Journal of Chromatography, 378 (1986) 430—436
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 3093

Note

Rapid separation of arachidonic acid metabolites by silicic acid chromatography for subsequent quantitative analysis by gas chromatography—mass spectrometry

BERND MAYER

Institut für Pharmakodynamic und Toxikologie, Universitätsplatz 2, A-8010 Graz (Austria)

and

ROBERT MOSER, HANS-JÖRG LEIS and HELMUT GLEISPACH*

Universitäts-Kinderklinik, Auenbruggerplatz 20, A-8036 Graz (Austria)

(First received October 28th, 1985; revised manuscript received January 22nd, 1986)

During the last few years, numerous reports have been published concerning the quantitative analysis of arachidonic acid (AA) metabolites in biological fluids [1]. Most of the work was done using gas chromatography—negative-ion chemical-ionization mass spectrometry (GC—NICI-MS), probably the best-suited method available. Extraction and purification of samples, however, is a lengthy procedure, including several chromatographic steps for sufficient specificity [2, 3].

The use of Sep-Pak cartridges [4] has greatly facilitated preparation of samples for GC—MS, but there have only been reports on the work-up of one class of compounds in each case using these columns [2, 3, 5, 6]. Total profiling of AA metabolites, however, requires a previous separation by column chromatography, usually a silicic acid [7] or XAD-7 resin [8]. These procedures are time-consuming and frequently require the evaporation of large volumes of organic solvent.

Thus, we developed a rapid and convenient method for separation of free AA, monohydroxyeicosatetraenoic acids (HETEs) and prostaglandins (PGs) on a special silica gel (Silicar CC-4) that considerably decreases elution volumes and analysis time.

The purity of the material eluted was sufficient for subsequent GC—NICI-MS. Additionally, this column chromatography step would greatly facilitate high-performance liquid chromatographic (HPLC) analysis, as the use of highly sophisticated and time-consuming gradient elutions [8] would become superfluous.

0378-4347/86/$03.50

EXPERIMENTAL

Arachidonic acid, prostaglandin standards, Ca-Ionophore A23187 and soybean lipoxygenase type IV were obtained from Sigma (Munich, F.R.G.). Deuterium-labelled prostaglandins were purchased from Merck Sharpe and Dohme (Munich, F.R.G.). This company also provided us with a generous gift of indomethacin (sodium salt). Silicar CC-4 was obtained from Mallinkrodt (St. Louis, MO, U.S.A.).

[1-^{14}C] AA (specific activity 55 mCi/mmol) was from New England Nuclear (Dreieich, F.R.G.). It was diluted with unlabelled arachidonic acid to a specific activity of 1 mCi/mmol and purified by silicic acid chromatography.

[1-^{14}C] 15-HETE was prepared by incubating arachidonic acid with soybean lipoxygenase and subsequent sodium borohydride reduction of the hydroperoxide [9]. [1-^{14}C] 6-keto-PGF$_{1\alpha}$ was prepared by incubating bovine aortic endothelial homogenates [10] with [1-^{14}C] AA and purification by thin-layer chromatography [11]. Platinum dioxide was purchased from Ventron (Karlsruhe, F.R.G.), methoxamine hydrochloride in pyridine (2%) and bis(trimethylsilyl)trifluoroacetamide from Pierce (Rockford, IL, U.S.A.) and pentafluorobenzylbromide from Supelco, supplied by ICT (Vienna, Austria). Other reagents and solvents of analytical grade were obtained from Merck (Darmstadt, F.R.G.).

Incubation procedures

Endothelial cells were obtained from bovine aortae as previously described [10]. For measurement of endogenously released products, the cells were incubated in the presence of 10 μM Ca-Ionophore A23187 for 5 min at 37°C in Dulbecco's phosphate-buffered saline (pH 7.4). For biosynthesis of ^{14}C-labelled 6-keto-PGF$_{1\alpha}$, cells were incubated for 5 min in the presence of 10 μM [1-^{14}C] AA (specific activity 1 mCi/mmol).

