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Journal of Chromatography B, 796 (2003) 11–19

JOURNAL OF CHROMATOGRAPHY B

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Determination of isoprostanes in urine samples from Alzheimer patients using porous graphitic carbon liquid chromatography–tandem mass spectrometry

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Received 1 May 2003; received in revised form 10 July 2003; accepted 23 July 2003

Abstract

F2-isoprostanes (F2-iPs) comprise four classes of isomers produced non-enzymatically by free radical attack on arachidonic acid, a component of the cell membrane. This paper describes a new method for the quantification of F2-isoprostanes in urine samples from thoroughly diagnosed Alzheimer's disease (AD) patients. The sample pretreatment consisted of liquid extraction of 900 μl urine with diethyl ether, its subsequent evaporation, and finally, reconstitution in 50 μl water. Of this, 20 μl was injected into a HPLC system with a 15 mm × 1 mm porous graphitic carbon column coupled to a triple quadrupole mass spectrometer running in negative electrospray ionization mode. The F2-isoprostanes were separated in 15 min using a linear solvent gradient comprising water, methanol, acetonitrile and ammonium hydroxide at a pH of 9.5. The average recovery obtained was approximately 75%. The limit of detection (3S/N) was calculated for iPF2α-III to be 0.7 pg injected on column, corresponding to 0.1 nM. The average level of iPF2α was 241 ± 163 pg/mg creatinine in the urine samples from AD patients (average ± standard deviation). The corresponding control values were 216 ± 101 pg/mg creatinine, i.e. no statistically significant difference was noticed. No correlation pattern specific to Alzheimer's disease was revealed by principal component analysis of the isoprostane peaks obtained either. The results from this study support earlier findings that levels of peripheral isoprostanes are not increased in patients with Alzheimer's disease.
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Keywords: Isoprostanes; Porous graphitic carbon

1. Introduction

Alzheimer's disease (AD) is a severe neurodegenerative disorder that affects millions of people worldwide, and as the average age of the population increases, so will this problem. Free radical

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doi:10.1016/S1570-0232(03)00600-7

damage and lipid peroxidation are believed to contribute to the neuronal death seen in the disease [1–4]. A suitable biomarker for these processes would therefore be very useful in the diagnosis of AD and subsequent therapeutic assessments. Several such biomarkers have been proposed, including the F2-isoprostanes (F2-iPs), first described in 1990 by Morrow et al., who suggested that they could be used as non-invasive markers of oxidant status in humans [5]. The F2-iPs constitute a family of lipids, isomers of the enzymatically-derived prostaglandins, that are formed in vivo by an enzyme independent, free radical catalyzed, peroxidation of arachidonic acid in membrane phospholipids [5]. Cleavage by phospholipases generates free F2-iPs that initially circulate in both unesterified and esterified forms, and are thereafter excreted in urine [6]. The F2-iPs can be divided into four subgroups of regioisomers: types III, IV, V and VI, with 16 possible isomers within each group [7] (see Fig. 1). Elevated levels of isoprostanes have been reported in various physiological states associated with enhanced lipid peroxidation and oxidative stress, such as cardiovascular and neurological disease [3,8–11]. However, there is no consensus in the literature as to whether levels of peripheral F2-iPs are increased in AD or not [12–16].

A large amount of work has been done in the field of isoprostane analysis since they were first described, and two main approaches have been adopted for their quantification in various biological samples. The first is an immunological approach involving radioimmunoassays (RIA) and enzyme immunoassays (EIA) that in many cases are inexpensive and easy to perform. These methods are considered to give only a semi-quantitative estimate of isoprostane levels, since the risk of cross reactivity is significant. The second approach is based on chromatographic separation and detection by mass spectrometry (MS). One of the most frequently used methods of this kind is gas chromatography–electron capture negative chemical ionization mass spectrometry (GC–ECNI-MS) detection [17]. This technique is very sensitive but

