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QUANTITATIVE ANALYSIS OF PROSTAGLANDINS IN CELL CULTURE MEDIUM BY HIGH-RESOLUTION GAS CHROMATOGRAPHY WITH ELECTRON-CAPTURE DETECTION

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SUMMARY

Prostaglandins have been shown to be important modulators of haemostatis, immune responses, and growth of normal and neoplastic cells. In order to investigate the cell origin and metabolic profile of the endogenous prostaglandins in human tumours, a convenient extraction and gas chromatographic method for measuring the various classes of prostaglandins was developed. Infiltrating macrophages from human tumours were isolated using adherence to plastic. Macrophage-enriched and macrophage-depleted cell populations were then cultured in vitro and the media supernatant was studied for the presence of prostaglandins E_1, E_2, $F_{2\alpha}$, and 6-keto-$F_{1\alpha}$ (the spontaneous breakdown product of prostacyclin, PGI_2). Routinely, 1 ml of medium containing 10^6 cells was studied. The eicosanoids were extracted using commercially available octadecylsilyl silica reversed-phase columns prior to derivatization. Standards and samples were prepared as pentafluorobenzyl ester (methoxime) trimethylsilyl ether derivatives for analysis on an OV-101 (25 m × 0.2 mm) fused-silica capillary column. Recovery of standards ranged from 93% to 37%, with linear recovery in all instances (regression coefficients greater than 0.98). Detection limits were 20 pg for each of the prostaglandins. Analysis of cell subpopulations from six human tumours revealed that infiltrating macrophages produce various prostaglandin profiles and are largely responsible for the prostaglandin production in human cancer. The described analytical method is the first application of high-resolution gas chromatography with electron-capture detection to the quantitative profiling of prostaglandins from human cell culture.

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INTRODUCTION

As the diversity of effects mediated by the various prostaglandins (PGs) becomes increasingly apparent, the need for a routine method capable of the simultaneous, quantitative determination of the profile of PGs is heightened. In biological systems, the different classes and types of PGs may cause different or opposing actions [1], thereby necessitating the simultaneous measurement of multiple PGs in the experimental setting. Additionally, the physiological concentration of PGs is in the nanomolar range, imposing strict demands on the sensitivity of the analytical method.

Detailed gas chromatographic profile analyses of PG standards have been done previously. Fitzpatrick [2] demonstrated the resolution of 24 different PG and thromboxane standards using glass capillary gas chromatography. This method was also used to study the metabolism of exogenously added PGH_2 by fibroblasts and lymphocytes in culture [3]. A similar method was used to analyze both cyclooxygenase and lipoxygenase products of arachidonic acid metabolism in platelets, endothelial cells, and mouse peritoneal macrophages [4]. Capillary column resolution of the various eicosanoids has been developed; however, quantitative measurements are not available. Arachidonic acid metabolism by mouse macrophages has been investigated using capillary chromatography and selected ion monitoring [5]; yet this methodology has also remained strictly qualitative in nature. Despite numerous developments in the analysis of PGs and other arachidonic acid metabolites by high-resolution gas chromatography, successful quantitative measurements from biological samples using this technology have yet to be performed.

We have developed a routine procedure for the quantitative analysis of a series of cyclooxygenase products using conventional fused-silica capillary gas chromatography with electron-capture detection. The method was employed for the detailed analysis of PG production in cell cultures derived from cells of cancer patients.

EXPERIMENTAL

Materials

Prostaglandin standards, obtained from Upjohn (Kalamazoo, MI, U.S.A.), were weighed, then dissolved in 70% ethanol; aliquots were stored in polypropylene tubes at −70°C. Organic solvents were of analytical grade, and purchased from Fisher Scientific (Fair Lawn, NJ, U.S.A.). Pentafluorobenzyl bromide and bis(trimethylsilyl)trifluoroacetamide were obtained from Pierce (Rockford, IL, U.S.A.). Methoxylamine · HCl in pyridine (4%, w/v) was purchased from Supelco (Bellefonte, PA, U.S.A.). Diisopropylethylamine was supplied by Aldrich (Milwaukee, WI, U.S.A.). Methyl formate was purchased from Eastman Kodak (Rochester, NY, U.S.A.). McCoy's 5A culture medium was supplied by Gibco (Santa Clara, CA, U.S.A.), and foetal calf serum was purchased from Flow Labs. (Inglewood, CA, U.S.A.). All in vitro culture was done using McCoy's 5A supplemented with 10% (v/v) foetal calf serum, which had been heat-inactivated at 56°C for 1 h. Tritiated prostaglandin E_2 {[5,6,8,11,12,14,15-^{3}H(N)]PGE_2, specific activity 160 Ci/m*M*} was obtained

from New England Nuclear (Boston, MA, U.S.A.). Bond Elut, octadecylsilyl silica columns, were purchased from Analytichem International (Harbor City, CA, U.S.A.).

