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QUANTITATIVE PROFILING OF PROSTAGLANDINS AND THROMBOXANE BY HIGH-RESOLUTION GAS CHROMATOGRAPHY–SELECTED-ION MONITORING

C. CHIABRANDO*, A. NOSEDA, M. N. CASTAGNOLI, M. ROMANO and R. FANELLI
Laboratory of Environmental Pharmacology and Toxicology, Istituto di Ricerche Farmacologiche "Mario Negri", Via Eritrea 62, 20157 Milan (Italy)

SUMMARY

The development and biological application of a rapid method for quatitative profiling of prostaglandins and thromboxane using high-resolution gas chromatography (HRGC) coupled with mass spectrometry in the selected-ion monitoring technique (SIM) are described. The method is based on the single-step extraction of prostaglandins from biological samples on C_{18} reversed-phase cartridges after addition of deuterated analogues as internal standards, followed by derivatization of functional groups and final analysis by HRGC–SIM with wall-coated open tubular persilanized capillary columns.

Biological applications include the determination of endogenous arachidonic acid cascade profiles in rat tissue homogenates and thromboxane synthetase inhibition studies in human serum.

INTRODUCTION

The metabolic conversion of arachidonic acid through the cyclooxyenase pathway leads to a family of compounds, prostaglandins (PGs) and thromboxane A_2 (TXA_2), which are potent mediators of various biological events. In spite of the outstanding advances that have followed the discovery of prostaglandins and the impressive expansion of this field, measuring endogenous PGs in biological samples still represents a major analytical challenge. These compounds, in fact, have very similar chemical structures, and very low concentrations may be present simultaneously in tissues, where they act as local hormones. The method of choice for the measurement of PGs must not only be sensitive and specific but also, if possible, be able to detect all the metabolites of arachidonic acid simultaneously. This feature is particularly relevant when studying physiological, pathological or pharmacological conditions that may influence one or more enzymes of the arachidonic acid cascade. Obviously, conventional methods that focus on the measurement of selected PGs cannot provide an adequate picture of altered metabolic profiles.

High-resolution gas chromatography (HRGC) coupled with mass spectrometry is recognized as the most specific and reliable method available for measuring

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PGs. In recent years a number of workers have used HRGC to solve the difficult problem of PG separation. They studied various derivatives[1,2], stationary phases[3] and coating techniques[4–6]. Several workers have used electron-capture detection[2,6,7], while others used mass spectrometry in the selected-ion monitoring (SIM) mode[5,8–10]. Promising results were recently obtained by HRGC–SIM for measuring PGs in urine[8] and cell incubation media[9].

This paper describes a rapid method for quantitative profiling of the arachidonic acid stable metabolites ($PGF_{2\alpha}$, PGE_2, PGD_2, 6-keto-$PGF_{1\alpha}$, TXB_2), endogenously formed in tissues or biological fluids. Rat tissue homogenates and human serum were chosen for testing the applicability of the method.

EXPERIMENTAL

Standards

$PGF_{2\alpha}$, PGE_2, PGD_2, 6-keto-$PGF_{1\alpha}$, TXB_2, 3,3′,4,4′-tetradeutero-$PGF_{2\alpha}$ ($PGF_{2\alpha}$-d_4), 3,3′,4,4′-tetradeutero-PGE_2 (PGE_2-d_4) and 3,3′,4,4′-tetradeutero-6-keto-$PGF_{1\alpha}$ (6-keto-$PGF_{1\alpha}$-d_4) were a generous gift from Dr. John Pike of the Upjohn Co., Kalamazoo, MI, U.S.A.

Derivatization

The pentafluorobenzyl ester trimethylsilyl ether (PFB-TMS) derivatives of $PGF_{2\alpha}$ and $PGF_{2\alpha}$-d_4 and the pentafluorobenzyl ester methyloxime trimethylsilyl ether (PFB-MO-TMS) derivatives of PGE_2, PGE_2-d_4, PGD_2, 6-keto-$PGF_{1\alpha}$, 6-keto-$PGF_{1\alpha}$-d_4 and TXB_2 were prepared as described previously[7].

Mass spectrometry

An LKB 2091-051 gas chromatograph–mass spectrometer, equipped with an LKB 2130 computer system for data acquisition and calculation, was used in the electron-impact mode. For SIM analysis the instrument was tuned on characteristic ions of PGs and the corresponding ions of the d_4-analogues: m/z 301 for TXB_2, 461 for PGE_2 (major isomer), 465 for PGE_2-d_4, 544 for PGD_2 and 6-keto-$PGF_{1\alpha}$, 548 for 6-keto-$PGF_{1\alpha}$-d_4, 589 for $PGF_{2\alpha}$ and 593 for $PGF_{2\alpha}$-d_4.