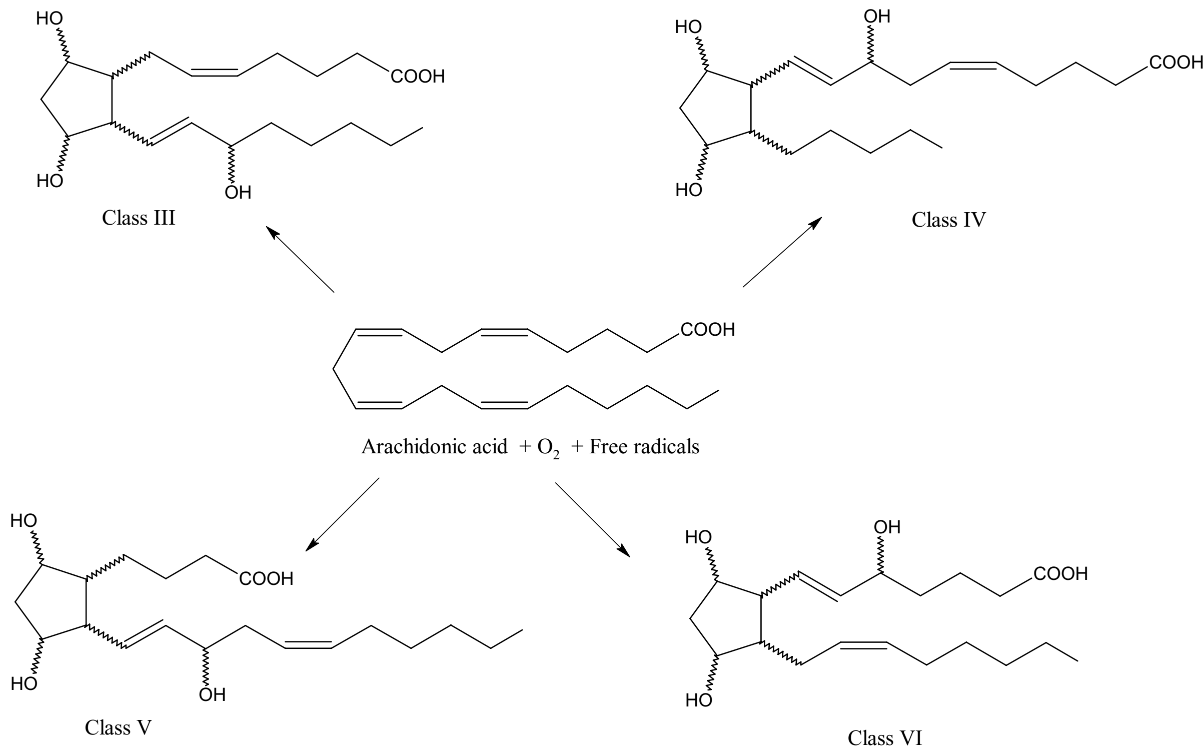

Fig. 1. Free radical attack on arachidonic acid yields four different classes of F2-isoprostanes.

time consuming and labor intensive due to the derivatization steps required, which also result in low recovery yields. Furthermore, it lacks the resolving power needed to fully isolate a single isoprostane isomer. LC–MS has been used as an alternative to GC–ECNI-MS for the determination of isoprostanes in urine and plasma samples [18–20]. A common feature of these methods is that they mainly focus on individual isomers of F2-iPs or metabolites thereof. It is probably advisable, or necessary even, to first study all four classes of F2-iPs and also as many isomers as possible within each class before selecting a single F2-iP isomer that can reflect the variations in formation of the entire F2-iP family. Li et al. have successfully separated F2-iP isomers in the four classes using a C_{18} stationary phase [18], but this separation is complex and the results have been difficult to repeat. Therefore, we decided to test whether the separation could be improved using porous graphitic carbon (PGC) as the stationary phase rather than alkyl-bonded silica. PGC, which was introduced as a stationary phase for HPLC by Knox et al. [21,22], is known to have strong reversed phase properties, but it also has other, unique, properties that are useful in separating polar and closely structurally related compounds. Further advantages of PGC are its physical and chemical robustness, and its stability over the entire pH range.

The aim of this study was to develop a sensitive, simple, and fast method for the separation and detection of isomers within the four classes of F2-iPs in urine samples from AD patients, employing porous graphitic carbon HPLC separation, and ESI-tandem MS detection. The developed method was applied on urine samples from AD patients to evaluate peripheral F2-iP levels. The effects of experimental parameters in extraction, separation, detection and data evaluation are discussed.

2. Experimental

2.1. Chemicals

Synthetic 8-iso Prostaglandin F2α, 8-iso Prostaglandin F2α-d4, 8-iso-15(*R*)-Prostaglandin F2α, 8-iso Prostaglandin F2β and Prostaglandin F2α were purchased from Cayman Chemicals (Ann Arbor, MI, USA) and were used without further purification. The molecular structures of the standards are presented in Fig. 2. Solutions were prepared with water from a Milli-Q system (Millipore, Billerica, MA, USA).

2.2. Patients and controls

The study population consisted of 23 patients with clinical AD diagnoses confirmed according to DSM-IV criteria [23]. Demographic data are shown in Table 1. These patients had been consecutively referred to the Memory Clinic at Huddinge University Hospital, for a complete dementia assessment, including a physical examination, neuropsychological evaluation, a cognitive screening test (Mini-Mental State Examination (MMSE) [24]), laboratory tests, ECG and EEG recordings, and a CT or MRI scan. Patients, referred to the memory clinic with subjective memory complaints, where comprehensive assessments showed normal brain scans and neuropsychological test results within normal limits, with regard to age and education, were considered controls. Thus, 30 non-demented, cognitively intact control patients were included in the study. The ethical committee at Karolinska Institute approved the study.