Apparatus

Collected eluates from extractions were dried using a Speed-Vac Concentrator (Savant Instruments, Hicksville, NY, U.S.A.). Gas chromatography was performed by microlitre injection with a Hamilton microlitre syringe, Model 701N (Pierce) into a Hewlett-Packard Model 3700 gas chromatograph equipped with a purged splitless injector and ^{63}Ni-electron-capture detector (Hewlett-Packard, Palo Alto, CA, U.S.A.). A fused-silica capillary column, 25 m × 0.2 mm, OV-101 WCOT, was obtained from Scientific Glass Engineering, Ringwood, Victoria, Australia). Instrument operating conditions were: injector temperature, 250°C; detector temperature, 300°C; column oven temperature programme: initial temperature, 50°C, maintained for 2 min after injection, followed by heating at 20°C/min to 200°C, immediately continuing at 10°C/min to 250°C, which was held isothermally for the remainder of the run. A post-run heating cycle of 300°C for 5 min was found to optimize the reproducibility of the chromatographic runs. Helium carrier gas was used at sufficient pressure to produce a linear column flow-rate of 28 cm/sec at 250°C. Make-up gas of argon—methane (90:10) was introduced at the detector base using a flow-rate of 20 ml/min. The detector signal was plotted on a strip chart recorder, as well as interfaced to a HP 3390A signal integrator (Hewlett-Packard).

Sample collection, preparation and storage

Human tumour ascites specimens were collected by paracentesis into preservative-free heparin (1000 units/ml). The cells were isolated by centrifugation at 900 *g* for 10 min, then washed once in culture medium. Cells were separated on the basis of adherence to plastic and phagocytosis of carbonyl iron particles. These methods generate subpopulations of cells depleted of macrophages (non-adherent and non-adherent—non-phagocytic) and a cell population enriched for macrophages (adherent). Cell-free supernatants from 24-h incubations of the collected cells were isolated by centrifugation, then stored at −70°C.

Sample clean-up

Prostaglandins were extracted from the culture medium by reversed-phase chromatography on octadecylsilyl silica [6]. A 1-ml volume of the supernatant was adjusted to pH 4.0 with 4% (v/v) formic acid. The sample was applied to a methanol-, and water-conditioned Bond Elut C_{18} column, which was slowly rinsed under vacuum sequentially with 10 ml each of distilled water, 15% ethanol, and hexane. The PGs were eluted into silanized conical tubes using two 0.5-ml flushes of methyl formate. The collected eluents were dried under vacuum by means of a Speed-Vac concentrator.

Recovery of prostaglandins from culture medium

To determine extraction recovery of the prostaglandins, [^{3}H]PGE_2 (25 pg) was added to 1 ml of culture medium, and processed using the extraction

protocol. Final recovery of the labelled PGE_2 was determined by scintillation counting, and compared to counts from the original aliquot. Additionally, authentic PG standards were used to verify the extraction recovery from culture medium. High-resolution gas chromatography of derivatized aliquots from standards and extracted standards was performed both to determine the purity of the extracted material and to measure the linearity of recovery.

Derivatization and sample analysis

For chromatographic stability and electron-capture response, the PGs were converted to their pentafluorobenzyl ester trimethylsilyl ether derivatives [7]. To secure stability of the keto group at the C-9 position of PGE_1, PGE_2, and 6-keto-$PGF_{1\alpha}$, a step producing the methoxime derivative was included prior to silylation [8]. Dried residues from extraction and standards were dissolved in 10 μl of pentafluorobenzyl bromide in acetonitrile (1:2, v/v) and 10 μl of diisopropylethylamine in acetonitrile (1:7, v/v), and the reaction vessel was securely capped. The solution was heated at 40°C for 5 min. The reactants were taken to dryness under nitrogen, and the esterification step was repeated again. To the dried residue were added 50 μl of methoxylamine · HCl in pyridine (4%, w/v), and the vial was heated at 60°C for 1 h, followed by evaporation under nitrogen. Sample purity was improved by partitioning the derivatized products in hexane (1 ml) and water (pH 3, 1 ml) twice. The hexane phases were combined and dried. Trimethylsilyl ether derivatives were made by reaction with 100 μl of bis(trimethylsilyl)trifluoroacetamide [9]. Routine chromatography was done after evaporating the silylating agent and dissolving the analyte in 100 μl of hexane to reduce the degree of solvent tailing. Verification of the final derivatized PGs was done by mass spectral analysis of standards.

