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Note

Picomole determination of 2,4-dimethoxyanilides of prostaglandins by high-performance liquid chromatography with electrochemical detection

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Prostaglandins (PGs) are metabolites of arachidonic acid and play an important role in the regulation of many physiological and pathophysiological functions[1]. Presently, the most commonly used methods for their quantification are radioimmunoassay (RIA) and gas chromatography–mass spectrometry (GC–MS). However, there is a need for a more convenient method. Both RIA and GC–MS offer excellent sensitivity in the low picogram range, but RIA is not well suited to the analysis of multiple components in a single experiment, while GC–MS requires multi-step derivatization.

Several methods which make use of high-performance liquid chromatography (HPLC) have been reported. Since PGs show only poor UV absorption[2] and no fluorescence, most of these methods include a derivatization reaction, leading to the formation of easily detectable compounds, prior to HPLC separation. Recently, a very sensitive HPLC method using electrochemical detection (ED) was reported for the determination of lipoxygenase metabolites of arachidonic acid[3]. However, anodic oxidation of PGs requires applied potentials above +1.6 V, so that this method is hardly applicable. Some methods involve the formation of UV-absorbing[4–6] or fluorescent[7–10] compounds. A few methods make use of ED after formation of an electrochemically active compound[11] or by using an indirect method[12], but they show only a limited sensitivity (detection limit in the low nanogram range). On the other hand, HPLC with ED is a more sensitive method for oxidizable compounds. This paper presents a method for the quantitation of PGE_2, $PGF_{2\alpha}$, PGD_2 and TXB_2, as their 2,4-dimethoxyanilides, by HPLC with ED. The derivatization reaction can be performed in an aqueous medium without protection from light. A clean-up step was included, thus allowing quantities as low as 10 ng of PG to be used. 16,16-Dimethyl-PGE_2, which is not present in biological samples, was used as the internal standard. The detection limit on the column was 50 pg.

EXPERIMENTAL

Reagents

PGE_2, $PGF_{2\alpha}$ and TXB_2 were generous gifts from Dr. C. O. Meese (Dr. Mar-

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garete Fischer-Bosch Institute, Stuttgart, F.R.G.). PGD_2 was obtained from Sigma (St. Louis, MO, U.S.A.), 16,16-dimethyl-PGE_2 from Paesel (Frankfurt, F.R.G.) and 1-ethyl-3-dimethylaminopropylcarbodiimide hydrochloride (EDC–HCl) from Merck (Darmstadt, F.R.G.).

The hydrochloride of 2,4-dimethoxyaniline (Merck) was prepared by introducing hydrogen chloride gas into an etheral solution and washing the precipitate with absolute diethyl ether. The hydrochloride was recrystallized from water before use.

Methanol (Riedel de Haen, Seelze, F.R.G.), acetonitrile (Baker, Deventer, The Netherlands) and ethanol (Merck) were of HPLC grade. Water (Ampuwa®) was obtained from Fresenius (Bad Homburg, F.R.G.). All other reagents used were of analytical grade or better.

The concentrations of the stock solutions of PGE_2 and 16,16-dimethyl-PGE_2 in methanol were determined with a Beckman DU 50 spectrophotometer by measuring the UV absorption at 278 nm (ε = 26 800) after converting these PGs into the analogous PGB derivatives with methanolic sodium hydroxide[13].

Reagent solutions

2,4-Dimethoxyaniline hydrochloride solution (0.02 *M*) was prepared by dissolving the reagent in water. To prepare the EDC solution, EDC–HCl was dissolved in ethanol and an equal volume of a pyridine solution (3%, v/v) in ethanol was added, so that the final concentration of EDC in the solution was 0.125 *M*.

Derivatization procedure

A 20-μl volume of a methanolic PG solution was placed in a screw-capped 1-ml vial. The cap seal used was punched from PTFE–perfluoroalkoxy (PFA) foil. A 2.5-μl volume of 2,4-dimethoxyaniline hydrochloride solution and 5 μl of EDC solution were added. The mixture was shaken on a vortex mixer before heating in a water-bath at 37°C for 1 h. The excess of reagent was removed using Baker C_{18} disposable columns. The method of Luderer *et al.*[14], who extracted PGs from biological samples, was modified to meet the present requirements. The column was first preconditioned with 2 ml of methanol and then with 2 ml of water. The reaction mixture was diluted in 300 μl of water and loaded on the column. The column was washed with 2 ml of 50% methanol, which was previously acidified with hydrochloric acid to pH 2.75. Then it was washed with 1 ml of 50% methanol (neutral) to remove the acid. Finally, the PG derivatives were eluted with 2 ml of absolute methanol. The eluate was evaporated to dryness under a stream of nitrogen at 37°C. The residue was resuspended in 100 μl of methanol. An aliquot (10 μl) of this solution was injected on the column.

