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PICOGRAM MEASUREMENT OF PROSTAGLANDIN E_2 SYNTHESIS BY GASTRIC MUCOSA BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

Prostaglandin biosynthesis by gastric mucosa was determined by a 1-min incubation, solvent extraction, and reaction with panacyl bromide. The prostaglandin ester was measured by normal-phase high-performance liquid chromatography. The prostaglandin E_2 levels of normal gastric mucosa of rats and swine were 90.6 ± 31.0 and 79.8 ± 39.8 pg/min/mg tissue, respectively. This method was sensitive to 40 pg and specific for prostaglandin E_2.

INTRODUCTION

Non-steroidal antiinflammatory drugs, such as aspirin and indomethacin, are known to inhibit prostaglandin synthetase activity. In both man and animals these drugs damage the gastric mucosa, and ulceration with bleeding occurs[1,2]. When prostaglandin E_2 (PGE_2) is administered, the degree of tissue damage is decreased[3]. This has been called the cytoprotective effect of prostaglandins. PGE_2 stimulates the secretion of mucus and bicarbonate, inhibits the release of histamine and acid, affects ion transport and membrane permeability, stimulates the growth of mucosa, and increases blood flow to the stomach[3–5].

Although it would seem that a change in either the gastric mucosal concentrations or the biosynthesis of the endogenous prostaglandin would be associated with a functional change leading to disease or cure, no such relationship has been established. Studies reporting the tissue content of PGE_2 are inaccurate, because cells do not store prostaglandins. What is actually measured is the amount synthesized during specimen collection and homogenation[1]. In other studies, the synthesis of PGE_2 was measured by radioimmunoassay[6,7], but there are difficulties with this method[8]. In particular, specific antibodies to PGE_2 are extremely hard to obtain[9].

High-performance liquid chromatography (HPLC) has been previously used to separate and quantitate prostaglandins. Unfortunately, methods using ultraviolet detection lacks the sensitivity necessary to measure PGE_2 biosynthesis from gastric

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mucosa. Recently, fluorescent techniques using panacyl bromide derivatization and reversed-phase HPLC have measured picogram levels of prostaglandins from tissue culture medium and human plasma[10,11]. Since normal-phase HPLC may be advantageous for the rapid separation of panacyl PGE_2 esters, the purpose of our study was to devise a specific and sensitive method for measuring the PGE_2 produced by gastric mucosa.

EXPERIMENTAL

Materials

PGE_2 and 13,14-dihydro-15-keto prostaglandin $F_{2\alpha}$ (13,14-dihydro-15-keto-$PGF_{2\alpha}$) were purchased from Upjohn (Kalamazoo, MI, U.S.A.). Panacyl bromide [*p*-(9-anthroyloxy)-phenacyl bromide] was kindly supplied by Walter Morozowich of Upjohn. [^{3}H]PGE_2, 160 Ci/mmol, rabbit anti-PGE_2, and Biofluor™ scintillation fluid were purchased from New England Nuclear (Boston, MA, U.S.A.). Ethyl acetate, acetone, water, dichloromethane, acetonitrile, tetrahydrofuran, and methanol were of HPLC-grade, and were purchased from Burdick & Jackson Labs. (Muskegon, MI, U.S.A.). Light petroleum (b.p. 37.2–57.8°C) and 88% formic acid were obtained from Mallinckrodt (St. Louis, MO, U.S.A.), triethylamine from J. T. Baker (Phillipsburg, NJ, U.S.A.) and Bacto gelatin from Difco Labs (Detroit, MI, U.S.A.). All other chemicals were purchased from Sigma (St. Louis, MO, U.S.A.). Silica gel Sep-Pak cartridges were obtained from the Waters Chromatography Division of Millipore (Milford, MA, U.S.A.), high-purity dry nitrogen from the T. W. Smith Corp. (Brooklyn, NY, U.S.A.) and polypropylene test tubes from Sarstedt (Princeton, NJ, U.S.A.).

Apparatus

An HPLC system (Model 590 delivery module, Model 420 fluorescence detector and Model 730 data module) was purchased from Waters. Samples were injected into a Rheodyne Model 7012 syringe loading sample injector (Rheodyne, Cotati, CA, U.S.A.) equipped with a 20-μl loop) with a 25-μl glass syringe (Hamilton, Reno, NV, U.S.A.). A prepacked, microparticulate silica gel column (Zorbax-Sil; Du Pont, Wilmington, DE, U.S.A.), 25 cm × 4.6 mm I.D. was employed at ambient temperature. The void volume of the HPLC system is 2.6 ml. Radioactivity was measured in a Model 3385 liquid-scintillation counter (Packard, Downer's Grove, IL, U.S.A.).

Animals

Holtzman rats, weighing 150–175 g, were purchased from Holtzman (Madison, WI, U.S.A.). Research-breed swine, weighing 25 kg, were purchased from Biomedical Assoc. (Friedensburg, PA, U.S.A.). Animals were fed a standard laboratory chow (Ralston Purina, St. Louis, MO, U.S.A.).

