Journal of Chromatography, 427 (1988) 209–218
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 4137

DETERMINATION OF PROSTAGLANDINS AND THROMBOXANE IN WHOLE BLOOD BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY WITH FLUORIMETRIC DETECTION

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(First received November 5th, 1987; revised manuscript received January 22nd, 1988)

SUMMARY

A highly sensitive and specific assay for the quantitation of prostaglandins (PGs) such as PGE_1, PGE_2, $PGF_{1\alpha}$, $PGF_{2\alpha}$, 6-keto-$PGF_{1\alpha}$, and including thromboxane B_2, is described. The method involves the addition of $PGF_{1\alpha}$ and PGE_1 as the internal standards, extraction from whole blood and purification by silica gel column chromatography. Following conversion into the methoximes, purification by reversed-phase chromatography and esterification with panacyl bromide, samples are analysed by high-performance liquid chromatography with fluorimetric detection. The lower limit of detection of the eicosanoids 6-keto-$PGF_{1\alpha}$, thromboxane B_2 and $PGF_{2\alpha}$ in blood is ca. 50 pg/ml and that of PGE_2 is 100 pg/ml. Assay linearity is demonstrated over a range from 60 pg to 60 ng of eicosanoid injected. The method allows simultaneous assessment of prostaglandins and thromboxane extracted from complex biological fluids at picogram levels.

INTRODUCTION

Eicosanoids have been shown to be involved in numerous physiological and pathological processes [1] and to possess potent biological activity, especially on the cardiovascular system [2,3]. Consequently, there is a widespread interest in the isolation and quantitation of these compounds. The extremely low concentration of prostaglandins (PGs) in biological fluids has necessitated the use of sensitive techniques for their estimation [4–6].

Analytical methods with high sensitivity for PGs in biological fluids were limited to radioimmunoassays [5,6], electron-capture gas chromatography [7,8] and

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gas chromatography–mass spectrometry [9]. Recently, however, high-performance liquid chromatographic (HPLC) methods have been described for the quantification of several PGs (6-keto-$PGF_{1\alpha}$, $PGF_{2\alpha}$, PGE_2 and PGD_2), as fluorescent derivatives, in standard solutions with subnanogram sensitivity [10–14].

Furthermore, Pullen and Cox [15] proposed the use of panacyl bromide derivatization for the determination of PGE_2 metabolites in human plasma. However, no method suitable for the assessment of a wide range of PGs, including thromboxane B_2 (TXB_2), in complex biological fluids, such as whole blood, has yet been reported. The determination of TXB_2 by reversed-phase HPLC is seriously hampered not only because this metabolite elutes as an asymmetrical and broad peak from reversed-phase columns owing to hemiacetal formation, but also because of its strong interference with $PGF_{2\alpha}$ [13,14]. Although the column efficiency for TXB_2 can be improved when it is chromatographed on a separate non-silica reversed-phase support at an alkaline pH [16], a simultaneous determination of TXB_2 and PGs is preferable. Therefore, it is necessary to convert the extracted samples into methoximes before derivatization with panacyl bromide in order to obtain an adequate separation between TXB_2 and $PGF_{1\alpha}$ on the one hand and a low detection limit of TXB_2 on the other. Moreover, to achieve a picogram detection limit a purification procedure was developed to remove interfering contaminants, yet ensure a high recovery.

EXPERIMENTAL

Materials

6-keto-$PGF_{1\alpha}$, $PGF_{2\alpha}$, PGE_2, and PGE_1 and TXB_2 were purchased from Cayman (Ann Arbor, MI, U.S.A.). Radiolabelled eicosanoids, such as 5,6,8,11,12,14,15-[^{3}H](N)-PGE_2 and 5,8,9,11,12,14,15-[^{3}H](N)-6-keto-$PGF_{1\alpha}$, were purchased from New England Nuclear (Boston, MA, U.S.A.). Panacyl bromide [*p*-(9-anthroyloxy)phenacyl bromide] was kindly provided by J. Cox, Pharmaceutical Research and Development, The Upjohn Company (Kalamazoo, MI, U.S.A.). Hexane, chloroform, methanol, dichloromethane, diethyl ether, acetonitrile, tetrahydrofuran and ethyl acetate were HPLC grade and were purchased from Rathburn (Walkerburn, U.K.). Triethylamine (99%), N,N-diisopropylethylamine and methoxylamine hydrochloride were obtained from Aldrich (Milwaukee, WI, U.S.A.). LiChroprep C_8 (40-μm particles), LiChroprep C_{18} (40-μm particles) and Kieselgel 60 (70–230 mesh) were purchased from Merck (Darmstadt, F.R.G.).