HPLC analysis

The HPLC equipment consisted of a Waters 590 pump, a Waters U6K injector and a Waters 460 electrochemical detector, equipped with a thin-layer glassy carbon electrode. The output signal was recorded on a Waters M 730 data module. To restore sensitivity to the glassy carbon electrode, its surface was treated with dichromate–sulphuric acid and alumina slurry according to the method of Herrmann *et al.*[3]. All potentials applied were referred to a silver–silver chloride electrode (filling: 3 *M* lithium chloride in 65% aqueous methanol).

HPLC separations were carried out using a Nucleosil® C_{18} column (particle size 5 μm, 250 mm × 4.6 mm I.D.) (Macherey-Nagel, Düren, F.R.G.). The solvent system was acetonitrile–methanol–water (35:22:43, v/v/v), containing an electrolyte (0.5 g/l lithium perchlorate). The pH of the solution was adjusted to 4.1 using trifluoroacetic acid.

RESULTS

The most commonly used mobile phase to separate cyclooxygenase products by reversed-phase HPLC is acetonitrile–water, containing either phosphoric or acetic acid[15]. Although an excellent separation of the PG derivatives can be achieved, this solvent system was not suitable when ED was used because of the high background current and noise level. Moreover a minor contaminant was coeluted with TXB_2. A mobile phase consisting of aqueous methanol was not able to separate the PGE_2-anilide from the PGD_2-anilide. We found that by using an acetonitrile–methanol–water solvent system satisfactory separation could be achieved. The baseline was also stable. At a potential of +1.10 V the background current was typically 4–7 nA, thus allowing measurements at a sensitivity of 100 pA full scale. A typical chromatogram is represented in Fig. 1.

It was found that the pH of the mobile phase exerted a strong influence on the peak height of TXB_2. Fig. 2 shows the increase in response of a constant amount of TXB_2, when the pH of the solvent system was changed from 3.15 to 4.5. The be-

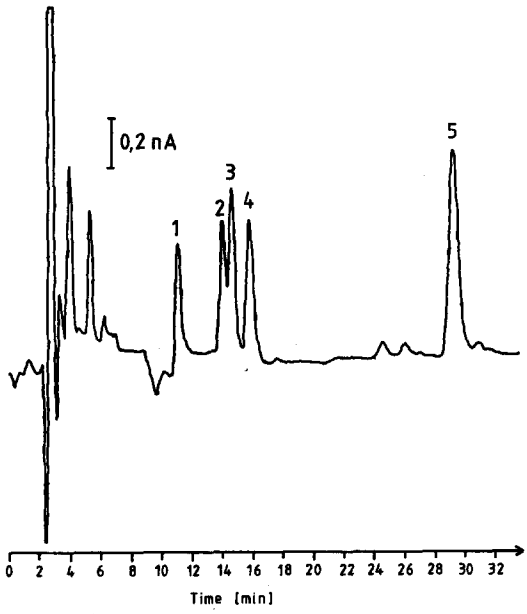

Fig. 1. Chromatogram of 2,4-dimethoxyanilides of prostaglandins. Eluent: acetonitrile–methanol–water (35:22:43, v/v/v), containing 0.5 g/l lithium perchlorate. The pH was adjusted to 4.1 using dilute trifluoroacetic acid. Flow-rate: 1.0 ml/min. Detector potential: +1.10 V (*vs.* silver–silverchloride). Peaks: 1 = TXB_2; 2 = $PGF_{2\alpha}$; 3 = PGE_2; 4 = PGD_2; 5 = 16,16-dimethyl-PGE_2 (approximately 2.5 ng each).

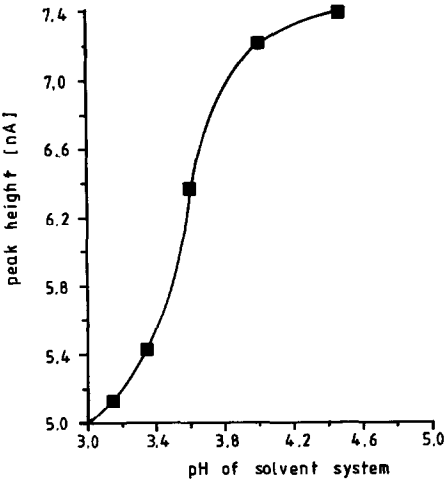

Fig. 2. Effect of the pH of the mobile phase on the response of TXB_2. A constant amount of TXB_2 (40 ng) was injected.

haviour above pH 4.5 could not be measured reliably, because the electrode was passivated. This passiviation resulted in a time-dependent diminution in response for all the compounds investigated. Simultaneously, the background current also decreased. The dependence of the response of TXB_2 on the applied pH could be explained by the fact that TXB_2 may exist in two isomeric forms. There exists an equilibrium between an open and closed hemiacetal ring, which is shifted to one side, when the pH value is in the neutral region, resulting in an increased peak sharpness for TXB_2 (Fig. 3)[16].