Sample preparation

Gastric mucosa samples were minced with a scapel and transferred to tared polypropylene 1.5-ml centrifuge tubes (Eppendorf, Hamburg, F.R.G.). The tissue weight, 150–200 mg, was determined with a Model 2474 analytical balance (Sartorius, Göttingen, F.R.G.). The biosynthesis of PGE_2 in 1 min was determined by the

method of Robert *et al.*[6]. A phosphate-buffered saline solution (0.5 ml), containing 0.9% sodium chloride, 0.01% sodium azide, 0.1% gelatin and 0.1 *M* sodium phosphate buffer at pH 7.4, was added to the tissue. After mixing, the tube was centrifuged in a Model 5414 fixed-speed centrifuge (Eppendorf) at 15 000 *g* for 1 min. The supernatant was discarded, and 0.5 ml of buffered saline was added to the pellet. After resuspending the tissue and vortex-mixing for 1 min at ambient temperature, 10 μg of indomethacin (1 mg/ml in 1% sodium bicarbonate) was added to inhibit further synthesis of prostaglandins from endogenous arachidonic acid. An additional 0.5 ml of buffered saline was added. The contents of the tube were mixed and centrifuged, as before. The supernatant was decanted into a 15-ml polypropylene centrifuge tube.

Although 13,14-dihydro-15-keto-$PGF_{2\alpha}$ is a natural oxidation product of $PGF_{2\alpha}$, the metabolite was not detectable in the prostaglandin-generating system used in this study for gastric mucosa. Since the retention times of the panacyl derivatives of 13,14-dihydro-15-keto-$PGF_{2\alpha}$ and PGE_2 were 2.1 and 2.8 min, the metabolite was used as the internal standard. After the addition of 50 ng of 13,14-dihydro-15-keto-$PGF_{2\alpha}$ to all samples and PGE_2 standards (0.04–100 ng), the eicosanoids were extracted by the method of Green *et al.*[12]. Protein was precipitated from the tissue medium with 1.5 ml of cold (−20°C) acetone. Then 1.5 ml light petroleum was added, and after mixing, the phases were allowed to separate. After discarding the light petroleum layer, which contains neutral lipids and most of the acetone, another 1.5-ml portion of light petroleum was added, mixed, and removed. The aqueous phase was then acidified with formic acid to pH 3.5, and prostaglandins were extracted twice with 1 ml of ethyl acetate. The ethyl acetate layers were pooled and evaporated at 45°C under a gentle stream of nitrogen.

Derivative formation

The residue was dissolved in 1 ml acetonitrile–tetrahydrofuran (4:1) which contained 10 μg of panacyl bromide. The reaction was initiated with 1 μl triethylamine, and proceeded for 2 h at room temperature. The reaction mixture was applied to a pre-packed silica gel (Sep-Pak) column, which had been equilibrated with dichloromethane. Unreacted panacyl bromide was eluted from the column with 20 ml dichloromethane. The panacylprostaglandin esters then were eluted with 2 ml of acetonitrile–methanol (85:15). The eluate was evaporated under nitrogen, and the residue was dissolved in 0.2 ml of acetonitrile.

Chromatographic conditions

The mobile phase, dichloromethane–acetonitrile–methanol (90:9:1) had a flow-rate of 2.3 ml/min. The fluorescence of the panacylprostaglandin esters was measured at an excitation of 280 nm with an emission filter having a 400-nm cutoff. Samples and standards were analyzed in duplicate.

Validation of method

In order to verify the elution of PGE_2 at a retention time of 2.8 min, two experiments were performed. In the first experiment, parallel analyses of the panacyl derivative of 230 pg of [^{3}H]PGE_2 was performed. Peak fluorescent areas were integrated and compared with standards. Void volumes were continuously collected in liquid scintillation vials every 12 s for 4 min, and the fractions were evaporated under

a nitrogen stream. The percentage of radioactive PGE_2 recovered as a single peak was calculated. In the second experiment, the amount of PGE_2 generated by rat gastric mucosa was divided into equal aliquots. Rabbit anti-PGE_2 in a saline buffer (0.9% sodium chloride, 10 m*M* EDTA, 0.3% bovine γ-globulin, and 0.005% Triton X-100 in 50 m*M* sodium phosphate buffer at pH 6.8) was added to one tube, and incubated at room temperature for 1 h. Then the antigen–antibody complex was precipitated with cold (2°C) 16% polyethylene glycol 6000 in 50 m*M* sodium phosphate buffer at pH 6.8. To the control tube only the saline buffer and precipitating solution was added. Tubes were placed in an ice bath for 30 min, and then were centrifuged at 3000 *g* for 10 min. The supernatants were decanted and panacyl derivatives were formed. Peak areas were compared with PGE_2 standards.

RESULTS

The internal standard used in the assay for PGE_2 was 13,14-dihydro-15-keto-$PGF_{2\alpha}$. The peak area ratio of PGE_2 to the internal standard was compared with the relative concentrations. The overall recovery of the internal standard was similar to PGE_2, indicating that the extraction and derivatization of the eicosanoids was identical. Thus the peak area ratios could be used to calculate the amount of PGE_2 present in the sample.

