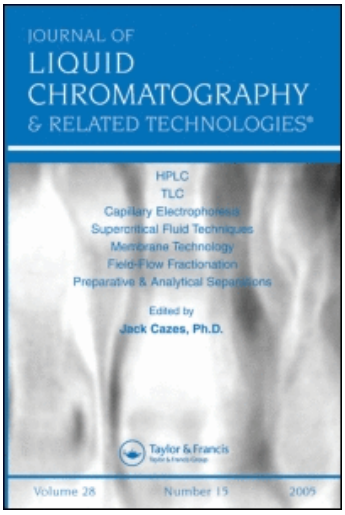

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MEASUREMENT OF PROSTAGLANDINS FROM BIOLOGICAL SAMPLES IN THE SUBNANOGRAM RANGE BY FLUORESCENCE LABELLING AND HPLC SEPARATION

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ABSTRACT

Small amounts of different prostaglandins can be detected in the subnanogram range after derivatisation with the reactive fluorescence dye 9 - anthroyldiazo - methane (ADAM), followed by specific purification and HPLC separation. This method allows the measurement of PGE2 from supernatants of the murine macrophage - like cell line P388 D1 with quantitative recovery.

INTRODUCTION

Prostaglandins are an important family of arachi - donic acid metabolites, synthesized by different tissues and involved in the regulation of cell - cell inter - actions (1), including immunoregulation (2) and inflam -

mation (2,3). It is well documented that prostaglandins are produced by macrophages and macrophage - like cell lines after several stimuli (4,5).

Several analytic methods are well established for the detection of prostaglandins, for example gas chroma - tography (GC), thin layer chromatography (TLC), high pressure liquid chromagraphy (HPLC) and radioimmunoassay (RIA). Although the RIA is a very sensitive and simple technique, specificity is often uncertain and allows only the detection of one single prostaglandin. An additional method for verifying RIA results is therefore often needed. Chromatographic techniques allow the detection of different prostaglandins simultaneously. Use of GC is hampered as it requires complicated derivatisation methods, and TLC failes in the sufficient separation of the main prostaglandins and in sensitivity. Although HPLC separation is superior to the other methods, HPLC is also low sensitive when using UV - absorbance of the separated prostaglandins for detection. In the absorbance range of 204 - 210 nm, the commonly used solvents show UV - cut off and many interferences from biological samples have also absorbance in this range.

Different approaches to solve the problem of sensi- tivity of HPLC have been published. Prostaglandins have been derivatised with strong UV - absorbing molecules like phenacylbromide (6) or, with better sensitivity,

with fluorescence dyes like 4 -bromomethyl - 7 - methoxy-coumarine (7), 3 - bromomethyl - quinoxalinone (8), Panacylbromide (9) and 9 - anthroyldiazomethane (ADAM) (10, 11). However, the amounts of prostaglandins in biological material are very low. It has still been a problem to handle such small quantities in a derivati - sation procedure and to detect them in the high back - ground of impurities from biological samples.

Our aim was to establish a procedure allowing us to extract very low levels of prostaglandins from superna - tants of cell cultures and to measure them by HPLC after derivatisation. The described procedure is able to detect prostaglandins from biological samples as low as 100 picogramm with quantitative recovery.

MATERIALS

Prostglandins were purchased from Sigma, Deisen - hofen, FRG, and tritiated prostaglandins from NEN, Drei - eich, FRG.

9 - anthraldehyde, activated manganese dioxide and hydrazine hydrate were from Aldrich, Steinheim, FRG.

RPMI - medium 1640 and PBS (Dulbecco, without calcium and magnesium,) were from Biochrom, Berlin, FRG, fetal calf serum (FCS) from Gibco, Karlsruhe, FRG and Costar 24 well flat bottom plates were from Tecnomara, Fernwald, FRG.

Merthiolate (N - ethylmercurythiosalicylate) was from Sigma, Deisenhofen, FRG.

Extraction equipment

1 ml Bond Elute extraction columns with 5 um ODS - or 5 um silicate material were from Analytichem, Harbour City, Ca, USA. All solvents were HPLC - grade and purchased from Baker, Groß - Gerau, FRG.

