Journal of Chromatography, 282 (1983) 435–442
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMSYMP. 115

HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC SEPARATION AND ULTRAVIOLET DETECTION OF PROSTAGLANDINS, OXIDIZED BY PYRIDINIUM DICHROMATE

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SUMMARY

A rapid UV derivatization procedure has been developed which allows complete separation of the prostaglandins E_1, E_2, A_1, A_2, B_1, and B_2 in less than 12 min. on a C_{18} reversed-phase column with an acetonitrile 10 m*M* aq. formic acid gradient as the mobile phase.

With pyridinium dichromate in acetonitrile, the prostaglandins could be completely and selectively oxidized to their corresponding 15-oxo derivatives in 8 min. The oxidized prostaglandins were found to have UV maxima at 228 nm (PGE_1, PGE_2, PGA_1 and PGA_2) and 298 nm (PGB_1 and PGB_2). This means that prostaglandins which otherwise need to be chromatographed by isocratic elution at 192 nm, where the detector noise is considerable, now can be analyzed by gradient elution at 228 nm.

Prolonged reaction of PGE_1 and PGE_2 with pyridinium dichromate gave rise to a second product, probably by oxidation of the non-allylic 11-hydroxyl group, but within 8 min peak heights were less than 3% relative to the major product.

The smallest detectable amount has been determined to be 30–80 pmol.

INTRODUCTION

In the course of physiological and biochemical research on prostaglandins (PGs) several methods of determination have been developed[1]. As the number of PGs and structurally related compounds is large, analysis depends on the availability of highly specific detection methods and/or high-resolution chromatography. Among the most widely applied analytical techniques are bioassays[2], radioimmunoassays (RIA)[3], gas chromatography–mass spectrometry (GC–MS)[4,5] and high-performance liquid chromatography (HPLC)[6–11]. The major advantages and drawbacks of these methods have been discussed elsewhere[1,12].

Quantitative determination of PGs in biological samples by HPLC has so far been impeded by the fact that the smallest detectable amounts have been at the 20–90 pmol level[11,13], which is too high for most samples. As many PGs lack conjugated chromophores, and natural fluorescence is absent, various derivatives have been investigated, which include fluorescent coumarins[9,10] and UV-absorbing phenacyl es-

0021-9673/83/$03.00

ters[6], *p*-nitro- and pentafluorobenzyloximes[7]. Reversed-phase HPLC followed by UV detection at 192–194 nm has been carried out on non-derivatized PGs with detection limits of 60–90 pmol[8,11]. Although the $\Delta^{13,14}$-double bond offers high absorptivity at this wavelength ($\varepsilon > 15{,}000$)[11], the detector noise level is considerable, owing to the solvent absorption, which prevents the application of gradient elution. A need for simple and rapid derivatization procedures therefore still seems to persist.

An analytical technique which includes oxidative formation of 15-oxo-PGs has been described[14]. The method makes use of a NAD^+-coupled enzymatic oxidation with subsequent UV detection of a transient absorption, appearing at 500 nm after treatment with alkali[15], or fluorimetric detection of the produced NADH[14]. A problem connected with the latter method seems to be the non-specific formation of background NADH.

Our report introduces a pre-column derivatization procedure, based on the oxidation of the 15-hydroxy group, not by enzymes, but by pyridinium dichromate (PDC) in a non-aqueous medium (acetonitrile). The oxidation creates an enone chromophore, which permits UV detection at higher wavelengths and, thus gradient elution is made possible. Oxidation of various compounds by PDC has been described by Corey and Schmidt[16], but contrary to our findings, this work excluded acetonitrile as solvent because of reagent instability.

EXPERIMENTAL

Apparatus

The HPLC equipment consisted of two Waters Assoc. Model 6000 A pumps, a Waters U6K valve/loop injector, a Waters Model 660 gradient programmer and a Perkin-Elmer LC-55 UV spectrophotometric detector. A new Perkin-Elmer LC-85 spectrophotometer was included in detector noise measurements for comparison.

