Available online at www.sciencedirect.com
SCIENCE DIRECT
Journal of Chromatography B, 827 (2005) 39–43
JOURNAL OF CHROMATOGRAPHY B
www.elsevier.com/locate/chromb

Porous graphitic carbon chromatography–tandem mass spectrometry for the study of isoprostanes in human cerebrospinal fluid

Kristina Claeson Bohnstedt [a], Bo Karlberg [a], Hans Basun [b], Staffan Schmidt [c,*]

[a] *Department of Analytical Chemistry, Stockholm University, SE-106 91 Stockholm, Sweden*
[b] *Clinical Science, AstraZeneca R&D, Södertälje, SE-151 85 Södertälje, Sweden*
[c] *Research DMPK and Biomarkers, AstraZeneca R&D, Södertälje, SE-151 85 Södertälje, Sweden*

Received 2 November 2004; accepted 10 February 2005
Available online 15 April 2005

Abstract

F2-isoprostanes are produced by the non-enzymatic peroxidation of arachidonic acid in membrane phospholipids. This paper describes a new method for the determination of all four classes of F2-isoprostanes in human cerebrospinal fluid (CSF) involving separation on a 1 mm × 150 mm porous graphitic carbon (PGC) column and detection by triple quadrupole mass spectrometry in negative-ion electrospray mode. The sample pre-treatment consisted of an ultrafiltration step, following which 300 μl of CSF sample could be injected directly onto a 1 mm × 10 mm PGC guard column functioning as a trap for the analytes. The loading solvent was Milli-Q water at 125 μl/min. After 3 min, the sample was switched into the separation column. The F2-isoprostanes were separated in 20 min using a linear solvent gradient comprising water, methanol, acetonitrile and ammonium hydroxide at a pH of 9.5 and a flow of 50 μl/min The limit of detection (calculated as 3S/N) was approximately 40 pM (14 pg/ml). The assay was linear within the examined range (18–450 pg/ml), using CSF spiked with iPF2α-III standard ($r^2 > 0.995$). Repeatability data were calculated for CSF spiked to 90 pg/ml and the relative standard deviation (RSD) obtained was 3% ($n = 6$).

Keywords: Isoprostanes; Porous graphitic carbon; Cerebrospinal fluid; Oxidative stress

1. Introduction

In 1990, Morrow et al. reported the formation of prostaglandin F2-like compounds, F2-isoprostanes, in vivo by the free radical-catalyzed, enzyme-independent peroxidation of a membrane constituent, arachidonic acid [1]. The F2-isoprostanes are formed in situ, esterified to phospholipids and subsequently released, by phospholipase action, into circulation [2,3]. F2-isoprostanes have attracted much attention as possible biomarkers for the lipid peroxidation process in various biological systems [4–6]. Further, they have also been shown to possess biological activity indicating that they may play a role as mediators of oxidative stress [7].

Depending on which of the arachidonic acid hydrogens is first abstracted by free radicals, four different regioisomers of F2-isoprostanes can be formed, generating the four structural classes, named III–VI [8]. Further, there are eight possible diaisostereoisomers within each class. In Fig. 1, the three commercially available isoprostanes from class III are shown, which differ only in the orientation of their hydroxylic groups. Also depicted is the deuterated internal standard used, 8-*iso*-prostaglandin F2α-d_4.

Various approaches to the determination of F2-isoprostanes have been reported, including the chromatographic techniques gas chromatography (GC) and high-performance liquid chromatography (HPLC) in combination with mass spectrometry (MS), or tandem MS detection [9–11]. The GC approach is still by far the most common, since it is sensitive, but the sample preparation required is tedious due to the derivatization and purification steps involved. The HPLC-based determination of isoprostanes requires less sample pretreatment and no derivatization, but has higher detection limits compared to GC. Immunoassay techniques have also been

* Corresponding author. Tel.: +46 8 553 28224; fax: +46 8 553 29024.
E-mail address: staffan.schmidt@astrazeneca.com (S. Schmidt).