HPLC - equipment

A HPLC system LC 41 from Bruker - Franzen, Bremen, FRG, was used. The fluorescence detector RF 530 was from Shimadzu, Düsseldorf, FRG. The column was from Bischoff, Leonhard, FRG, with the dimension of 4.6 mm I.D., 250 mm length and filled with 5 um nucleosil ODS - silica. The radioactivity detector was from Packard, Downers Grove, IL, USA. All solvents, including the Rialuma scintil - lation cocktail, were HPLC - grade and purchased from Baker, Groß - Gerau, FRG.

Methods

9 - anthroyldiazomethane synthesis

Although ADAM is now commercially available (Funakoshi, Tokyo, Japan, or Molecular Probes, Eugene, OR, USA.), we synthesized it because ADAM is very unstable and transport may be a great problem. We modified a reported method (12). 10 g of 9 - anthral -

dehyde were dissolved in 150 ml of ethanol and stirred with 10 g of 80 % hydrazine hydrate at room temperature for 4 hours. The solvent changed its colour from yellow to dark yellow and solid crystalls of 9 - anthroyl - hydrazone fell out. The crystalls were filtered and dried. 1 g of the hydrazone was dissolved in 100 ml anhydrous diethylether and stirred together with 1 g of activated manganese dioxide and 0.5 ml of a saturated potassium hydroxide solution in ethanol for 3 hours. The manganese dioxide was filtered and the eluate solution was dried. The red crystalls (0.8 g) were identified as 9 - anthroyldiazomethane with UV -, fluorescence - and IR - spectra (data not shown).

Cell stimulation

200000 cells from the P 388 D1 cell line were cultered overnight in 1 ml RPMI - medium 1640 with 0.5 % FCS in 24 well flat bottom plates. The next day, the adherent cells were washed twice with 1 ml PBS and finally 1 ml of RPMI - medium with 0.5 % FCS was given to the cells again. Different concentrations of merthiolate were added for an incubation time of one hour to the cells.

Prostaglandin extraction

The cell - supernatants were centrifuged at 12000 g for 10 minutes at 4 °C, acidified to pH 5.5 with 1 %

acetic acid and applied on the Bond Elute columns ODS 5 um 1 ml volume, which had been prewashed with 2 ml of diethylether, 2 ml methanol and equilibrated with 2 ml of water with 0.0001 % acetic acid adjusted to pH 5.5 with 1 % sodium hydroxide solution. The supernatants had to ooze through the columns without being sucked in order to avoid bad recoveries. The columns were washed with 2 ml water pH 5.5 and with 2 ml of a mixture of 85 % water/ 15 % methanol with an apparent pH 5.5, adjusted in the above described manner. Then the columns were sucked to dryness for 2 minutes under vaccuum. Finally the columns were washed with 2 ml of petrolether and eluted with 2 ml of diethylether.

Derivatisation

The diethylether extracts of standards or cell - supernatants were dried under a stream of nitrogen in Eppendorf polypropylene vials and the prostaglandins were resolved in 20 ul of methanol. 20 ul of ADAM (1 mg/ml in diethylether) were added to these solutions. These mixtures were allowed to react at 37 °C for 6 hours in the darkness.

Purification of the Prostaglandin - ADAM - Derivates

The reaction mixture of the ADAM derivatisation was dried under a stream of nitrogen and redissolved in 200

ul chloroform. This solution was loaded on 1 ml Bond Elute columns with 5 um silica material. The columns were prewashed first with 2 ml of a mixture of 15 % methanol, 85 % acetonitrile, then with 2 ml of acetone and equili - brated with 2 ml of chloroform. After applying the samples on the columns, they were washed with 3 ml of chloroform to remove ADAM impurities. The ADAM - prosta- glandin - esters were eluted from the columns with 2 ml of a mixture of methanol and acetonitrile (15:85, v/v). The eluat was dried under a stream of nitrogen, resolved in 50 ul of the eluent and diluted with 50 ul water prior to HPLC separation.

HPLC procedure

The mobile phase consisted of 62 % acetonitrile, 37.9 % water and 0.1 % acetic acid, adjusted to an apparent pH of 5.5 with sodium acetate. The flow rate was 1 ml/min. Fluorescence excitation was adjusted to 367 nm and emission to 413 nm. Tritiated prostaglandins were measured by collecting fractions of the eluent and determining their radioactivity in a Packard β - counter.

RADIOIMMUNOASSAY (RIA)

The RIA was performed as described elsewhere in detail (16).