Extraction of arachidonic acid metabolites

The incubation medium was immediately diluted with 1 vol. of ice-cold water–ethanol (1:1) and acidified to pH 3.5 with 0.1 M hydrochloric acid. After addition of stable isotope-labelled analogues as internal standards for isotope dilution MS, the medium was extracted twice with 2 vols. of ethyl acetate. The solvent was removed under nitrogen, the residue dried with a small volume of ethanol and the dry sample redissolved in 100 μl of diethyl ether—light petroleum (25:75). If large volumes of aqueous media have to be extracted, Sep-Pak C_{18} reversed-phase extraction columns [12] are an advantage compared to solvent extraction.

Column chromatography

Columns were prepared with 0.3 g of dry Silicar CC-4 in pasteur pipettes and washed with 2 ml of diethyl ether–light petroleum (25:75). The sample was applied onto the column, and AA together with neutral lipids was eluted with 3 ml of the same solvent (fraction I). Subsequently, monohydroxy fatty acids were eluted with 3 ml of diethyl ether—light petroleum (75:25) (fraction II), and finally prostaglandins were recovered together with dihydroxylated

metabolites by elution with 3 ml of ethyl acetate- methanol (90:10) (fraction III).

If a total lipid extraction had been performed, peptide leukotrienes and phospholipids could be eluted by increasing the methanol concentration in the solvent.

Each fraction was then dried under nitrogen and derivatized for GC—MS.

Derivatization procedure

For positive-ion detection, the conventional methyl ester-methyl oxime-trimethylsilyl (TMS) ether derivatives were prepared as previously described [13]. Monohydroxy fatty acids were analysed in most cases after catalytic hydrogenation with hydrogen gas using platinum dioxide as the catalyst in methanol. For negative-ion detection, pentafluorobenzyl (PFB) esters instead of methyl ester derivatives were prepared by treating the sample with 10 μl of N,N-diisopropylethylamine and 40 μl of 7% pentafluorobenzyl bromide in acetonitrile for 10 min at room temperature [14]. Catalytic hydrogenation of hydroxy fatty acids was always performed prior to esterification in order to avoid partial reduction of the aromatic ring.

Instrument

A Finnigan gas chromatograph 9610 coupled to a Finnigan 4500 mass spectrometer with positive electron impact and a pulsed positive–negative chemical-ionization device combined with an Incos data system was used. GC separation on a DB-5 fused-silica capillary column and instrumental parameters have been described in detail previously [10, 13].

RESULTS AND DISCUSSION

Fig. 1. shows, schematically, the complete extraction and purification procedure for AA metabolites prior to GC—MS analysis. The small columns have been found to be very effective for separation of different lipid classes and required only small elution volumes.

As shown in Table I, recoveries of radiolabelled standards averaged 90%. So far, silicic acid chromatography of these compounds has commonly resulted in recoveries of $< 70\%$ (15, 16]. Additionally, these procedures were time-consuming, since they require the evaporation of large elution volumes. The acidic Silicar CC-4 seems to facilitate the elution of the various compounds, whereby separation seems to be based on filtration combined with adsorption according to the polarity of the substances.

Since the columns can be prepared with dry Silicar CC-4 and are completely insensitive to drying in the course of the elutions, we handle up to 30 columns simultaneously. After the elution process, the columns can be washed with 5 ml of methanol and be reused for the next run.

We used this separation technique for the MS analysis of monohydroxy-eicosatetraenoic acids and prostaglandins produced by bovine aortic endothelial cells from endogenously released AA. For this purpose, the cells were incubated in the presence of 10 μM Ca-Ionophor A23187 for 5 min at 37°C. AA metabolites were extracted and separated as described in Experimental. The hydroxy

