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SIMULTANEOUS DETERMINATION OF THE PRIMARY PROSTANOIDS PROSTAGLANDIN E_2, PROSTAGLANDIN $F_{2\alpha}$ AND 6-OXOPROSTAGLANDIN $F_{1\alpha}$ BY IMMUNOAFFINITY CHROMATOGRAPHY IN COMBINATION WITH NEGATIVE ION CHEMICAL IONIZATION GAS CHROMATOGRAPHY–TANDEM MASS SPECTROMETRY

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SUMMARY

The simultaneous determination of prostaglandin E_2 (PGE_2), prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) and 6-oxoprostaglandin $F_{1\alpha}$ (6-oxo-$PGF_{1\alpha}$) in urine using immunoaffinity chromatography in combination with negative ion chemical ionization gas chromatography–tandem mass spectrometry (NICI-GC–MS–MS) is described Monoclonal antibodies against PGE_2 (100% cross-reactivity with 6-oxo-$PGF_{1\alpha}$) and $PGF_{2\alpha}$ were both coupled to a derivatized agarose matrix After extraction with a C_{18} cartridge the sample was applied to the immunoaffinity column The prostaglandins were eluted with acetone–water and the methoxime-pentafluorobenzyl-trimethylsilyl (MO-PFB-TMS) derivatives (PGE_2 and 6-oxo-$PGF_{1\alpha}$) and the PFB-TMS derivative ($PGF_{2\alpha}$) were quantified by GC–MS–MS For reproducibility experiments, spiked urine samples were analysed several times The correlation coefficients were 0 997 (6-oxo-$PGF_{1\alpha}$) and 0 999 (PGE_2 and $PGF_{2\alpha}$) and the slopes were 0 99 and 1 03, respectively The inter-assay coefficient of variation ranged from 8 6 to 9 5% for the unspiked urine samples and from 2 0 to 5 2% for the spiked samples This method offers several advantages, e g , high specificity and sensitivity good reproducibility and an increase in sample throughput

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INTRODUCTION

The kidney has been recognized as one of the main sources of prostanoid synthetic activity Urinary prostaglandin E_2 (PGE_2), prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) and 6-oxoprostaglandin $F_{1\alpha}$ (6-oxo-$PGF_{1\alpha}$) levels are thought to reflect renal prostaglandin synthesis Urinary excretion rates of these prostanoids are therefore used as indices of renal prostaglandin activity [1] The determination of these urinary prostanoids is increasingly used for monitoring patients with a variety of renal diseases [2]

There are several techniques for the determination of prostaglandins in urine and other biological materials, including bioassay, thin-layer chromatography (TLC), high-performance liquid chromatography (HPLC), radioimmunoassay (RIA), enzyme-linked immunosorbent assay (ELISA), gas chromatography (GC), GC–mass spectrometry (GC–MS) and GC–tandem mass spectrometry (GC–MS–MS) GC–MS and GC–MS–MS are considered to be the most specific and reliable techniques However, the disadvantage of these methods is the time-consuming purification prior to quantification Immunoaffinity chromatography reduces the time required

For prostanoids, the immunoaffinity technique was first used to extract the prostacyclin analogue iloprost from plasma Iloprost antiserum was coupled to cyanogen bromide-activated Sepharose 4B and the pentafluorobenzyl-trimethylsilyl (PFB-TMS) derivative was quantified by negative ion chemical ionization (NICI)-GC–MS [3] A similar method was described for purification of urine samples, using a polyclonal anti-thromboxane B_2 (TxB_2) antibody immobilized on N-hydroxysuccinimidyl-silica gel The methoxime (MO)-PFB-tris-*tert*-butyldimethylsilyl derivative was quantified by NICI-GC–MS [4] Antibody-mediated extraction combined with NICI-GC–MS was employed for TxB_2 and 2,3-dinor-TxB_2 in human and rat urine [5] and for 6-oxo- $PFG_{1\alpha}$ in human urine and canine plasma [6] The MO-PFB-TMS derivatives were quantified by NICI-GC–MS

This paper describes the simultaneous determination of PGE_2, $PGF_{2\alpha}$ and 6-oxo-$PGF_{1\alpha}$ using an immunoaffinity column with monoclonal antibodies (MABs) combined with NICI-GC–MS–MS