1570-0232/$ – see front matter © 2005 Elsevier B.V. All rights reserved.
doi:10.1016/j.jchromb.2005.02.023

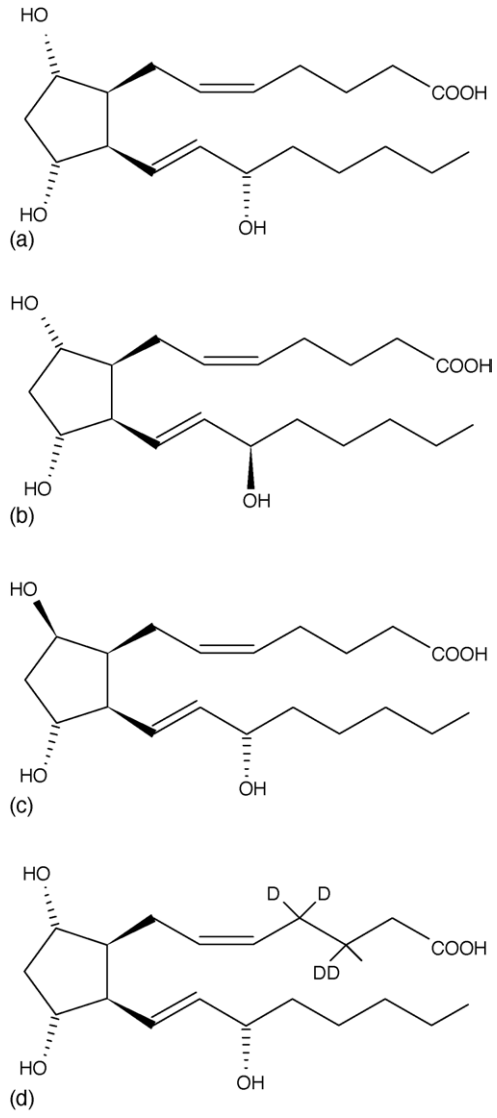

Fig. 1. Stereospecific molecular structures of the available standards: (a) 8-*iso*-prostaglandin F2α; (b) 8-*iso*-15(*R*)-prostaglandin F2α; (c) 8-*iso*-prostaglandin F2β; (d) 8-*iso*-prostaglandin F2α-d_4 (used as internal standard).

developed for F2-isoprostane determination that, if further refined, could offer simple analyses in the future. Currently, cross-reactivity within the group of F2-isoprostanes or their metabolites cannot be ruled out for these immunological methods. Cracowski et al. [4] have presented a survey of isoprostane determinations in various types of samples representing different disease states.

As oxidative stress and lipid peroxidation have been suggested to play a role in several neurological diseases, human cerebrospinal fluid (CSF) is a potentially important matrix for studies of isoprostane levels. The sampling of CSF is more invasive than, for example, blood and (especially) urine collection. However, it is possible that information regarding oxidative damage in isolated diseased regions in the brain may be distorted by the larger basal production in all tissues if peripheral isoprostane levels are determined. The corresponding levels in CSF might therefore provide more relevant information. Alzheimer's disease is an example of a condition where peripheral levels of F2-isoprostanes do not seem to reflect central nervous system levels [12]. There is an agreement within the literature that F2-isoprostane levels are elevated in CSF from patients with probable Alzheimer's disease [13,14]. However, the literature regarding the peripheral levels is less consistent in this respect.

To our knowledge, HPLC–tandem MS has not yet been used for the determination of isoprostanes in CSF, probably because the limits of detection are deemed to be too high. The GC–MS approach has been exclusively used and the reported levels of isoprostanes in CSF range between approximately 10 and 50 pg/ml in healthy control subjects with an average around 23 pg/ml [15,16].

The final determination step is equally rapid (roughly) for most of the methods discussed above, leaving little scope for time or labor savings, but much time and manual work could be saved if the sample preparation could be simplified and automated to a greater extent. We have earlier described a convenient HPLC–tandem MS method for the determination of isoprostanes in human urine involving robotized liquid–liquid extraction and separation on a porous graphitic carbon (PGC) column followed by tandem MS detection [17]. PGC is a material developed by Knox and co-workers [18,19] that has unique properties for separating polar and closely structurally related compounds. Here, we describe the use of this material for the separation of isoprostanes in human CSF, employing an even further simplified sample pre-treatment regime.

2. Experimental

2.1. Chemicals

Synthetic (>99% pure) class III isoprostanes comprising 8-*iso*-prostaglandin F2α, 8-*iso*-prostaglandin F2α-d_4 (used as internal standard), 8-*iso*-15(*R*)-prostaglandin F2α and 8-*iso*-prostaglandin F2β were purchased from Cayman Chemicals (Ann Arbor, MI) and were used without further purification. Solutions were prepared with water from a Milli-Q system (Millipore, Billerica, MA, USA). All other chemicals were of analytical grade and were purchased from local suppliers.

