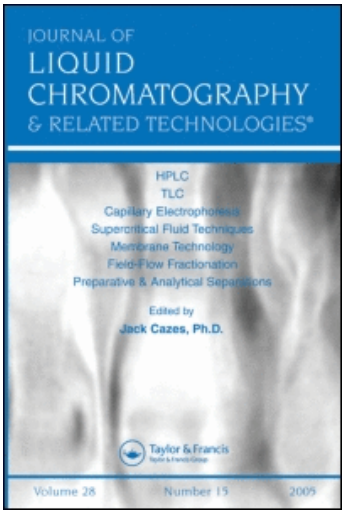

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Rapid Isolation of Ecdysteroids from Crustacean Tissues and Culture Media Using Sep-Pak C18 Cartridges

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RAPID ISOLATION OF ECDYSTEROIDS FROM CRUSTACEAN TISSUES AND CULTURE MEDIA USING SEP-PAK C18 CARTRIDGES

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ABSTRACT

A liquid chromatographic method for isolating ecdysteroids from crab tissues and crustacean tissue culture media is reported. The method employs commercially-available Sep-Pak C18 cartridges containing a reversed phase packing that retains ecdysteroids. The technique is simple and rapid; it typically yields recoveries in the range of 85-90% for extractions of tissues, and 90-95% for extractions of media.

INTRODUCTION

Ecdysteroids are steroid hormones that regulate growth and molting in arthropods (cf. 1). Among the ecdysteroids, ecdysone, the apparent secretory product of arthropod molting glands, and ecdysterone, a 20-OH metabolite which stimulates the cellular events that lead to growth and molting, are of primary interest.

A reliable quantitative assay for ecdysteroids, in terms of specificity, resolution, and sensitivity, is UV absorbance at 254 nm coupled with high pressure liquid chromatography

(HPLC) (2). However, the method applied to extracts of arthropod tissues requires removal of numerous contaminants prior to HPLC. The clean-up procedure, usually solvent partition, is tedious, time-consuming, and expensive, with losses of ecdysteroids attending the several steps.

We report a rapid, efficient method for isolating ecdysteroids from crustacean tissues and culture media using Sep-Pak C18 chromatographic cartridges. Coupled with HPLC, the procedure reduces the preparation steps by 50% and the time involved by 75%.

MATERIALS AND METHODS

Chemicals and Supplies

Sep-Pak C18 cartridges (approximately 1 cm x 1 cm, containing octadecylsilane bonded phase retained between two filters), were purchased from Waters Associates, Inc. (Milford, Mass.). High purity water and glass distilled MeOH were from Burdick and Jackson Laboratories, Inc. (Muskegon, Mich.). Ecdysone was from Simes (Milano, Italy) and ecdysterone from Rhoto Pharmaceutical (Osaka, Japan); both were purified on HPLC before use. Medium 199 (10X concentrate with Hank's salts and L-glutamine) was purchased from GIBCO (Grand Island, N.Y.). The salts used in Pantin's crustacean saline (3) were reagent grade. Fluoropore filters were from Millipore (Bedford, Mass.).

Experimental Animals

Cancer antennarius crabs (male, 5-10 cm in breadth) were purchased from Pacific Bio-Marine (Venice, Cal.). They were maintained in artificial sea water in compartmented marine trays, exposed to a photoperiod of 15L:9D, and fed pieces of fish every other day.

High Pressure Liquid Chromatography

A Waters Model 6000 pump with Waters U6K injector and ISCO UA5 absorbance monitor and Type 6 optical unit (ISCO, Lincoln, Neb.) were used for HPLC analysis. Quantitation of ecdysteroids was at 254 nm using a LDC Model 308 computing integrator (LDC, Riviera Beach, Florida). A uPorasil column from Waters was used with a solvent system of $CHCl_3$/ 95% EtOH (86:14) pumped at 2 ml/min (1200 psi). HPLC solvents were Burdick and Jackson glass distilled and were filtered before use.

Procedure

Standard procedure was to connect a Sep-Pak C18 cartridge to a luer-tip glass syringe, and to prime the cartridge by washing sequentially with 2 ml of methanol (MeOH) and 5 ml of H_2O. A sample was then placed in the syringe and injected through the cartridge. Subsequent washing and elution varied with experimental objective and is described below.

Recovery of ecdysteroids was monitored by adding ecdysone and ecdysterone standards to samples intended for extraction and Sep-Pak processing (all standards were made up in $CHCl_3$/ 95% EtOH (86:14)). Recoveries were calculated by dividing yield by the starting amount of standard, each determined by HPLC. In the case of blood and tissue extractions, a control blank (containing blood or tissue but not standards) was included; values of any endogenous ecdysteroid were subtracted from yields of the experimental samples before calculating recovery. Control blanks were not included with the two media extracted since repeated measurements beforehand showed the media did not contain endogenous ecdysteroids.