Quantitation and peak identification

Precision of the assay was evaluated by repeated injections of standards. Linearity of extraction was measured by the analysis of diluted PG standards in culture medium, followed by the routine extraction and derivatization procedures. Peak identification was based on the retention times of sample chromatograms compared with standards which were run daily.

RESULTS

The mass spectrum for each prostaglandin standard was consistent with the fragmentation pattern of the anticipated derivative, as well as with reports in the literature [8].

A representative chromatogram from a 350-pg injection of each of the PG standards is displayed in Fig. 1. The major chromatographic peaks for PGE_1, PGE_2, $PGF_{2\alpha}$, and 6-keto-$PGF_{1\alpha}$ are clearly resolved from one another, and demonstrate good peak shape. The precision of the assay was determined by repeated 450-pg injections of the standards. The retention times and peak heights measured are presented in Table I. Retention times for the four prostaglandins tested are constant, with a coefficient of variation for each below 0.03%. The detector response to repeated injections of the same

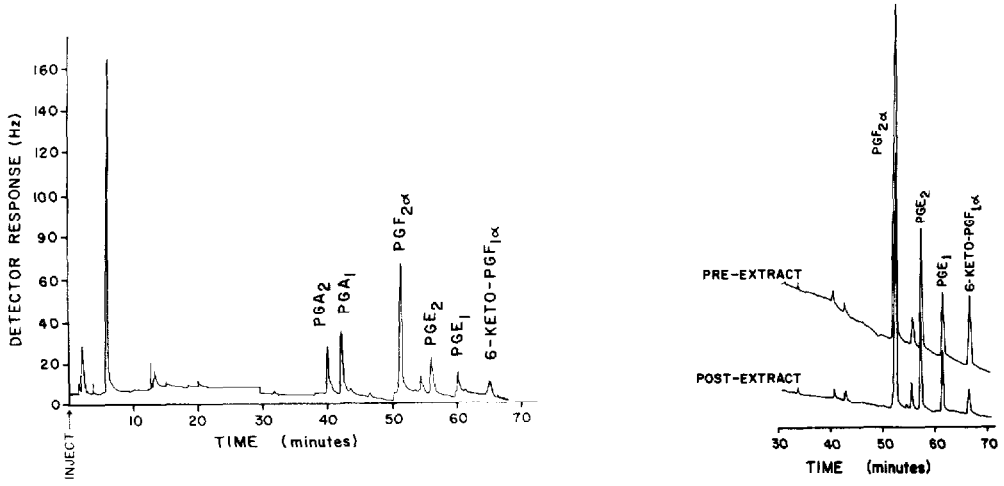

Fig. 1. Capillary chromatography of prostaglandins as pentafluorobenzyl ester (methoxime) trimethylsilyl ether derivatives. Splitless injection of 350 pg of each in hexane. Column: 25 m × 0.2 mm, OV-101, WCOT. ^{63}Ni-Electron-capture detector. Helium carrier gas at 28 cm/sec. Column temperature: 50°C × 2 min, 50°C/min to 200°C, 10°C/min to 250°C, 250°C isothermal for remainder of run.

Fig. 2. Extraction of prostaglandins from serum-supplemented culture medium. Equal aliquots of prostaglandin standards were analyzed before and after extraction using C_{18} reversed-phase. Chromatographic conditions as in Fig. 1. Approximately 450 pg.

TABLE I

PRECISION OF CAPILLARY CHROMATOGRAPHY AND ELECTRON-CAPTURE DETECTION OF PROSTAGLANDINS

Values shown are the means from four 450-pg injections. The coefficients of variation are given in parentheses.

	Retention time (min)	Peak height (mm)
PGE_1	61.50 ± 0.01 (0.008%)	32.5 ± 4.6 (14.2%)
PGE_2	57.31 ± 0.01 (0.017%)	25.3 ± 3.2 (12.6%)
$PGF_{2\alpha}$	52.39 ± 0.01 (0.019%)	72.0 ± 3.5 (4.9%)
PGI_2	66.51 ± 0.02 (0.027%)	69.3 ± 4.7 (6.8%)

standards was found to have the following coefficients of variation: PGE_1, 14.15%; PGE_2, 12.78%; $PGF_{2\alpha}$, 4.81%; and 6-keto-$PGF_{1\alpha}$, 6.84%.