RESULTS

Extraction conditions

On the basis of a described procedure (13), we developed a purification method for prostaglandins useful for later derivatisation with ADAM. To have a good re - tention of prostaglandins on the Bond Elute ODS columns, mild acidification of the supernatants is necessary as well as to avoid the sucking of the samples through the columns. To prevent acid destruction of ADAM in the fol - lowing derivatisation procedure, a pH of 5.5 was found to be optimal in prostaglandin extraction. The samples were purified by washing the columns after applying the probe with 2 ml of water and with 2 ml of a mixture of water and methanol (85/15, v/v) to remove very polar components and by washing with 2 ml of petrolether to remove very unpolar components. Prostaglandins were retained on the ODS - columns under these conditions and were eluted with diethylether.

Reaction conditions and purification

To minimize impurities, it is essential to use only small amounts of ADAM in the reaction mixture. We found that the best conditions for esterification of prosta - glandins with small quantities of ADAM were a reaction solution of one part of methanol and one part of diethyl- ether (14) and mild temperature for 6 hours. The reaction

rates were measured by esterification of tritiated prosta glandins with ADAM and separating them from unesterified prostaglandins by HPLC under the conditions described above. The coupling rates were always in the range of at least 90 %. The same results were achieved by adding tritiated prostaglandins to biological probes or to prostaglandin standards. By measuring temperature kinetics, time kinetics and ADAM concentration kinetics (Figure 1 for PGE2, same results for other prosta - glandins, data not shown) it was found to be optimal to dissolve prostaglandin extracts in 20 ul methanol, add 20 ul of a solution of ADAM in diethylether (1 mg/ml) and let the mixture react at 37 °C for 6 hours. Eppendorf polypropylene vials showed no surface adsorbance of pro - staglandin - ADAM - esters.

To minimize impurities in the later chromatographic separation, the prostaglandin - ADAM - esters were purified using Bond Elute silica columns. The polar esters were retained on the column and could be separated from unpolar unreacted ADAM and impurities with chloro - form as washing liquid. Finally the coupled prosta - glandins were eluted by a mixture of methanol and aceto - nitrile (15/85, v/v).

Recovery of prostaglandins

To determine the recovery of prostaglandins after the extraction, derivatisation and purification before

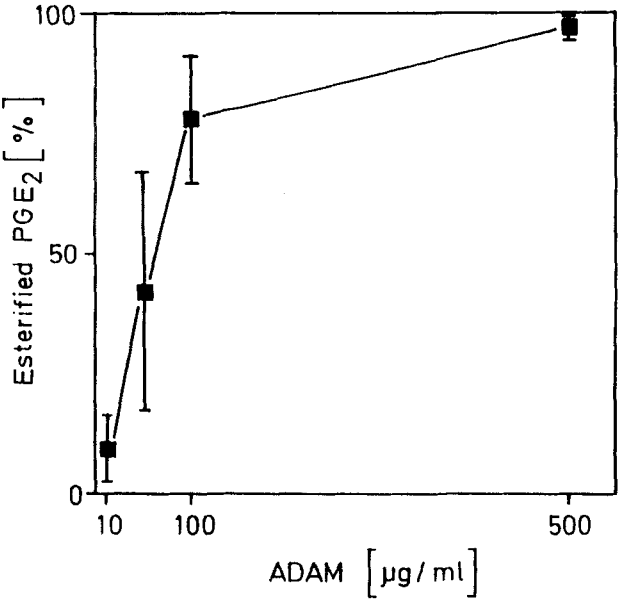

FIGURE 1

PGE2 esterification rate with ADAM in dependence of ADAM concentration in the reaction mixture. The reaction temperature was 37 °C and the reaction time was 4 hours. n = 4

HPLC - separation, tritiated prostaglandins were given to the cell supernatants. Recovery was calculated by measuring radioactivity in the supernatants and in the ADAM - derivatisation mixture in a Packard β - counter. As shown in table 1, recoveries were in the range of 90 % for all prostaglandins.

HPLC and Calibration

The samples were extracted, derivatised and purified in the described manner and then separated by

TABLE 1

Percent Recovery of 3H-Prostaglandins

Prostaglandin	% Recovery	Deviation
6-keto-PGF1$_\alpha$	81.2	+- 5.6
PGF2$_\alpha$	84 .6	+- 7.2
PGE2	88.1	+- 3.3
PGD2	91.4	+- 5.1
TXB2	78.9	+- 8.2

Aliquots from the supernatants and from the HPLC - injection samples were counted. Recovery studies were done for each single prostaglandin with 3 - 5 experi - ments. The radioactivity prior to derivatisation was set to 100 %. Care was taken to have equal quench in all probes.

