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Note

Determination of prostaglandins D_2, E_2, $F_{2\alpha}$ and 6-ketoprostaglandin $F_{1\alpha}$ as well as thromboxane B_2 in rat brain by gas chromatography–mass spectrometry

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In the ischemic brain, the liberation of arachidonic acid leads to increased formation of eicosanoids [1,2]. The inhibition of cyclooxygenase prior to ischemia seems to improve post-ischemic reflow in the brain [3–5].

A quantitative determination of cyclooxygenase products (COPs) by gas chromatography–mass spectrometry (GC–MS) with stable isotope dilution has been considered to be a reference standard [6]. A corresponding method for the quantitative determination of COPs in brain and other organs of the rat was described in detail several years ago [7,8]. In these papers the ionization mode used was electron impact (EI). Other authors have shown that negative-ion chemical ionization (NICI) yields far better detection limits than EI [9–11]; however, it has yet to be shown whether this technique is suitable for determination in brain tissue.

This paper describes a method for determination of various prostaglandins (PGs) and thromboxane B_2 (TXB_2) in lyophilized rat brain using GC–NICI-MS. Three reagent gases and two extraction procedures [7,12,13] were compared.

The brain was lyophilized before analysis, because other compounds of intermediary metabolism, which are prone to hydrolysis, were also determined from the same tissue [14].

EXPERIMENTAL

Reagents and solvents

[1-^{14}C]PGE_2 (specific radioactivity, 2.21 GBq/mmol) was obtained from

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Amersham Buchler (Braunschweig, F.R.G.). TXB_2, PGE_2, PGD_2, $PGF_{2\alpha}$ and 6-keto-$PGF_{1\alpha}$ were purchased from Sigma (Munich, F.R.G.). $[^2H_4]PGE_2$ and $[^2H_4]PGF_{2\alpha}$ were from IC Chemikalien (Munich, F.R.G.). $[^2H_4]$6-keto-$PGF_{1\alpha}$ and $[^2H_4]TXB_2$ were purchased from Dr. C.O. Meese, Dr. Margarete-Fischer-Bosch-Institut (Stuttgart, F.R.G.). Standard solutions of COPs were prepaired in acetonitrile and kept at −70°C.

Glassware was silanized with 2% (v/v) trimethylchlorosilane in toluene.

Tissue preparation

Rat brains were lyophilized and pulverized at the temperature of liquid nitrogen. As internal standards 5 ng each of $[^2H_4]PGF_{2\alpha}$, $[^2H_4]PGE_2$, $[^2H_4]$6-keto-$PGF_{1\alpha}$ and $[^2H_4]TXB_2$ were added to 90–100 mg of tissue powder (1 g dry weight corresponds to 4.3 g wet weight).

For the extraction of the COPs from the brain two different methods were used and compared with respect to recovery and quality of chromatograms.

Extraction procedure I

The spiked brain tissue powder was extracted using a modification of the method of Powell [12]. The tissue was treated twice with 1.5 ml of ethanol–water (9:1, v/v), the combined organic phases were evaporated to dryness and the residue was dissolved in ethanol–water (15:85, v/v) (pH adjusted to 3.5 with formic acid). In some experiments the solution was washed twice with 10 ml of petroleum ether. The aqueous phase was applied to a Sep-Pak C_{18} cartridge. After sample application, the cartridge was washed with 10 ml of ethanol–water (15:85, v/v; pH 3.5) and 10 ml of petroleum ether. COPs were eluted with 6 ml of ethyl acetate. The eluate was evaporated to dryness and the COPs were derivatized as described below.

Extraction procedure II

The extraction was carried out according to Mayer et al. [13]. The spiked tissue was extracted twice with 1.5 ml of methanol–water (9:1, v/v). The suspensions were centrifuged at 5°C; the pellet was discarded and the supernatants were combined and added to 14 ml of water, acidified to pH 3.5 with formic acid. The solution was applied to a Bond Elut RP-18 cartridge, prewashed with 20 ml of methanol, 20 ml of water and 5 ml of methanol–water (15:85, v/v; pH 3.5 with formic acid).

After sample application, the column was washed with 5 ml of methanol–water (15:85, v/v; pH 3.5) and the COPs were eluted with 5 ml of ethanol. The eluate was evaporated to dryness under nitrogen, and the residue dissolved in 0.5 ml of diethyl ether–petroleum ether (25:75, v/v).