Extraction recovery of the prostaglandins from the culture medium was tested with respect to isolation of the peaks of interest from interfering substances, as well as the linearity of extraction. Fig. 2 shows overlapping chromatograms, one from the stock mixture of the PG standards, the other from the same standards following extraction from culture medium. It is evident that the PGs are well isolated from any interfering peaks. Additionally, the PGs were recovered in a linear fashion from the culture medium which had been supplemented with decreasing concentrations of standards ranging from

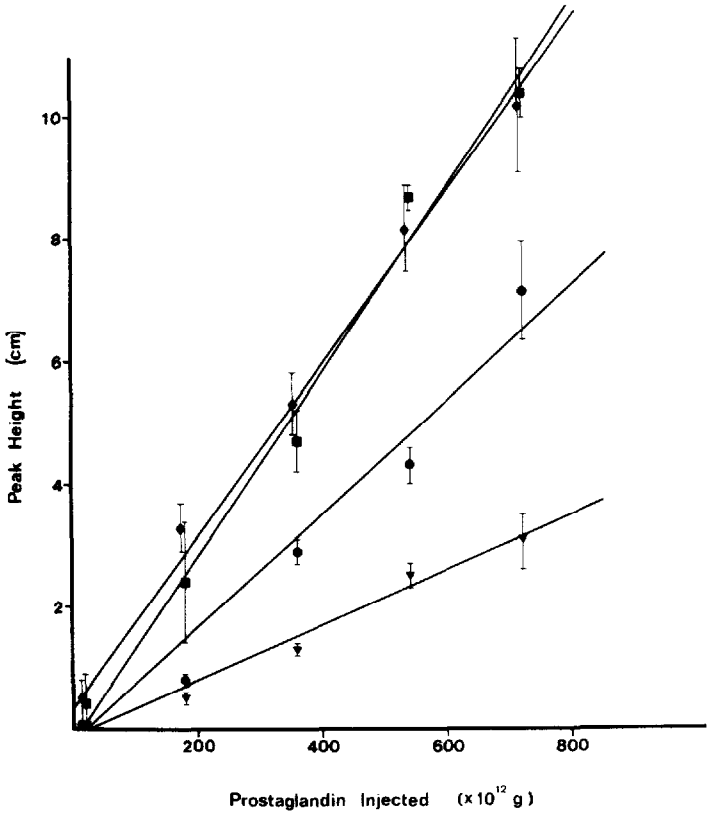

Fig. 3. Linearity of extraction and detector response of decreasing amounts of prostaglandins. Regression coefficients given in text. PGE_1 (◆); PGE_2 (●); $PGF_{2\alpha}$ (■); 6-keto-$PGF_{1\alpha}$ (▲).

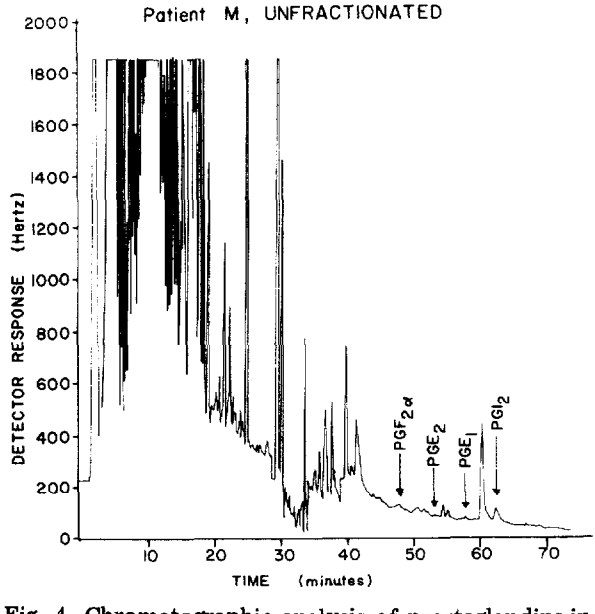

Fig. 4. Chromatographic analysis of prostaglandins in culture supernatant from 24-h incubation of dispersed cells from human ovarian cancer specimen.