HPLC and detected by fluorescence. Good separation of prostaglandin -ADAM - esters were found by using a mobile phase consisting of 62 % acetonitrile, 37.9 % water and 0.1 % acetic acid, adjusted to pH 5.5 with sodium acetate The prostaglandin esters were identified by their re - tention times. To verify the identification of prosta - glandins, trace amounts of tritiated prostaglandins were given to the prostaglandin standards or biological samples prior to derivatisation. Tritiated prostaglandin - ADAM - esters showed identical retention times compared to standards or biologically formed prostaglandins (Fig. 2). From each prostaglandin 0.1, 1, 10, 50 and 100 nano-

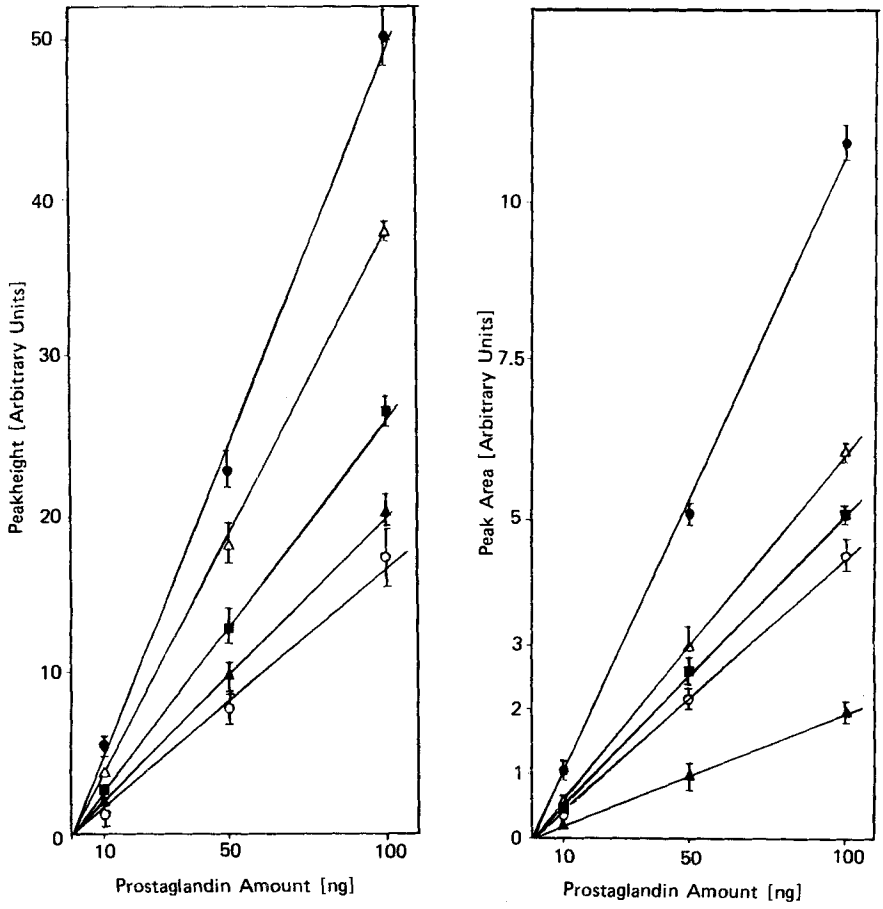

FIGURE 2

Prostaglandin weights prior to derivatisation were plotted against their peakheight and peak area. The column was ODS - silica, the mobile phase consists of acetonitrile : water : acetic acid (62 : 37.9 : 0.1, v/v) pH 5.5, the flow rate was 1 ml/min. Fluorescence was measured with an excitation of 367 nm and an emission of 416 nm. ▲ 6 - keto - $F1_{\alpha}$, Δ $PGF2_{\alpha}$, ○ TXB2, ■ PGE2, ● PGD2 .

