Journal of Chromatography, 129 (1976) 447–450

CHROM. 9359

Note

Separation of ecdysterone, inokosterone, makisterone A, α-ecdysone and ponasterone A by a combination of adsorptive and reversed-phase liquid chromatography

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(Received May 19th, 1976)

Due to their high degree of polar group substitution, the ecdysteroids present special separation difficulties, particularly when it is necessary to recover the compounds for further analysis. For example, the combination of ecdysterone and inokosterone, has been difficult to separate, but could be resolved by two very slow chromatographic methods[1,2]. Despite repeated efforts, it has not been possible to resolve this pair by rapid, adsorptive liquid chromatography and the previous report of such chromatography was not concerned with this separation[3].

The principal interest in this study was to be able to separate rapidly those ecdysteroids which might be constituents of crustaceans and to recover the resolved mixture of steroids. To date four ecdysteroids have been identified in crustacean extracts: ecdysterone (β-ecdysone, 20-hydroxyecdysone, crustecdysone) [4–6], inokosterone[6], makisterone A[6] and 2-deoxycrustecdysone[7]. Since 2-deoxycrustecdysone was not available but α-ecdysone shows similar polarity[7], and may also be a constituent of crustaceans, α-ecdysone was included as a reference compound. To resolve such a mixture the following combination of adsorptive and reversed-phase liquid chromatography was developed.

EXPERIMENTAL

All solvents were analytical grade and redistilled in an all-glass still. Water was distilled. Ecdysterone, inokosterone (Schwarz/Mann), α-ecdysone (Steraloids), makisterone A and ponasterone A (gifts from Prof. K. Nakanishi) were dissolved in methanol and when possible diluted to 1 mg/ml. Corasil II and Bondapak phenyl–corasil (Waters Assoc.) columns were packed by vertical rapping[8] in stainless-steel columns (1000 × 3 mm I.D.) equipped with outlet fittings containing 5-μm stainless-steel frits. Origin ends were plugged with glass wool. All samples were injected through a septum injection port directly into the column.

Solvent was delivered at pressure (up to 68 atm) by a reciprocating pump (Milton Roy) equipped with a pulse dampener (Laboratory Data Control). Gradients were provided by delivering the modifying solvent to the gradient mixing chamber, containing a measured volume of the starting solvent, at one half the flow-rate at

which the gradient mixture was delivered to the column (linear gradient[9]). All solvents were vacuum degassed before use. The column effluent was monitored at 254 nm using a Chromatronix Model 200 spectrophotometer with 8-μl cells.

RESULTS AND DISCUSSION

It is apparent from the elution pattern of the ecdysteroids shown in Fig. 1, that ecdysterone and inokosterone, while not resolved from each other, were readily separated from the other two ecdysteroids on a semi-preparative Corasil II column. Other adsorptive phases will give similar separations[3].

It is illustrated in Fig. 2 that inokosterone and ecdysterone were separated by reversed-phase chromatography on Bondapak phenyl–corasil but that inokosterone was not completely separated from makisterone A. The inadequate separation of inokosterone and makisterone A was not markedly improved by gradient elution (Fig. 3), but this procedure does permit separation of all of the other ecdysteroids tried, in a reasonable time and with high sensitivity. The solvent combinations given yield the best separations so far achieved.

For the separation and recovery of a crude ecdysteroid mixture, the first separation would best be on Bondapak phenyl–corasil equilibrated with water. This would allow the crude sample to be applied in high concentration in alcohol and minimize salt contamination problems, as salts are not retarded by Bondapak phenyl–corasil. The mixture would then be resolved and eluted with a solvent gradient. The gradient would most efficiently separate the necessary broad range of polarities in a short time and allow an initial water wash when necessary. The unresolved makisterone A–inokosterone combination could then be quickly separated on an adsorptive