The instrumental conditions were as follows: ion source, 260°C; electron energy, 22.5 eV; trap current, 100 μA; accelerating voltage, 3.5 kV; source slitwidth, 0.1 mm; collector slitwidth, 0.3 mm; and resolution, 650. The mass spectra of PGs derivatives were presented in a previous paper[5].

High-resolution gas chromatography

The gas chromatograph was a DANI 3800, equipped with a temperature-programmed vaporizer (TPV) injector (DANI). OV-1 or SE-54 wall-coated open tubular (WCOT) persilanized Pyrex glass columns (20 m × 0.35 mm I.D.) were prepared according to Grob *et al.*[11].

The instrumental conditions were as follows: carrier gas (helium) head-pressure, 1.3 atm; oven temperature programming, from 150 to 280°C at 20°C min^{-1}; TPV temperatures, 40–280°C for solvent-split injections and 280°C isothermal for splitless injections.

Extraction

Rat (male CD COBS, Charles River, 200 g) tissues were homogenized for 20 sec in phosphate buffer [0.05 *M*, pH 7.4, 1:10 (w/v), Ultraturrax homogenizer], immediately incubated at 37°C for 15 min, then centrifuged at 200,000 *g* for 30 min.

Known amounts (200–2000 ng) of PG tetradeutero analogues were added to the supernatant (10 ml) as internal standards. Human serum samples (2 ml) were prepared by low-speed centrifugation of blood clotted (37°C, 30 min) in the presence or absence of thromboxane synthetase inhibitor (dazoxiben, Pfeizer, 40 μM). Amounts of 200 ng each of $PGF_{2\alpha}$-d_4, PGE_2-d_4 and 6-keto-$PGF_{1\alpha}$-d_4 were added to these samples as internal standards.

All samples were then acidified (pH 3.5) with 1 *N* hydrochloric acid and extracted on Sep-Pak C_{18} cartridges (Waters Assoc.) according to Powell[12].

The fraction (methyl formiate) containing prostaglandins was evaporated to dryness on a rotary evaporator and then submitted to the derivatization reactions, as described previously[7].

Quantitation

Tetradeutero analogues of $PGF_{2\alpha}$, PGE_2 and 6-keto-$PGF_{1\alpha}$ were used for quantitative analysis. Deuterated analogues of PGD_2 and TXB_2 were not available.

Calibration graphs were drawn to test the linearity of the d_0/d_4 intensity ratio over a wide concentration range. Amounts of 10–1000 ng of each d_0 standard PG were added to 200 ng of each d_4 analogue. The d_0/d_4 intensity ratios were plotted against the amount in nanograms of the d_0 form added to each sample. For $PGF_{2\alpha}$, PGE_2 and 6-keto-$PGF_{1\alpha}$ the ratios were calculated by using the corresponding fragment ion pairs of the d_0/d_4 analogues (*i.e.*, *m/z* 589/593 for $PGF_{2\alpha}$, 461/465 for PGE_2 and 544/548 for 6-keto-$PGF_{1\alpha}$), whereas for TXB_2 (*m/z* 301) and PGD_2 (*m/z* 544) the d_4 ion of 6-keto-PGF_1 (*m/z* 548) was used for internal standardization. The resulting correlation coefficients were 0.9999 for $PGF_{2\alpha}$, 0.9998 for PGE_2, 0.9976 for PGD_2, 0.9987 for TXB_2 and 0.9987 for 6-keto-$PGF_{1\alpha}$. Similar plots were drawn for extracted samples (10 ml of 0.05 *M* phosphate buffer, pH 7.4, spiked with the d_0/d_4 standard mixtures), and superimposable curves were obtained, with the exception of PGD_2 and TXB_2, which gave similar correlation coefficients but lower slope values (0.0019 *versus* 0.0026 for PGD_2; 0.006 *versus* 0.011 for TXB_2), indicating a lower overall recovery for these two products than for 6-keto-$PGF_{1\alpha}$. The results reported here for PGD_2 and TXB_2 can thus be considered underestimates, as no corrections were made to compensate for this phenomenon (see discussion below).

Quantitation was therefore performed for all PGs by direct comparison of the sample *versus* standard d_0/d_4 ratio.