TABLE 2

Calibration Data for Prostaglandin - ADAM - Esters

Peak Height

ng	6 keto $F1_\alpha$	$PGF2_\alpha$	TXB2	PGE2	PGD2
0.1	40	50	45	50	60
1.0	241	405	188	325	605
10.0	2162	3946	1674	2707	5217
50.0	9802	18133	7936	12940	23084
100.0	21064	38002	17255	26355	50049
Corr. coeff.:	0.998	0.998	0.997	0.998	0.999

Peak Area

0.1	220	650	410	490	920
1.0	2030	6201	4024	4955	10154
10.0	19255	61392	42271	50950	105206
50.0	98368	310472	219078	255593	510047
100.0	190540	601167	444829	510649	1094006
Corr. coeff.:	0.998	0.998	0.997	0.998	0.999

The weights of prostaglandin standards prior to derivatisation were correlated with peak height and peak area of derivatised prostaglandins determined by HPLC - separation and fluorescence detection. The table shows the mean of 4 different experiments, maximal deviation was 35 % in the low concentration range. ODS - column 250 mm 4.6 mm ID, mobile phase acetonitrile : water : acetic acid (62 : 37.9 : 0.1, v/v) pH 5.5, flow rate 1 ml/min. Excitation 367 nm, emission 416 nm.

gram in RPMI medium with 0.5 % FCS were used. The peak areas and peak heights were plotted against amounts of prostaglandins. The calibration curves were linear in the observed range (Table 2 and Figure 3).

Stimulation of Prostaglandin Synthesis in Macrophage Tumor Cells

In relation to control cells, P 388 D1 cells show enhanced release of PGE2 after exposure to merthiolate

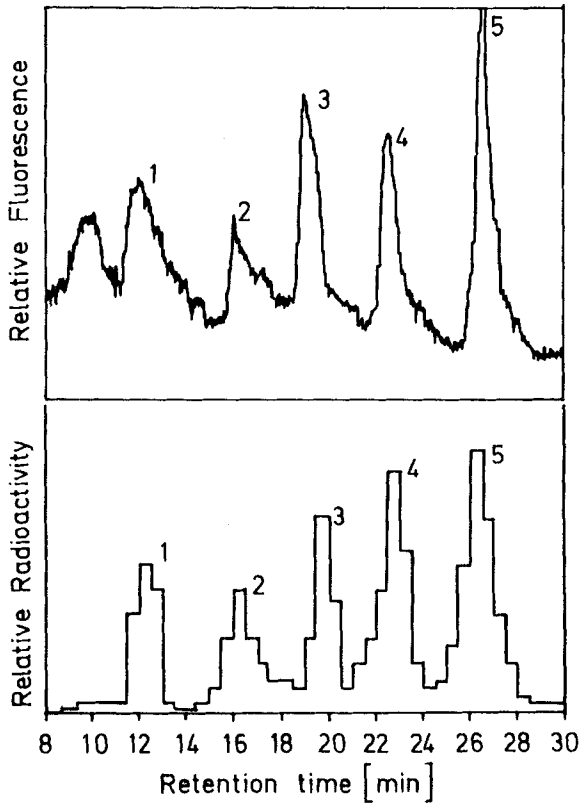

FIGURE 3

1 ng of PG - 6 - keto - $F1_{\alpha}$ (1), TXG2 (2), $PGF2_{\alpha}$ (3), PGE2 (4), PGD2 (5) and trace amounts of tritiated prostaglandins were esterified with ADAM after extraction from RPMI 1640 - medium with 5 % FCS. HPLC - separation was done with a ODS -silica column with 250 mm 4.6 mm ID and a mobile phase consisting of acetonitrile : water : acetic acid (62 : 37.9 : 0.1, v/v) with pH 5.5. Fluorescence was measured with an excitation of 367 nm and an emission of 416 nm. Radioactivity of fractions was determined in a Packard β - counter.

(Figure 4). The supernatants of 1 million cells were divided and one part was measured for PGE2 by RIA (15), the other part by HPLC. By the RIA in the control 1 ng and 6 ng after merthiolate treatment (50 uM) for 1 hour were detected. The HPLC quantification showed 0.7 ng and 4.5 ng PGE2. $PGF2_{\alpha}$ and PGD2 were not detectable in this experiment. It is difficult to achieve this result with a RIA alone, because prostaglandin antibodies often show cross reactivity to other prostaglandins.