2.2. Cerebrospinal fluid samples

As a matrix, commercially available pooled human CSF obtained by lumbar puncture was used (US Biological, Swampscott, MA). The samples were stored at −70 °C until analysis.

2.3. Ultrafiltration

After thawing on ice, the CSF sample was mixed with internal standard and subsequently ultra-filtered at 2000 × *g*,

10 °C, for 10 min through a Centricon centrifugal device (30 MW cut-off, Millipore, Billerica, MA, USA). The resulting filtrate was then injected into the LC–MS/MS system.

2.4. Chromatography

A 300 μl sample portion was loaded, in three consecutive 100 μl injections, onto a trap consisting of a 1 mm × 10 mm Hypercarb guard column (ThermoHypersil-Keystone, Bellefonte, PA, USA), and a Rheos 4000 LC pump (Flux Instruments, Basel, Switzerland) was used to deliver the loading solvent (water) at a flow rate of 125 μl/min. An electrically actuated six-port valve was used to switch the sample into the separation column and the time between the last sample injection and back-flushing was 3 min. The chromatographic separation system included an HTS PAL autosampler (CTC Analytics AG, Zwingen, Switzerland), two connected LC-10AD pumps (Shimadzu, Kyoto, Japan) and a 1 mm × 150 mm, 5 μm Hypercarb porous graphitic carbon column (ThermoHypersil-Keystone, Bellefonte, PA, USA) as the separation column. The mobile phase consisted of water with 0.5% ammonia (pH 9.5) (solvent A) and acetonitrile–methanol (60:40) with 0.5% ammonia (solvent B). The flow rate was 50 μl/min. The separation was carried out using a linear solvent gradient that started at 50% B and increased to 100% B over 20 min before a 5 min hold at 100% B.

2.5. Mass spectrometry

A Micromass Ultima (Micromass, Beverly, MA, USA) triple quadrupole mass spectrometer was used in these studies. The instrument was operated in negative-ion electrospray mode with the ESI voltage set at 2.5 kV, the cone voltage at 40 V, the source temperature at 110 °C, and the desolvation temperature and gas flow at 300 °C and 600 1/h, respectively. The collision energy was 25 eV and the argon gas pressure in the radiofrequency-only region was 2.2 μbar. The analyzer resolution parameters were all set to give unit resolution at half peak height. Multiple reaction-monitoring mode was used during analysis. All four groups of isoprostanes generate the same quasi-molecular ion (M − H) at *m*/*z* 353 in negative mode ESI conditions. Class-specific daughter ions were selected, as suggested in [9], i.e., *m*/*z* 193, 127, 151 and 115 ions for classes III–VI, respectively.

3. Results and discussion

3.1. Method performance

With the instrument settings described above, the limit of detection (LOD, 3S/N) for iPF2α-III, without the column-switching system, calculated for a more normal (20 μl) injection volume, was approximately 500 pM. With the trapping procedure the LOD was lowered to approximately 40 pM (14 pg/ml). CSF spiked with iPF2α-III standard at a range of concentrations (18, 32, 90, 180 and 450 pg/ml) were analyzed, and the assay was found to be linear within this range, with a correlation coefficient (r^2) >0.995. Repeatability data were calculated for a CSF sample spiked to 90 pg/ml and the relative standard deviation (RSD) obtained was 3% ($n = 6$).

3.2. Analysis of the cerebrospinal fluid samples

Previously, isoprostanes in CSF samples have only been analyzed by GC–MS, but here the HPLC–MS method described above was used for this purpose. Fig. 2 shows a CSF sample spiked with the three available standards, the iPF2α-III peak corresponds to 32 pg/ml. None of the class III isoprostanes that are available as standards could be detected. One peak appeared a few minutes after the standards and its concentration was estimated to be approximately 25 pg/ml. This level is in accordance with the results previously reported in literature. The class VI isoprostanes were represented by a number of slightly more intense peaks than the other classes in the samples' ion chromatogram. This tendency was also seen in urine samples that we reported previously [17]. Even though the signals obtained from CSF samples rise only moderately above the noise level the results are repeatable and the peak pattern is similar from run to run.

