Journal of Chromatography, 349 (1985) 431–438
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMSYMP. 650

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

OPTIMIZATION AND APPLICATIONS

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SUMMARY

Determination of prostaglandins by UV detection after chromatographic separation requires derivatization if detection at low wavelengths (190–195 nm) is to be avoided. The present study includes optimization of a previously reported one-step UV derivatization procedure in which prostaglandins are oxidized to their corresponding 15-oxo derivatives with pyridinium dichromate in acetonitrile. A dection limit for PGE_1 and PGE_2 of 0.14 pmol was obtained by using a photometric detector equipped with a cadmium UV emission source. As the emission energy maximum of cadmium coincides with the UV absorption maxima of the prostaglandin enone chromophores, signal-to-noise ratios were improved relative to conventional deuterium lamp spectrophotometric detectors. Further reduction of detection limits was obtained by using C_{18} narrow- and micro-bore columns. 15-Oxo-$PGF_{2\alpha}$, -$PGF_{1\alpha}$, -PGE_2 and -PGE_1 were separated by isocratic elution on C_{18} columns with acetonitrile–aqueous 10 m*M* phosphoric acid. Signal-to-noise ratios resulting from columns of different internal diameter as well as flow-cells of different volumes were compared.

INTRODUCTION

The continued efforts to elucidate the physiological and biochemical significance of prostaglandins (PGs) have led to the application of numerous analytical techniques. Methods for determination of PGs after separation by high-performance liquid chromatography (HPLC) mainly involve pre-column derivatization with UV[1–5], fluorescence[6–10] or electrochemical [11,12] detection. Detection limits of 0.14 pmol have been obtained with some fluorescent derivatives[8].

The isolated double bonds of non-derivatized PGs can be detected by UV absorbance at low wavelengths (190 nm)[13,14]. The use of narrow-bore columns can lower detection limits for non-derivatized PGs to 0.70 pmol[14].

In a previous study we reported a rapid one-step UV derivatization procedure

0021-9673/85/$03.30

in which PGs A, B and E are oxidized to the corresponding 15-oxo-derivatives with pyridinium dichromate (PDC) in acetonitrile[15].

This paper reports the optimization of this procedure by means of narrow- and micro-bore C_{18} columns, connected to a photometric detector equipped with a cadmium emission source. The emission energy maximum of cadmium (228.6 nm) closely matches the UV absorption maximum of the 15-oxo-PG enone chromophore. Preliminary results from the oxidation of $PGF_{1\alpha}$ and $PGF_{2\alpha}$ are also included.

EXPERIMENTAL

Apparatus

The HPLC equipment consisted of a Waters Model 590 pump, a Rheodyne 7520 fixed-loop (1 μl) injector and a Waters EWM Cd-lamp photometric detector. The electronic unit of a Waters Model 440 detector served as signal amplifier for the Cd-lamp assembly. For detection at 190 nm and recording of spectral scans, a Hewlett-Packard 1040 A diode array detector was used.

Chemicals

All PGs were obtained from Sigma (St. Louis, MO, U.S.A.), PDC (98%) from Aldrich (Milwaukee, WI, U.S.A.) and phosphoric acid (p.a.) from Merck (Darmstadt, F.R.G.). Acetonitrile and ethyl acetate were HPLC grade from Rathburn (Walkerburn, U.K.). Deionized water was distilled once. PG standards were dissolved in acetonitrile ($2.8 \cdot 10^{-4}$ *M*) and stored at −20°C.

Columns and mobile phases

All separations were carried out on C_{18} columns with acetonitrile–aqueous 10 m*M* phosphoric acid (pH 2.7) as mobile phase. Four different column dimensions were investigated: a 200 × 3.0 mm I.D. glass cartridge column and a 250 × 1.3 mm I.D. column, both 8 μm porous C_{18} silica from Chrompack (Middelburg, The Netherlands), a 220 × 2.1 mm I.D. column and a 200 × 4.6 mm I.D. column, both cartridges with 5 μm porous C_{18} silica from Brownlee (Santa Clara, CA, U.S.A.). The first and fourth columns consisted of two 100-mm cartridges, connected in series.

Derivatization with PDC

The prostaglandins were oxidized with PDC in 10–20 μl of acetonitrile, as previously described[15], except that the oxidations were terminated by adding 90–180 μl of water. In this way, the reaction mixture could be directly injected without further dilution. To avoid PDC interferences at PG levels of less than 50 ng, extractions with ethyl acetate were performed by thoroughly mixing the two phases in the reaction vial with a syringe followed by centrifugation at 2000 *g* for 3 min to obtain complete phase separation.

