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Short Communication

Reversed-phase high-performance liquid chromatographic separation of 5β,20-dihydroxyecdysone and 20-hydroxyecdysone on a β-cyclodextrin-bonded stationary phase

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ABSTRACT

For the high-performance liquid chromatographic separation of two ecdysteroids, 20-dihydroxyecdysone and 5β,20-dihydroxyecdysone which are hardly or not at all resolved on classical normal or reversed stationary phases, we have used a β-cyclodextrin stationary phase, prepared in our laboratory. Easy preparation was obtained in reversed-phase mode, either with methanol or with acetonitrile as an organic modifier in the aqueous mobile phase. The separation of these two ecdysteroids in mixture with two other ecdysteroids by gradient elution is also shown.

INTRODUCTION

The ecdysteroids control a number of important physiological functions, *i.e.* periodic moulting, interruption of larval or pupal diapause, or they can influence control of embryonic development in insects [1]. In the late 1960s the first analogues of these compounds were found in some plants (phytoecdysones), and among others also in the fern species *Polypodium vulgare* and *Pteridium aqulinium* [2].

During our experiments on the production of ecdysteroids by plant tissue cultures [3] we aimed to develop a suitable method for their separation. As we needed to inject the liquid media without any further purification, a reversed-phase system with C_{18} stationary phase and aqueous methanol or acetonitrile mobile phase was shown to be suitable [4]. Behavior of ecdysteroids under reversed-phase conditions was extensively studied from a structural point of view [5]. It was established that the position of hydroxy groups is more important for their retention than their number, *e.g.* a hydroxy group in the aliphatic chain has greater effect compared with C-2 or C-11 hydroxy groups. A hydroxy group in the 5β position has the least effect on retention

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and the separation of 20-hydroxyecdysone from 5β,20-dihydroxyecdysone is therefore a difficult one. However, separation of these two ecdysteroids with supercritical fluid chromatography has been described previously [6].

Recently many applications of cyclodextrin-bonded stationary phase to reversed-phase liquid chromatography have emerged [7,8]. In these papers many separations of isomeric or closely related compounds have been shown. We applied our prepared cyclodextrin stationary phase 7CDSIL [9] to the separation of ecdysteroids to achieve a more selective separation than on common C_{18} reversed-phase.

EXPERIMENTAL

Chemicals

Methanol and acetonitrile (Lachema, Brno, Czechoslovakia) were redistilled from glass, water was twice deionized and redistilled from glass. Standards of ecdysteroids were obtained from The Department of Natural Compounds in our Institute.

Apparatus

Chromatographic experiments were performed on Gilson liquid chromatograph (France) comprising two Model 303 pumps, a Model 802C manometric module, a Model 811 dynamic mixer and a Holochrome UV-detector. The system was controlled by an IBM PC-AT via a Gilson data module Model 621 Data Master. Samples were injected through a Rheodyne 7125 (USA) sampling valve.

Chromatographic conditions

The cyclodextrin-bonded stationary phase (7CDSIL) was slurry packed into a stainless-steel column (250 × 4 mm I.D.). The mobile phases were mixtures of acetonitrile or methanol with water. The flow-rate was 1.0 ml/min and detection was UV at 245 nm.

RESULTS AND DISCUSSION

Separations on 7CDSIL were at first performed in an aqueous methanol mobile phase. Retention of solutes in 80% methanol was low, resembling on a C_{18} reversed phase in similar mobile phase. A remarkable increase in retention, however, was observed with increasing polarity of mobile phase, and with 50% methanol separation of solutes was observed. Further decrease in the methanol portion improved separation such that at 35% methanol almost baseline separation was obtained (resolution, R_s = 1.56) (Fig. 1). Decreasing the amount of methanol below 30% did not bring about any remarkable improvement of resolution; on the contrary, peak broadening was observed. These results suggest a strong dependence of host–guest interaction on the amount of water in the mobile phase. The elution order 20-hydroxyecdysone (B) > 5β,20-dihydroxyecdysone (A) was determined from the retention times of pure solutes.

It has been noted elsewhere [5] that better results in separations of ecdysteroids on reversed phase were obtained with acetonitrile as the organic modifier in the mobile phase, so we performed experiments with this solvent using the same methods as with methanol. A reasonable separation was observed with 40% acetonitrile be-

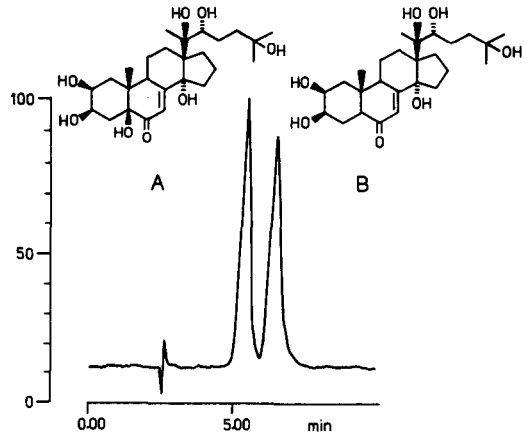

Fig. 1. Separation of 20-hydroxyecdysone (B) and 5β,20-dihydroxyecdysone (A) in a methanol–water (35:65) mobile phase. Flow-rate, 1.0 ml/min; detection, UV at 245 nm; injection, 10 μl of methanolic solution, approximately 1 μg of each compound.

cause of the greater elution strength of this mobile phase, and baseline separation with 15% acetonitrile (R_s = 1.87) was achieved. A further decrease of the acetonitrile portion again caused peak broadening. However, observed resolution and peak shape were better with acetonitrile than with methanol, and, moreover, a lower amount of organic modifier was necessary for baseline separation.

Separation of these two ecdysteroids in mixture with two other ecdysteroids (ecdysone and ponasterone B) under gradient elution conditions, as indicated in the figure legend, is shown in Fig. 2.

These investigations have shown that a cyclodextrin-bonded stationary phase

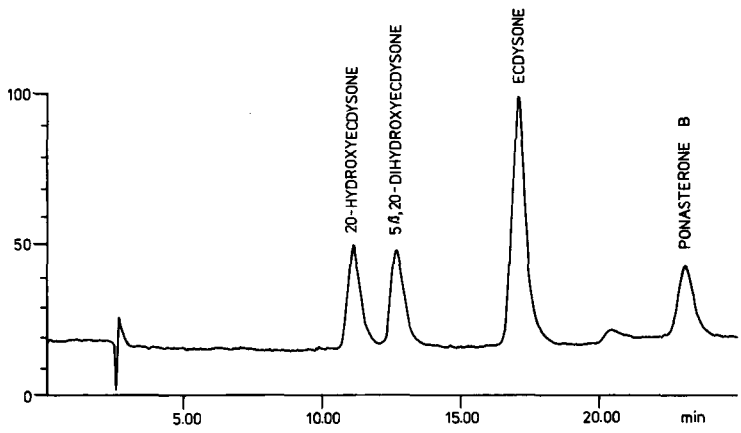

Fig. 2. Gradient separation of four ecdysteroids: 0–25 min, 15–50% methanol in water. Flow-rate, 1.0 ml/min; detection, UV at 245 nm; injection, 10 μl of methanolic solution, approximately 1 μg of 20-hydroxyecdysone, 5β,20-dihydroxyecdysone and ponasterone B and 2 μg of ecdysone.

with aqueous acetonitrile and aqueous methanol mobile phases can be recommended for separations of 20-hydroxyecdysone and 5β,20-dihydroxyecdysone. Further use of this methodology in the analysis of ecdysteroids is under investigation.

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