Journal of Chromatography, 253 (1982) 271–275
Elsevier Scientific Publishing Company, Amsterdam — Printed in The Netherlands

CHROM. 15,297

Note

9-Anthryldiazomethane derivatives of prostaglandins for high-performance liquid chromatographic analysis

MAKIKO HATSUMI*, SHIN-ICHI KIMATA and KOSHICHIRO HIROSAWA
The Heart Institute Japan, Tokyo Women's Medical College, 10 Ichigaya-kawada-cho, Shinjuku-ku, Tokyo, 162 (Japan)
(Received August 17th, 1982)

Prostaglandins (PGs) have several physiological actions at trace levels. Multiple micro-analysis of PGs is required in clinical and experimental medicine. In order to analyse such PGs, high-performance liquid chromatography (HPLC) is a useful method. Though there have been many papers[1–10] on the separation and determination of PGs by HPLC, studies in which 6-keto-$PGF_{1\alpha}$, thromboxane B_2 (TXB_2) and other PGs are simultaneously determined have been reported only by Turk *et al.*[5], Inayama *et al.*[8] and Terragno *et al.*[10]. None of these methods has sufficient sensitivity for the quantitative determination of PGs in biological materials.

For the analysis of PGs at higher sensitivities, we attempted to determine the PGs labelled with a fluorescent reagent, 9-anthryldiazomethane[11] (ADAM) (Fig. 1), which is highly reactive towards carboxyl groups without the need for a catalyst and which has been used for the determination of fatty acids at high sensitivity. The present paper describes the HPLC separation and quantitative determination of 6-keto-$PGF_{1\alpha}$, PGD_2, PGE_1, PGE_2 and $PGF_{2\alpha}$ esterified with ADAM. Since ADAM contains many impurities which interfere with the HPLC determination, the purification of PGs esterified with ADAM (PG–ADAMs) was attempted using gel-permeation chromatography (GPC).

EXPERIMENTAL

Materials

6-Keto-$PGF_{1\alpha}$, PGD_2, PGE_1, PGE_2 and $PGF_{2\alpha}$ were provided by the Ono Pharmaceutical Co. (Osaka, Japan). A 1-mg sample of each PG was dissolved in 5 ml of ethyl acetate and this solution was then further diluted accordingly. Solutions were stored in a freezer.

ADAM was purchased from Funakoshi (Tokyo, Japan). The reagent was dissolved in ethyl acetate and stored in a freezer. Solutions were used within 5 days.

Acetonitrile for HPLC was purchased from Katayama (Osaka, Japan). Ethyl acetate and tetrahydrofuran for HPLC were purchased from Kanto (Tokyo, Japan).

Instruments

Gel permeation chromatography. An HLC-803A high-pressure pump (Toyo

0021-9673/82/0000–0000/$02.75

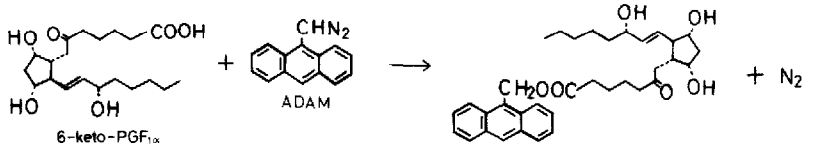

Fig. 1. Esterification of PG with 9-anthryldiazomethane (ADAM).

Soda) was used to deliver ethyl acetate. Two columns (600 × 7.5 mm I.D.) packed with TSK-GEL G1000H8 using ethyl acetate (Toyo Soda) were used in series. A UV-8 Model II spectrophotometer (Toyo Soda) was used to measure the absorbance at 254 nm. Chromatograms were recorded on a Hitachi 056 recorder with a 10-mV span set at a chart speed of 5 mm/min.

High-performance liquid chromatography. An HLC-803D high-pressure pump (Toyo Soda) was used. A column (250 × 4.6 mm I.D.) was packed with LS 410 ODS SIL (5 μm particle size) by the method of Yamauchi and Kumanotani[12]. The number of theoretical plates of the column was 17,900. FS-950 fluoromat (Toyo Soda) was used as a detector, the fluorescence being measured at 418 nm with excitation at 365 nm. The recorder was the same as that used for GPC. The solvent system used for elution was acetonitrile–water (5:3).