RESULTS AND DISCUSSION

*Oxidation of PGE** and PGF

As previously described[15], oxidation of PGE for 8 min with an excess of PDC

* PG without a subscript denotes PG_1 and PG_2 (PGE = PGE_1 and PGE_2).

(> 10 eq.) resulted in the formation of 15-oxo-PGE in high yields. As shown in Fig. 1, oxidation of PGF under similar conditions was less selective, owing to the appearance of secondary oxidation products. Additional oxidation of non-allylic hydroxyl groups was indicated by the higher capacity factors (*i.e.* lower polarity), by the presence of absorption maxima between 226 nm and 236 nm typical for an enone chromophore, and by the increase of the secondary peaks and decrease of the primary peak following prolonged reaction and/or increased PDC concentration. The earliest-eluted secondary compound emerged together with 15-oxo-PGE. In addition, the spectroscopic homogeneity of the chromatographic peak, measured by UV absorption ratio scans at four different wavelength sets by the diode array detector, indicated that the two compounds were identical.

Until the formation of secondary oxidation compounds from PGF can be controlled, these reactions limit the usefulness of PDC oxidations for determination of PGF.

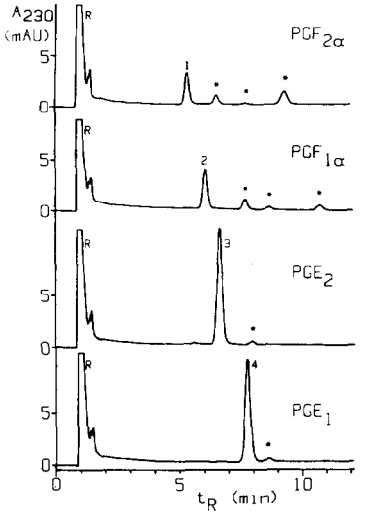

Fig. 1. Oxidation of $PGF_{2\alpha}$, $PGF_{1\alpha}$, PGE_2 and PGE_1 with PDC. Separation of reaction mixtures in a C_{18} column (Brownlee 5 μm C_{18}, 220 × 2.1 mm I.D.) with acetonitrile–aq. 10 m*M* phosphoric acid (36:64) at 0.40 ml/min after 10 min oxidation with 20 eq. PDC. Injected sample: 10 μl, corresponding to 50 ng non-oxidized PG in acetonitrile–water (1:9). Detector: UV at 230 nm. Peaks: 1, 2, 3 and 4: 15-oxo-$PGF_{2\alpha}$, -$PGF_{1\alpha}$, -PGE_2 and -PGE_1, respectively; * = secondary oxidation products; R = reagent (PDC).

Separations of oxidized PGE and PGF

The elution order of the four PGs 15-oxo-$F_{2\alpha}$, -$F_{1\alpha}$, -E_2 and -E_1 by reversed-phase chromatography followed simple polarity considerations, assuming that polarity is primarily governed by the number of hydroxyl groups and secondly by the number of double bonds. This is in accordance with our previous report on separations of oxidized and non-oxidized PGE, PGA, and PGB[15], as well as the observations on several PGs by Terragno *et al.*[13]. The isocratic separation of the reaction mixture of the two PG pairs PGF and PGE is illustrated in Fig. 2. The relationship