Chemicals

All PGs were obtained from Sigma (St. Louis, MO, U.S.A.), PDC (98%) from Aldrich (Milwaukee, WI, U.S.A.), formic and acetic acid (p.a.) from E. Merck (Darmstadt, F.R.G.), and ethanol (absolute) from Vinmonopolet (Oslo, Norway). Acetonitrile, methanol, dichloromethane, and 1,2-dichloroethane were HPLC grade from Rathburn (Walkerburn, U.K.). Deionized water was distilled once. The solvents were filtered through a 0.45-μm Millipore filter. PG standards were dissolved in absolute ethanol ($3 \cdot 10^{-4}$ *M*) prior to use and stored at -20°C.

Columns and mobile phases

The separations were carried out on two sets of two Brownlee MPLC cartridge columns from Brownlee Labs. (Santa Clara, CA, U.S.A.), 100 × 4.6 mm each, connected in series. Guard columns were dry-packed in our laboratory. The two sets were:

C_{18}: Guard column: 50 × 4.6 mm I.D., 40-μm pellicular C_{18} silica (Supelco). Pair of analytical columns: 200 × 4.6 mm I.D., 5-μm porous C_{18} silica. Mobile phase: acetonitrile–10 m*M* aq. formic acid.

Silica: Guard column: 60 × 4.6 mm I.D., 30–38-μm pellicular silica (Whatman). Pair of analytical columns: 200 × 4.6 mm I.D., 5-μm porous silica. Mobile phase: dichloromethane–methanol–acetic acid (995:6.0:1.1).

The silica guard column served to remove excess PDC and reduced chromium salts from the injected oxidation mixtures. Salts are strongly adsorbed on the silica and would, in the absence of a guard column, accumulate at the analytical column inlet.

Oxidations with PDC

PDC solutions were prepared by stirring 0.10 g of PDC with 50 ml of acetonitrile for 60 min at ambient temperature. The reagent solution, which was *ca.* 5.0 m*M* (1.9 mg/ml), was used after removal of excess solid PDC by centrifugation.

Prior to oxidation, the PG samples were dried under a nitrogen stream in 1-ml glass vials fitted with PTFE valve caps. The reagent solution (10 equiv. of PDC) was added to the samples through the PTFE valve by use of a volumetric syringe and mixed in a Fision Scientific Whirlimixer (60 sec). After 8 min at ambient temperature, the oxidation was terminated by adding 10% (v/v) water.

Separations on the C_{18} column were accomplished by injecting the reaction mixture directly, but separations on silica had to be preceded by evaporation of aq. acetonitrile, addition of a less polar solvent (1,2-dichloroethane) and removal of precipitated PDC by centrifugation. Without this solvent change, column efficiency tended to be low.

No loss of reactivity of the PDC–acetonitrile solution was observed within 2 days, if it was stored at 5°C.

Noise measurements

Detector noise levels at different wavelengths were measured in the C_{18} column system by recording baseline sequences of *ca.* 2-min. duration at normal solvent flow (1.5 ml/min, isocratic at 40% acetonitrile). Attenuation was chosen so as to give baseline bandwidths of 5–10 mm.

Detection of 15-oxo-PGB$_1$ and -PGB$_2$

In order to detect 15-oxo-PGB* together with 15-oxo-PGA and/or -PGE in a single run, the wavelength was manually changed from 228 to 298 nm during the elution. When the wavelength was changed between peaks eluted closely together, this had to be done rapidly in order not to miss the recording of the next peak. On the Perkin-Elmer LC-55 this requirement was met by precalibrating the new baseline level, which could then be momentarily established after a rapid manual wavelength change by simply pushing a button. In contrast, the automated wavelength-change option of the Perkin-Elmer LC-85 performed too slowly to give satisfactory recordings.

