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Note

Separation of veratrine using high-performance liquid chromatography or droplet countercurrent chromatography

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Several ceveratrum alkaloids have become useful in clinical medicine[1], in pharmacology[2] and in neurophysiological studies of the structure and mechanism of the voltage-dependent sodium channels[3]. Veratridine is particularly important as it acts on the sodium ion channels that mediate the electrical excitability of nerve, heart and skeletal muscles[4]. In cultured neuroblastoma cells cevadine[5], another ceveratrum alkaloid, was found to inhibit the stimulatory effect of veratridine on Na^+ influx.

In 1973 Honerjäger expressed concern that misleading results were being reported because "the preparations still used for experimental work are ill-defined, *e.g.* "veratrine" is a mixture of veracevine, cevacine, cevadine, veratridine and vanilloylveracevine"[6].

To date several thin-layer chromatographic systems[7,8,9] have been developed to analyse and isolate the ceveratrum alkaloids. These methods present many problems. Most seriously, the alkaloids show a degree of instability under the conditions required to achieve separation. The visualisation of the alkaloids is destructive and quantitation is difficult.

Recently we required pure samples of veratridine (VT), cevadine (CD) and veracevine (VC). Quantitative analysis of our commercial samples of veratrine was not possible with the methods available and neither was a quick determination of the purity of the VC prepared[9]. Published methods[8,10] of isolation of VT and CD from commercial mixtures were cumbersome and accompanied by high losses in yield.

We now report the isolation of the two major components of commercial veratrines, namely VT and CD, by preparative droplet countercurrent chromatography (DCCC) and two reliable quantitative methods of analysis using reversed-phase and normal-phase high-performance liquid chromatography (HPLC). Both of these HPLC methods can also be extended to the preparative isolation of these alkaloids.

EXPERIMENTAL

DCCC

The DCCC system used was made up of a Model 670 DCC-chromatograph (Büchi), a Model 100-40 UV spectrophotometer (Hitachi) fitted with a preparative 0.5-mm flow-cell (Altex), and an electronic filter device which gave a 10-sec time constant on the recorder output and a Model 7000 Ultrorac fraction collector (LKB).

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The chromatograph was fitted with 294 glasstube columns (2.7 mm I.D.), containing 673 ml of stationary phase.

The solvent system used was prepared as follows: a mixture of 0.5 *M* phosphate buffer, pH 5 (200 ml), water, distilled and deionised (200 ml), methanol (400 ml), chloroform (Ajax Chemicals, Unichrom; 800 ml) and *n*-propanol (BDH Analar; 80 ml) was stirred vigorously for 30 min, its pH adjusted to 5, filtered through a 0.5-μm PTFE filter and allowed to settle in a separating funnel for 1 h. After separation the aqueous layer (upper) was used for the stationary phase and the organic layer (lower) for the mobile phase. The sample was introduced in a mixture of mobile and stationary phases (1:1, 10 ml).

Isolation of alkaloids

The small amount of aqueous phase present was separated from the combined fractions eluted from the DCCC. An equal amount of dichloromethane was added and the organic phase was again separated. The organic phase was washed with 1% sodium bicarbonate solution (3 × 100 ml), dried over anhydrous sodium sulphate and concentrated. The products were dried under vacuum and identified by ^{1}H and ^{13}C nuclear magnetic resonance, ultraviolet spectroscopy and mass spectroscopy.

HPLC

The HPLC system used was made up of the following Waters Assoc. components: a M6000 solvent delivery system, a U6K injector, a RCM 100 radial compression module and a R 401 differential refractometer; plus a Model 100-40 UV spectrophotometer (Hitachi) with an analytical 10-mm flow-cell (Altex) and a Model HP5880A GC terminal (Hewlett-Packard), fitted with an external analogue–digital interface for integration of peak areas.

The chromatographic columns used were (A) stainless steel, 300 × 3.9 mm I.D., 10 μm, μPorasil™ thermostated to 30°C, (Waters Assoc.); (B) 100 × 8 mm I.D., CN-8 Radial-PAK™ Cartridge (Waters Assoc.); (C) stainless steel, 600 × 20 mm I.D., flotation-separated silica gel[11], *ca.* 7–20 μm; (D) stainless steel, 500 × 9.4 mm I.D., Partisil™ 10 ODS2 M9 (Whatman).

Water was distilled (all-glass system), deionised and filtered with a Milli-Q 3 cartridge purification system. Its mixtures with methanol (Mallinckrodt ChromAR) and Reagent D-4 (Waters Assoc., dibutylamine phosphate mobile phase modifier, 10 ml/l) were equilibrated for 5 min (magnetic stirrer) and filtered through a 0.5-μm PTFE filter (Millipore), petroleum spirit (60–80°) (Ajax Chemicals, Univar) was fractionally distilled over $LiAlH_4$, then together with ethanol (Ajax Chemicals, Univar absolute) and diethylamine (BDH, Analar) filtered through a 0.5-μm PTFE filter. The solvent reservoirs were maintained at 30°C by immersion in a constant temperature bath.