RESULTS AND DISCUSSION

SE-54 or OV-1 WCOT persilanized capillary columns (0.15-μm film thickness) were prepared according to Grob *et al.*[11]. These columns are stable for months and show no loss of efficiency after several hundred injections of biological samples. Columns 20 m long showed typical "Trennzahl" (*TZ*) values of about 30, and could easily separate our test mixture ($PGF_{2\alpha}$, PGD_2, PGE_2, TXB_2 and 6-keto-$PGF_{1\alpha}$) in a relatively short time (6 min) in an isothermal chromatogram. For biological sample

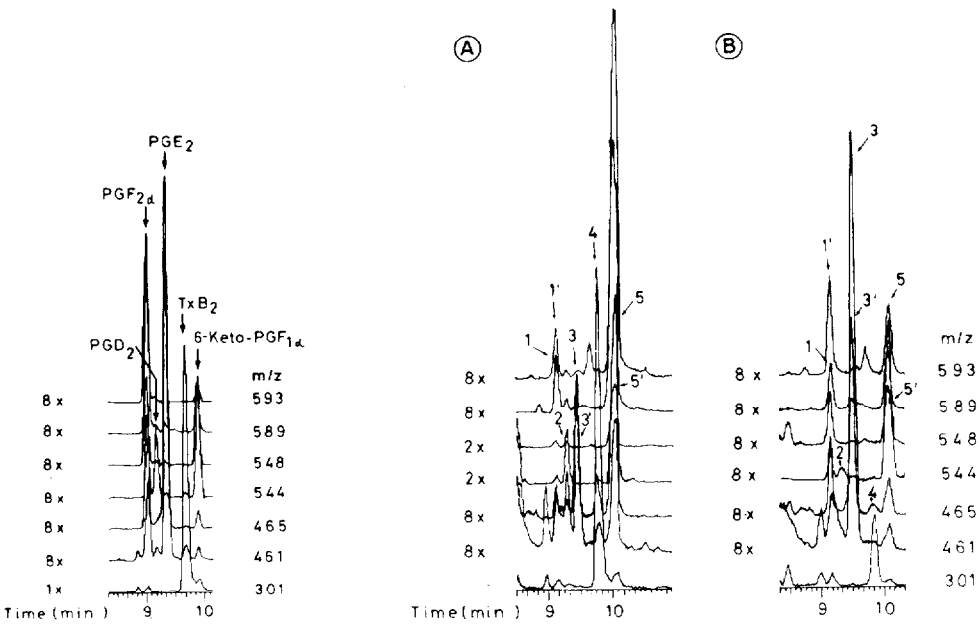

Fig. 1. Typical SIM analysis of a d_0/d_4 PG standard mixture.

Fig. 2. SIM profiles of (A) lung and (B) seminal vesicles homogenates. 1 = $PGF_{2\alpha}$; 1′ = $PGF_{2\alpha}$-d_4; 2 = PGD_2; 3 = PGE_2; 3′ = PGE_2-d_4; 4 = TXB_2; 5 = 6-keto-$PGF_{1\alpha}$; 5′ = 6-keto-$PGF_{1\alpha}$-d_4.

analysis, a temperature programmed vaporizer is used for the splitless technique, which requires a longer analysis time (about 10 min). SE-54 should be used when the lesser of the *syn–anti* isomer pair of PGE_2 derivative must be separated from $PGF_{2\alpha}$. This is not necessary when SIM is used as a detector, as the minor PGE_2 isomer gives no detectable signal at *m/z* 589, which is used to monitor $PGF_{2\alpha}$. For biological experiments 20-m OV-1 columns were used.

The TPV injector was used both in the solvent-split(injector temperature programmed from 40 to 280°C) and splitless modes (280°C isothermal or programmed from 40 to 280°C) with no significant difference in response. The pre-column was changed after 20–30 injections.

Erratic results, in terms of absolute intensity response, were sometimes observed when small amounts of PGs were injected. Nevertheless, this problem, probably due to adsorption and/or decomposition of the derivatized PGs, did not affect the quantitative results, which are independent of the amount injected, but are related to the d_0/d_4 ratio. For this reason, the quantitation of PGD_2 and TXB_2, based on the 6-keto-PGF_1-d_4 response, because of the lack of corresponding analogues, is less precise and the overall recovery (extraction plus SIM analysis) could not be calculated exactly. However, the variability was low when identical biological samples were analysed (coefficient of variation, C.V. = 5% for 60 ng/g levels for TXB_2 and C.V. = 7% for 90 ng/g levels for PGD_2; $n = 4$). It is likely that for high-sensitivity, high-precision PG profiling, deuterated TXB_2 and PGD_2 will be essential.