RESULTS AND DISCUSSION

Oxidation of PGE, PGA and PGB

When the A and B PGs were individually oxidized, separation of each reaction mixture on C_{18} and silica columns revealed a single reaction product in each case. With PGE a second, minor product appeared, but with 10 equiv. of PDC and reaction

* PG without subscript denotes PG_1 and PG_2 (PGB = PGB_1 and PGB_2).

TABLE I

UV ABSORPTION MAXIMA OF PROSTAGLANDINS IN A PDC–ACETONITRILE REACTION MIXTURE, MEASURED BY STOP-FLOW SCANS OF CHROMATOGRAPHIC PEAKS

C_{18} column, acetonitrile 10 m*M* aq. formic acid.

Prostaglandin	λ_{max} *15-hydroxy-PG (nm)*	λ_{max} *15-oxo-PG (nm)*
E_1, E_2	<195	228
A_1, A_2	217	228
B_1, B_2	278	298

times less than 8 min, peak heights were less than 3% relative to the major product.

By use of stop-flow UV scans of the chromatographic peaks the absorption maxima of the 15-oxo-PGs were found to be shifted towards longer wavelengths compared to the parent compound (Table I).

Absorption maxima for non-oxidized PGE, PGA, PGB and 15-oxo-PGE_2 were in accordance with the measurements of Terragno *et al.*[11], except that the detector noise level below 195 nm prevented confirmation of the reported common PG λ_{max} at *ca.* 192 nm.

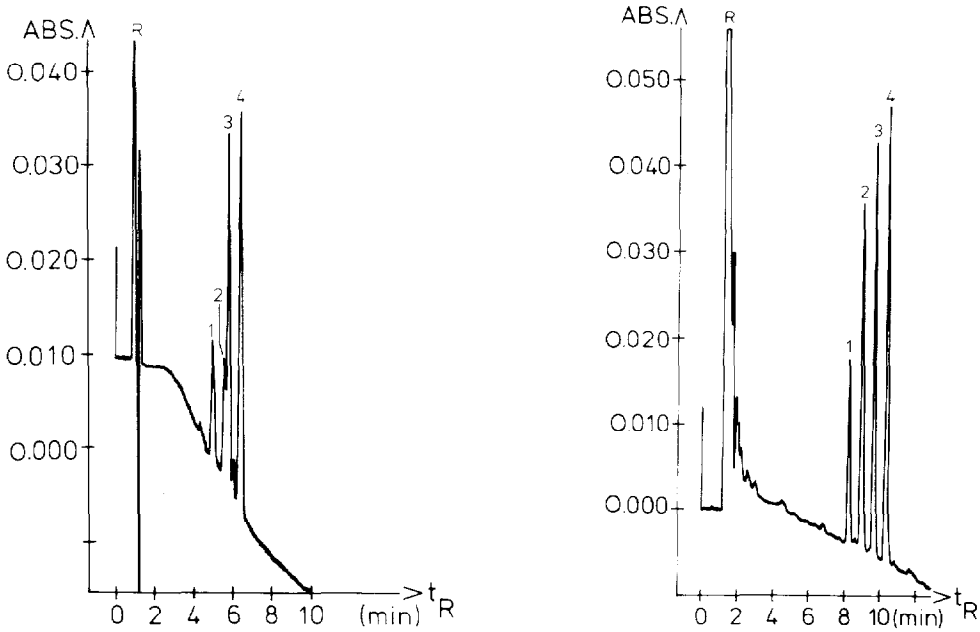

Fig. 1. Separation of PGE_2(1), PGE_1(2), 15-oxo-PGE_2(3) and 15-oxo-PGE_1(4) on a C_{18} column (Brownlee 5-μm RP-18, 200 × 4.6 mm I.D. with a 40-μm pellicular C_{18} guard column (Supelco), 50 × 4.6 mm I.D.) with acetonitrile–aq. 10 m*M* formic acid linearly from (40:60) to (60:40) in 10 min at 1.5 ml/min. Detection at 210 nm; injected sample, 5 μl, each oxidized PG corresponding to 2.2 nmol non-oxidized PG, 2.2 nmol non-oxidized PGE_1 and PGE_2 added. R = reagent (PDC).