Samples

Blood samples (3 ml) from healty volunteers were collected in plastic syringes containing 200 μl of an EDTA solution (25 mg/ml). Subsequently, the blood samples were spiked with standard solutions of eicosanoids in acetonitrile, keeping the acetonitrile concentration of the resulting blood standards less than 1% by volume. Standards were prepared over a concentration range of 0–10 ng/ml. After mixing, 1-ml portions were pipetted into 2 ml of methanol (containing 10

ng of PGE_1 and 10 ng of $PGF_{1\alpha}$ as internal standards) under vigorous mixing. If necessary, the samples were stored at $-70°C$ until further analysis.

Extraction and purification

The denatured blood samples were acidified with acetic acid to pH 4.0–4.5, and 4 ml of chloroform were added. After vigorous mixing for 1 min, the mixtures were centrifuged at 4000 *g* for 10 min. The aqueous phase and the interphase were reextracted with 6 ml of methanol–chloroform (1:2, v/v). The organic phases were collected and dried under a stream of nitrogen at 40°C. The residue was dissolved in diethyl ether–acetic acid (100:0.5, v/v) and fractionated by silica gel chromatography according to Claeys et al. [17]. Small columns of 0.4 g of silica gel (Kieselgel 60, Merck) were prepared with Pasteur pipettes and were eluted stepwise after application of the residue to the column with 6 ml of each of the following mixtures of increasing polarity: *n*-hexane–diethyl ether–acetic acid (90:10:0.5, v/v/v; system A), *n*-hexane–diethyl ether–acetic acid (60:40:0.5, v/v/v; system B), diethyl ether–acetic acid (100:0.5, v/v; system C) and diethyl ether–methanol–acetic acid (90:10:0.5, v/v/v; system D).

Derivatization

The PG/TXB_2 fraction, eluted with solvent system D from the silica gel column, was dried under a stream of nitrogen and methoximated with 100 μl of methoxylamine hydrochloride in pyridine (5 mg/ml) at room temperature for 16 h. After removal of pyridine under a stream of nitrogen, 0.5 ml of water was added and the methoxime derivatives were extracted twice with 2 ml of freshly distilled dietyl ether. After removal of diethyl ether under a stream of nitrogen, the residues were dissolved in methanol and diluted with water to 15% (v/v) methanol. After acidification with hydrochloric acid to pH 3.5, the samples were applied to small reversed-phase chromatography columns, prepared in Pasteur pipettes containing 100 mg of LiChroprep C_8. After elution of the columns with 3 ml of methanol–water (15:85, v/v) and 3 ml of water, the PG/TXB_2 fraction was eluted with 4 ml of ethyl acetate and collected into a pointed glass tube. A small amount of water (ca. 0.5 ml) was allowed to settle out. The ethyl acetate layer was transferred into Biovials (Beckmann, Palo Alto, CA, U.S.A.), and the solvent was evaporated under a stream of nitrogen at 40°C. The residue was redissolved in 0.4 ml of acetonitrile and esterified with 75 μl of panacyl bromide in tetrahydrofuran (1.5 mg/ml) and 2 μl of triethylamine as the catalyst at 40°C for 2 h. The panacyl esters were purified by silica gel chromatography to remove excess of unreacted panacyl bromide, according to Watkins and Peterson [13]. Briefly, small columns of 0.4 g of silica gel (Kieselgel 60, Merck) were prepared with Pasteur pipettes and were eluted after the application of the sample with 3.5 ml of dichloromethane–methanol (100:1, v/v) to remove excess of unreacted panacyl bromide. The panacyl esters were recovered by elution with 3 ml of acetonitrile–methanol (85:15, v/v). After removal of the eluent under a stream of nitrogen at 40°C, the sample was dissolved in 100 μl of acetonitrile.

Assessment of linearity and sensitivity

The relationship between the amount of methoximated eicosanoid panacyl esters injected on-column and their relative peak heights was assessed as follows. To aliquots of 1 ml of blood, various amounts (25 pg to 50 ng) of 6-keto-$PGF_{1\alpha}$, TXB_2, $PGF_{2\alpha}$ and PGE_2 were added in the presence of 2 ng of the internal standards $PGF_{1\alpha}$ and PGE_1. After extraction and derivatization of the eicosanoids, the panacyl esters were solubilized in 100 μl of acetonitrile. Following injection of 50 μl of these samples and separation by HPLC, the absolute peak heights of the individual eicosanoids were transformed into relative peak heights by correction for recovery based on a peak height of 80 mm for the internal standards.