The solution was applied to a silica gel column prepared in the following manner: 0.5 g of Silicar CC-4 was placed into a Pasteur pipette, washed with 2 ml of methanol, dried overnight at 80°C and washed with 2 ml of diethyl ether–petroleum ether (25:75, v/v). After application of the sample, the column was washed with 3 ml of diethyl ether–petroleum ether (75:25, v/v). COPs were eluted with 3 ml of ethyl acetate–methanol (9:1, v/v). The eluate was evaporated to dryness under nitrogen, and the residue was derivatized.

Derivatization

For NICI-MS the COPs were derivatized to methoxime pentafluorobenzyl ester tris(trimethylsilyl) ethers (MO–PFB–TMSi derivatives) according to ref. 11.

Methoximation. The samples obtained after evaporation were dissolved in 100 μl of redistilled anhydrous pyridine and treated with 25–35 mg of methoxyamine hydrochloride. The reaction continued at 40°C for 1 h, then the pyridine was removed under nitrogen. The residue was dissolved in 200 μl of water, and the solution was extracted three times with 400 μl of diethyl ether. The organic phases were combined and evaporated to dryness.

Pentafluorobenzyl esterification. The methoxime derivative was treated with 30 μl of acetonitrile–pentafluorobenzyl bromide (2:1. v/v) and 10 μl of diisopropylethylamine (freshly purified on basic aluminium oxide). Reaction time was 10 min at 40°C. Excess of reagents was removed under a stream of nitrogen.

Silylation. A 50-μl volume of bis(trimethylsilyl)trifluoroacetamide (BSTFA) was added to the dry residue and left to react for 1 h at 40°C. Samples were stored at 4°C until analysis.

Gas chromatography–mass spectrometry

A Varian GC 3700 equipped with a cooled injection system (Gerstel, Mülheim, F.R.G.) and an Ultra 2 fused-silica capillary column (12 m×0.2 mm I.D., 0.33 μm film thickness; Hewlett-Packard) was used. A retention gap (1 m×0.2 mm I.D.) was coupled to the analytical column. The injection was made in the splitless mode. The temperature of the injection port was programmed: starting temperature, 60°C; heating rate, 10°C/s up to 250°C; hold time, 100 s. The GC oven temperature started from 140°C with a heating rate of 35°C/min up to 290°C; this temperature was held for 5 min. Helium was used as carrier gas; the column head pressure was 150 kPa.

The derivatized samples were injected in BSTFA. Aliquots of 0.5 μl out of 50 μl were injected.

The capillary column was directly introduced into the ion source of the mass spectrometer (Finnigan MAT 8230). The derivatives of the COPs were determined by NICI-MS as $[M-PFB]^-$ ions.

Three reagent gases, namely ammonia, isobutane and methane, were compared with respect to the detector response. For this purpose, standard solutions of PGE_2, PGD_2, $PGF_{2\alpha}$ and 6-keto-$PGF_{1\alpha}$ (each 100 pg per 0.5 μl) were used. For the determination of COPs in brain tissue, ammonia was used as reagent gas.

Registration and quantification were carried out by multiple ion detection. Perfluorotributylamine was used as reference substance. The ion source temperature was 200°C, the emission current 0.1 mA, and the electron energy 92 eV.

Recovery and quantification

The recovery of PGE_2 from extraction procedures I and II was calculated. For this purpose, 900-Bq radioactively labelled PGE_2 was added to ca. 100 mg of dry brain tissue, which was worked up as described above.

For the determination of calibration curves ca. 100 mg of dry brain tissue were

spiked with various amounts (0.2–50 ng) of each COP as well as with 5 ng of each deuterated standard. The content of endogenous COP was determined and subtracted as blank value.

Deuterated PGD_2 was not available; for the quantification of PGD_2, the internal standard for PGE_2, namely $[^2H_4]PGE_2$, was used.

RESULTS AND DISCUSSION

Comparison of methane, isobutane and ammonia as reagent gases

For the determination of COPs by NICI-MS, methane is generally used as reagent gas [9,11,15,16]. Recently, methane, isobutane and ammonia were compared with respect to sensitivity and linearity for the determination of several PGs out of plasma [14]; however, a clear statement was only made with respect to ammonia, which seemed to be the preferred reagent gas.

In the present experiments the NICI mass spectra obtained from standard solutions were very similar and exhibited the same base peak, $[M-PFB]^-$ with each of the three gases. No statistically significant differences were obtained with respect to reproducibility and sensitivity for each COP. This held true also for the determination from freeze-dried brain tissue. Hence, following to Miyazaki et al. [10], ammonia was used for the determination of COPs in brain tissue.