2.3. Urine sample preparation

Urine was collected between 10 and 12 a.m. and immediately frozen and stored at −70 °C until analy-

Table 1
Demographic and clinical characteristics of AD patients and control subjects

	AD	Control
Number	23	30
Male/female ratio	11/12	12/18
Age (years)	75.5 (10)[a]	63.1 (10)[a]
ApoE alleles		
ε4+/ε4−	14/9	8/19
NA[c]	–	3
MMSE[b]	22.4 (4.5)[a]	28.9 (1.1)[a]
Smokers	4	7
Duration (months)	30 (19)[a]	–
NA[c]	3	–

[a] Average (standard deviation).
[b] Mini-Mental State Examination.
[c] Not assessed.

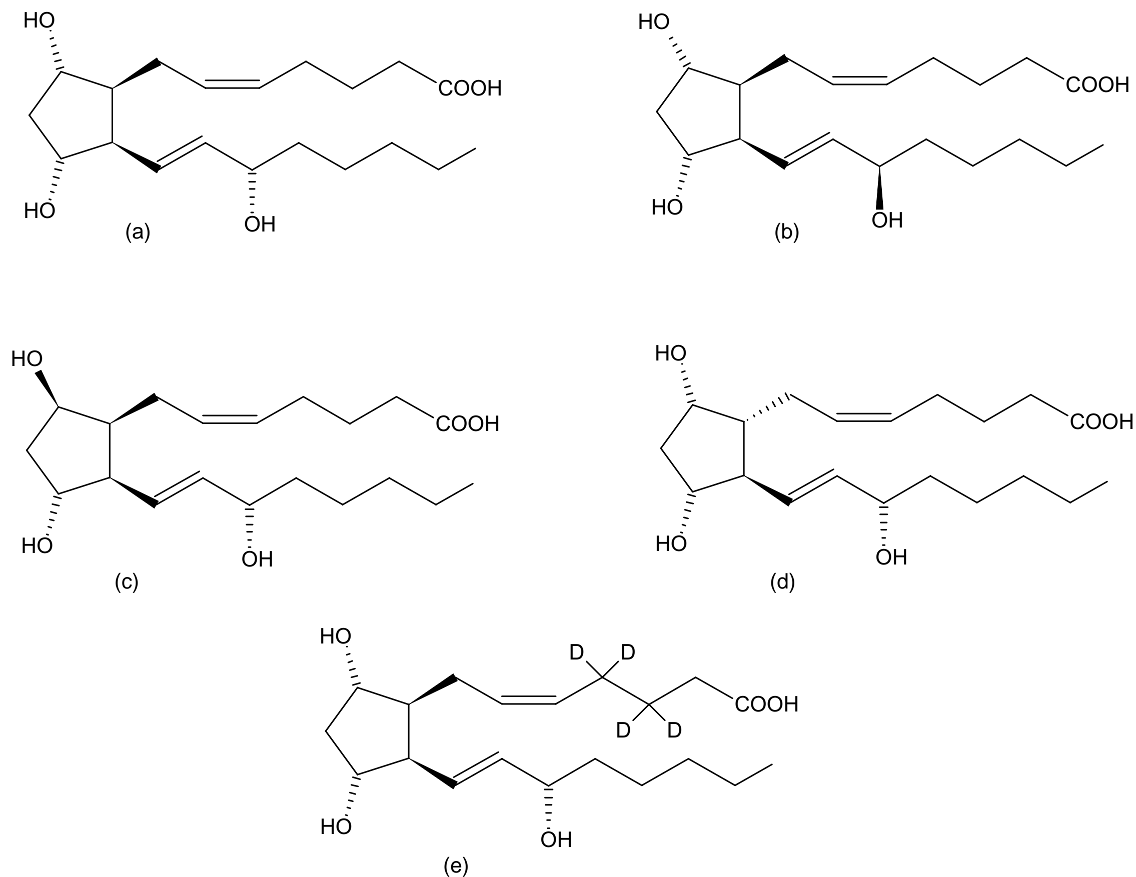

Fig. 2. Stereospecific molecular structures of the available standards: (a) 8-iso Prostaglandin F2α; (b) 8-iso 15(*R*)-Prostaglandin F2α; (c) 8-iso Prostaglandin F2β; (d) Prostaglandin F2α and (e) 8-iso Prostaglandin F2α-d4 (IS).