DISCUSSION

Prostaglandins can be derivatised by esterification of their carboxylic group. Fluorescence dyes thereby allow better detection limits than absorbing dyes. Bromo-activated dyes like 4 - bromo -7 - methoxycoumarin (7), 7 - bromo -quinoxalinone (8) and panacylbromide (9) need catalysts like diisopropylethanolamine or di - benzo - crown - 6 in combination with powdered potassium carbonate for their reaction with prostaglandins. Our own experiments with these dyes showed that catalysts and high amounts of the bromo - activated dyes had to be used in the reaction mixture because these dyes are not very reactive and the reaction mixtures had to be heated to 60 °C or more. By using lower concentrations of dyes and catalysts to reduce impurities or mild conditions in order to avoid prostaglandin destruction, we found low

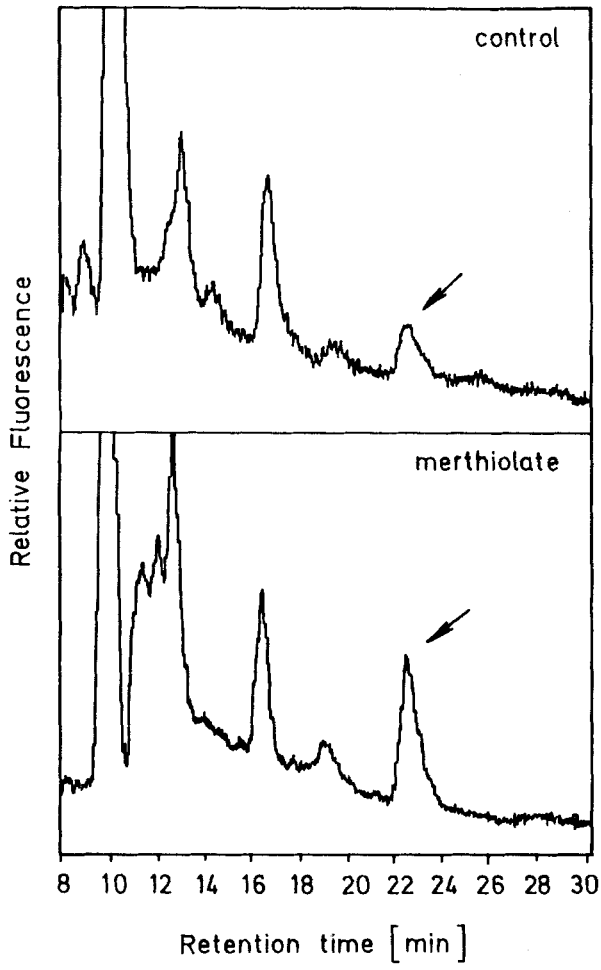

FIGURE 4

1 million P 388 D1 cells were incubated for one hour in RPMI 1640 medium with 0.5 % FCS (control) or additional exposed to 50 uM merthiolate (mer - thiolate). Then the supernatants were extracted and the derivatisation and purification procedure were performed. HPLC - separation was done on a ODS - silica - column 250 mm 4.6 mm ID and a mobile phase of acetonitile : water : acetic acid (62 : 37.9 : 0.1, v/v). The flow rate was 1 ml/min. Fluorescence was measured with an excitation of 367 nm and an emission of 416 nm. PGE2 peaks are marked with an arrow.

and unreproducable coupling rates. For these reasons, these dyes failed, in our hands, to esterify very small amounts of prostaglandins.

By using ADAM with the high reactive diazo - group for esterification, the reaction could be done without the need of a catalyst under mild reaction conditions and with small amounts of the dye itself. The result was a reducing of the impurities. Nevertheless it was necessary to clean up the reaction mixture after derivatisation. It has been shown (10, 11) that this could be done by gel permeation chromatography and post - column collecting of fractions to separate prostaglandin - ADAM - esters from impurities, but this method is very complicated and limits the number of probes to be analysed. Simple solid phase extraction on Bond Elute silica columns, where prostaglandin - ADAM - esters are retained and unpolar ADAM and impurities are eluted with chloroform, is also possible. The prostaglandin esters could then be eluted with a mixture of methanol and acetonitrile (15/85, v/v).

The described procedure allows to reach nearly the same detection limit with prostaglandins from biological samples as with prostaglandin standards by reducing the background of impurities by a multi - step sample puri - fication method and by using only small amounts of a high reactive dye to minimize derivatisation dependend sample soiling, followed by a second purification step.

With the demonstrated HPLC analytic method it is possible to show that exposure of P 388 D1 cells to merthiolate enhances production of PGE2 without producing $PGF2_{\alpha}$ or PGD2 in a similar quantity. This is consistent with the results of other investigators (15, 16), who proposed that merthiolate induces prostaglandin synthesis by blocking the reacylation of free arachidonic acid into cellular lipid pools. By this way, the concentration of free arachidonic acid in the cells is increased and the higher availability of substrate enhances the prostaglandin synthesis (17).

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