column contents were collected into a graduated cylinder by connecting the inlet of the column to a nitrogen line at 80 p.s.i. (1 p.s.i. = 6894.76 Pa).The retention of the stationary phase relative to the total column capacity was computed from the volume of the stationary phase collected from the column.

2.5. *Analysis of fractions*

The pH value of each fraction was determined manually with a portable pH meter (Accumet Portable Laboratory, Fisher Scientific, Pittsburgh, PA, USA).

Alkaloids were identified by TLC (Kieselgel 60 F_{254}) using a solvent mixture composed of chloroform–methanol–32% acetic acid (16:4:1, v/v/v) (R_F values: 0.64 for crinine; 0.74 for powelline; and 0.85 for crinamidine) as well as by comparison of MS and NMR spectrum analyses with standard compounds[1].

3. Results and discussion

Fig. 1A shows a typical chromatogram obtained from 3 g of the crude alkaloid extract of *C. moorei Hook f.* by the reverse-displacement mode. Alkaloids were eluted as an irregular rectangular peak where three absorbance plateaus are observed at retention times of 42–90, 91–136 and 140–148 min. The pH measurement of the collected fractions also revealed three flat pH zones, I, II and III which respectively correspond to the above absorbance plateaus, suggesting the successful separation of three components. Lack of an ideal detector for these specific compounds (all bore similar UV absorption) means that fractions showing subtle changes in UV or pH must be examined carefully (TLC) to determine the onset of elution of the various species. Considerable amounts of impurities were eluted in the front and the back of the main peak, forming multiple peaks. TLC analysis of fractions corresponding to each zone boundary revealed that the mixing zones were no more than several milliliters. MS and NMR analyses of fractions corresponding to the main plateaus identified fractions of pure crinine (zone I), powelline (zone II) and crinamidine (zone III) as indicated in the diagram.

Fig. 1B shows a similar chromatogram obtained from 3 g of the same sample by the displacement mode of pH-zone-refining CCC. Three alkaloids (crinamidine, powelline and crinine) were also eluted together as rectangular peaks but, as expected, in the reverse order due to the reverse-displacement mode (Fig. 1A).

Each mode of pH-zone-refining CCC has its own specific advantages depending on the nature of the analytes. In the displacement mode, each analyte is collected as a free base (or acid) in the organic phase which can be easily evaporated. In the reverse-displacement mode, all analytes are eluted as their salts with the aqueous mobile phase. Although evaporation of fractions requires a longer time, some alkaloids are more stable in the salt form than in the free base and therefore have less risk of decomposition. The reverse-displacement mode also facilitates accurate pH monitoring through the aqueous mobile phase and further provides more stable retention of the stationary phase for low-interfacial-tension solvent systems suitable for the separation of polar compounds.

The overall results of our studies demonstrated that both reverse-displacement and displacement modes of pH-zone-refining CCC produced efficient separations of three alkaloids from gram quantities of *C. moorei* crude extract. The present method may be applied to various other alkaloids from natural products.

[1] One of us (H.M.F.) maintains a reference collection of several hundred amaryllidaceae alkaloids and their chemical degradation products. Inquiries are welcome.