EXPERIMENTAL

Materials

The characteristics of the MABs used in this study are described elsewhere [7] the very specific MAB-$PGF_{2\alpha}$, and MAB-PGE_2 (5A-E_2R_1) showing high cross-reactivity with 6-oxo-$PGF_{1\alpha}$ (100%) and with $PGF_{2\alpha}$ (20%) MAB-secreting hybridomas were cultivated in serum-free medium according to the method of Iscove and Melchers [8] The culture supernatants were concentrated and the MABs purified employing protein A–Sepharose CL-4B (Phar-

macia, Freiburg, F R G) in affinity chromatography The purity of the MABs was controlled by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE), staining with Coomassie Brilliant Blue The immunoglobulin content of the purified MABs, lyophilized after dialysis against phosphate-buffered saline (PBS), was 0 17 mg for MAB-PGE_2 and 0 15 mg for MAB-$PGF_{2\alpha}$ per milligram of the lyophilized powder

The following solvents of analytical-reagent grade were used chloroform, hexane, ethyl acetate (Promochem, Wesel, F R G), water, dichloromethane (Baker, Gross-Gerau, F R G), acetone (Riedel-de Haen, Seelze, F R G), pyridine (99 9%) (Aldrich, Milwaukee, WI, U S A), N,N-diisopropylethylamine (>99 5% for GC) (Pierce, Rockford, IL, U S A) and formic acid (Merck, Darmstadt, F R G) Pentafluorobenzyl bromide (PFBB) was obtained from Serva (Heidelberg, F R G), bis(trimethylsilyl)trifluoroacetamide (BSTFA) from Macherey and Nagel (Duren, F.R G) and O-methylhydroxylamine hydrochloride from Pierce (Bender & Hobein, Heidelberg, F R G) PBS (buffer 1) consisted of 0 05 *M* KH_2PO_4 and 0 15 *M* NaCl adjusted to pH 7 4 and buffer 2 consisted of 1 2 g of tris(hydroxymethyl)aminomethane, 8 76 g of NaCl, 1.0 g of polyvinylpyrrolidone 40 (PVP-40) per litre of water and was adjusted to pH 7 4 PGE_2, $PGF_{2\alpha}$ and 6-oxo-$PGF_{1\alpha}$ and their 3,3′,4,4′-[$^2H_4$]-analogues were kind gifts from Drs U Axen and J Pike (Upjohn, Kalamazoo, MI, U.S.A) Tritiated prostaglandins were obtained from Amersham Buchler (Braunschweig, F R G) Sep-Pak C_{18} cartridges were from Waters Assoc (Gross-Gerau, F R.G.), Affi-Gel and Econo columns from Bio-Rad Labs (Munich, F R G.), Sephadex LH-20 from Pharmacia (Uppsala, Sweden) and Extrelut from Merck. Opti-Fluor (Packard, Frankfurt, F R G.) was used as a scintillation cocktail in a Minaxi β liquid scintillation counter (Packard)

Preparation of the immunoaffinity columns

A 2-ml amount of Affi-Gel 10 (N-hydroxysuccinimide ester of a derivatized agarose) was first washed with 100 ml of cold (4 °C) distilled water and then with the same amount of 0.1 *M* sodium hydrogencarbonate (4 °C) The wet gel was mixed with the MAB solution, containing 20 mg of lyophilized MAB-PGE_2 (3 4 mg IgG) and 20 mg of lyophilized MAB-$PGF_{2\alpha}$ (3 0 mg of IgG) redissolved in 3 ml of water This mixture was rotated end-over-end for 16 h at 4 °C, 200 μl of 1 *M* ethanolamine (pH 8 0) were subsequently added to the gel and rotated for an additional 1 h in order to cap unreacted functional groups of the matrix The gel was then pipetted into an Econo column The column was washed with all solvents used in the assay (see below) It was stored under PBS containing 0 1% sodium azide (bacteriostatic agent) at 4 °C

Binding capacity of the affinity column

The binding capacity of the column for PGE_2, $PGF_{2\alpha}$ and 6-oxo-$PGF_{1\alpha}$ was determined as follows. Solutions of 100 ng, 500 ng, 1 μg, 3μg and 5 μg of syn-

thetic prostaglandins and the corresponding tritiated analogues (about 0 05 μCi) in 0 5 ml of buffer 2 were made These solutions were incubated successively on the affinity column for 30 min at room temperature The column was washed with 10 ml of buffer 2 and then with 10 ml of distilled water The bound prostaglandins were eluted with 10 ml of 95% (v/v) acetone in water Aliquots of 1 ml were evaporated to dryness and the residue was dissolved in scintillation cocktail and counted in a liquid scintillation counter The results were compared with the counts of the original radioactive tracer solutions (about 0.05 μCi)