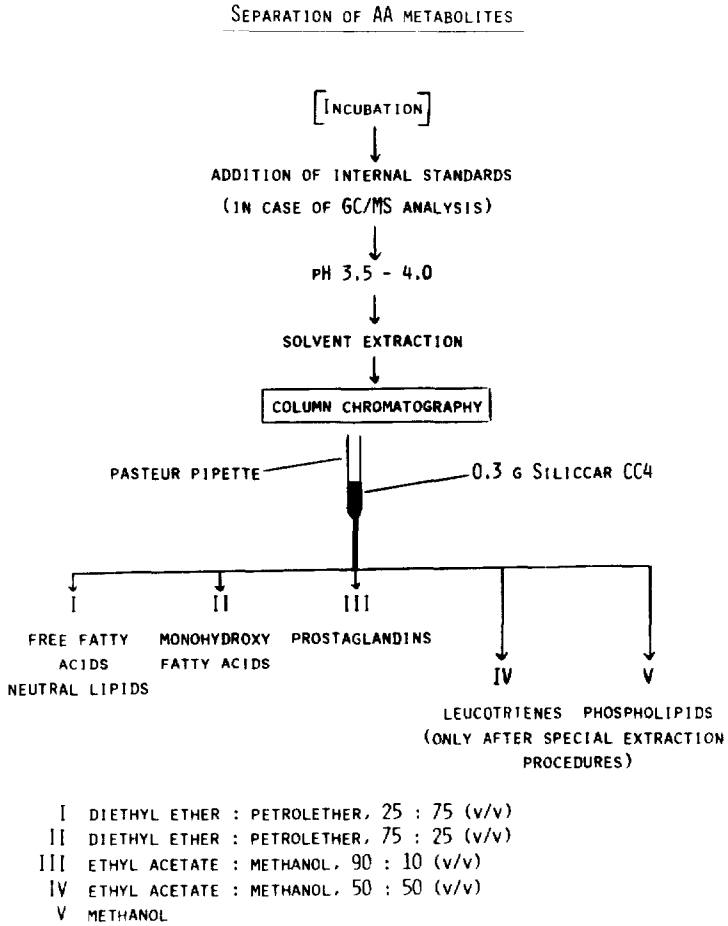

Fig. 1. Schematic illustration of the described extraction and purification procedure.

TABLE I

RECOVERIES OF RADIOLABELLED STANDARDS AFTER COLUMN CHROMATOGRAPHY ON 0.3 g OF SILICAR CC-4

^{14}C-Labelled standards were obtained and worked-up as described in Experimental. Each fraction represents a 3-ml volume of the appropriate solvent.

Compound	Recovery (mean ± S.D., n = 3) (%)			
	Fraction I	Fraction II	Fraction III	Methanol
Arachidonic acid	92 ± 3	1.5 ± 1.0	N.D.*	N.D.
15-HETE	N.D.	87 ± 6	2.5 ± 1.5	N.D.
6-Keto-PGF$_{1\alpha}$	N.D.	N.D.	88 ± 4	1.5 ± 0.5

*N.D. = Not detectable.

acids were subjected to catalytic hydrogenation prior to GC—MS analysis since the chain fragmentation of the saturated derivatives is defined by the position of the hydroxy function and leads to two intense ions specific for each

positional isomer. The mass chromatogram of material isolated from an endothelial cell incubation is shown in Fig. 2 and clearly demonstrates the presence of 15-HETE and 11-HETE accompanied by small amounts of 5-HETE. Incubations performed in the presence of 10 μM indomethacin showed that 11-HETE, in contrast to 15- and 5-HETE, was a cyclooxygenase product of AA. This confirmed the results obtained previously with cultured human skin fibroblasts [11].

The mass chromatogram in Fig. 2 further shows that in spite of a rapid temperature-programmed GC run, purification after one chromatographic step was sufficient for MS analysis of monohydroxy fatty acids.

The detection limit, however, in the electron-impact mode was very low (ca. 10 ng per injection). A considerable increase in sensitivity was observed on performing negative-ion detection with methane as the reagent gas. With this mode of detection, however, it is not possible to distinguish between the various hydroxy isomers.

Prostaglandins (fraction III) were quantified with tetradeuterated analogues as internal standards by GC–NICI-MS. Since fatty acid derivatives are observed