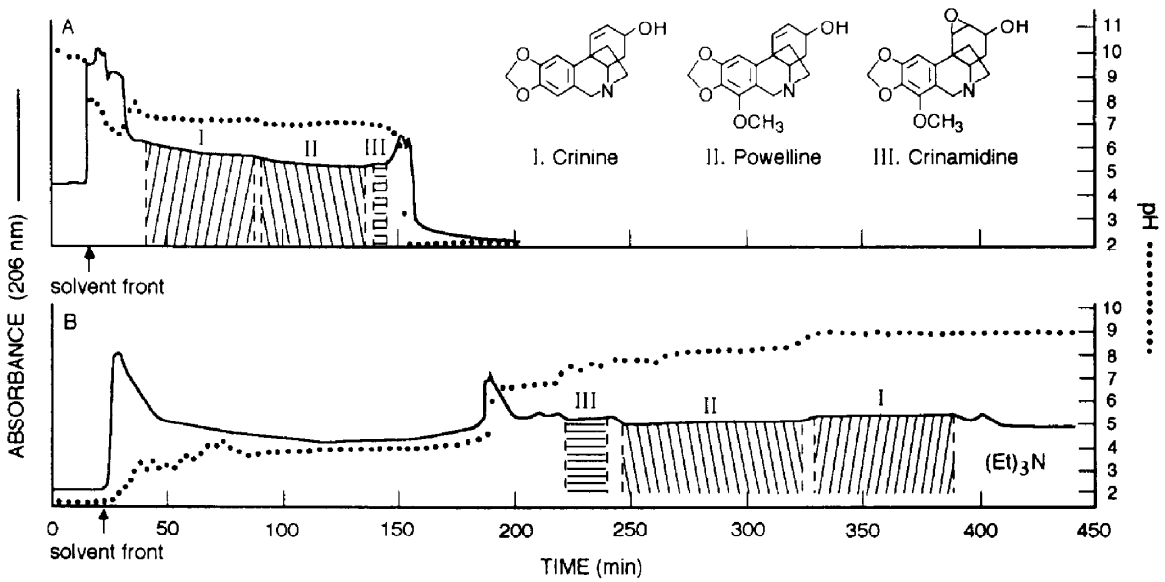

Fig. 1. Chromatograms of crude alkaloid extract of *Crinum moorei* obtained by reverse-displacement mode (A) and displacement mode (B) of pH-zone-refining CCC. Experimental conditions were as follows: apparatus: high-speed CCC centrifuge equipped with a multilayer coil of 1.6 mm I.D. and about 300 ml capacity; solvent system: methyl *tert.*-butyl ether–water; stationary phase: (A) upper phase (5 m*M* triethylamine) and (B) lower phase (10 m*M* HCl); mobile phase: (A) lower phase (5 m*M* HCl) and (B) upper phase (10 m*M* triethylamine); flow-rate: 3.3 ml/min; sample: crude alkaloid extract of *Crinum moorei*, 3 g dissolved in 30 ml of each phase; revolution: (A) 800 rpm (600 rpm until 66 ml of mobile phase was eluted) and (B) 600 rpm throughout.

References

[1] Y. Ito, in N.B. Mandava and Y. Ito (Editors), *Countercurrent Chromatography —Theory and Practice*, Marcel Dekker, New York, 1988, pp. 79–442.

[2] W.D. Conway, *Countercurrent Chromatography —Principle, Apparatus and Applications*, VCH, New York, 1990.

[3] Y. Ito, in E. Heftmann (Editor), *Chromatography —Fundamentals and Applications of Chromatography and Related Differential Migration Methods, Part A: Fundamentals and Techniques (Journal of Chromatography Library*, Vol. 51A), Elsevier, Amsterdam, 1992, Ch. 2.

[4] Y. Ito, *CRC Crit. Rev. Anal. Chem.*, 17 (1986) 65–143.

[5] A. Weisz, A.L. Scher, K. Shinomiya, H.M. Fales and Y. Ito, *J. Am. Chem. Soc.*, 116 (1994) 704–708.

[6] Y. Ito, in Y. Ito and W.D. Conway (Editors), *High-Speed Countercurrent Chromatography*, Wiley-Interscience, New York, in press.

[7] Y. Ito, K. Shinomiya, H.M. Fales, A. Weisz and A.L. Scher, in W.D. Conway and R. Petroski (Editors), *Countercurrent Chromatography*, American Chemical Society, Washington, DC, in press.

[8] A.L. Scher, in W.D. Conway and R. Petroski (Editors), *Countercurrent Chromatography*, American Chemical Society, Washington, DC, in press.

[9] Cs. Horváth, A. Nahum and J.H. Frenz, *J. Chromatogr.*, 218 (1981) 365–393.

[10] Y. Ito and Y. Ma, *J. Chromatogr. A*, 672 (1994) 101.

[11] Y. Ma and Y. Ito, *J. Chromatogr. A*, 678 (1994) 233.

[12] A. Weisz, D. Andrzejewski, R.J. Highet and Y. Ito, *J. Chromatogr. A*, 658 (1994) 505–510.

[13] A. Weisz, in Y. Ito and W.D. Conway (Editors), *High-Speed Countercurrent Chromatography*, Wiley-Interscience, New York, in press.

[14] H.-G. Boit, *Chem. Ber.*, 87 (1954) 1704.