The chromatographic method for PGE_2 was validated by the parallel quantitation of radioactive PGE_2, which showed a 60–75% recovery of the sample as a single peak at the retention time of PGE_2 (Fig. 1). The retention time for PGE_2 was

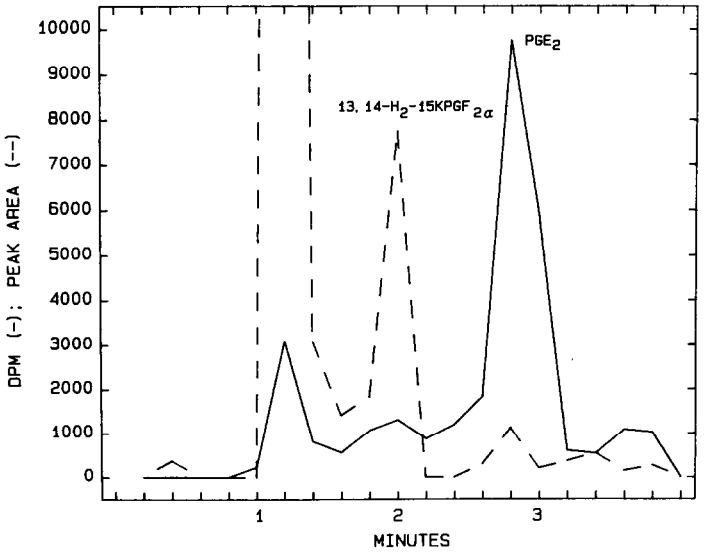

Fig. 1. Separation of [^{3}H]prostaglandin E_2 (PGE_2) and 13,14-dihydroxy-15-keto-prostaglandin $F_{2\alpha}$ (13,14-H_2-15KP$GF_{2\alpha}$) panacyl esters. Volumes were collected at 12-s intervals for 4 min. Radioactivity was measured in disintegrations per minute (DPM) (—), and the peak fluorescent areas (- - - -) were determined. 60–75% of the tritium was recovered in a single peak.

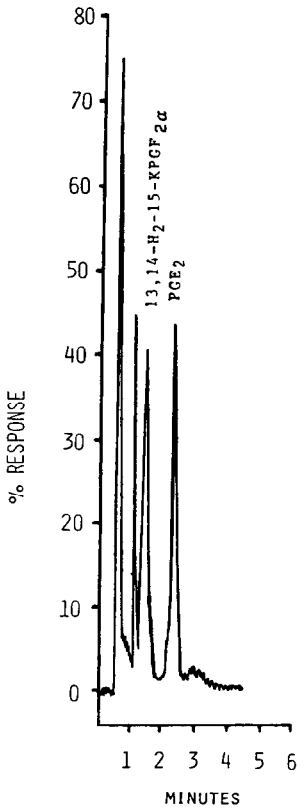

Fig. 2. Chromatogram of the panacyl esters of prostaglandin E_2 (PGE_2), extracted from rat gastric mu cosa, and 13,14-dihydroxy-15-keto-prostaglandin $F_{2\alpha}$ (13,14-H_2-15-$KPGF_{2\alpha}$), the internal standard.

2.8 min, and for the internal standard it was 2.1 min. Only a small amount of tritium migrated with the internal standard.

When anti-PGE_2 was incubated with prostaglandins, synthesized by rat gastric mucosa, the peak area corresponding to PGE_2 was reduced by 90%. Peak areas were 508 ± 115 (S.D.) mV · min with anti-PGE_2, and 7066 ± 540 (S.D.) with the buffer solution. There was no significant reduction of the internal standard peak; the peak areas were 9799 ± 1656 (S.D.) with the antibody, and 11 400 ± 1612 (S.D.) with the buffer. Both of these experiments indicate that the HPLC method was specific for PGE_2.

PGE_2 concentrations were determined from standard curves of 1–50 ng. In 22 rats, the biosynthesis of PGE_2 from normal gastric mucosa in 1 min was 90.6 ± 31.0 (S.D.) pg/min/mg tissue. A typical chromatogram shows the separation of panacyl prostaglandin esters in Fig. 2; the peak areas represent 50 ng of the internal standard and 78 ng of PGE_2. The sensitivity of the method was to 40 pg of PGE_2. In two pigs the amount of PGE_2 produced was 79.8 ± 39.8 (S.D.) pg/min/mg tissue.

DISCUSSION

We have described an HPLC method for measuring PGE_2 synthesis in biological specimens. A normal-phase column was used because the panacyl derivatives of both, the internal standard and PGE_2, could be eluted rapidly with good separation of the peaks from the extracted mucosa. This method should also be useful for PGE_2 measurements in other tissues.

Prostaglandins appear to be important metabolites in many tissues, and may determine the susceptibility of a cell for disease. However, many previous studies may have been inaccurate in the measurement of prostaglandins, and their conclusions may be in error. Thus the role of endogenous prostaglandins in the gastric mucosa is still unclear. HPLC methods based on the use of panacylprostaglandin esters can provide a sensitive and accurate measurement of the eicosanoids in biological specimens. The physiological importance of prostaglandins is currently being elucidated.

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