Procedure for sample preparation

Each of the PG solutions was put into a 2-ml brown test tube. Ethyl acetate was removed with a stream of nitrogen. A portion (50 μl) of 0.1% ADAM solution was added to the tube which was then stoppered tightly using silicone rubber. The mixture was warmed to 40°C, kept at this temperature for 30 min, cooled and then diluted with 70 μl of ethyl acetate. A 100-μl portion of the resulting mixture was applied to the GPC columns. A fraction containing the PG–ADAMs was dried under nitrogen at 40°C using a rotary evaporator. The residue was dissolved in 180 μl of acetonitrile. A portion (50 μl) of the solution was analysed by HPLC.

RESULTS AND DISCUSSION

Esterification with ADAM

The optimal volume of ADAM solution (150 μl) with which 6-keto-$PGF_{1\alpha}$ (5.6 ng), PGD_2 (1.49 ng), PGE_1 (2.3 ng), PGE_2 (1.64 ng) and $PGF_{2\alpha}$ (2.0 ng) were esterified was examined. The concentrations of ADAM solution used were 0.025, 0.05, 0.1 and 0.2% (w/v). With 0.2% ADAM solution, 6-keto-$PGF_{1\alpha}$, PGE_1, PGE_2 and $PGF_{2\alpha}$ were detected at the highest sensitivity. The sensitivity used for PGD_2 was not changed for all concentrations of ADAM solution. However, with a solution greater than 0.1%, the chromatogram was dirty because the baseline had risen with the impurities. We therefore used 50 μl of 0.1% ADAM solution. After the reaction, 70 μl of ethyl acetate was added to dilute the ADAM solution.

Although esterification was accelerated at higher temperatures, a little ethyl acetate evaporated from the reaction mixture at 50°C. The volume therefore became inaccurate, and the interfering peaks arising from ADAM became large. Consequently, PGs were esterified at 40°C for 30 min with 50 μl of 0.1% ADAM solution.

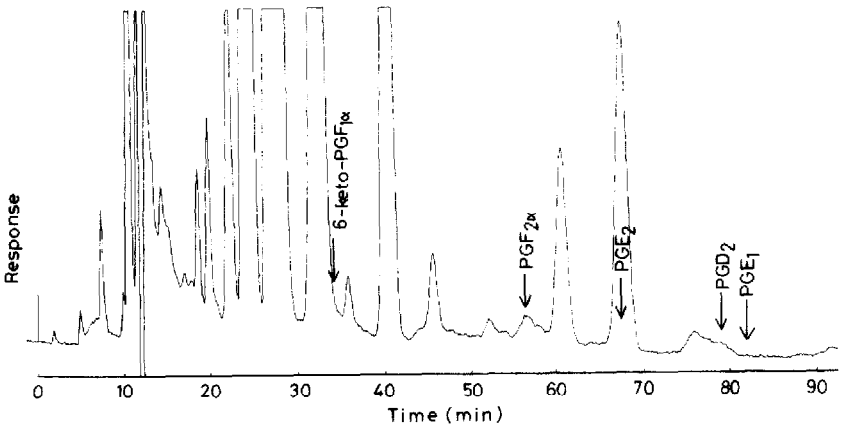

Fig. 2. HPLC chromatogram of an ADAM solution. The arrows (↓) indicate the retention times of the PG-ADAMs. Sample size, 5 μl. Solvent system, acetonitrile–tetrahydrofuran–water (9:2:11). Flow-rate, 1.0 ml/min.

Purification of PG–ADAMs from the reaction mixture by GPC and determination of PGs by HPLC

To examine the separation of five PG–ADAMs a large quantity of PG (*ca.* 1 μg) was individually esterified with a small amount of ADAM, and each reaction mixture was applied directly to the LS 410 ODS column. In this case, the interfering peaks which arose from ADAM were not so large, because of the use of the relatively minor amount of ADAM, and each PG–ADAM was well separated by HPLC. Thus, five PG–ADAMs were separated from each other using acetonitrile–tetrahydrofuran–water (9:2:11) as eluent.

