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HPLC-FLUOROMETRIC ANALYSIS OF PROSTAGLANDINS FROM MARINE ORGANISMS

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A selective and sensitive method for determining prostaglandins (PG) in marine organisms is proposed. The method includes four stages: extraction and purification of PG, preparation of fluorescent derivatives using 4-bromomethyl-7,8-benzocoumarin (Br-MBC), and two-stage HPLC on silica gel and C-8 columns. HPLC on the silica-gel column is used for preliminary screening of PG in the extracts and also for purification and separation of the Br-MBC ethers of PG. The Br-MBC ethers of PG are placed on a C-8 column with subsequent fluorescent detection. Analysis of ten species of marine organisms demonstrated that they contain a mixture of various PG.

Biologically active compounds from marine organisms have recently been attracting increasing attention. Among these, prostaglandins (PG) are of particular interest. Quantitative analysis of PG and related eicosanoids in multicomponent mixtures addresses several problems. One of the methods for high-sensitivity analysis of PG is the preparation of fluorescent ethers. A number of reagents are used for this: methylmethoxycoumarin (MMC) [1], 4-bromomethyl-7-methoxycoumarin (Br-MMC) [2-4], 4-bromomethyl-7-acetoxycoumarin [5], 9-anthryldiazomethane [6], and *p*-(9-anthryloxy)phenacylbromide [7].

Fluorescent reagents should satisfy several requirements. The yield of reaction products should be consistently high. The reaction conditions should be mild. The reagents should be stable over time and during chromatographic separation. The most common reagents are Br-MMC and MMC ethers, which give practically quantitative yields. However, these are highly photolabile. The fluorescence quantum yield of MMC ethers is highly dependent on the solvent polarity [1]. This lowers the sensitivity and complicates quantitative calculations if HPLC is used. We used 4-bromomethyl-7,8-benzocoumarin (Br-MBC) in the present work to increase the sensitivity of fluorometric analysis of PG. This compound has the advantages of a high fluorescence quantum yield of the methylbenzocoumarin (MBC) ethers that is less dependent on the solvent polarity, high photostability, and quick reaction kinetics.

We describe an improved analytical procedure for determining PG that includes their extraction from the natural material, preparation of derivatives with a fluorescent marker, and two-stage HPLC on silica-gel and C-8 columns.

We previously demonstrated that many marine organisms exhibit PG-like activity [8]. Subsequent detailed research of echinoderms found that the PG-like activity is concentrated mainly in the internal organs [9]. The literature indicates that many researchers also emphasize the study of PG in animal organs [10, 11]. Therefore, we used only animal internal organs in the present work. Some doubt can be cast on the necessity for developing new or modifying known analytical methods for PG. However, the enormous variety of PG that are simultaneously present in biological material and the need for a universal analytical method justify the present work.

Quantitative analysis of PG in biological material, where their content is extremely low, presents special demands. The literature methods proposed previously were developed mainly to analysis PG standards [12-14] and infrequently to apply to biological specimens. For example, PG determination in insects [15], the mucous lining of the human stomach [16, 17], and biological samples [18] has been reported.

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TABLE 1. PG MBC-Derivatives in Extracts of Marine Organisms (% in the Organic Portion of the Extract)*

Sample, family, class, species**	Collection time	B_2	E_2	$F_{2\alpha}$
Annelida				
Sipunculidea				
Phascolosoma japonica	June 1986	-	0.4±0.01	-
	July 1986	-	5.1±0.02	8.9±0.27
Mollusca				
Gastropoda				
Haliotis ovina	March 1986	24.2±0.73	6.2±0.18	7.4±0.22
Aplysia sp.	March 1986	-		4.4±0.13
Bivalvia				
Crenomytilus grayanus	June 1986	0.5±0.02	0.4±0.01	-
	October 1986	-	1.3±0.04	1.2±0.04
*Mytilus edulis****	October 1986	26.7±0.80	-	-
Mytilus edulis	October 1986	12.1±0.36	-	-
*Modiolus difficilis****	October 1986	-	0.8±0.02	5.1±0.15
Modiolus difficilis	October 1986	15.6±0.48	-	30.3±0.90
Echinodermata				
Holothurioidea				
*Stichopus japonicus****	April 1985	32.3±1.03	0.5±0.01	1.5±0.04
Stichopus japonicus	April 1985	8.1±0.24	3.4±0.10	
Asteroidea				
*Distolasterias nippon****	January 1984	-	-	4.2±0.12
Distolasterias nippon	January 1984	19.1±0.57	-	-
Chordata				
Ascidiasea				
Halocynthia aurantium	March 1984	-	-	9.2±0.27
	August 1984	1.4±0.04	10.9±0.33	1.7±0.05
Pisces				
Carangidae				
Seriola quinqueradiata	August 1986	30.0±0.90	-	-

*Samples were analyzed within a month of collection despite the fact that the table contains data from different years.