The hydrodynamic voltammograms of PGE_2, $PGF_{2\alpha}$, PGD_2, TXB_2 and 16,16-dimethyl-PGD_2 are presented in Fig. 4. The half-wave potential was +1.03 V for all the compounds investigated. The maximum response was obtained at +1.20 V. At +1.10 V the response was 90% of the maximum. Since there was an increase in background current at higher potentials, a potential of +1.10 V was chosen.

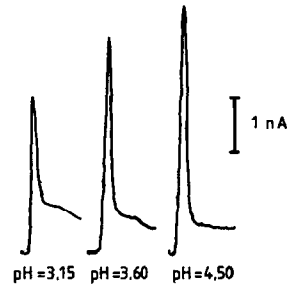

Fig. 3. Chromatograms of TXB_2 (amount injected: 25 ng) at different eluent pH values. All other chromatographic conditions as in Fig. 1.

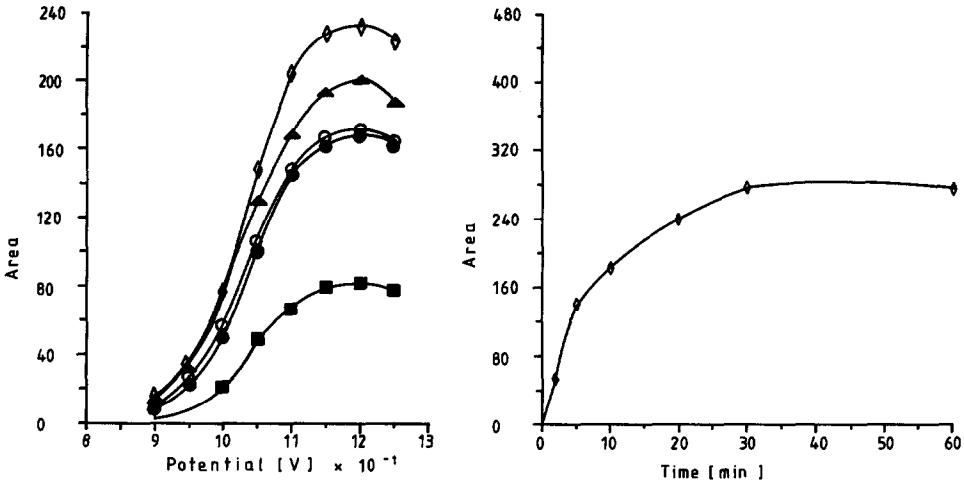

Fig. 4. Hydrodynamic voltammograms of TXB_2 (■), $PGF_{2\alpha}$ (●), PGE_2 (◇), PGD_2 (○) and 16,16-dimethyl-PGE_2 (▲), each corresponding to 20 ng.

Fig. 5. Effect of the reaction time on derivatization of PGE_2. A 100-ng amount of PGE_2 was treated by the derivatization procedure using various reaction times. One-tenth of the eluate obtained (10 μl) was injected on the column.

The derivatization reaction was carried out according to the method of Miwa *et al.*[17], who synthesized 2-nitrophenylhydrazides of fatty acids by using EDC–HCl as a catalyst. The cyclooxygenase metabolites reacted with 2,4-dimethoxyaniline and EDC–HCl to form the corresponding anilides in a similar way. Treatment with ethanolic potassium hydroxide was not necessary. In order to ensure maximum derivatization, 100 ng of PGE_2 were incubated under standard conditions, except that the reaction period was varied from 2 to 60 min and the time-dependent increase in the peak height was monitored by HPLC (Fig. 5). The peak height became constant after a reaction period of 30 min.

The clean-up procedure was verified by reloading a part of the eluate on the Baker column, and comparing the peak heights. The recoveries in the 100% methanol fraction are presented in Table I.

TABLE I

MEANS OF RECOVERIES IN % ± S.D. OF PG DERIVATIVES EXTRACTED FROM THE REACTION MIXTURE USING BAKER C_{18} COLUMNS

n = Number of samples.

Compound	*Recovery (%) (Mean ± S.D., n = 3)*
PGE_2	102 ± 11
$PGF_{2\alpha}$	106 ± 11
PGD_2	103 ± 12
TXB_2	102 ± 8
16,16-Dimethyl-PGE_2	100 ± 12

TABLE II

MEANS OF PEAK HEIGHTS IN nA (y) ± S.D. AS A FUNCTION OF THE AMOUNT INJECTED IN ng (x) OF EACH PG

n = Number of samples.