Instrumentation and chromatographic conditions

The HPLC system consisted of a Spectroflow 400 pump (Kratos Analytical, Ramsey, NJ, U.S.A.), a Rheodyne 7125 loop injector (Cotati, CA, U.S.A.) and a Perkin-Elmer LS-1 fluorescence detector (Beaconsfield, U.K.). A LiChrocart Superspher 100-RP-18 column (250 mm$\times$4 mm I.D., 4-μm particles) (Merck, Darmstadt, F.R.G.) was used in conjunction with a Chrompack guard column (75 mm$\times$2.1 mm I.D.) filled with pellicular RP-18 material (Chrompack, Vlissingen, The Netherlands). The mobile phase was, unless otherwise indicated, acetonitrile–water–acetic acid (72:28:0.1, v/v/v). To obtain optimal response of the fluorescence detector and a low noise level, the eluent was degassed daily by sparging helium gas for 15 min at a flow-rate of 250 ml/min for each 1000 ml of eluent. The injection volume was 20 μl (20% of the total sample volume), unless otherwise indicated, and the flow-rate was 0.5 ml/min. The fluorescence detector had the following settings: excitation wavelength, 360 nm; emission wavelength, 419 nm (cut-off).

RESULTS AND DISCUSSION

Sample extraction, derivatization and chromatography

A generalized flow scheme of the whole assay procedure is given in Fig. 1. The rationale and results of each individual step will be presented and discussed below.

Step 1. Whole blood samples rather than plasma samples were used since eicosanoids have been shown to bind considerably to various blood components, such as erythrocytes and fibrinogen [18,19]. According to Sinzinger et al. [19], EDTA was used as anticoagulant since the use of citrate or heparin both result in higher eicosanoid values in blood samples [19]. Repeated freezing and thawing of the blood samples should be avoided since this is an additional source of error in assessing prostaglandin levels. Moreover, if precipinated fibrin (after thawing) is removed by centrifugation, eicosanoid values might decrease significantly [19].

Step 2. For the reasons mentioned in step 1, immediately after collection of blood, the samples were denatured with methanol and were, if necessary, stored at -70°C or lower to maintain the initial levels of eicosanoids. A 12-ml volume of chloroform–methanol (2:1, v/v) appeared to be sufficient to extract quantitatively 1.06 ml of whole blood in EDTA. With ^{3}H-labelled 6-keto-$PGF_{1\alpha}$, TXB_2

Step

1 whole blood collection in EDTA

2 denaturation and extraction

3 silica gel column chromatography

4 methoxime formation

5 reversed-phase C_8 column chromatography

6 panacyl ester formation

7 silica gel chromatography

8 reversed-phase C_{18} HPLC with fluorimetric detection

Fig. 1. Flow scheme for the assay of 6-keto-$PGF_{1\alpha}$, $PGF_{2\alpha}$, $PGF_{1\alpha}$, PGE_2, PGE_1 and TXB_2 with HPLC on a reversed-phase C_{18} silica column with fluorescence detection.

and PGE_2, recoveries were found to be 91 ± 5, 92 ± 4 and $93 \pm 4\%$ during this step, respectively.

Step 3. Since extraction of whole blood resulted in rather "dirty" extracts, an additional purification procedure with silica gel column chromatography was required prior to derivatization and analysis. In addition, inclusion of silica gel chromatography allows the separation of PGs and TXB_2 from hydroxyeicosatetraenoic acids (HETE), di-HETE, non-esterified fatty acids, triglycerides, cholesterol esters and phospholipids [17]. Average recoveries of radioactivity for extraction and silica gel chromatography (steps 2 and 3) were 76, 69 and 77% for 6-keto-[^{3}H]$PGF_{1\alpha}$, [^{3}H]TXB_2 and [^{3}H]PGE_2, respectively.