A SIM profile obtained from a standard mixture of $PGF_{2\alpha}$ (d_0/d_4), PGE_2

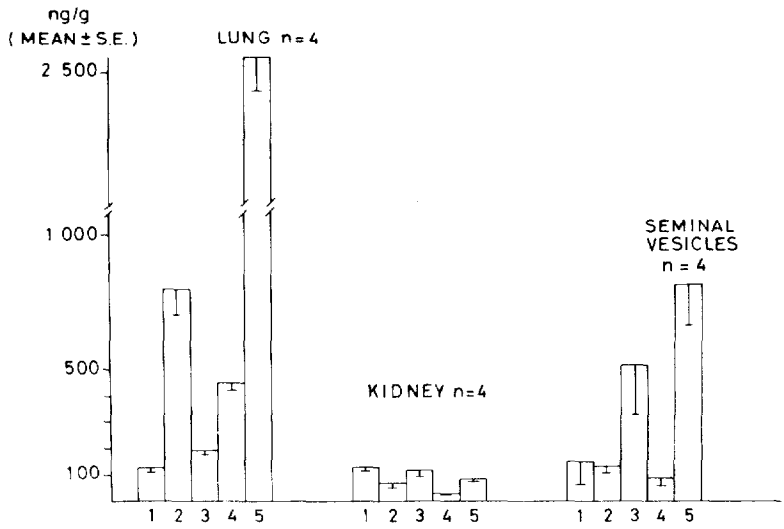

Fig. 3. PG levels in rat tissue homogenates. 1 = $PGF_{2\alpha}$; 2 = PGD_2; 3 = PGE_2; 4 = TXB_2; 5 = 6-keto-$PGF_{1\alpha}$.

(d_0/d_4), PGD_2, 6-keto-$PGF_{1\alpha}$ (d_0/d_4) and TXB_2 is shown in Fig. 1. The SIM profiles of rat lung and seminal vesicles homogenates are shown in Fig. 2.

To test the applicability of the method described, quantitation was performed on rat lung, kidney and seminal vesicles homogenates. The results of four determinations are shown in Fig. 3.

The method is sufficiently sensitive (10–50 ng of PG per gram of tissue, depend-

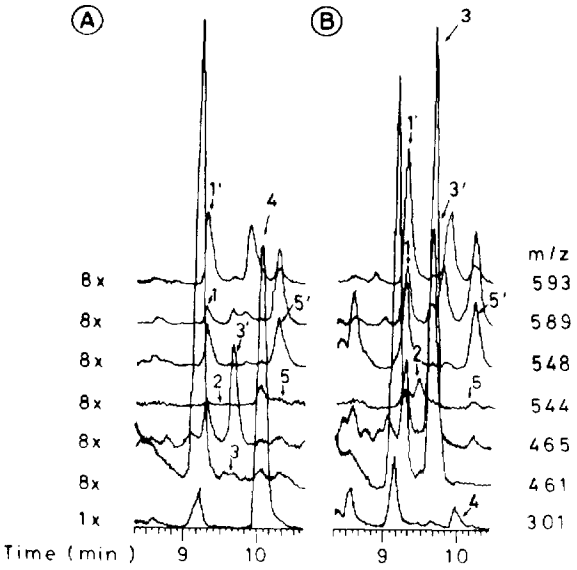

Fig. 4. SIM profiles of (A) control and (B) dazoxiben-treated human serum. 1 = $PGF_{2\alpha}$; 1′ = $PGF_{2\alpha}$-d_4; 2 = PGD_2; 3 = PGE_2; 3′ = PGE_2-d_4; 4 = TXB_2 5 = 6-keto-$PGF_{1\alpha}$; 5′ = 6-keto-$PGF_{1\alpha}$-d_4.

ing on the specific tissue background) to detect the five stable cyclooxygenase products of arachidonic acid in the tissues so far tested (preliminary screening included the stomach, spleen and liver).

Application studies also included an experiment to assess any shift in PG metabolic profiles. Serum samples (2 ml) were obtained from blood, clotted in the presence ($n = 4$) or absence ($n = 4$) of 40 μM dazoxiben, a known thromboxane synthetase inhibitor[13]. Typical SIM profiles obtained in this experiment are shown in Fig. 4. TXB_2 was found to be the major arachidonic acid metabolite (78.6 $\pm$ 3.6 ng/ml) in control human serum, while rearrangement in the metabolic profile was noted in the dazoxiben-treated samples. TXB_2 formation was, in fact, almost completely inhibited (6.6 $\pm$ 2.3 ng/ml), and high levels of PGE_2 (68.7 $\pm$ 3.4 ng/ml) and PGD_2 (45.4 $\pm$ 8.1 ng/ml) were found.

Further studies are in progress to assess the applicability of this method to other experimental conditions.

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