**Five organisms of each studied species were used.

***Elution by hexane—ethylacetate (1:1).

The uniqueness of marine material (large assortment of pigments, neutral lipids, phospholipids, etc.) was considered in analysis of PG in marine organisms. The method enables the highly reproducible separation of fractions of nonpolar lipid MBC-derivatives and MBC-PG with subsequent quantitative analysis by HPLC on C-8 columns. MBC-derivatives of other lipids are formed in addition to MBC-PG. These interfere with the analysis of PG. We found experimentally that MBC-PG do not undergo photolysis and remain completely intact during fractionation with UV-detection at 280-310 nm. The structural integrity was monitored by recording spectra at given time intervals (one week to six months).

The method for purifying total PG extracts was developed using extracts of internal organs of *Stichopus japonicus* and *Seriola quinqueradiata*. The purification scheme is given in the Experimental section. It employs elution on a silica-gel column with fluorometric detection and was developed to screen samples from marine organisms for PG content. Sometimes the complexity of the lipid content (according to TLC) made it necessary to use more than one mobile phase. An additional solvent mixture, in particular, hexane—ethylacetate (1:1), was used. Table 1 gives results from fractionation of a mixture using hexane—ethylacetate (1:1) and ethylacetate. The chromatograms were interpreted by comparing retention times with MBC-PG standards. Figure 1 shows the fractionation of a mixture of MBC standards with PG B_2, PG E_2, PG F_{2a}, and two samples of marine echinoderms.

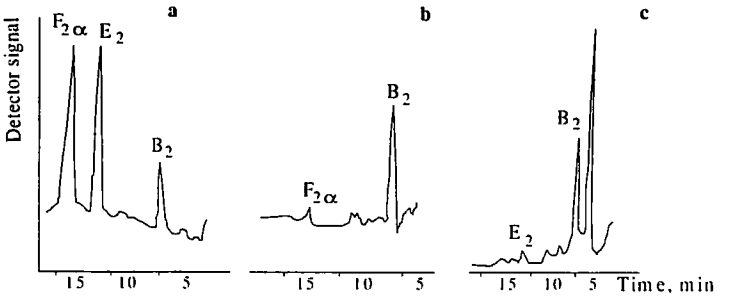

Fig. 1. HPLC of MBC-ethers of PG standards (a), PG from *Distolasterias nippon* (ethylacetate fraction) (b), and PG from *Stichopus japonicus* (ethylacetate fraction) (c). Sepharon-Silica column (7 μm, 150 × 3 mm); eluent: linear gradient of ethylacetate in hexane from 30 to 90% over 15 min; fluorometric detection at λ_{ex} 275 and λ_{fl} 430 nm.

The studied echinoderm species contain three PG: E_2, $F_{2\alpha}$, and B_2. Extracts of the marine organisms listed in Table 1 were analyzed for PG content using this method.

The majority of the studied species of marine organisms characteristically contain PG B_2, PG E_2, and PG $F_{2\alpha}$. The collection time had a noticeable influence on the quantitative content and composition of PG. Thus, the PG E_2 content varies over wide ranges both from organism to organism and in one species. The amount of PG B_2 in the studied organisms is greater than that of other PG. This is probably due to the conversion of PG E_2 to PG B_2 during sample treatment and storage.

Thus, the described fluorometric analytical method can identify and quantitatively determine PG in natural material.

EXPERIMENTAL

Reagents. Standard PG (E_2, $F_{2\alpha}$, and B_2) were purchased from the pilot plant at the Institute of Organic Synthesis (Tallin, Estonia). Each sample was crystalline and pure according to TLC.

Synthesis of 4-Bromomethyl-7,8-benzocoumarin (Br-MBC). The following changes were made to the synthesis method previously reported [3]. The solvents were changed. A purification step using a silica-gel (40-100 μm, Chemapol) column with elution by toluene-acetone (95:5 by volume, here and throughout this article) at low pressure (1.5-2 atm Ar or compressed air) was added in addition to recrystallization of Br-MBC.