One tenth of the amount of ADAM which was described in "Procedure for sample preparation" was applied to the column under the same conditions, and the chromatogram which was obtained is shown in Fig. 2. If a practical-sized volume of ADAM was applied, the peaks of all the PG–ADAMs except PGE_1 overlapped those

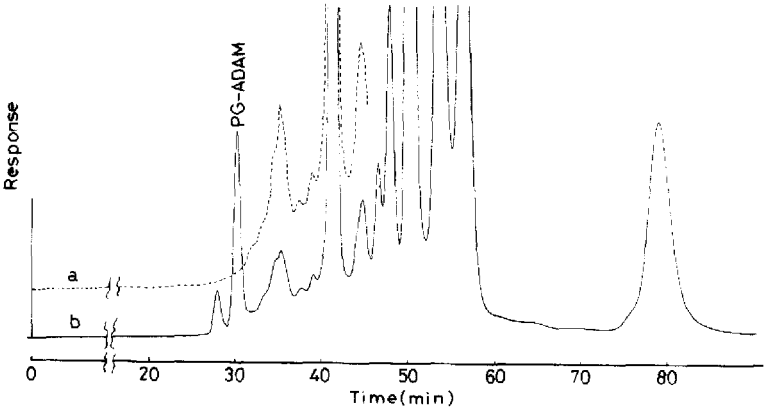

Fig. 3. GPC chromatograms of ADAM solution and the mixture of PG and ADAM solution. a (---) = 100 μl of 0.025% ADAM solution; b (— · —) = 2.16 μg $PGF_{2\alpha}$ esterified with 0.025% ADAM. Sample size, 50 μl. Solvent, ethyl acetate. Flow-rate, 1.0 ml/min.

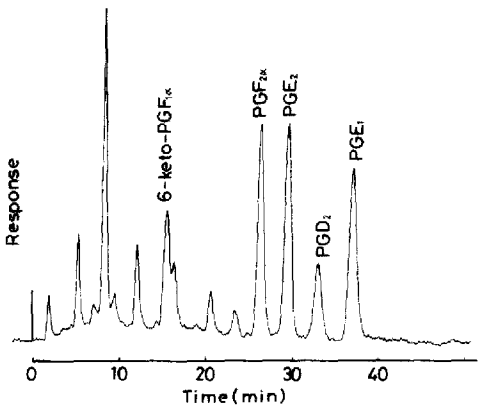

Fig. 4. Separation of PG–ADAMs with an LS 410 ODS column. 6-keto-$PGF_{1\alpha}$ (18 ng), $PGF_{2\alpha}$ (8.5 ng), PGE_2 (8 ng), PGD_2 (6 ng) and PGE_1 (8.5 ng) were esterified with 0.1% ADAM. Solvent system, acetonitrile–water (5:3). Flow-rate, 1.0 ml/min.

of ADAM. Therefore it was necessary to purify the PG–ADAM reaction mixture.

The GPC chromatogram of the reaction mixture of ADAM and esterified $PGF_{2\alpha}$ is shown in Fig. 3. All PG–ADAMs were eluted together at 30 min by GPC. Thus, the impurities were removed by purifying the reaction mixture using GPC. The peak of the PG–ADAMs could not be detected with less than 70 ng of PGs. In such a case, *ca.* 100 ng of PG was esterified and applied prior to GPC to ensure sufficient time to fractionate. Five PG–ADAMs, purified as mentioned above, were separated cleanly by HPLC during 40 min using acetonitrile–water (5:3), as eluent (Fig. 4).