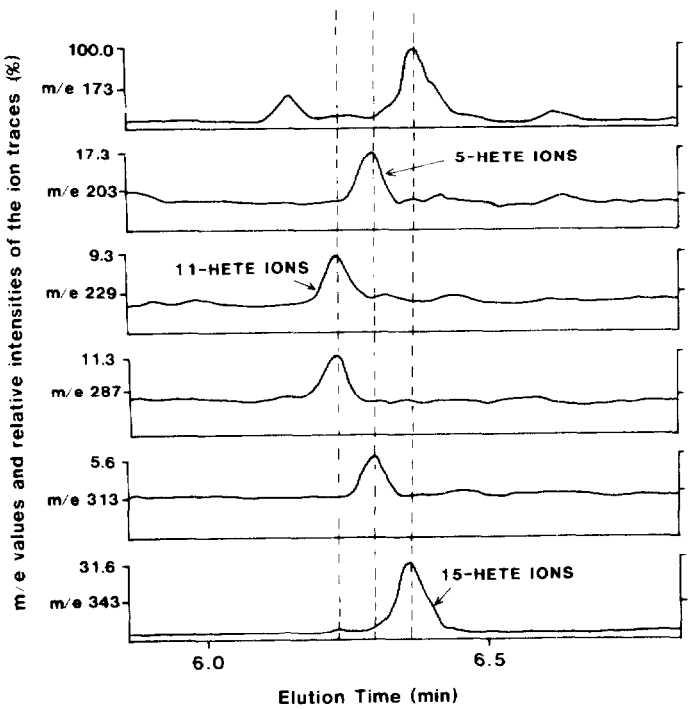

Fig. 2. Selected-ion monitoring of 15-HETE, 11-HETE and 5-HETE produced by freshly isolated bovine aortic endothelial cells. The cells (1 mg protein per ml) were incubated in the presence of 10 μM Ca-Ionophore A23187 for 5 min at 37°C and the acidified incubation medium was worked-up as described in Experimental. GC—MS analysis of the hydrogenated methyl ester-TMS ether derivatives was performed on a 30-m DB-5 fused-silica capillary column. The oven temperature was 100°C for 1 min and then increased at 40°C/min to a final temperature of 320°C. In the course of mass spectrometric detection, the following ions were monitored under electron impact: *m/e* 173 and *m/e* 343 for 15-HETE, *m/e* 203 and *m/e* 313 for 5-HETE, and *m/e* 229 and *m/e* 287 for the 11-HETE derivative.

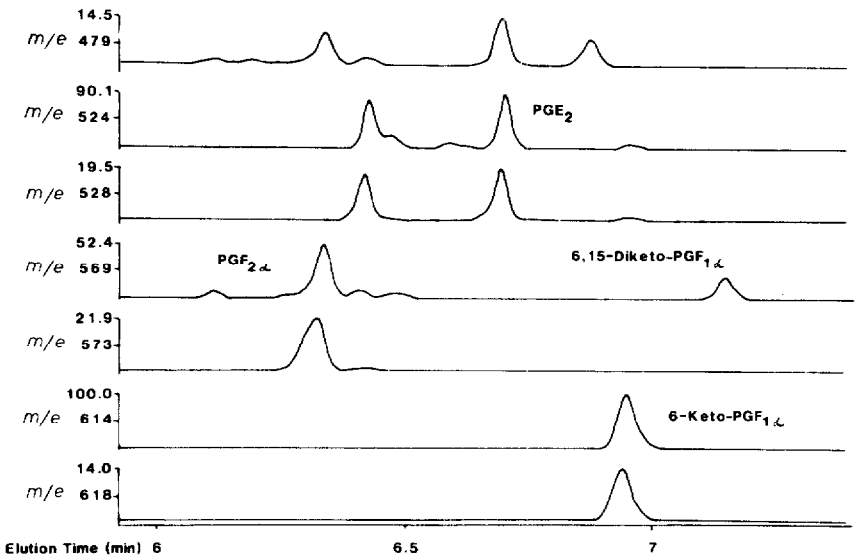

Fig. 3. Selected-ion monitoring of prostaglandins produced by freshly isolated bovine aortic endothelial cells. The cells (0.1 mg protein per ml) were incubated in the presence of 10 μM Ca-Ionophore A23187 for 5 min at 37° C and the acidified incubation medium was worked-up as described in Experimental. GC—MS analysis was performed with 5 ng of tetra-deuterated analogues as internal standards in the NICI mode with methane as the reagent gas. GC separation was achieved on a 30-m DB-5 fused-silica capillary column. The oven temperature was 170° C for 1 min and then increased at 40° C/min to a final temperature of 320° C. Compounds were analysed as pentafluorobenzyl ester—methyloxime-TMS ether derivatives and the following ions were monitored: *m/e* 524 and *m/e* 528 for PGE_2 and $[^2H]_4$-PGE_2, *m/e* 569 and *m/e* 573 for $PGF_{2\alpha}$ and $[^2H]_4$-$PGF_{2\alpha}$, and *m/e* 614 and *m/e* 618 for 6-keto-$PGF_{1\alpha}$ and $[^2H]_4$-6-keto-$PGF_{1\alpha}$.