Fig. 2. Separation of PGA_2(1), PGA_1(2), 15-oxo-PGA_2(3) and 15-oxo-PGA_1(4) on a C_{18} column. Conditions as in Fig. 1, except: detection at 228 nm; injected sample, 5 μl, each oxidized PG corresponding to 3.0 nmol non-oxidized PG, 5.8 nmol PGA_1 and 3.0 nmol PGA_2 added. R = reagent (PDC).

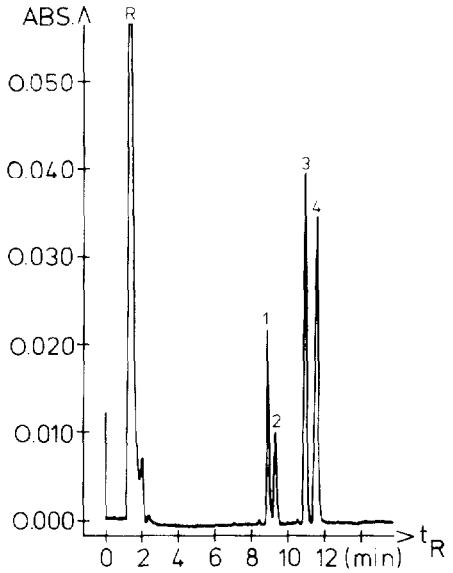

Fig. 3. Separation of PGB_2 (1), PGB_1 (2), 15-oxo-PGB_2 (3) and 15-oxo-PGB_1 (4) on a C_{18} column. Conditions as in Fig. 1 except: detection at 298 nm; injected sample, 5 μl, each oxidized PG corresponding to 2.3 nmol non-oxidized PG, 1.0 nmol PGB_1 and 2.3 nmol PGB_2 added. R = reagent (PDC).

Oxidation of the 15-hydroxyl group to a ketone was expected to reduce the polarity of the PGs. This was confirmed by the chromatographic data, showing the appearance of earlier eluted products on silica and later eluted products on C-18. Further characterization of the 15-oxo-PGs will be published elsewhere[17]. Separa-

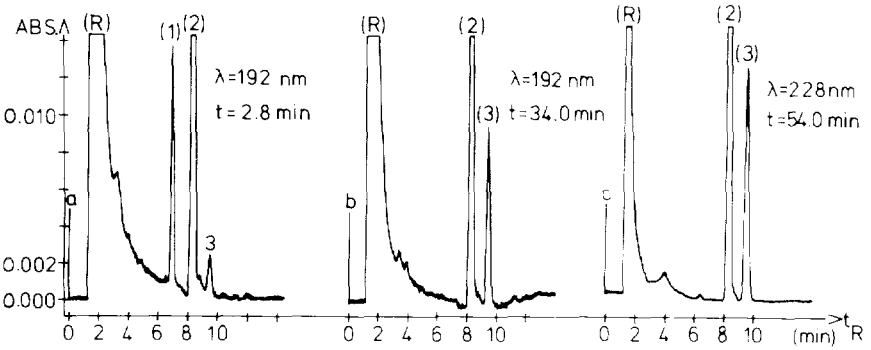

Fig. 4. PGE_2 (1), 15-oxo-PGE_2 (2) and the assumed 11,15-dioxo-PGE_2 (3) at different reaction times during oxidation with PDC–acetonitrile. Conditions as in Fig. 1, except: detection at wavelength as indicated; mobile phase, acetonitrile–10 m*M* aq. formic acid (37.5:62.5); injected sample, 5 μl, corresponding to 3.2 nmol non-oxidized PGE_2.