TABLE II

PROSTAGLANDIN PRODUCTION BY CELL SUBPOPULATIONS FROM HUMAN TUMOUR SPECIMENS

Patient	Cell fraction*	Prostaglandin production (ng/ml)			
		PGE_1	PGE_2	$PGF_{2\alpha}$	PGI_2
M	UN	—	—	—	75.8
	NA	0.7	4.0	4.7	43.9
	AD	—	33.2	6.2	193.0
N	UN	—	9.5	—	3.0
	NA	—	4.4	—	8.5
	NA—NP	—	5.4	—	3.5
	AD	—	16.7	—	—
O	UN	—	13.7	—	3.6
	NA	1.1	10.5	—	—
	NA—NP	2.1	32.2	—	36.0
	AD	1.1	22.5	—	1.2
P	NA	14.1	210.0	75.8	1963.0
	AD	2.9	78.8	50.9	334.3
Q	UN	14.6	235.2	88.5	247.7
	NA	11.4	38.1	134.6	4.9
	NA—NP	—	51.2	—	—
	AD	60.4	14.0	—	34.4
R	UN	21.8	64.3	10.8	7.5
	NA	18.7	47.6	53.8	—
	NA—NP	—	51.8	45.5	—
	AD	14.5	240.6	48.2	—

*UN = unfractionated cells; NA = non-adherent cells; NA—NP = non-adherent—non-phagocytic cells; AD adherent cells.

40 to 1 ng/ml (Fig. 3). The regression coefficients for the extracted standards were: PGE_1, 0.997; PGE_2, 0.982; $PGF_{2\alpha}$, 0.995; and 6-keto-$PGF_{1\alpha}$, 0.993. Each of the regression lines has a negative y-intercept with the exception of PGE_1, which shows a positive intercept.

The analytical method was used to measure PGs in culture medium supernatants from cell populations isolated from human tumours (Table II). Chromatograms of the extracted supernatants from three subpopulations from one patient sample are shown in Figs. 4—6. The unfractionated cells (original, washed cells) produce low levels of PGs, with moderate levels of prostacyclin (PGI_2).

The non-adherent cells from the same patient produced marginally detectable amounts of the three PGs and also a low level of prostacyclin. Adherent cells from this specimen, however, showed significant PG synthesis compared to the other two cell subpopulations. This trend for predominant macrophage production of PGs is also evident in samples from patients N, O,

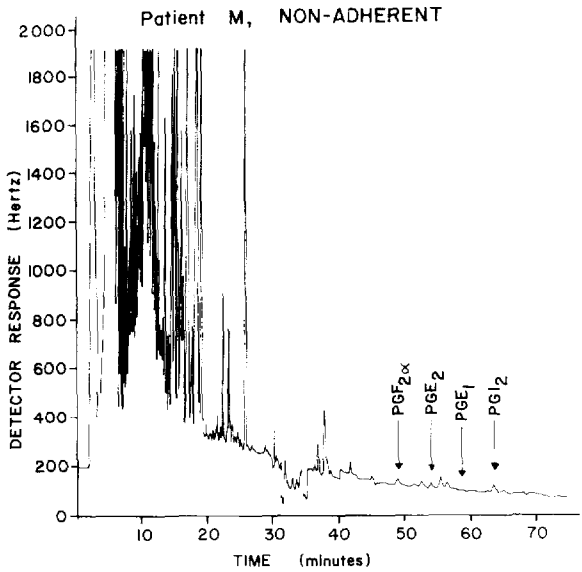

Fig. 5. Chromatogram from culture supernatant of non-adherent cells of human ovarian cancer specimen.

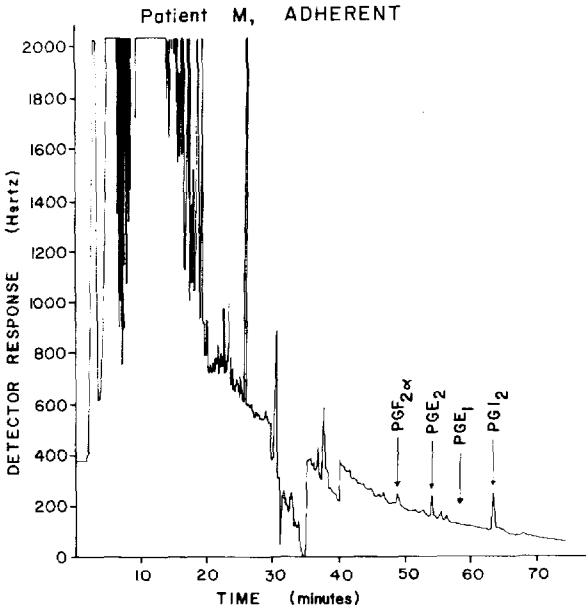

Fig. 6. Chromatogram from culture supernatant of adherent cells of human ovarian cancer specimen.