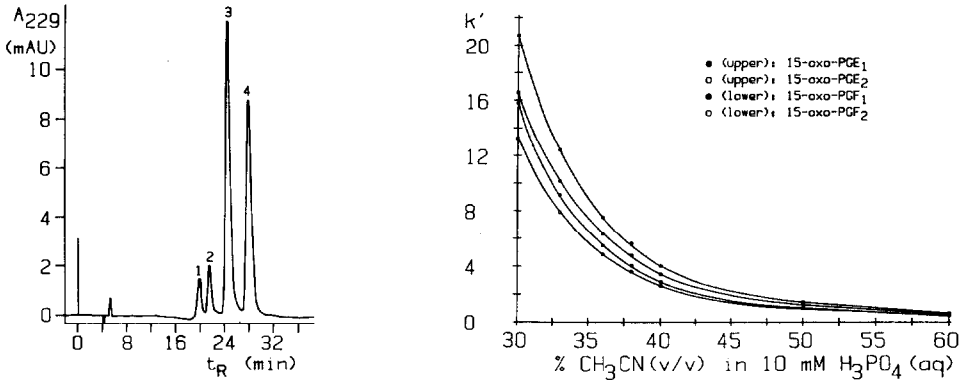

Fig. 2. Separation of 15-oxo-$PGF_{2\alpha}$, -$PGF_{1\alpha}$, -PGE_2 and -PGE_1 in a micro-bore C_{18} column (Chrompack 8 μm C_{18}, 250 × 1.3 mm I.D.) with acetonitrile–aq. 10 m*M* phosphoric acid (38:62) at 60 μl/min. Oxidation: 10 min/10 eq., extracted by ethyl acetate. Injected sample: 1 μl, corresponding to 25 ng PG in acetonitrile–water (1:9). Detector: UV at 229 nm (Cd-lamp) with 16-μl flow-cell. Peaks: 1, 2, 3 and 4: 15-oxo-$PGF_{2\alpha}$, -$PGF_{1\alpha}$, -PGE_2 and -PGE_1, respectively.

Fig. 3. Relationship between capacity factor (k') of different 15-oxo-PGs and content of acetonitrile in the mobile phase. Column: Chrompack 250 × 1.3 mm I.D., 8-μm porous C_{18} silica at 60 μl/min.

between capacity factor (k') and acetonitrile content of the mobile phase is shown in Fig. 3.

Purification of reaction mixtures

At low PG concentrations, purification of the reaction mixture prior to injection may be advantageous, since the reagent can interfere with PG detection in two ways. Excess PDC eluted in the void volume will tail badly and delay baseline stability. Pyridine, which inevitably is present in the reagent mixture, can also interfere with PG peaks. As illustrated in Fig. 4, both interferences can be reduced to a minimum by a single extraction of the dilute reaction mixture with an equal volume of

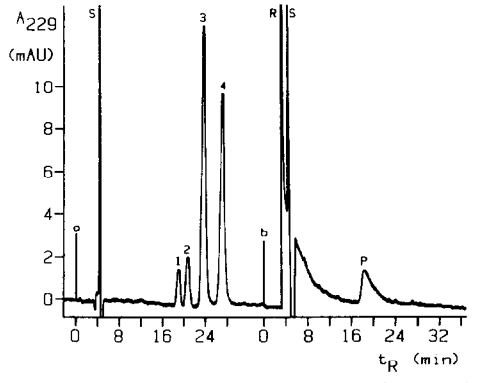

Fig. 4. Extraction of PG/PDC reaction mixtures with ethyl acetate. (a) Organic phase, (b) aqueous phase, both reconstituted after removal of ethyl acetate under nitrogen. Conditions as in Fig. 2, except detector with 2-μl flow-cell. Peaks: 1, 2, 3 and 4: 15-oxo-$PGF_{2\alpha}$, -$PGF_{1\alpha}$, -PGE_2 and -PGE_1, respectively; R = reagent; S = solvent; P = pyridine.

ethyl acetate. PDC remained in the aqueous phase, whereas pyridine was removed in part by the extraction and in part by evaporation under nitrogen. Extractions with ethyl acetate can be made of aqueous solutions with an upper limit of 80% acetonitrile (v/v), where phase separation disappears. However, in order to optimize the removal of PDC, extractions are best performed on solutions diluted to 20% acetonitrile (v/v) or less with aqueous 10m*M* phosphoric acid, resulting in 99% recovery of 15-oxo-PGE with only 1% PDC remaining in the organic phase. Recoveries of 15-oxo-PGE and PDC from solutions of various acetonitrile contents are given in Table I.

TABLE I

RECOVERIES OF 15-OXO-PGE AND PDC FROM THE ORGANIC PHASE

Ethyl acetate extraction of acetonitrile solutions diluted with various amounts of aq. 10 m*M* phosphoric acid. Samples contained 5.7 μg of PDC and 1.0 μg of each 15-oxo-PGE in 100 μl of solution, and were extracted with equal volumes of ethyl acetate. Recoveries were measured with reference to peak heights.