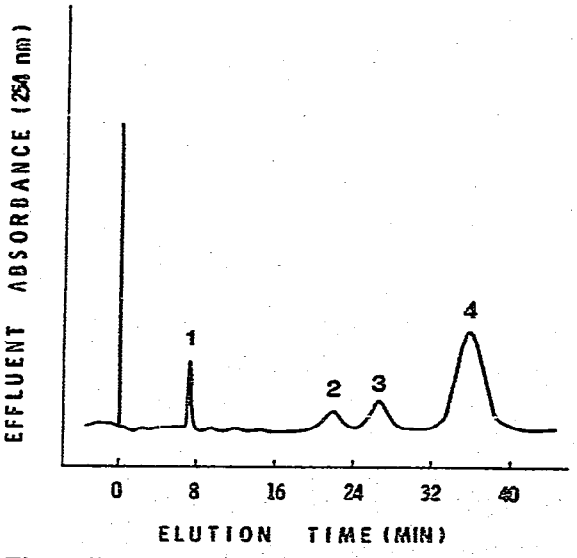

Fig. 1. Elution of 1–5 μg of ecdysterone triacetate (1, front), α-ecdysone (2), makisterone A (3), ecdysterone + inokosterone (4) from a Corasil II column (1000 × 3 mm I.D.) with chloroform–ethanol 95% (8:1) at a flow-rate of 1.1 ml/min. Particle size, 37–50 μm; pressure, 47 atm; temperature, 20°.

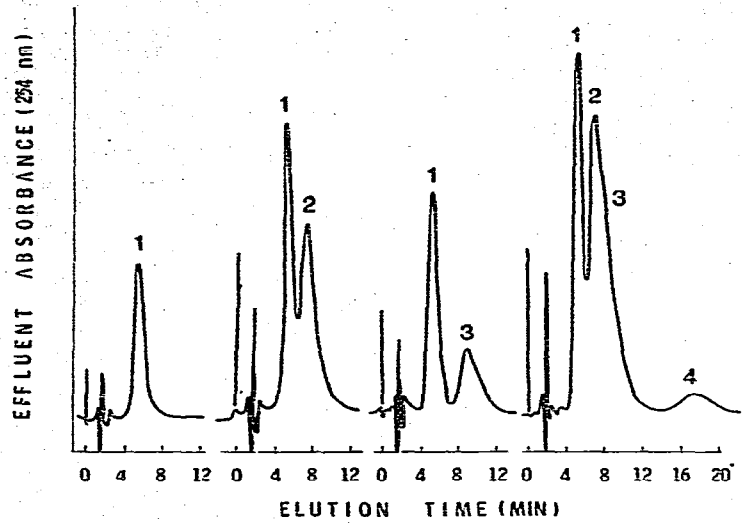

Fig. 2. Elution of 1–5 μg of ecdysterone (1), inokosterone (2), makisterone A (3) and α-ecdysone (4) from a Bondapak phenyl–corasil column (1000 × 3 mm I.D.) with 5% ethanol, 2% isopropanol in water at a flow-rate of 1.1 ml/min. Particle size, 37–50 μm; pressure, 30 atm; temperature, 20°.

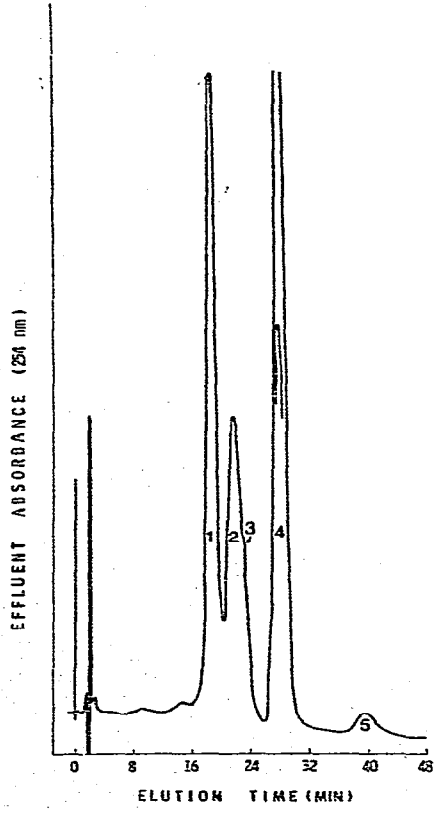

Fig. 3. Elution of 0.1–5 μg of ecdysterone (1), inokosterone (2), makisterone A (3), α-ecdysone (4) and ponasterone A (5) from a Bondapak phenyl–corasil column (1000 × 3 mm I.D.) at a flow-rate of 1.0 ml/min with a linear gradient from water (initial volume 10 ml) to 20% ethanol. Particle size, 37–50 μm; pressure, 16 atm; temperature, 20°.

column such as Corasil II. Preliminary work has shown that this reversed-phase system also can be applied readily to at least some of the polar ecdysterone metabolites of a crustacean.

While the UV detection is not as sensitive for ecdysteroids as electron capture detection of the TMS-ethers in gas chromatography[10] it is about as sensitive as flame ionization detection[11] and has the advantage that it allows simple recovery of the original compound in high yield. To obtain high sensitivities one would require smaller, more efficient columns than the semi-preparative ones used in this study, thus the elutions would be more rapid.

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