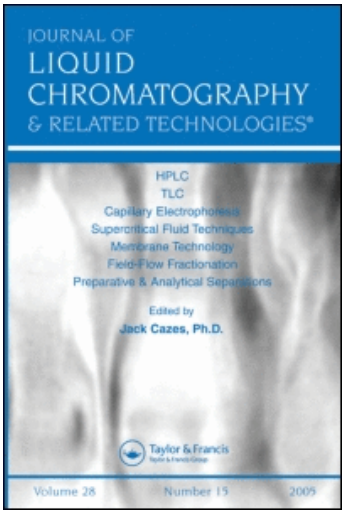

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G. A. Hines[a]; S. A. Watts[a]; S. A. Sower[b]; C. W. Walker[b]
[a] Department of Biology, University of Alabama at Birmingham, Birmingham, Alabama [b] Department of Zoology, University of New Hampshire, Durham, New Hampshire

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SEX STEROID EXTRACTION FROM ECHINODERM TISSUES

G. A. HINES[1], S. A. WATTS[1],
S. A. SOWER[2], AND C. W. WALKER[2]
[1]*Department of Biology*
University of Alabama at Birmingham
Birmingham, Alabama 35294
[2]*Department of Zoology*
University of New Hampshire
Durham, New Hampshire 03824

ABSTRACT

A method for sex steroid extraction from selected echinoderm tissues is presented. This method utilizes solvent and solid based extraction techniques and is reliable for small tissue samples. Tissue homogenates were incubated in warm acidic conditions prior to organic extraction of steroids. Further isolation of steroids using Sep-Pak cartridges and a simple elution scheme resulted in percent recoveries ranging from 56%-90% depending on steroid and tissue type.

INTRODUCTION

Previous investigations have demonstrated the presence of steroids in tissues of several classes of echinoderms (1, 2, 3, 4, 5). In addition, seasonal changes in the levels of sex steroids have been observed in some of the Asteroidea which exhibit annual

reproductive cycles (6, 7, 8). These steroids, which are known to regulate reproduction in many vertebrates, may be also involved in the regulation of reproduction in echinoderms.

Methods used to isolate steroids from echinoderm tissues in previous studies have utilized techniques based on biological fluid steroid extraction. These methods have often proven to be unreliable. These analyses usually required large amounts of tissue, resulting in labor-intensive procedures. Also, previous extraction techniques did not remove potential biological contaminants which could interfere with many current steroid assay techniques. As tissue preparations may contain more protein than biological fluids, a higher potential for extraction artifacts due to steroid-protein interactions exists. Steroid binding proteins (steroid receptors) which are necessary to elicit a response in any target tissue may also be present in echinoderm tissues (9).

In this paper we present a method for steroid extraction from echinoderm tissues. This method utilizes solvent and solid based extraction techniques, is rapid and reliable for the echinoderm tissues tested, and can be adapted easily for other lipid-rich and protein-rich invertebrate tissues.

MATERIALS AND METHODS

Chemicals and Supplies

Sep-Pak C_{18}-cartridges with octadecyl-silane bonded reversed phase packing were purchased from Waters Associates, Milford, MA. Sterile Luer Lok plastic syringes (10cc) were purchased from

Beckton Dickinson & CO. Glass distilled "Baker Resi-Analyzed" methanol and "Baker Analyzed" hydrochloric acid were purchased from J.T. Baker Chemical CO., Phillipsburg, NJ. Reagent grade sodium chloride and sodium phosphate and Optima grade methylene chloride were purchased from Fisher Scientific, Fair Lawn, NJ. Radiolabeled progesterone (1,2,6,7 ^{3}H: 97 Ci/mmol), testosterone (1,2,6,7,16,17 ^{3}H; 141 Ci/mmol) and estradiol (2,4,6,7 ^{3}H; 105 Ci/mmol) were purchased from Amersham Corp., Arlington Hts. IL. and purified by Sep-Pak techniques before use. Water (used for washes, buffer and salts solution) was double distilled.

Experimental Animals

Reproductively mature sea stars, Asterias vulgaris, and sea urchins, Strongylocentrotus droebachiensis, were purchased from Ocean Resources (Portland Harbor, ME.). Animals were collected by SCUBA and sent via overnight air freight to the laboratory where tissues were excised and placed on ice. Wet mounts of gonads were viewed by microscopy to determine sex.