and R. In all patient samples tested, PGE_2 and PGI_2 were the dominant cyclo-oxygenase product measured, although for samples P, Q, and R, PGE_1 and $PGF_{2\alpha}$ were also found in moderately high concentrations. While the adherent cell population is the most homogeneous among the various samples with respect to cell composition, there is conspicuous lack of conformity between the PG profiles and levels of the PGs produced by the adherent cells from these

six patient specimens. Adherent cells from samples N, O, and R produced PGE_2 almost to the exclusion of the other compounds; the PG profiles of samples M, P, and Q show relative dominance by prostacyclin.

DISCUSSION

Prostaglandins in cell culture medium can be measured at the subnanogram level using reversed-phase extraction and electron-capture detection following derivatization to the pentafluorobenzyl ester (methoxime) trimethylsilyl ether derivatives. High-resolution capillary gas chromatography allows the simultaneous determination of prostaglandins E_1, E_2, $F_{2\alpha}$, and 6-keto-$F_{1\alpha}$ (the spontaneous breakdown product of prostacyclin, PGI_2). Furthermore, extraction using octadecylsilyl silica shows linear recovery characteristics sufficient for quantitative measurements of these important compounds. Although the extraction method does not isolate the PGs to the exclusion of other compounds, the precision of the chromatography is such that qualitative determination based on retention times is reasonable. The described method uses no instrument modifications, and is potentially amenable to automation.

The method has been successfully applied to the measurement of PGs in complex biological fluids. Cell culture supernatants from 24-h incubations of various cell subpopulations from human tumours were analyzed. These results indicate that there is considerable variation among the levels and profiles of PG production by human malignant tumours. The present study, however, includes insufficient numbers of patients to evaluate the possible clinical significance of prostaglandin synthesis by human tumours. Other investigators have reported that PG levels in human breast tumours may relate to the metastatic potential [10].

In the present investigation it was found that tumour macrophages display a wide range of cyclooxygenase activity which results in diverse levels and profiles of the PGs. It is known that macrophages possess a wide range of biological activities, one of which is prostaglandin synthesis [11]. Cyclooxygenase activity of macrophages has also been shown to be dependent on the level of macrophage activation [12] and the in vitro culture conditions [13]. The macrophages studied in this present report were isolated and cultured under identical experimental conditions; consequently, the divergent profiles and levels of PG production most likely reflect different functional states of these infiltrating host cells.

The convenient extraction and gas chromatographic analysis are presently used to investigate PG production by cell populations from a wide variety of human tumours including leukaemia, carcinomas, and sarcomas. The analytical procedure should prove useful in laboratories interested in routine studies of the profile of cyclooxygenase products. Attempts are under way to automate parts of the analysis.

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REFERENCES

1 B. Samuelsson, M. Goldyne, E. Granström, M. Hamberg, S. Hammarström, and C. Maimstem, Ann. Rev. Biochem., 47 (1978) 997.
2 F.A. Fitzpatrick, Anal. Chem., 50 (1978) 47.
3 F.A. Fitzpatrick, D.A. Stringfellow, J. Maclouf and M. Rigaud, J. Chromatogr., 177 (1979) 51.
4 J. Maclouf, H. de la Baume, J. Caen, H. Rabinovitch and M. Rigaud, Anal. Biochem., 109 (1980) 147.
5 J. Rosellό, E. Gelpi, M. Rigaud, J. Durand and J.C. Breton, Biomed. Mass Spectrom., 8 (1981) 149.
6 W.S. Powell, Prostaglandins, 20 (1980) 947.
7 J. Mai, S.K. Goswami, G. Bruckner and J.E. Kinsella, J. Chromatogr., 230 (1982) 15.
8 C. Chiabrando, A. Noseda and R. Fanelli, J. Chromatogr., 250 (1982) 100.
9 F. Vane and M.G. Horning, Anal. Lett., 2 (1969) 357.
10 P.H. Rolland, P.M. Martin, J. Jacquemier, A.M. Rolland and M. Toga, J. Nat. Cancer Inst., 64 (1980) 1061.
11 C.F. Nathan, H.W. Murray and Z.A. Cohn, N. Engl. J. Med., 303 (1980) 622.
12 J.H. Passwell, J.-M. Dayer and E. Merler, J. Immunol., 123 (1979) 1151.
13 R.S. Bockman, Prostaglandins, 21 (1981) 9.