3.3. Analysis system

Ultrafiltration is a rapid and simple technique that was used to improve the robustness of the analytical system. Although it was possible to inject 300 μl of untreated CSF directly into the system, pressure slowly increased over the trap. When ultrafiltration was used, no significant pressure changes occurred over the trap during the course of at least 100 injections. In order to verify that no isoprostanes were lost due to adsorption in the ultrafiltration device, the responses of standards with and without filtration were compared. The calculated average recovery over the filtration step was 99% ($n = 6$).

The levels of isoprostanes in CSF are lower than those in urine. This places high demands on the analytical system, and our ambition was to establish a sensitive yet robust technique, allowing both automated, quantitative transfer of the analytes of interest by the column-switching system, and as much of the sample as possible to be injected. Our earlier experiences of porous graphitic carbon and isoprostanes have shown that the analytes can be very strongly retained on the PGC material and that elution is promoted by adding ammonia to the mobile phase [17]. When pure water was used as the sample-loading medium, the analytes were retained on the trap and no peak broadening was noticed as a result of injecting the three sample aliquots. In fact, injecting three aliquots seemed beneficial, since the pressure fluctuations over the trap during injection were weaker than those that occurred when one large injection was made. Injection into the trap allows the sample to be focused and desalted before it reaches the an-

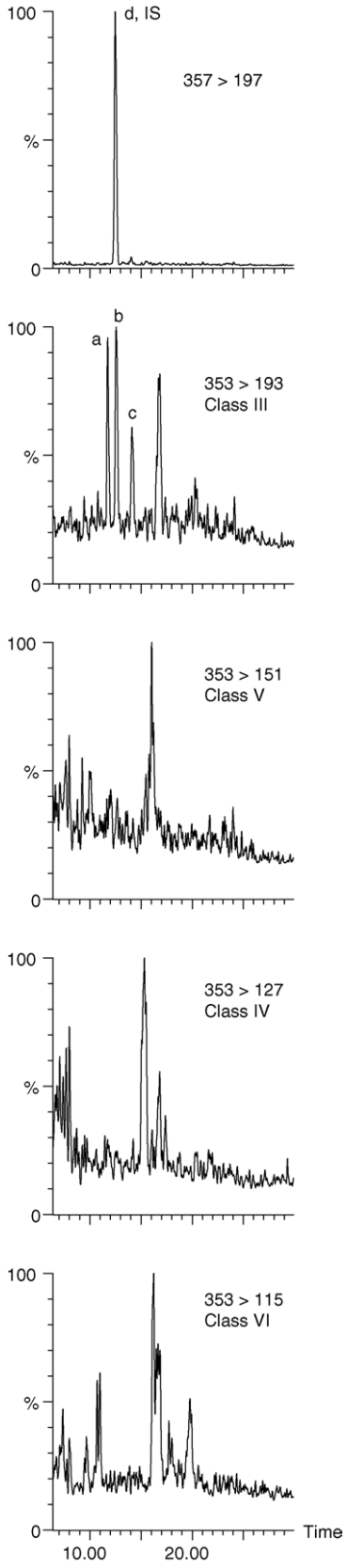

Fig. 2. HPLC–MS/MS analysis of a CSF sample spiked with isoprostane standard: (a) 8-*iso*-15(*R*)-prostaglandin F2α; (b) 8-*iso*-prostaglandin F2α; (c) 8-*iso*-prostaglandin F2β; (d) 8-*iso*-prostaglandin F2α-d_4 (IS). The 8-*iso*-prostaglandin F2α peak corresponds to 32 pg/ml.

alytical column, allowing a much larger volume of the sample to be injected, thereby improving concentration detection limits.

In order to detect pM concentrations of analyte in a complex matrix, a sensitive and selective detector is required. All F2-isoprostanes have the same mass (354) but, fortunately, the four different classes of isoprostanes generate class-specific daughter ions [9]. Negative mode electrospray tandem mass spectrometry offers both sensitivity and selectivity. Further, the composition of the mobile phase is an important factor that strongly affects the ESI process and thus detection limits. A high content of organic solvents in the mobile phase in combination with well-ionized analytes reaching the MS interface is beneficial for the analysis. The chemical robustness and strong retention of the PGC material thus allow experimental settings to be used that are very suitable for the ESI process. These features, in combination with the on-line enrichment and desalting offered by column switching over the trap, enable the direct injection of cerebrospinal fluid.