Steroid Extraction from Tissue Homogenates

Subsamples (0.1g) of testes, ovaries or pyloric caecal tissues were homogenized on ice in 0.5 ml of water for ca 5 sec using a Wheaton glass and teflon homogenizer. Six replicate homogenates of each tissue for each steroid of interest (progesterone, testosterone and estradiol) were prepared for determination of standard errors. Homogenates were then pulse-sonicated on ice for 10 sec using a

Branson Sonifier Cell Disruptor 350 with micro tip (output level = 6, duty cycle = 40%). Aliquots of purified radioactive steroid standards were added to homogenates for determination of recovery efficiencies. Next, 0.4 ml of prewarmed 0.025 M HCL was added to homogenates for a final pH ~ 2.0, vortexed, and incubated for 15 min. at 40 ^{o}C to enhance the dissociation of steroids from binding proteins. After incubation, 1.25 ml of 0.07 M Na_2HPO_4 buffer (pH 7.4) was added to neutralize homogenates.

Three methylene chloride (MC) extractions were performed on each sample to extract steroids. Seven mls of MC was added to each homogenate and vortexed thoroughly. Samples were centrifuged at 2500 rpm for 10 min to separate aqueous and organic phases. Organic (steroid-containing) phases were removed by pasteur pipet, pooled and dried under nitrogen gas in a 40 ^{o}C water bath. Steroids were resuspended in 0.5 ml MeOH, vortexed and 4.5 mls buffer (as above) added to prepare samples for Sep-Pak processing. Aliquots of this solution were counted for radioactivity to determine steroid recovery using MC.

Preparation of Sep-Pak Cartridges

New Sep-Pak cartridges were fitted to syringes and primed with three sequential washes of 10 ml MeOH, 10 ml water and 5 ml 0.15 M NaCl at a flow rate of 15 ml/min. Subsequent washes were eluted at the same flow rate. Sep-Pak cartridges could be reconditioned with sequential washes of 10 ml water, 5 ml MeOH and 10 ml water and were used to process optimally five samples (10).

Steroid Isolation with Sep-Pak Cartridges

Prepared samples were applied to either primed or reconditioned Sep-Pak cartridges and washed sequentially with 5 mls 0.15 M NaCl, 10 mls water and 5 mls MeOH. Aliquots of the MeOH fraction were counted for radioactivity to determine steroid recovery from Sep-Pak cartridges.

Elution efficiencies of steroids from Sep-Pak cartridges were determined based on recoveries of radiolabeled steroid standards. Aliquots of stock radiolabeled steroid standards were dried and resuspended in MeOH and buffer (as above) and processed using the same elution scheme. Eluants from each wash (0.15 M NaCl, water and MeOH) were collected. The efficiency of recovery of steroids was determined based on radioactivity present in each eluant.

RESULTS

Steroid Extraction from Tissue Homogenates

Extraction of steroids from aqueous tissue homogenates using MC resulted in % recoveries of radiolabeled steroid standards ranging between 66-94% (TABLE 1). Progesterone recovery was similar in sea star testes and ovaries at ~74% recovery and relatively lower in pyloric caeca at 68% recovery. Progesterone recovery in sea urchin was highest in testes at 87% and lowest in ovaries at 66%. Testosterone recovery was similar in all tissues with recoveries ranging from 87-88% except sea urchin testes showing a higher recovery of 94%. Estradiol recoveries in sea star tissues ranged from

TABLE 1

Percent Recoveries of Methylene Chloride-Extracted Steroids from Sea Star and Sea Urchin Tissues (mean ± SE, n=6).

Tissue	Progesterone	Testosterone	Estradiol
Sea star			
Testis	74.6 ± 1.9	88.0 ± 1.0	84.0 ± 1.9
Ovary	74.4 ± 2.1	87.0 ± 3.2	88.7 ± 1.5
Pyloric caecum	68.3 ± 6.5	87.9 ± 1.8	78.4 ± 4.1
Sea urchin			
Testis	87.0 ± 2.4	93.7 ± 1.4	94.3 ± 1.4
Ovary	65.8 ± 3.6	88.7 ± 2.9	82.2 ± 0.7

a high of 88% in ovaries, 84% in testes and 78% in pyloric caeca. Estradiol recoveries in sea urchin tissues were highest in testes at 94% and relatively lower in ovaries at 82%.