Urine samples

Urine was collected from normal healthy volunteers and kept at 4 °C during the collection period Aliquots of 10 ml were spiked with the deuterated analogues (3,3′, 4,4′-[$^2H_4$]prostanoids) of PGE_2 (13 1 ng), $PGF_{2\alpha}$ (11 55 ng) and 6-oxo-$PGF_{1\alpha}$ (24 28 ng) as internal standards and with 5, 10 or 20 ng of the natural prostaglandins for reproducibility experiments (see below) Then they were frozen and stored at −80 °C until analysis

Extraction and purification

A 10-ml aliquot of urine was adjusted to pH 3.2 with 2 *M* formic acid This solution was extracted using a Sep-Pak C_{18} cartridge preconditioned with methanol (5 ml) and distilled water (10 ml) The cartridge was washed with 10 ml of distilled water, 5 ml of methanol–water (10 90, v/v) and finally with 10 ml of hexane The prostaglandins were eluted with 8 ml of chloroform–ethyl acetate (4 1, v/v) and evaporated to dryness The residue was solved in 900 μl (3 × 300 μl) of buffer 2, adjusted to pH 7 4 and loaded on the affinity column, which had been previously washed with the same buffer After incubation for 30 min, the affinity column was first washed with 10 ml of buffer 2 and then with 10 ml of distilled water The column was rotated end-over-end with 5 ml of acetone–water (95 5, v/v) for about 3 min The eluate was collected in a glass flask and the affinity column was washed with an additional 3 ml of acetone–water (95 5, v/v) The combined eluates were evaporated to dryness The affinity column was immediately washed with about 30 ml of acetone–water (95 5, v/v), 50 ml of distilled water and 30 ml of buffer 2 and then stored at 4 °C under PBS containing 0 1% sodium azide The evaporated eluate was transferred into a plastic vial and derivatized to the PFB ester by adding 60 μl of acetonitrile, 20 μl of PFBB (35% in acetonitrile) and 20 μl of diisopropylethylamine at 35 °C for 25 min The solvent was removed at room temperature under a stream of nitrogen and the residue dissolved in dichloromethane (0 5 ml) Excess of PFBB was removed by elution through a Sephadex LH-20 column preswollen with dichloromethane (3 ml) The sample was evaporated to dryness under a stream of nitrogen

The methoxime derivative (PGE_2 and 6-keto-$PGF_{1\alpha}$) was prepared by adding 200 μl of O-methylhydroxylamine hydrochloride in anhydrous pyridine

(20 mg/ml). The sample was allowed to stand overnight at room temperature and the pyridine was then removed under a stream of nitrogen. The residue was dissolved in chloroform and the excess of O-methylhydroxylamine was removed using a short Extrelut column. After evaporation, 25 μl of BSTFA were added and the reaction mixture was heated at 60°C for 1 h and 2 μl of this solution were injected on to the GC column.

Gas chromatography–tandem mass spectrometry

A Finnigan-MAT TSQ45 gas chromatograph–tandem mass spectrometer was employed. GC was carried out on a J&W DB-1701 capillary column (20 m × 0.25 mm I.D., 0.25-μm film thickness) in the splitless mode with helium as carrier gas at an inlet pressure of 100 kPa. The initial temperature of 100°C was held for 2 min, then increased at 30°C/min to 280°C and at 5°C/min to 310°C, the final temperature being held for 3 min. The MS conditions were interface temperature 300°C, source temperature 120°C, methane CI gas pressure 50 Pa, electron energy 70 eV, emission current 0.2 mA and electron multiplier 2800 V. The argon collision cell pressure was 1.8 mTorr and the collision energy was 15 eV (for $PGF_{2\alpha}$ and 6-oxo-$PGF_{1\alpha}$) and 10 eV (for PGE_2).