4. Conclusions

In this paper, we describe a method for the separation and determination of isoprostanes in human CSF. The method involves injecting a relatively large sample volume (300 μl) after an ultrafiltration step of just 10 min. The very low levels of the isoprostane analytes in the samples are slightly above or close to the LOD levels, resulting in signals for the analytes that are only just possible to determine. Due to the lack of commercially available standards, positive identification of F2-isoprostanes in the classes IV–VI could not be performed.

However, the use of a very selective analytical set-up including a PGC chromatographic column, negative-ion electrospray, and tandem mass spectrometry monitoring highly specific daughter ions strongly indicates the identity of the unknown peaks as F2-isoprostanes. Current developments in mass spectrometers are rapidly producing more sensitive instruments, which should allow the detection limits to be further improved. The fast, convenient methodology presented here could give increased knowledge about isoprostane levels in CSF from various disease states where lipid peroxidation and oxidative stress are suspected.

Acknowledgements

Carina Norsten-Höög is acknowledged for allowing us to use the MS instrument and Anette Mörtberg for providing the CSF samples.

References

[1] J.D. Morrow, K.E. Hill, R.F. Burk, T.M. Nammour, K.F. Badr, L.J. Roberts 2nd, Proc. Natl. Acad. Sci. U.S.A. 87 (1990) 9383.

[2] J.D. Morrow, J.A. Awad, H.J. Boss, I.A. Blair, L.J. Roberts 2nd, Proc. Natl. Acad. Sci. U.S.A. 89 (1992) 10721.
[3] J.D. Morrow, J.A. Awad, T. Kato, K. Takahashi, K.F. Badr, L.J. Roberts 2nd, R.F. Burk, J. Clin. Invest. 90 (1992) 2502.
[4] J.-L. Cracowski, T. Durand, G. Bessard, Trends Pharmacol. Sci. 23 (2002) 360.
[5] D. Pratico, J. Rokach, J. Lawson, G.A. FitzGerald, Chem. Phys. Lipids 128 (2004) 165.
[6] S.S. Fam, J.D. Morrow, Curr. Med. Chem. 10 (2003) 1723.
[7] D.J. Crankshaw, P.K. Rangachari, Mol. Cell. Biochem. 253 (2003) 125.
[8] R.J. Waugh, J.D. Morrow, L.J. Roberts 2nd, R.C. Murphy, Free Radic. Biol. Med. 23 (1997) 943.
[9] H. Li, J.A. Lawson, M. Reilly, M. Adiyaman, S.W. Hwang, J. Rokach, G.A. FitzGerald, Proc. Natl. Acad. Sci. U.S.A. 96 (1999) 13381.
[10] J.D. Morrow, L.J. Roberts 2nd, Methods Enzymol. 300 (1999) 3.
[11] D. Pratico, J.A. Lawson, G.A. FitzGerald, J. Biol. Chem. 270 (1995) 9800.
[12] T.J. Montine, J.F. Quinn, D. Milatovic, L.C. Silbert, T. Dang, S. Sanchez, E. Terry, L.J. Roberts 2nd, J.A. Kaye, J.D. Morrow, Ann. Neurol. 52 (2002) 175.
[13] T.J. Montine, M.F. Beal, M.E. Cudkowicz, H. O'Donnell, R.A. Margolin, L. McFarland, A.F. Bachrach, W.E. Zackert, L.J. Roberts, J.D. Morrow, Neurology 52 (1999) 562.
[14] D. Pratico, C.M. Clark, V.M. Lee, J.Q. Trojanowski, J. Rokach, G.A. FitzGerald, Ann. Neurol. 48 (2000) 809.
[15] K.S. Montine, J.F. Quinn, J. Zhang, J.P. Fessel, L.J. Roberts 2nd, J.D. Morrow, T.J. Montine, Chem. Phys. Lipids 128 (2004) 117.
[16] D. Pratico, V.M.Y. Lee, J.Q. Trojanowski, J. Rokach, G.A. Fitzgerald, FASEB J. 12 (1998) 1777.
[17] K.C. Bohnstedt, B. Karlberg, L.-O. Wahlund, M.E. Jonhagen, H. Basun, S. Schmidt, J. Chromatogr. B: Biomed. Sci. Appl. 796 (2003) 11.
[18] M.T. Gilbert, J.H. Knox, B. Kaur, Chromatographia 16 (1982) 138.
[19] J.H. Knox, B. Kaur, G.R. Millward, J. Chromatogr. 352 (1986) 3.