selectively by this technique, the background was very low and sensitivity was considerably increased as compared to positive-ion detection. Fig. 3 shows a typical mass chromatogram of prostaglandins obtained from endothelial cell incubations. 6-Keto-$PGF_{1\alpha}$, the stable hydrolysis product of PGI_2, was by far the main metabolite of AA. Biosynthesis of 6,15-diketo-$PGF_{1\alpha}$ from PGI_2, possibly by a 15-hydroxyprostaglandin dehydrogenase, is presently investigated in more detail and will be published elsewhere. Smaller amounts of PGE_2 and $PGF_{2\alpha}$ were also present, but these compounds were, in contrast to PGI_2 metabolites, not produced by unstimulated cells. Probably high substrate concentrations, as may occur when phospholipase A_2 is stimulated, might lead to an accumulation of PGH_2 and a subsequent increase in PGE_2 and $PGF_{2\alpha}$ generation.

We also applied the described method for the determination of cyclooxygenase products in other biological fluids, including supernatants of cultured cells, sputum, plasma, tissue homogenates and inflammatory exudate. As shown in Fig. 3, the presented procedure results in a purity of samples that is sufficient for a rapid GC—NICI-MS analysis.

ACKNOWLEDGEMENT

This work was supported by Grant No. 5617 from the "Fonds zur Förderung der wissenschaftlichen Forschung", Vienna, Austria.

REFERENCES

1 I.A. Blair, Br. Med. Bull., 39 (1983) 223.
2 P.M. Woolard and A.I. Mallet, J. Chromatogr., 306 (1984) 1.
3 C.R. Pace-Asciak and S. Micallef, J. Chromatogr., 310 (1984) 233.
4 K.A. Waddell, J. Wellby and I.A. Blair, Biomed. Mass Spectrom., 10 (1983) 83.
5 C. Chiabrando, A. Noseda, M.N. Castagnoli, M. Salmona and R. Fanelli, Biochim. Biophys. Acta, 794 (1984) 292.
6 C. Chiabrando, A. Noseda, M.N. Castagnoli, M. Romano and R. Fanelli, J. Chromatogr., 279 (1983) 581.
7 L. Santebin, D. Caruso, G. Galli and R. Paoletti, FEBS Lett., 157 (1983) 173.
8 D.C. Henke, S. Konzan and T.E. Eling, Anal. Biochem., 140 (1984) 87.
9 M. Hamberg and B. Samuelsson, J. Biol. Chem., 242 (1967) 5329.
10 B. Mayer, R. Moser, H. Gleispach and W.R. Kukovetz, Biochim. Biophys. Acta, 875 (1986) 641.
11 B. Mayer, L. Rauter, E. Zenzmaier, H. Gleispach and H. Esterbauer, Biochim. Biophys. Acta, 795 (1984) 151.
12 J.R. Luderer, D.L. Riley and L.M. Demers, J. Chromatogr., 273 (1983) 402.
13 H. Gleispach, R. Moser, B. Mayer, H. Esterbauer, U. Scriletz, L. Zierman and H.-J. Leis, J. Chromatogr., 344 (1985) 11.
14 I.A Blair, S.E. Barrow, K.A. Waddell, P.J. Lewis and C.T. Dollery, Prostaglandins, 23 (1983) 579.
15 L.M. Demers and D.D. Derck, in B. Samuelsson, P. Ramwell and R. Paoletti (Editors), Advances in Prostaglandin and Thromboxane Research, Vol. 6, Raven Press, New York, 1978, pp. 1—18.
16 E. Granstrom and H. Kindahl, in J.C. Frölich (Editor), Advances in Prostaglandin and Thromboxane Research, Vol. 5, Raven Press, New York, 1978, pp. 119–210.