RESULTS

In binding experiments, the recovery of labelled prostaglandins in the acetone–water eluate was 80.2 ± 3.7% (mean ± S.D., $n = 4$) and the binding capacity was 1.9 μg for PGE_2, 2.2 μg for $PGF_{2\alpha}$ and 2.1 μg for 6-oxo-$PGF_{1\alpha}$. Although these capacities are extremely high compared with physiological levels of pros-

TABLE I

DAUGHTER IONS OF THE $[M-PFB]^-$ ION OF NON-LABELLED AND $[^2H_4]PGF_{2\alpha}$, PGE_2 AND 6-OXO-$PGF_{1\alpha}$ DERIVATIVES USED FOR QUANTIFICATION

Daughter ions can be explained by loss of three TMSOH ($PGF_{2\alpha}$ derivative) and two TMSOH (PGE_2 and 6-oxo-$PGF_{1\alpha}$ derivatives), respectively.

	m/z	
	Parent ion	Daughter ion
$PGF_{2\alpha}$	569	299
$[^2H_4]PGF_{2\alpha}$	573	303
PGE_2	524	344
$[^2H_4]PGE_2$	528	348
6-Oxo-$PGF_{1\alpha}$	614	434
$[^2H_4]$-6-oxo-$PGF_{1\alpha}$	618	438

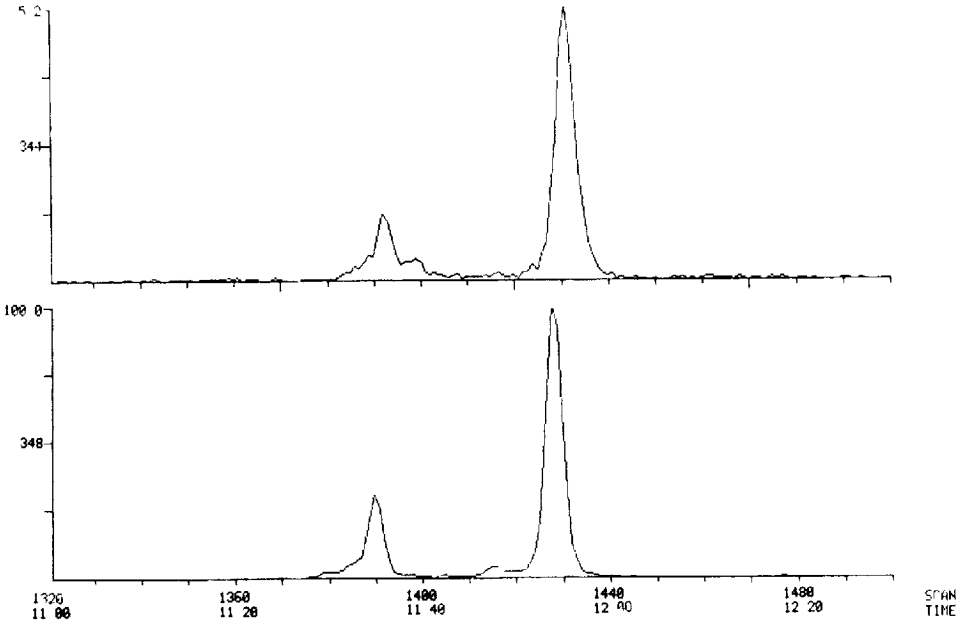

Fig 1 Daughter-ion chromatograms of PFB-MO-TMS derivatives of endogenous PGE_2 [upper trace, scan 1392 and 1431 (isomers)] and [$^2H_4$]PGE_2 [lower trace, scan 1390 and 1428]