RESULTS

DCCC

A commercial sample of veratrine (Baird and Tatlock) gave complete separation of CD and VT in a single run. These compounds separated well from a complex mixture of highly coloured contaminants which eluted first (see Fig. 1). The

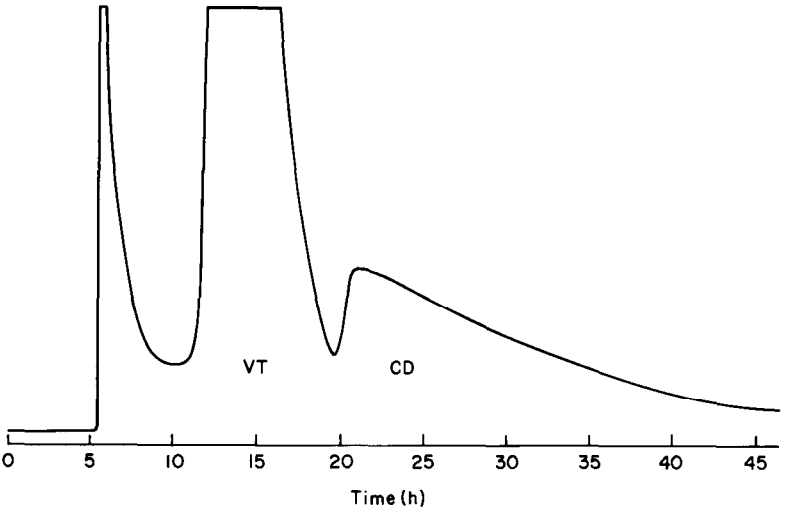

Fig. 1. DCC chromatogram of Veratrine (Baird and Tatlock). Samples: 1.0 g in 5 ml mobile phase. Flow-rate: 15 ml/h (pump stroke 7.5, pump speed 20). Detection: UV at 250 nm.

highly polar bases, (*ca.* 5%), which were also present in veratrine, remained in the stationary phase. It is worth noting that the reported Craig countercurrent system for veratrine[12], needed extensive modification for use on DCCC.

HPLC

Several commercial samples of veratrine were analysed by HPLC (see Table I). A sample chromatogram is presented for veratrine (Sigma) on normal phase (Fig. 2a) and reversed-phase (Fig. 2b).

In reversed-phase HPLC, the relative response factors on the RI detector were 1.0 ± 0.02 for all three alkaloids. Reversed-phase HPLC was the more convenient method for quantitation, allowing easy quantitation of the more polar minor component VC, as well as the two major components (VT and CD). It was important that the veratrine samples were freshly prepared as VT was not completely stable in methanol due to some transesterification. This resulted in up to a 10% conversion

TABLE I

COMPOSITION OF COMMERCIAL VERATRINES BY REVERSED-PHASE HPLC

Commercial source	*% (w/w)*			
	CD	*VT*	*VC*	*Other*
Baird and Tatlock (London, U.K.)	54	39	2	5
Hopkin and Williams (London, U.K.)	59	41		
Sigma (St. Louis, MO, U.S.A.)	59	38	0.3	2.7
ICN Pharmaceuticals (Plainview, NY, U.S.A.)	49	23	5	23

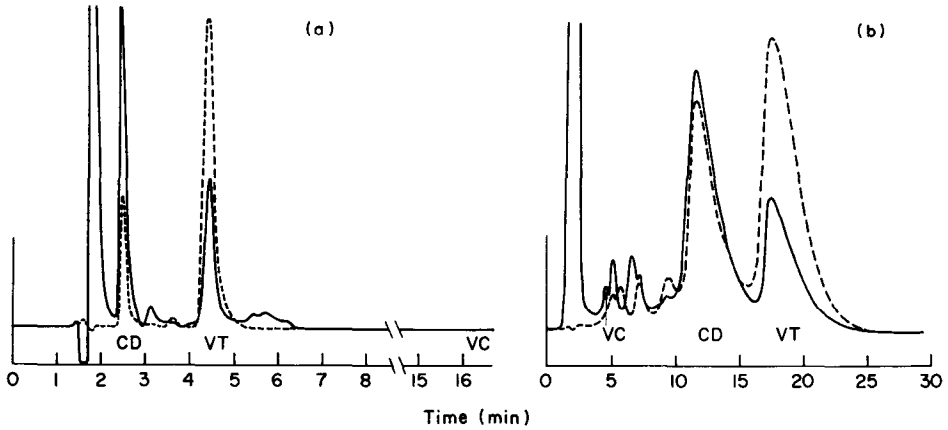

Fig. 2. HPLC chromatograms of Veratrine (Sigma). Detection: refractive index at 30°C (——), UV at 245 nm (----). (a) Normal phase (column A, 30°C) with light petroleum–ethanol–diethylamine (950:50:4) at 2 ml/min. (b) Reversed phase (column B) with methanol–water (40:60) + modifier D-4. Flow-rate was 2 ml/min.

of VT to VC over a 2.5-h period. However, we saw no evidence for decomposition during chromatography.

On normal-phase HPLC, VC has a response factor of 1.3. Since it is present in $< 1\%$, no peak could be distinguished from the noise in our analysis.

Total recovery runs for CD gave 100% recovery on reversed-phase [column D, methanol–water (1:1) + D-4, 5 ml/min] and 95% recovery on normal phase [column C, light petroleum (b.p. 60–80°C)–ethanol–diethylamine (94:5:1) 14 ml/min].

CONCLUSIONS

The alkaloids present in commercial veratrine were separated satisfactorily on both normal- and reversed-phase HPLC. Even the minor, more polar components can be quantified by reversed-phase HPLC. Both methods, as well as DCCC, can be employed for preparative isolation of pure components of veratrine.

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