For calibration, five PGs (65–280 ng) were worked up as described above and the solution which was obtained was diluted accordingly. The correlation of the PG–ADAM peak height with the amount of PG was examined. The calibration graphs were straight lines passing through the origin. Quantities greater than 90 pg (injected) of 6-keto-$PGF_{1\alpha}$, 120 pg of PGE_1 and 30 pg of PGD_2, PGE_2 and $PGF_{2\alpha}$ were

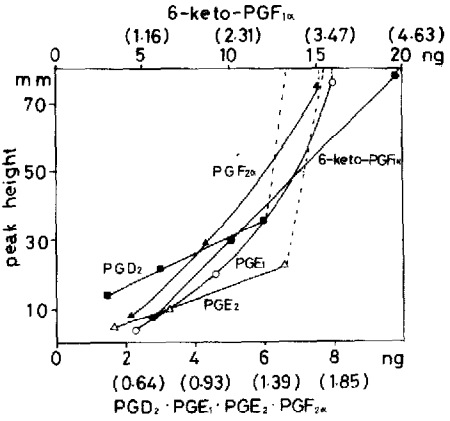

Fig. 5. Calibration graphs of PG-ADAM peak height *vs.* amount of PG. Amounts of PG applied to the LS 410 ODS column are shown in parentheses.

detected and determined by the present method. Terragno *et al.*[10] analysed 30 ng of PG utilizing a UV detector at 192.5 nm while Turk *et al.*[5] analysed 20 ng of PG labelled with 4-bromomethyl-7-methoxycoumarin. Thus our determination method is advantageous for biological materials which contain small amounts of PGs.

In addition, as an approach to actual biological materials, we examined the relationship of the peak height to the amount of each PG when small amounts of PGs (1–28 ng) were esterified. The results are shown in Fig. 5. For the cases of 6-keto-$PGF_{1\alpha}$ (5.6–19.6 ng), PGD_2 (1.49–5.96 ng) and PGE_2 (1.64–6.5 ng), a linear relationship was observed between the peak height and the amount of PG. For $PGF_{2\alpha}$ (2.16–7.56 ng) and PGE_1 (2.3–8.05 ng), the relationship of the peak height to the amount was almost linear, the plot being gently curved. Reproducibility was observed for all five PGs, the standard deviation of three runs being ± 1.98 mm (± 0.0–± 6.2 mm). When 6-keto-$PGF_{1\alpha}$ (28 ng), PGD_2 (9 ng), PGE_1 (11 ng), PGE_2 (10 ng) and $PGF_{2\alpha}$ (10 ng) were esterified, the peak heights of these compounds increased suddenly. It seems that the efficiency of esterification increases with increasing the amount of PG.

ACKNOWLEDGEMENT

We thank Mr. Yamauchi (Institute of Industrial Science, University of Tokyo) for advice and Dr. Onodera (Faculty of Pharmaceutical Science, Tokyo University of Science) for helpful comments regarding the manuscript.

REFERENCES

1 W. Morozowich and S. L. Douglas, *Prostaglandins*, 10 (1975) 19.
2 K. Carr, B. J. Sweetman and J. C. Frölich, *Prostaglandins*, 11 (1976) 3.
3 M. V. Merritt and G. E. Bronson, *Anal. Chem.*, 48 (1976) 1851.
4 M. V. Merritt and G. E. Bronson, *Anal. Biochem.*, 80 (1977) 392.
5 J. Turk, S. J. Weiss, J. E. Davis and P. Needleman, *Prostaglandins*, 16 (1978) 291.
6 F. A. Russell and D. Deykin, *Prostaglandins*, 18 (1979) 11.
7 A. R. Whorton, K. Carr, M. Smigel, L. Walker, K. Ellis and J. A. Oates, *J. Chromatogr.*, 163 (1979) 64.
8 S. Inayama, H. Hori, T. Shibata, Y. Ozawa, K. Yamagami, M. Imazu and H. Hayashida, *J. Chromatogr.*, 194 (1980) 85.
9 S. Ikenoya, O. Hiroshima, M. Ohmae and K. Kawabe, *Chem. Pharm. Bull.*, 28 (1980) 2941.
10 A. Terragno, R. Rydzik and N. A. Terragno, *Prostaglandins*, 21 (1981) 101.
11 N. Nimura and T. Kinoshita, *Anal. Lett.*, 13 (1980) 191.
12 Y. Yamauchi and J. Kumanotani, *Seisan Kenkyu*, 32 (1980) 255.