To determine the MeOH concentration required to elute ecdysteroids from Sep-Pak cartridges, 10 ul each of ecdysone

(181.5 ug/ml) and ecdysterone (179.0 ug/ml) were added to test tubes and allowed to air dry. Five ml of 1 of 11 test solvents were added to each tube: H_2O, MeOH diluted with water to concentrations of 10-100%. After priming a Sep-Pak cartridge, a test solvent was injected, and the resultant elutant collected, dried down on a rotary evaporator (BUCHI Rotavapor, Switzerland), and taken up in 1 ml $CHCl_3$/ 95% EtOH (86:14). Each sample was then injected through a 0.5 um fluoropore filter, dried under a stream of N_2, taken up in 250 ul $CHCl_3$/95% EtOH (86:14), and the recovery of ecdysteroids quantitated on HPLC.

For the extraction of ecdysteroids from Pantin's saline, standards were added in 3 quantities: 5, 10, or 15 ul each of ecdysone (181.5 ug/ml) and ecdysterone (179.0 ug/ml). After allowing the standards to dry, 5 ml of Pantin's saline were added to each tube and vortexed. The Sep-Pak procedure: 1) prime cartridge, 2) inject sample, 3) rinse with 10 ml of H_2O, and 4) elute ecdysteroids with 10 ml of 100% MeOH. The elutant was dried on a rotary evaporator and prepared for HPLC analysis as described above. Individual assays were performed on 5 separate tubes for each set of standards.

Three sets of standards were also used in the extraction of Medium 199: 10, 20, and 30 ul each of ecdysone (101.7 ug/ml) and ecdysterone (107.5 ug/ml). After the standards had dried, 2 ml of Medium 199 were added to each tube. The Sep-Pak procedure: 1) prime cartridge, 2) inject sample, 3) rinse with 10 ml of H_2O, 3) rinse with 15 ml of 20% MeOH, and 5) elute with 10 ml of 100% MeOH. Five trials were conducted on each set of standards.

For the extraction of ecdysteroids from crab tissues, a male Cancer antennarius (intermolt, body wt.: 488.5 g) was exsanguinated and sacrificed. A window was cut in the

dorsal carapace with a bench dental drill (EMESCO, N.Y., N.Y.), and tissue samples dissected free and weighed on a Roller-Smith balance. Tissues were homogenized by hand in 10 ml of 100% MeOH using a ground glass tissue homogenizer. A one ml aliquot of the homogenate was pipeted into a centrifuge tube to which ecdysteroid standards had been added (20 ul ecdysone (101.7 ug/ml) and 20 ul ecdysterone 107.5 ug/ml)), and one ml was pipeted into a control tube to which no standards had been added. This was repeated 5X for each tissue, with vigorous vortexing between each pipeting. The homogenate was centrifuged for 15 min at 2000 RPM, and the supernatant removed and saved. After adding another ml of MeOH to the pellet, the sample was recentrifuged and the second ml of supernatant added to the first. The combined supernatants were dried on a rotary evaporator, and the dried residue taken up in 5 ml of H_2O and added to a primed Sep-Pak cartridge. After rinsing with 10 ml of H_2O and 10 ml of 20% MeOH, the cartridge was eluted with 10 ml of 100% MeOH and the elutant dried down and prepared for HPLC.

The crab blood was allowed to clot, centrifuged for 15 min at 2000 RPM, and the serum removed. One ml of serum was pipeted into a centrifuge tube to which standards had been added (20 ul ecdysone (101.7 ug/ml) and 20 ul ecdysterone (107.5 ug/ml)), and one ml was pipeted into a control tube. Blood samples were then injected into a primed Sep-Pak cartridge. The rinse and elution procedure was as for crab tissues.

RESULTS

The effect of MeOH concentration on the elution of ecdysteroids from Sep-Pak C18 cartridges is shown in Table 1. Water, 10% MeOH and 20% MeOH do not elute ecdysteroids

TABLE 1

The Effect of MeOH Concentration on the Elution of Ecdysteroids From Sep-Pak C18 Cartridges

Solvent	Percent Recovery	
	Ecdysone	Ecdysterone
H_2O	0	0
MeOH 10%	0	0
20%	0	0
30%	6.2 ± 8.3	24.0 ± 7.0
40%	42.5 ± 2.8	74.2 ± 4.9
50%	69.1 ± 2.3	83.1 ± 4.5
60%	85.4 ± 3.2	92.1 ± 10.3
70%	90.2 ± 5.9	93.9 ± 3.9
80%	89.7 ± 13.9	90.2 ± 10.0
90%	88.6 ± 0.2	95.5 ± 8.4
100%	89.7 ± 0.6	90.2 ± 2.5

from the cartridges. Accordingly, these more polar solvents can be used to de-salt samples, and to wash components such as sugars, amino acids, hydrophilic proteins, and other polar organics from biological extracts that contain ecdysteroids.

As the MeOH concentration of the solvent is increased, the recovery of ecdysteroids in the elutant also begins to increase (TABLE 1). The difference in percent recovery between ecdysone and ecdysterone seen with solvents of intermediate polarity (e.g. 40% MeOH) is a reflection of the relative polarities of the two ecdysteroids, i.e., the less polar ecdysone is retained more strongly by the octadecylsilane bonded phase of the cartridge than is the more polar ecdysterone.