Acetonitrile (v/v, %)	*PG recovery (%)*	*PDC recovery (%)*
10	99	1
20	99	1
30	99	2
50	98	5
70	97	24

Optimization of detection limits

In order to reduce the detection limit, two measures were taken. Firstly, a cadmium lamp photometer was used to detect the oxidized PGs. As the emission energy maximum of the cadmium lamp (228.6 nm) coincides with the enone chromophore absorption of oxidized PGs (λ_{max} = 228–234 nm), an increased signal-to-noise (S/N) ratio was expected. Secondly, columns with reduced I.D. were employed to obtain increased concentration of eluted components. As described in Table II, maximum peak heights were observed with the 1.3-mm I.D. column, connected to the micro-bore flow-cell. However, as the noise level of the conventional-bore flow-

TABLE II

EFFECT OF REDUCED-DIAMETER COLUMNS ON PEAK HEIGHTS OF 15-OXO-$PGF_{2\alpha}$, -$PGF_{1\alpha}$, -PGE_2, -PGE_1

Mobile phase, acetonitrile–aq. 10 m*M* phosphoric acid (38:62); linear velocity: 0.10 cm/s. Noise measured as time-averaged peak-to-peak values (10 min). Columns: all porous C_{18} silica; (1) and (3) Chrompack 8 μm, (2) and (4) Brownlee 5 μm.

Column		*k'_{min}–k'_{max} ($PGF_{2\alpha}$–PGE_1)*	*Flow-cell (μl)*	*Mean relative peak height*	*Noise (10^{-5} a.u.f.s.)*	*Relative S/N*
No.	*Dimensions (mm)*					
1	250 × 1.3	2.9–5.2	2	20	20	1.0
1	250 × 1.3	2.9–5.2	16	17	3.0	5.7
2	200 × 2.1	2.3–3.5	16	15	3.0	5.0
3	200 × 3.0	2.7–4.0	16	4	3.0	1.3
4	200 × 4.6	2.5–3.7	16	3	3.0	1.0

cell was 6.7 times lower, the best S/N ratios were obtained when this cell was used. Thus, to our slight surprice, an S/N gain factor of 5.3 was measured when we operated the 1.3-mm I.D. micro-bore column with the conventional-bore flow-cell. The increased noise level of the micro-bore cell is in part caused by the reduced light energy reaching the photodiode. Both pathlengths were identical (10 mm), but the cross-sectional areas differed by a factor of 4.7. The remaining noise difference is suggested to be caused by geometrical differences between the two cells.

Fig. 5 illustrates an external calibration for PGE_1 and PGE_2, obtained by oxidizing 1 μg of each PGE with 10 eq. PDC for 10 min and injecting diluted samples. Good linearity was observed for both components from 0.09 pmol to 14 pmol. The detection limit, defined as S/N = 2, was determined to be 0.14 pmol at a k' of *ca.* 3.0.

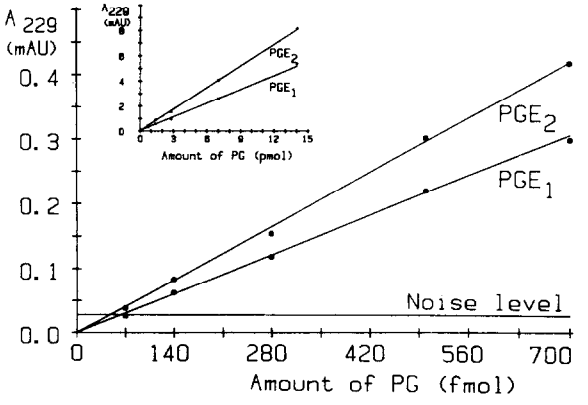

Fig. 5. External calibration of oxidized standard PGE_2 and PGE_1. Noise level as indicated at $3 \cdot 10^{-5}$ a.u.f.s. peak-to-peak. Chromatographic conditions as in Fig. 2. Injected volume, 1 μl.

Spectral considerations

UV absorption scans of non-oxidized PGE and PGF and their corresponding 15-oxo derivatives, obtained with the diode array detector, are illustrated in Fig. 6. The two pairs of 15-oxo-PGE and 15-oxo-PGF exhibit absorption maxima at 228 and 234 nm, respectively, consistent with the enone chromophore[16].

No absorption maximum was observed for native PGs at wavelengths higher than 190 nm, contrary to earlier observations[13]. When the spectral scans of 15-oxo-PG_1 and 15-oxo-PG_2 are compared, it is also evident that the isolated $\Delta^{5,6}$-*cis*-double bond contributes considerably to the absorption at 190 nm. 15-Oxo-PG_1 will absorb only weakly at this wavelength owing to the bathochromic shift of the π–π^* transition of the conjugated enone. This is in accordance with our observation that the relative chromatographic response, of the diene PGs were 1.8 times the response of monoene PGs at 190 nm when equal amounts of PGF_2/PGF_1 and PGE_2/PGE_1 were compared at equal capacity factors.