Step 4. The rationale for the inclusion of the methoximation step in the extraction procedure was to obtain sharp, adequately separated peaks of the eicosanoids in the final HPLC assay. When a standard mixture of 6-keto-$PGF_{1\alpha}$, $PGF_{2\alpha}$, $PGF_{1\alpha}$, PGE_2, PGE_1 and TXB_2 was derivatized with panacyl bromide (step 6), deleting the preceding methoximation procedure, analysis by HPLC showed a broad TXB_2 peak interfering with the $PGF_{2\alpha}$ and $PGF_{1\alpha}$ peaks (Fig. 2). Since TXB_2 contains a hemiacetal ring instead of a cyclopentane ring, like other prostaglandins, this substance exists in an open and a closed form in equilibrium [20]. At an eluent pH of 3.8, the presence of these two forms of the TXB_2 panacyl ester resulted in a broad, poorly defined peak. When the eluent pH was raised from 3.8 to 4.9, an adequate separation between TXB_2 and $PGF_{2\alpha}$ was obtained, owing to a shift in the equilibrium between the open and closed hemiacetal ring of TXB_2 completely to the open form [20]. A disadvantage of the rise in pH was that 6-keto-$PGF_{1\alpha}$ was eluted as a small and tailing peak (data not shown). This undesirable effect could be overcome by addition of tetrahydrofuran to the elution fluid to a final concentration of 5% (by volume), with a concomitant reduction of the concentration of acetonitrile (Fig. 3).

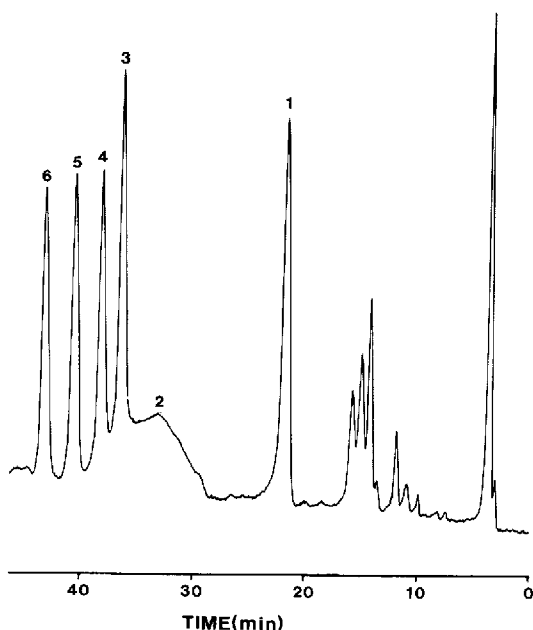
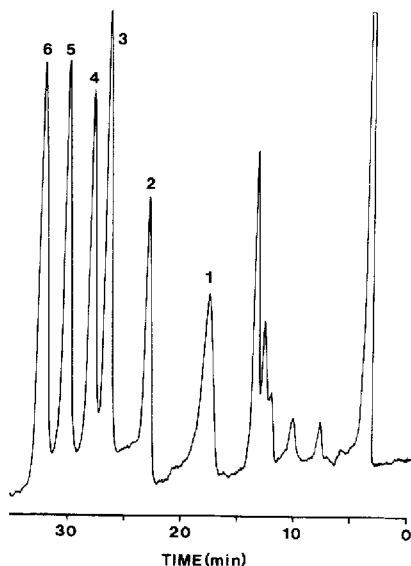


Fig. 2. Reversed-phase chromatogram of a mixture of standard eicosanoid panacyl esters. Each peak represents 4 ng of eicosanoid. The mobile phase was acetonitrile–water–actic acid (70:30:0.1, v/v/v) at pH 3.8. Peaks: 1 = 6-keto-$PGF_{1\alpha}$; 2 = TXB_2; 3 = $PGF_{2\alpha}$; 4 = $PGF_{1\alpha}$; 5 = PGE_2; 6 = PGE_1.

Injection of TXB_2 alone showed a rather tailing peak profile characterized by a stepping baseline (data not shown). Although this separation is acceptable for qualitative purposes, for quantitative determinations higher peaks with a smaller width are required. Another drawback of a direct derivatization of the extracted samples with panacyl bromide was the frequent appearance of an interfering peak with a retention time identical with that of 6-keto-$PGF_{1\alpha}$. An alternative for a complete separation of TXB_2 and $PGF_{2\alpha}$ is to form an oxime of TXB_2 [20]. Since $PGF_{2\alpha}$ does not contain a carbonyl group, it will elute with the same retention time, whereas the retention time of the oxime of TXB_2 will be prolonged. Therefore, a methoximation step was introduced before derivatization with panacyl bromide. Consequently the retention time of 6-keto-$PGF_{1\alpha}$ increased and the TXB_2 peak became sharp and symmetrical. Fig. 4 shows a complete separation of symmetrical peaks of various PGs and TXB_2.