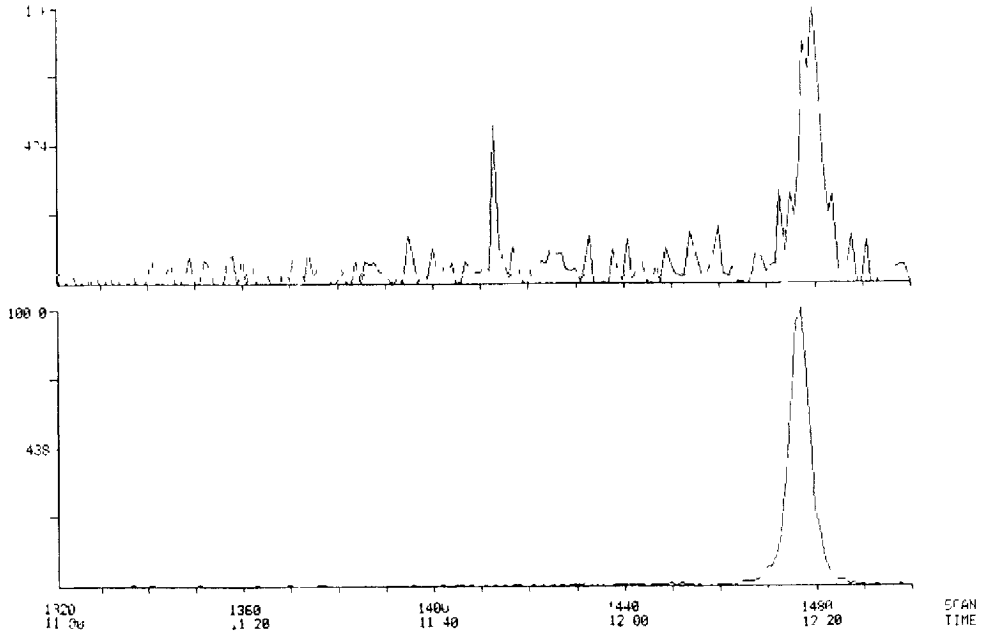

Fig 2 Daughter-ion chromatograms of PFB-MO-TMS derivatives of endogenous 6-oxo-$PGF_{1\alpha}$ (upper trace, scan 1480) and [$^2H_4$]-6-oxo-$PGF_{1\alpha}$ (lower trace, scan 1477)

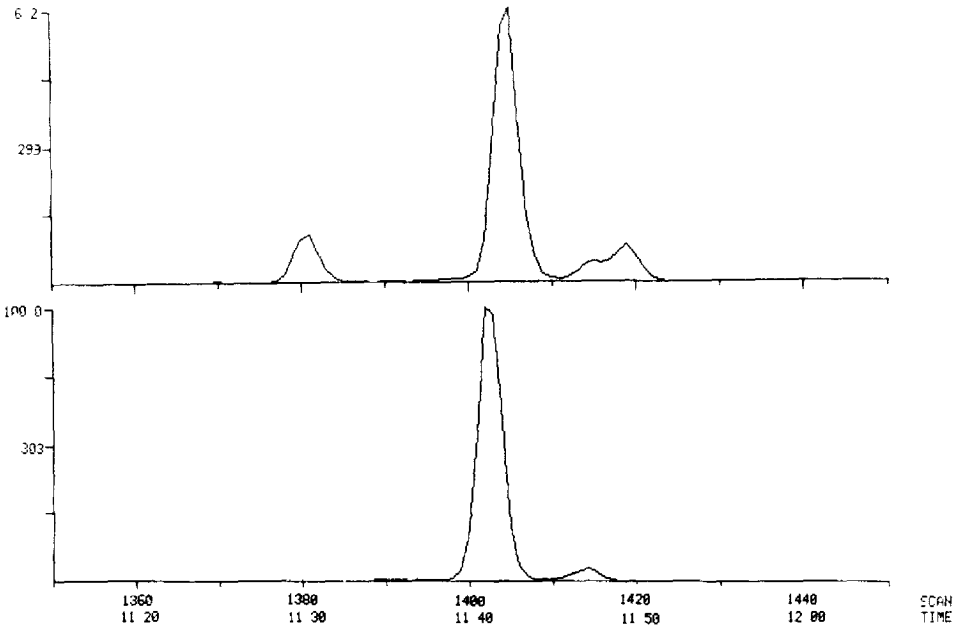

Fig 3 Daughter-ion chromatograms of PFB-TMS derivatives of endogenous $PGF_{2\alpha}$ (upper trace, scan 1405) and $[^2H_4]PGF_{2\alpha}$ (lower trace, scan 1402)

TABLE II

RESULTS OF THE ANALYSIS OF THE URINES SPIKED WITH 5, 10 AND 20 ng OF SYNTHETIC PROSTAGLANDINS

Values in parentheses are inter-assay coefficients of variation (%)

Sample	Concentration (mean) (ng per 10 ml)		
	PGE_2	$PGF_{2\alpha}$	6-Oxo-$PGF_{1\alpha}$
Control	0 805 (8 6)	0 818 (9 5)	0 440 (9 4)
Control + 5 ng	5 665 (5 4)	5 940 (5 3)	5 710 (7 9)
Control + 10 ng	11 364 (4 2)	11 208 (4 6)	10 682 (3 9)
Control + 20 ng	21 266 (2 0)	21 376 (2 7)	20 288 (5 2)

taglandins, the affinity gel was not diluted, because of a loss of capacity after numerous clean-up procedures (about 25% after 100 cycles) It is necessary to wash the columns after elution of the antigens with some more acetone–water (we used 30 ml), because otherwise prostaglandins would be retained on the column, leading to a carryover effect (cumulation) and thus falsifying the results