Concentrations of MeOH greater than 70% can be used to elute ecdysteroids quantitatively from Sep-Pak cartridges. (TABLE 1). 100% MeOH was chosen for use in future experiments

because, compared to solvents containing water, it dries more quickly and is less prone to loss of yield through bumping during rotary evaporation.

Pantin's saline and Medium 199 are among the culture media that have been utilized for *in vitro* studies on the synthesis and secretion of ecdysteroids by crustacean molting glands (4,5). That Sep-Pak cartridges can be used for the extraction of ecdysteroids from these media is shown in TABLE 2. The Sep-Pak rinse and elution procedure for Pantin's saline involves only a rinse with 10 ml of H_2O to remove the constituent salts, followed by the elution of ecdysteroids with 10 ml of 100% MeOH. Medium 199 is a complete, defined tissue culture medium; when only a water rinse is used before elution of ecdysteroids with 100% MeOH and subsequent HPLC analysis, a large contaminating

TABLE 2

Extraction of Ecdysteroids From Crustacean Culture Media

Culture Medium	Ecdysone		Ecdysterone	
	Standard (ug)	Percent Recovery	Standard (ug)	Percent Recovery
Pantin's Saline	0.9075	97.6 ± 6.9	0.8950	89.9 ± 1.9
	1.815	92.5 ± 5.9	1.790	96.4 ± 7.1
	2.7225	96.8 ± 3.8	2.685	97.2 ± 3.9
Medium 199	1.017	95.6 ± 9.7	1.075	102.7 ± 6.1
	2.034	91.0 ± 3.7	2.150	94.5 ± 5.7
	3.051	96.2 ± 3.1	3.225	87.9 ± 4.3

peak, migrating with a retention time similar to ecdysone standard, is found on the chromatographs. A wash with 15 ml of 20% MeOH effectively removes this contaminant and allows quantitation of the ecdysteroid standards.

Sep-Pak C18 cartridges can also be used to extract ecdysteroids from aqueous solutions of tissue extracts (TABLE 3). After injecting the aqueous solution through the cartridge, a rinse with 10 ml of H_2O, followed by 10 ml of 20% MeOH effectively cleans up these samples in preparation for HPLC. The decrease in percent recovery of ecdysteroids from tissue samples compared to the percent recovery from culture media is apparently due to the additional steps involved in tissue extraction per se (i.e., homogenization, centrifugation, evaporation and resuspension), which precede the Sep-Pak procedure itself.

DISCUSSION

The Sep-Pak technique is a simple and effective method for cleaning up biological samples prior to analysis of ecdysteroids by HPLC. It is a useful alternative to the usual solvent extraction and partition methods. Prior to the development of this technique, analysis of ecdysteroids in our laboratory was accomplished by the method outlined in FIGURE 1. This method was developed for use on crustacean tissues; it is a modification of the methods of Horn et al. (6) and of Kaplanis et al. (7), and is characteristic of similar techniques used by others. It requires a chloroform/methanol (2:1) extraction, two solvent partitions, a silica gel preparatory column preceding HPLC, and two HPLC steps. The method has proven effective, but is labor-intensive and extremely time-consuming. In our hands, recovery of ecdysteroids using this system is typically in the range

TABLE 3

Extraction of Ecdysteroids From Crab Tissues

Tissue	Wet Weight (mg)	Percent Recovery	
		Ecdysone	Ecdysterone
Gonad	893.0	92.9 ± 7.2	85.5 ± 6.6
Gill	943.6	87.2 ± 1.4	87.1 ± 5.1
Muscle	965.0	84.7 ± 2.6	83.7 ± 2.9
Hepato-pancreas	864.8	79.5 ± 15.0	88.2 ± 8.2
Blood	25.0[a]	90.0 ± 3.2	87.8 ± 9.5

[a]milliliters

of 40-50%. In contrast, the Sep-Pak procedure is rapid, easily performed, uses limited organic solvents, and requires minimal laboratory equipment. Moreover, recovery is typically 85-90% (TABLE 3). The utility of the method is even more apparent when one is extracting ecdysteroids from culture media. In this case, the preliminary solvent extraction can be eliminated, and the media injected directly through the cartridge. This decrease in the number of experimental steps is accompanied by an increase in recovery to 90-95%.

Because Sep-Pak C18 cartridges are relatively inexpensive, many workers will be dissuaded from attempting to reuse them. However, we have reused cartridges as many as five times with no decrease in recovery. Cartridges were routinely washed with 10 ml of 100% MeOH and equilibrated with 10 ml of H_2O before reuse.

The Sep-Pak method reported here should have widespread applications for both *in vitro* and *in vivo* studies on ecdysteroid physiology and arthropod growth and molting.

FIGURE 1. Extraction and purification of ecdysteroids for HPLC analysis using (a) solvent partition and (b) Sep-Pak C18 cartridges

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