Steroid Isolation with Sep-Pak Cartridges

Elution of MC-extracted steroids (from tissue homogenates) using Sep-Pak cartridges resulted in recoveries of radiolabeled steroids ranging from 56-90% (TABLE 2). Progesterone recoveries were similar in sea star tissues ranging from 64-67%. Progesterone recoveries in sea urchin tissues were higher in testes at 72% and relatively lower in ovaries at 56%. Testosterone recoveries in sea star tissues were highest in ovaries and pyloric caeca at 90% and 86% respectively and relatively lower at 82% in testes. Testosterone

TABLE 2

Percent Recoveries of Sex Steroids Extracted from Sea Star and Sea Urchin Tissues with Methylene Chloride and Isolated with Sep-Pak Cartridges (mean ± SE, n=6).

Tissue	Progesterone	Testosterone	Estradiol
Sea star			
Testis	67.7 ± 2.2	82.3 ± 1.5	81.6 ± 2.3
Ovary	64.5 ± 2.2	90.2 ± 2.4	88.3 ± 2.8
Pyloric caecum	66.7 ± 2.3	86.2 ± 4.5	84.5 ± 2.2
Sea urchin			
Testis	72.5 ± 2.0	82.3 ± 1.2	80.7 ± 1.1
Ovary	56.0 ± 3.2	79.4 ± 3.4	71.2 ± 0.5

recoveries in sea urchin tissues were similar at 82% in testes and 79% in ovaries. Estradiol recoveries in sea star tissues were 88% in ovaries, 84% in pyloric caeca and 81% in testes. Estradiol recoveries in the sea urchin were 81% and 72% in testes and ovaries respectively.

Elution efficiencies of radioactive steroid standards from Sep-Pak cartridges ranged between 91-97%. Steroids applied to cartridges were not eluted with initial salt and water washes. Subsequent washing with 5 ml of MeOH eluted most steroids from the cartridges. Percent recovery of radioactive steroid standards in MeOH washes was 92.7%, 91.8 % and 97.6% for progesterone, testosterone and estradiol respectively.

DISCUSSION

Incubation of tissue homogenates in warm, acidic conditions should release steroids from steroid binding proteins (11) and will increase the efficiency of steroid extraction. A variety of organic solvents (combined or used alone) were tested previously for their abilities to extract progesterone, testosterone and estradiol. These included chloroform, hexane, hexane:benzene (2:1) and diethyl ether. Methylene chloride proved the best universal solvent for extracting these steroids from lipid-rich echinoderm tissues. This organic solvent should also inactivate any steroid metabolic enzymes present in the tissue homogenates. Using methylene chloride, the average percent recoveries for radiolabeled progesterone, testosterone and estradiol in the sea star were 72%, 88% and 84% and in the sea urchin 76%, 91% and 88% respectively.

Steroids extracted initially from echinoderm tissue homogenates via methylene chloride may be isolated further using commercially-available Sep-Pak cartridges. These cartridges have been used in the direct extraction of steroids from biological fluids surch as urine (10), milk (12) and serum (13). This technique is also useful for separating biological contaminants from tissue-extracted steroids. The proposed elution scheme allows the preferential isolation of steroids from amino acids, salts or other non-steroidal polar products which may interfere with subsequent steroid analysis. Average percent recoveries for radiolabeled progesterone, testosterone and estradiol after methylene chloride extraction and Sep-Pak processing were 66,% 86% and 85% in the sea star and 64%, 81% and 76% in the sea urchin respectively.

Warm acid incubation of tissue homogenates, followed by methylene chloride extraction and Sep-Pak purification techniques can be used to effectively extract steroids from echinoderm tissues. These procedures result in relatively high recoveries of partially-purified steroids. In addition, these procedures yield reliable results using small tissue samples. This is useful considering many echinoderms have only small amounts of tissue available for steroid analysis. Thus, high recoveries of steroids from small tissue samples would be desirable for subsequent steroid analysis techiniques, such as radioimmunoassay. This method offers a reliable and efficient means of isolating sex steroids from tissues of the sea star Asterias vulgaris, the sea urchin Stongylocentrotus droebachiensis and, presumably, from other echinoderm and invertebrate tissues.

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