PGE_2			$PGF_{2\alpha}$			PGD_2			TXB_2		
x	y	n	x	y	n	x	y	n	x	y	n
1	0.27 ± 0.02	5	1	0.23 ± 0.03	5	1	0.16 ± 0.02	5	1	0.11 ± 0.03	5
2.5	0.68 ± 0.02	6	2.5	0.54 ± 0.02	6	2.5	0.45 ± 0.03	6	2.5	0.30 ± 0.04	6
5	1.40 ± 0.18	3	5	1.12 ± 0.15	3	5	0.88 ± 0.12	3	5	0.74 ± 0.08	3
10	2.58 ± 0.02	3	10	2.08 ± 0.05	3	10	1.61 ± 0.09	3	10	1.45 ± 0.22	3

TABLE III

EQUATIONS CORRESPONDING TO THE CALIBRATION GRAPHS AND CORRELATION COEFFICIENTS, r, FOR THE 2,4-DIMETHOXYANILIDES

The equation is defined as $y = ax + b$, where y is the peak height and x is the amount (ng) of sample.

	t_R (min)	Equation	r
TXB_2	11.27	$y = 0.15229x - 0.05434$	0.99879
$PGF_{2\alpha}$	14.34	$y = 0.20602x + 0.03964$	0.99915
PGE_2	14.99	$y = 0.25580x + 0.05293$	0.99893
PGD_2	16.21	$y = 0.15952x + 0.03723$	0.99828

Calibration graphs were prepared by derivatizing increasing amounts, 10–100 ng each of PGE_2, $PGF_{2\alpha}$, PGD_2 and TXB_2, in the presence of 50 ng of 16,16-dimethyl-PGE_2 as the internal standard. An aliquot corresponding to one tenth of each derivatized amount (1–10 ng) was injected on the column. The means of the peak heights (in nA) and the standard deviations (S.D.) corresponding to the amount of each PG injected are listed in Table II. Table III presents the equations of these graphs and the correlation coefficients for the derivatives, which were obtained by calculating the results shown in Table II by the least-squares method. The detection limits of the PGs, based on a peak height *versus* baseline noise ratio of 5:1, were determined to be 40–70 pg in an injection volume of 2 μl.

The method presented shows that HPLC with ED allows sensitive detection even at higher potentials. Based on the fact that reagents of high reactivity were used for the simple derivatization procedure and an effective clean-up step was included, relatively small amounts of PGs could be detected.

ACKNOWLEDGEMENTS

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REFERENCES

1 C. Pace-Asciak and E. Granstroem (Editors), *Prostaglandins and Related Substances*, Elsevier, New York, Amsterdam, 1983.
2 A. Terragno, R. Rydzik and N. A. Terragno, *Prostaglandins*, 21 (1981) 101–112.
3 T. Herrmann, D. Steinhilber and H. J. Roth, *J. Chromatogr.*, 416 (1987) 170–175.
4 W. Morozowich and S. L. Douglas, *Prostaglandins*, 10 (1975) 19–39.
5 F. A. Fitzpatrick, *Anal. Chem.*, 48 (1976) 499–502.
6 F. A. Fitzpatrick, M. A. Wynalda and D. G. Kaiser, *Anal. Chem.*, 49 (1977) 1032–1035.
7 W. D. Watkins and M. B. Peterson, *Anal. Biochem.*, 125 (1982) 30–40.
8 R. A. Kelly, D. S. O'Hara and V. Kelley, *J. Chromatogr.*, 416 (1987) 247–254.
9 M. Hatsumi, S.-I. Kimata and K. Hirosawa, *J. Chromatogr.*, 253 (1982) 271–275.
10 M. Yamaguchi, K. Fukuda, S. Hara, M. Nakamura and Y. Ohkura, *J. Chromatogr.*, 380 (1986) 257–265.
11 S. Ikenoya, O. Hiroshima, M. Ohmae and K. Kawabe, *Chem. Pharm. Bull.*, 28 (1980) 2941–2947.
12 W. P. King and P. T. Kissinger, *Clin. Chem.*, 26 (1980) 1484–1491.
13 S. Bergstroem, R. Ryhage, B. Samuelsson and J. Sjoevall, *J. Biol. Chem.*, 238 (1963) 3555–3564.
14 J. R. Luderer, D. L. Riley and L. M. Demers, *J. Chromatogr.*, 273 (1983) 402–409.
15 W. S. Powell, *Anal. Biochem.*, 148 (1985) 59–69.
16 M. Moonen, G. Klok and M. J. N. C. Keirse, *Prostaglandins*, 26 (1983) 797–803.
17 H. Miwa, C. Hiyama and M. Yamamoto, *J. Chromatogr.*, 321 (1985) 165–174.