Step 5. Reversed-phase column chromatography after the methoximation step was introduced to purify the methoxime. Since a reproducible derivatization with panacyl bromide was found to be possible only when the sample was eluted with ethyl acetate (see also ref. 15), C_8 in stead of C_{18} material had to be used. Elution with ethyl acetate gave recoveries of 100% and 40% for 6-keto-$PGF_{1\alpha}$ on C_8 and C_{18} material, respectively. When methoximation (step 4) was omitted, the reversed-phase column chromatography step remained necessary since the last elution step in the silica gel chromatography was performed with an eluent containing methanol and acetic acid, both substances interfering with the derivatization. In



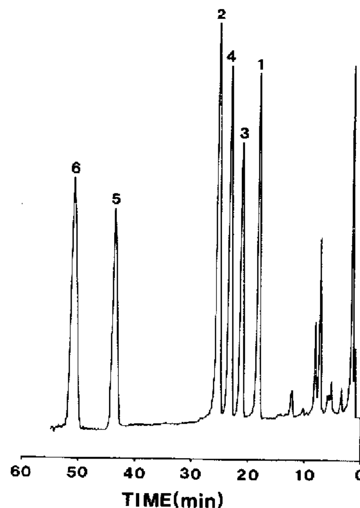


Fig. 3. Reversed-phase chromatogram of a mixture of standard eicosanoid panacyl esters. Each peak represents 4 ng of eicosanoid. The mobile phase was acetonitrile–tetrahydrofuran–water–acetic acid (65:5:30:0.1, v/v) at pH 4.9. Peaks: 1=6-keto-$PGF_{1\alpha}$; 2=TXB_2; 3=$PGF_{2\alpha}$; 4=$PGF_{1\alpha}$; 5=PGE_2; 6=PGE_1.

Fig. 4. Reversed-phase chromatogram of standard methoxime derivatives of eicosanoid panacyl esters. Each peak represents 4 ng of eicosanoid. The mobile phase was acetonitrile–water–acetic acid (72:28:0.1, v/v/v) at pH 3.8. Peaks: 1=6-keto-$PGF_{1\alpha}$; 2=TXB_2; 3=$PGF_{2\alpha}$; 4=$PGF_{1\alpha}$; 5=PGE_2; 6=PGE_1.

contrast, ethyl acetate did not influence the panacyl ester formation procedure. The overall recovery for the entire procedure (steps 1–5) was 65±6, 58±4 and 72±4% (n=5), when processing 25 pg of [^{3}H]6-keto-$PGF_{1\alpha}$, [^{3}H]TXB_2 and [^{3}H]PGE_2, respectively, in 1 ml of EDTA blood. When a range of 25 pg to 50 ng of eicosanoid in the presence of 25 pg of ^{3}H-labelled eicosanoid, added to 1 ml of blood, was processed, a constant recovery was found over the entire range (data not shown).

Step 6. For panacyl ester formation, triethylamine rather than diisopropylethylamine was used as the catalyst. Although both catalysts gave an almost complete conversion of the eicosanoids to their panacyl esters after 2 h at 40°C, in our hands triethylamine gave less interfering peaks in the chromatograms than diisopropylethylamine.

Step 7. The removal of the excess non-reacted panacyl bromide appeared to be absolutely necessary as was already indicated by Watkins and Peterson [13] and Cox and Pullen [21]. Addition of methanol to a final concentration of 1% (v/v) to the dichloromethane washing step increased the removal of unreacted panacyl bromide without loss of panacyl esters (data not shown). As a consequence there was less contamination of the HPLC column with panacyl bromide, resulting in a better signal-to-noise ratio.

Step 8. Although a sensitive analysis of panacyl esters of PGs other than PGE_2 derivatives would be possible using normal-phase HPLC with fluorescence detection, as was proposed by Cox and Pullen [21], we did not succeed in developing a stable and reproducible analytical system based on normal-phase chromatography. The major complication was the interference by the panacyl bromide derivatizing agent. Therefore, for PG profiling applications, an HPLC system based on reversed-phase chromatography was elaborated. For an optimal separation between $PGF_{2\alpha}$, $PGF_{1\alpha}$ and TXB_2, a relatively long column (250 mm×4 mm I.D.) filled with 4-μm particles was necessary. As a consequence, the retention times of PGE_2 and PGE_1 became relatively long, but could be reduced by increasing the concentration of acetonitrile in the element from 71 to 75% (v/v) by a stepwise gradient, starting after the appearance of the TXB_2 peak (data not shown). It has to be noticed that with reversed-phase HPLC the low detection limit for PGE_2 could not be achieved as reported by Cox and Pullen [21], owing to a higher quenching of the fluorescence signal by the polar components of the eluent.