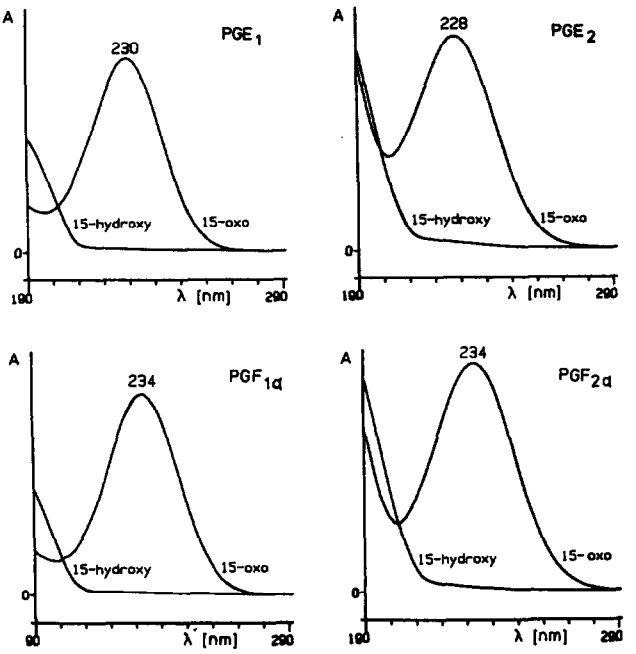

Fig. 6. UV scans of chromatographic peaks of oxidized and non-oxidized PGs.

CONCLUSIONS

The one-step UV derivatization of PGs by PDC in acetonitrile, coupled with the Cd-lamp photometric detector and micro-bore columns, affords detection limits for PGE_2 and PGE_1 comparable with those achieved with the most sensitive fluorescence methods reported. A detection limit for standard PGE of 0.14 pmol (50 pg) is promising with respect to further application on biological samples.

Further investigations are necessary to control the formation of secondary oxidation products from PGF.

A single ethyl acetate extraction conveniently purifies the reaction mixture with high yields (>95%) of 15-oxo-PGs.

With 200–250 mm micro-bore columns the best S/N ratios were obtained with a detector equipped with a conventional-size flow-cell. Thus, even with a standard 10-mm light-path, micro-bore flow-cells may increase detection limits drastically, contrary to presumptions.

REFERENCES

1 F. A. Fitzpatrick, *Anal. Chem.*, 48 (1976) 499.
2 F. A. Fitzpatrick, M. A. Wynalda and D. G. Kaiser, *Anal. Chem.*, 49 (1977) 1032.
3 W. Morozowich and S. L. Douglas, *Prostaglandins*, 10 (1975) 19.
4 M. V. Merritt and G. E. Bronson, *Anal. Chem.*, 48 (1976) 1851.
5 M. V. Merritt and G. E. Bronson, *Anal. Chem.*, 49 (1977) 392.

6 H. Tsuchiya, T. Hayashi, H. Naruse and N. Takagi, *J. Chromatogr.*, 231 (1982) 247.
7 J. Turk, S. J. Weiss, J. E. Davies and P. Needleman, *Prostaglandins*, 16 (1978) 291.
8 W. D. Watkins and M. B. Peterson, *Anal. Biochem.*, 125 (1982) 30.
9 M. Hatsumi, S. Kimata and K. Hirosawa, *J. Chromatogr.*, 253 (1982) 271.
10 K. Yamadu, M. Onodera, and Y. Aizawa, *J. Pharm. Methods*, 9 (1983) 93.
11 W. D. King and P. T. Kissinger, *Clin. Chem.*, 26 (1980) 1484.
12 S. Ikenoya, O. Hiroshima, M. Ohmae and K. Kawabe, *Chem. Pharm. Bull.*, 28 (1980) 2941.
13 A. Terragno, R. Rydzik and N. A. Terragno, *Prostaglandins*, 21 (1981) 101.
14 R. Rydzik, A. Terragno, R. Tackett, *J. Chromatogr.*, 308 (1984) 31.
15 J. Døhl and T. Greibrokk, *J. Chromatogr.*, 282 (1984) 435.
16 A. I. Scott, *Interpretation of the Ultraviolet Spectra of Natural Products*, Pergamon, Oxford, New York, 1964.