Comparison of different methods of extraction of COPs from brain tissue

Extraction method I and extraction method II were both modifications of methods published earlier for GC–NICI-MS of PGs. However, method I was developed for measurements of PGs from bovine lung, human plasma and human urine [7], whereas method II was applied for determination in various biological materials but not brain tissue [8].

Using extraction method I, the recovery of $[^{14}C]PGE_2$ from brain tissue was $73.4 \pm 7.8\%$ (mean $\pm$ S.D., $n=5$). However, the extracts were considerably contaminated. Peak tailing and broadening as well as variable GC retention times were observed. To remove the contaminants, the aqueous tissue extract was washed twice with petroleum ether prior to application to the Sep-Pak column. GC of these samples resulted in sharp and symmetrical peaks with reproducible retention times. However, the recovery was drastically reduced, to $37.5 \pm 2.4\%$ (mean $\pm$ S.D., $n=6$). The tissue extracts obtained after extraction method II were very clean and even less contaminated than the samples washed with petroleum ether in extraction method I. The recovery of $[^{14}C]PGE_2$ was $88.2 \pm 3.2\%$ (mean $\pm$ S.D., $n=6$). Therefore method II is preferred for the extraction of COPs from rat brain.

Calibration curve, detection limit and reproducibility

The calibration curve and detection limit of COPs from brain tissue were determined using extraction method II; the curves were linear for each of the five COPs mentioned (concentration range 0.2–50 ng per 100 mg dry weight). The resulting correlation coefficients were 0.9993 for $PGF_{2\alpha}$ and 6-keto-$PGF_{1\alpha}$, 0.9991 for PGE_2, 0.9983 for PGD_2 and 0.9977 for TXB_2. The absolute detection limit

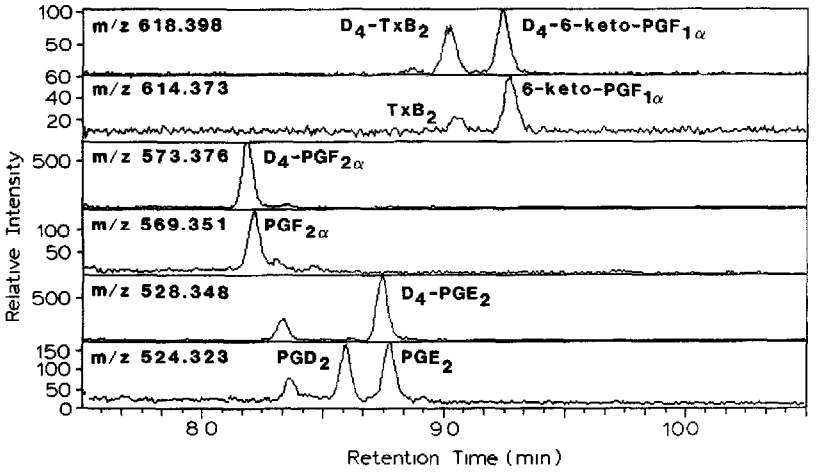

Fig. 1. Typical chromatogram of endogenous COPs in normoxic rat brain. Concentrations (per 100 mg dry weight): TXB_2, 1.4 ng; 6-keto-$PGF_{1\alpha}$, 1.2 ng; $PGF_{2\alpha}$, 2.0 ng; PGE_2, 0.6 ng; PGD_2, 1.2 ng.

was 0.5 pg per injection from standard solutions for each COP. For determination in brain tissue the limit is 100 pg per sample. This is lower than the concentrations usually found with the present method in the normoxic brain (0.3–4 ng per 100 mg) [17]. A typical chromatogram of a tissue extract from normoxic brain tissue is given in Fig. 1.

In measurements of brain concentrations of ca. 4 ng per 100 mg dry weight, the highest reproducibility was obtained for 6-keto-$PGF_{1\alpha}$ (1.6%) and the lowest for PGD_2 (5.7%) ($n=6$). However, as mentioned above, no deuterated standard was available for the latter compound.

To summarize, the method presented here gives a specific and sensitive determination of PGD_2, PGE_2, $PGF_{2\alpha}$, 6-keto-$PGF_{1\alpha}$ and TXB_2 from rat brain. The basal levels of the COPs mentioned can be easily determined in non-ischemic brain tissue. For an experienced analyst, the work-up and GC–MS analysis of ten samples and a calibration curve with four concentrations take two days.

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