The following were studied: one species of worm (*Phascholosoma japonica*), five species of mollusks (*Haliotis ovina*, *Aplysia* sp., *Crenomytilus grayanus*, *Mytilus edulis*, and *Modiolus difficilis*), two species of echinoderms (*Stichopus japonicus* and *Distolasterias nippon*), one species of ascidian (*Halocynthia aurantium*), and one species of fish (*Seriola quinqueradiata*).

Preparation of MBC Ethers of PG Standards and Fatty Acids. Stearic acid, the coumarin derivative of which gave one fluorescent spot by TLC and one peak by HPLC using CH_3CN-H_2O (60:40), was used as a model compound for preparing the coumarin derivatives.

Next the conditions for preparing the MBC derivatives of the PG standards and separating them by TLC at 25°C using ethylacetate—hexane—acetic acid—water (80:50:20:150, upper organic phase) were investigated.

Standard PG E_2, PG $F_{2\alpha}$, and PG B_2 (100 μg each) or PG samples from marine organisms were treated successively with K_2CO_3 (10 μg, anhydrous), Na_2SO_4 (10 μg, anhydrous), Br-MBC (100 μg in CH_3CN), crown ether (50 μg in CH_3CN) and incubated for 15 min in a glycerine bath at 60°C (in the dark with vigorous stirring). The formation of the coumarin derivatives of PG was monitored by UV-TLC (254 nm).

A mixture of standard MBC-ethers of various PG (E_2, $F_{2\alpha}$, B_2) was separated by semi-preparative HPLC on a C-8 column.

Preparation and Purification of PG Extracts. Only internal organs of marine organisms were used. Tissue samples (0.5-1.0 g) were extracted with ethylacetate (2 × 25 ml) in a homogenizer for 2 min. The combined supernatants were centrifuged for 10 min at 3000 rpm at 4°C, washed with water, dried over anhydrous Na_2SO_4, filtered, and evaporated in vacuum. The solid was dissolved in hexane—ethylacetate (1:1, 0.5 ml) and chromatographed on a low-pressure silica-gel

column (5 × 1 cm) or on an analogous cartridge using hexane—ethylacetate (1:1, 20 ml) (fraction 1) and ethylacetate (20 ml) (fraction 2). The evaporated fractions were dissolved in hexane—ethylacetate (1:1, 250 µl) and filtered (45-µl Gelman Sciences filter, USA). The filtered samples (100 µl) were placed on a liquid chromatograph (Sepharon Silica HPLC column, 150 × 3 mm, Covo, Czech Republic). A linear gradient was used: 2 ml/min from 30% ethylacetate in hexane to 90% ethylacetate in hexane over 15 min, isocratic regime at this ratio for 5 min, and column regeneration with 30% ethylacetate for 5 min. Fractions eluting over 2-3 min were collected. The solvent was evaporated. The solid was dissolved in CH_3CN (50 µl) and used for quantitative PG analysis.

Rapid HPLC Analysis of PG from Marine Organisms. The analysis used an Altex gradient liquid chromatograph equipped with a Shimadzu fluorometric detector. Samples of PG MBC ethers standards or extracts (5-20 µl) were chromatographed on a Sepharon-Silica column (SiO_2 6 µm, 150 × 3 mm, Covo, Czech Republic). The detector was set at λ_{ex} 275 and λ_{fl} 430 nm. Peaks were identified by comparing retention times with those of PG MBC ethers standards.

Quantitative Analysis of PG MBC Ethers by HPLC. Fractions of MBC-PG (5-20 µl) were analyzed on an Altex gradient liquid chromatograph equipped with a fluorometric detector and an Ultrasphere C-8 (5 µm, 250 × 4 mm) reverse-phase column.

The elution used an isocratic regime [0.8 ml/min, 0-15 min, CH_3CN—H_2O (60:40)] and a linear gradient (up to 100% CH_3CN over 10 min). The column was regenerated for 5 min by CH_3CN—H_2O (60:40) after isocratic elution by CH_3CN for 5 min.

Peaks were detected using fluorescence at λ_{ex} 275 and λ_{fl} 430 nm and were identified by comparing retention times with those of PG MBC standards. The detection limit was 0.1 ng PG. The detector response was linear with PG content in the range 0.1-50 ng and was independent of the ether structure.

All analyses were performed in triplicate. In all instances the standard deviations were within ±3% of the average.

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