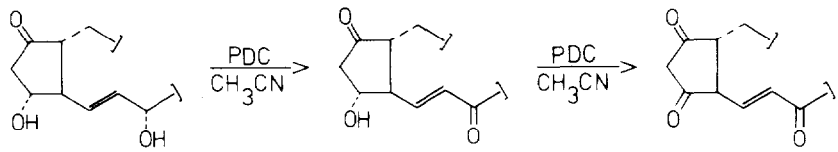

Fig. 5. Suggested formation of two products from oxidations of PGE with PDC-acetonitrile.

tions of oxidation mixtures of the three PG pairs PGE, PGA and PGB on the C_{18} column are illustrated in Figs. 1–3.

The formation of a second oxidation product from PGE_2 is illustrated in Fig. 4. A possible explanation for this could be the non-allylic oxidation of 15-oxo-PGE_2, leading to 11,15-dioxo-PGE_2, as shown in Fig. 5. The suggested formation of 11,15-dioxo-PGE is in accordance with the allylic selectivity in the PDC oxidation of alcohols described by Corey and Schmidt[16], as the second product is formed at a considerably lower rate than 15-oxo-PGE.

Separation on the C_{18} column

The separation of the six PGs 15-oxo-E_1, -E_2, -A_1, -A_2, -B_1, and -B_2 on the

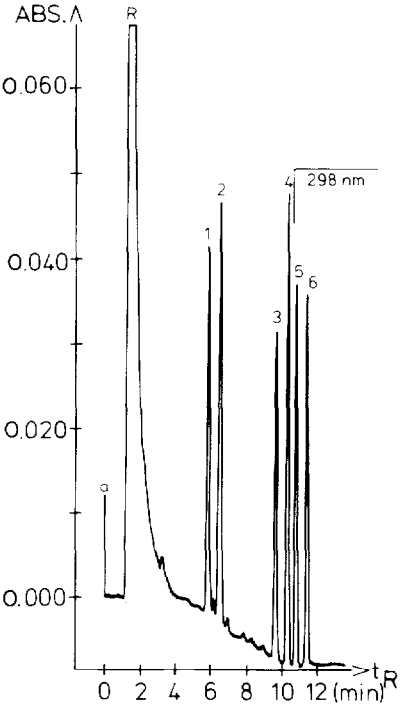

Fig. 6. Separation of 15-oxo-PGE_2(1), -PGE_1(2), -PGA_2(3), -PGA_1(4), -PGB_2(5) and -PGB_1(6) on a C_{18} column. Conditions as in Fig. 1, except: detection at 228 nm, changed to 298 nm as indicated; injected sample, 5 μl, corresponding to 2.7 nmol PGB_1 and PGB_2, 3.2 nmol PGA_2, 2.2 nmol PGA_2 and 2.4 nmol PGE_1 and PGE_2. R = reagent (PDC).