In NICI mass spectra of $PGF_{2\alpha}$ PFB-TMS, PGE_2 PFB-MO-TMS and 6-oxo-$PGF_{1\alpha}$ PFB-MO-TMS and their deuterated analogues, $[M-PFB]^-$ is

always the most intense fragment ion [9] For quantification, we used daughter ions of these $[M-PFB]^-$ ions obtained by collisionally activated decomposition (CAD) [10] The most intense daughter ions of $[M-PFB]^-$ are the fragments of *m/z* 268 (PGE_2) and *m/z* 272 ($[^2H_4]PGE_2$), respectively These should be the most suitable fragments for quantification However, a simultaneous fragmentation to the ion of *m/z* 271 (about 35% of the intensity of *m/z* 272) that can be explained by loss of two TMSOH, CO_2 and CH_3OD was observed for the deuterated PGE_2 For this reason we used the fragment ions *m/z* 344 and 348, respectively (Table I) The same effect could be seen in the most intense daughter ions of 6-oxo-$PGF_{1\alpha}$ (*m/z* 268) and $[^2H_4]$-6-oxo-$PGF_{1\alpha}$ (*m/z* 272) Therefore, we used the daughter ions of *m/z* 434 and 438, respectively (see Table I)

Figures 1, 2 and 3 show the daughter-ion chromatograms of PGE_2, 6-oxo-$PGF_{1\alpha}$ and $PGF_{2\alpha}$ derivatives The detection limits are about 10 pg of PGE_2, 40 pg of 6-oxo-$PGF_{1\alpha}$ and 5 pg of $PGF_{2\alpha}$ injected on-column with a signal-to-noise ratio of 5 1

The reproducibility of the immunoaffinity technique was determined by repeated analysis of the same spiked urine using 5, 10 or 20 ng of each synthetic prostaglandin per 10 ml of urine (Table II) The range of added prostaglandins corresponds to the actual concentrations in urine samples from patients measured in our laboratory over the past three years

The correlation coefficients were 0 997 (6-oxo-$PGF_{1\alpha}$) and 0 999 (PGE_2 and $PGF_{2\alpha}$) and the slopes were 0 99 (6-oxo-$PGF_{1\alpha}$) and 1 03 (PGE_2 and $PGF_{2\alpha}$)

The inter-assay coefficient of variation ranged between 8 6 and 9 5% for the unspiked urine samples and between 2 0 and 5 2% for the urine samples spiked with 20 ng of prostanoids

DISCUSSION

There have been many efforts to improve the determination of arachidonic acid metabolites in biological matrices For prostanoids, immunoaffinity chromatography in combination with GC–MS is a sensitive method [3–6] The specific antigen–antibody interaction is used for the purification of complex samples such as urine or plasma When coupling different antibodies to the same affinity column, extractions of several prostaglandins can be carried out in one step and the time for the clean-up procedure is shortened This simplifies the preparation of the sample, a basic requirement for use in routine analysis GC–MS–MS increases the specificity compared with GC–MS Chromatograms are obtained which are almost free from interferences Cross-reactivity of antibodies does not pose problems when using GC–MS–MS In fact, it is an advantage when the cross-reacting antigen is also to be determined The use of MABs leads to reproducible results, as shown in this paper Another advan-

tage of immunoaffinity is that there is no longer any need for radioactive tracers The handling of the immunoaffinity columns is easy compared with HPLC and the results obtained by the immunoaffinity technique are very reliable However, there is some loss of binding capacity when using the same column over a long period, possibly there is some 'bleeding' of the bound antibodies from the column It is therefore advisable to construct columns with excess binding capacity The efficiency of coupling depends on, among other factors, the ratio of the antibody concentration and affinity support Incomplete coupling might also be an explanation for an apparent loss of capacity Unbound antibodies could be gradually eluted and for this reason it is important to perform several wash cycles after coupling

Many supports are used for affinity chromatography and it would be of interest to establish which is the most suitable We used Affi-Gel, which shows high stability and permits coupling over a wide pH range (3 0–10 0) The preferred solvent systems for elution of the prostaglandins are acetonitrile–water and acetone–water We chose acetone–water because it is less noxious and can easily be evaporated to dryness However, the denaturating properties of acetone have to be considered and this could also be a reason for the loss of capacity of the affinity column The amount of the elution mixture has to be as small as possible The time of the elution procedure should be short and the following wash cycles with buffer and water have to be carried out immediately after the elution of the prostaglandins The stability of the columns could perhaps be improved when working in a cold-storage chamber

Because of the obvious advantages of the combination of immunoaffinity chromatography and (tandem) MS, we are planning to extend the use of this method in our laboratory to determine other arachidonic acid metabolites in various biological fluids

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