Linearity

The linearity of the fluorescence detector response versus the blood concentration of the various prostaglandins and TXB_2 standards added was evaluated by extracting and analysing a range of 0 pg/ml to 50 ng/ml of each eicosanoid. Each amount was measured in quadruplicate. In this range, plots of relative peak heights versus picograms of eicosanoid derivatized were linear. Regression lines relating the relative peak height (y) to pg of PG injected on column (x) were: 6-keto-$PGF_{1\alpha}$, $y=3.25+4.40\ x$ ($r=0.997$); TXB_2, $y=8.76+5.37\ x$ ($r=0.999$); $PGF_{2\alpha}$, $y=-0.01+4.83\ x$ ($r=997$); PGE_2, $y=-2.29+3.64\ x$ ($r=0.998$). Since the samples were individually extracted, purified and derivatized for this experiment, the results indicate that the procedure has a constant efficiency that is independent of the amount of eicosanoid derivatized over the indicated range.

Sensitivity

The lower limit of detection under these assay conditions was estimated to be 50 pg of 6-keto-$PGF_{1\alpha}$, TXB_2 and PGE_2, and 100 pg of PGE_2 on-column. These values were estimated from the regression curves constructed to assess the linearity of the assay (see above). With a short-term (1 min) noise level of 1.8 mm, a peak height of 5 mm (signal-to-noise ratio of 3:1) was considered to represent the lower limit of detection. These values corresponded to an overall limit of detection of 100 and 200 pg/ml of blood. When the final sample volume is decreased from 100 to 50 μl, a lower limit of detection can be achieved of 50 pg/ml of blood for 6-keto-$PGF_{1\alpha}$, TXB_2 and $PGF_{2\alpha}$ and of 100 pg/ml for PGE_2. Further gains in assay sensitivity require the processing of larger volumes of blood. This feature has not been explored in detail. It has to be remarked that in non-spiked blood, eicosanoids could not be detected. This is in accordance with the observations that under physiological conditions the concentrations of 6-keto-$PGF_{1\alpha}$, TXB_2, $PGF_{2\alpha}$ and PGE_2 in human blood are below 20 pg/ml [18,22–24].

Stability

The stability of the methoxime derivatives of eicosanoid panacyl esters stored at −20°C was high. There was no loss in peak area after 180 days of storage at this temperature.

CONCLUSION

The applicability of an HPLC fluorescence assay was demonstrated for the analysis of various PGs, including TXB_2, added to human whole blood. The internal standards for this analysis were $PGF_{1\alpha}$ and PGE_1. When PGE_1 has to be detected, 15-methyl-PGE_2 can be used as the internal standard. The samples were prepared for analysis by a combination of extraction, silica gel chromatography, methoximation, reversed-phase chromatography and esterification with panacyl bromide. Complete separation was obtained for 6-keto-$PGF_{1\alpha}$, $PGF_{2\alpha}$, $PGF_{1\alpha}$, TXB_2, PGE_2 and PGE_1. The lower detection limit (signal-to-noise ratio of 3:1) of the method was 50 pg/ml of blood for 6-keto-$PGF_{1\alpha}$, $PGF_{2\alpha}$ and TXB_2, and 100 pg/ml of blood for PGE_2, when processing 1 ml of whole blood. The precision of the method at 250 pg/ml was ca. 5%, and linearity was demonstrated for a range from 50 pg to 50 ng of eicosanoid panacyl ester injected on-column. Although similar HPLC methods have been described with picogram level sensitivity for PGs [13,15], there are no reports dealing with the assessment of low pg/ml levels of PGs including TXB_2 in complex biological samples such as whole blood.

ACKNOWLEDGEMENTS

The authors are indebted to Dr. W. Morozowich and Dr. J. Cox for providing panacyl bromide and for helpful advice, to G. Kivits for his help with the silica gel chromatography and to Dr. D. Nugteren for critically reading the manuscript. This study was supported by Medigon/ZWO.

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