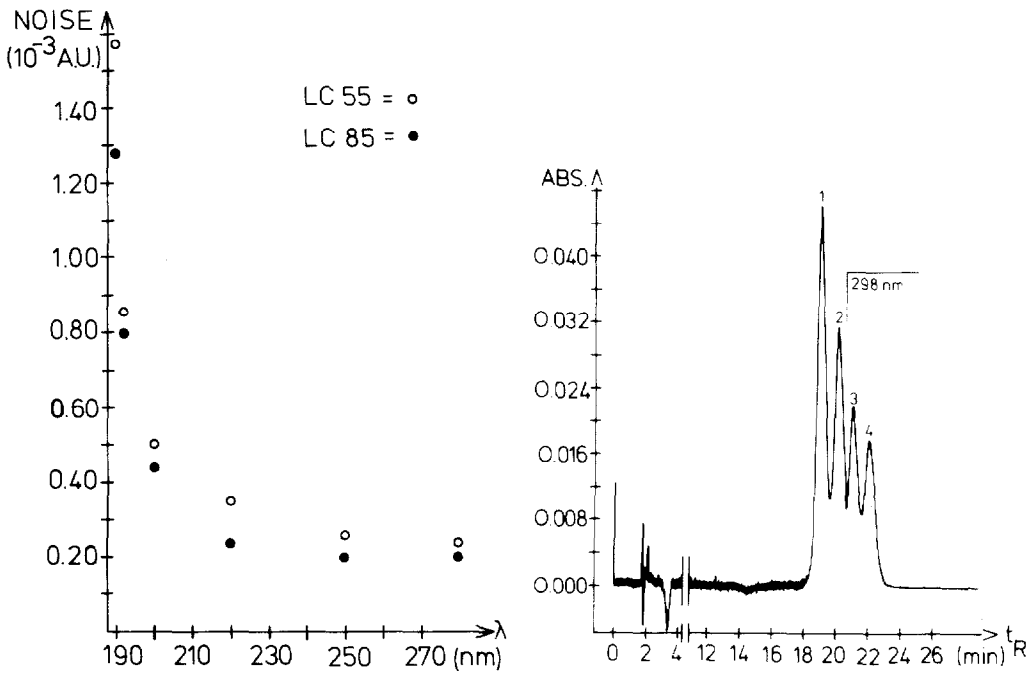

Fig. 7. Noise level at different wavelengths of two UV spectrophotometers, Perkin-Elmer LC-55 ($^2H_2$ lamp used *ca.* 1000 h) and LC-85 (new) with acetonitrile 10 m*M* aq. formic acid (40:60) at 1.5 ml/min.

Fig. 8. Separation of 15-oxo-PGA_1(1), -PGA_2(2), -PGB_1(3) and -PGB_2(4) on silica (Brownlee 5-μm Spheri-5, 200 × 4.6 mm I.D. with a 30–38-μm pellicular silica guard column (Whatman, 60 × 4.6 mm I.D.) with dichloromethane–methanol–acetic acid (995:6.0:1.1) at 1.5 ml/min. Detection, at 228 nm, changed to 298 nm as indicated; injected sample, 5 μl, corresponding to 3.0 nmol PGB_1 and PGB_2, 6.3 nmol PGA_1 and 4.5 nmol PGA_2.

C_{18} column with gradient elution is shown in Fig. 6. Within 12 min all six components were completely separated. By increasing the steepness of the acetonitrile gradient from 2.0%/min to 3.3%/min, the separation was completed in 10 min with a minor loss in resolution of 15-oxo-PGA_1 and -PGB_2.

Low-wavelength detection of non-oxidized PGs gave low signal-to-noise ratios, due to detector noise. Measured noise levels at 192 nm were 2.7 times higher than at 228 nm (Fig. 7).

The smallest detectable amount in this chromatographic system was found to be 30–80 pmol.

Separation on the silica column

In order to compare the separation obtained by the reversed-phase chromatography with an adsorption system, separation of 15-oxo-PGA_1, -PGA_2, -PGB_1, and -PGB_2 was carried out on silica. It is evident from Fig. 8 that the silica system is inferior to the C_{18} column in separating these compounds. The non-ideal behaviour on silica is expected to be due to interactions with the carboxyl group.

CONCLUSIONS

Oxidation of PGs with PDC in acetonitrile represents a rapid one-step derivatization procedure under mild reaction conditions, suitable for UV detection in HPLC. Gradient elution with detection at 228 nm (298 nm for PGB) produced an excellent separation of the six 15-oxo-PGs. As capacity factors for different PGs on the C_{18} column show a considerable difference between the PGE eluted early and the PGA and PGB eluted late[11], gradient elution is indicated to lower the detection limits and reduce the analysis time.

The smallest detectable amount obtained by the present method is comparable to that in other methods, but it is still too high for most biological samples. In order to make this method applicable to determination of biological PG levels, further development is in process. This includes the use of microbore columns as well as a cadmium UV emission source (with energy maximum at 228.6 nm), which is expected to improve signal-to-noise ratios. Application to other PGs, including $PGF_{1\alpha}$ and $PGF_{2\alpha}$, is part of this effort.

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