sis. The labeled internal standard, 8-iso Prostaglandin F2α-d4, was added to 900 μl of urine, and the pH was subsequently adjusted to approximately 2.5 by the addition of 10 μl 6 M HCl. From this mixture, 900 μl was extracted with 2.7 ml diethyl ether for 10 min using a Reax 2 overhead mixer (Heidolph, Germany) and the ether phase was then washed with 900 μl of acidified water. The resulting organic layer was evaporated under a stream of nitrogen at 30 °C. The residue was dissolved in 50 μl water and filtered through a 0.5 μm disposable syringe sample filter (Upchurch Scientific, Oak Harbor, WA, USA) before injection into the LC–ESI-MS system. Liquid handling operations were performed with a Genesis robotic sample processor (Genesis RSP 150, Tecan AG, Männedorf, Switzerland). A flow diagram of the liquid–liquid extraction is presented in Fig. 3. The urine creatinine levels were determined at Huddinge University Hospital, Stockholm, Sweden.

2.4. High performance liquid chromatography

The chromatographic system included an autosampler (HTS PAL, CTC Analytics AG, Zwingen, Switzerland), two connected pumps (Shimadzu LC-10AD, Kyoto, Japan) and a Hypercarb, 5 μm, 1 mm × 150 mm, porous graphitic carbon column (ThermoHypersil-Keystone, Bellefonte, PA, USA). The mobile phase consisted of water with 0.5% ammonia pH 9.5 (solvent A) and acetonitrile:methanol 40:60 with 0.5% ammonia (solvent B). The flow rate

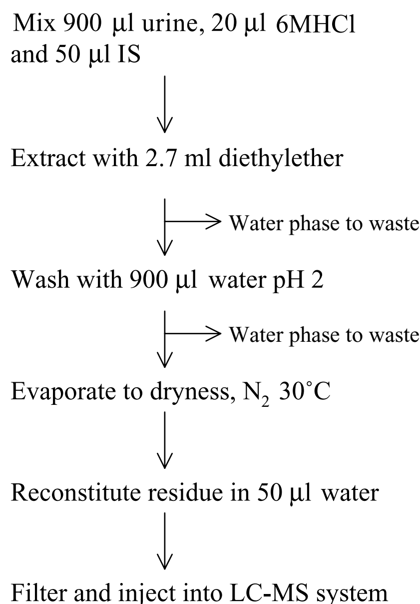

Fig. 3. Flow diagram of the sample pretreatment procedures.

was 50 µl/min, and the sample volume injected was 20 µl. The separation was carried out using a linear solvent gradient program 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 mode electrospray with the ESI voltage set at 2.5 kV, the cone voltage at 40 V, the source temperature at 100 °C, and the desolvation temperature and gas flow at 200 °C and 600 l/h, respectively. The collision energy was 25 eV and the argon gas pressure in the radiofrequency-only region was 2.2 µbar. The analyzer parameters were set to give 1 amu resolution at half peak height.

2.6. Data analysis

Principal component analysis (PCA) was applied to the data to elucidate the correlation patterns of the chromatogram peaks. Unscrambler 7.01 (Camo A/S, Oslo, Norway) software was used for this purpose.

3. Results and discussion

3.1. Method development

3.1.1. Extraction from urine

Initially, a solid phase extraction procedure from [18] was tried. This approach worked more as a pre-concentration than as a sample purification step, and was insufficiently selective to prepare samples for separation and detection using the PGC-LC–MS system. As an alternative, liquid–liquid extraction was tested as described in Section 2.3. A Tecan liquid handling robot performed the pipetting involved in the extraction, and various extraction solvents were tested; namely, ethyl acetate, toluene, diisopropyl ether, and diethyl ether. Extraction with diethyl ether gave the best recovery and it could be rapidly evaporated at a low temperature. The acidified water-washing step reduced matrix effects otherwise observed at the beginning of the chromatograms.

3.1.2. Separation and mass spectrometry

It is possible that the batch-to-batch variations of the C_{18} stationary phases were responsible for the difficulties encountered in reproducing the results published by Li et al. [18]. Unshielded silanol groups in the polymeric backbone of the C_{18} stationary phase seem to affect the separation of F2-iPs. By using a column packed with a silanol-rich C_{18} stationary phase, Spherisorb ODS1, 120 Å, 3 µm, 150 mm × 0.3 mm i.d. (packed in-house) and the mobile phase system suggested in [18], some separation of the F2-iP standards was achieved. However, this approach was discontinued due to the more favorable results obtained using PGC.

The PGC material behaves somewhat like a strong reversed phase stationary phase, usually requiring stronger eluting mobile phases with higher levels of an organic modifier than C_{18}. PGC also tends to retain molecules containing polar groups, via a mechanism called the “polar retention effect on graphite” (PREG). This makes PGC very suitable for the separation of ionizable molecules such as acids. Due to its flat surface, as compared to the more fluid nature of octadecyl-bonded silica, PGC has a greater ability to

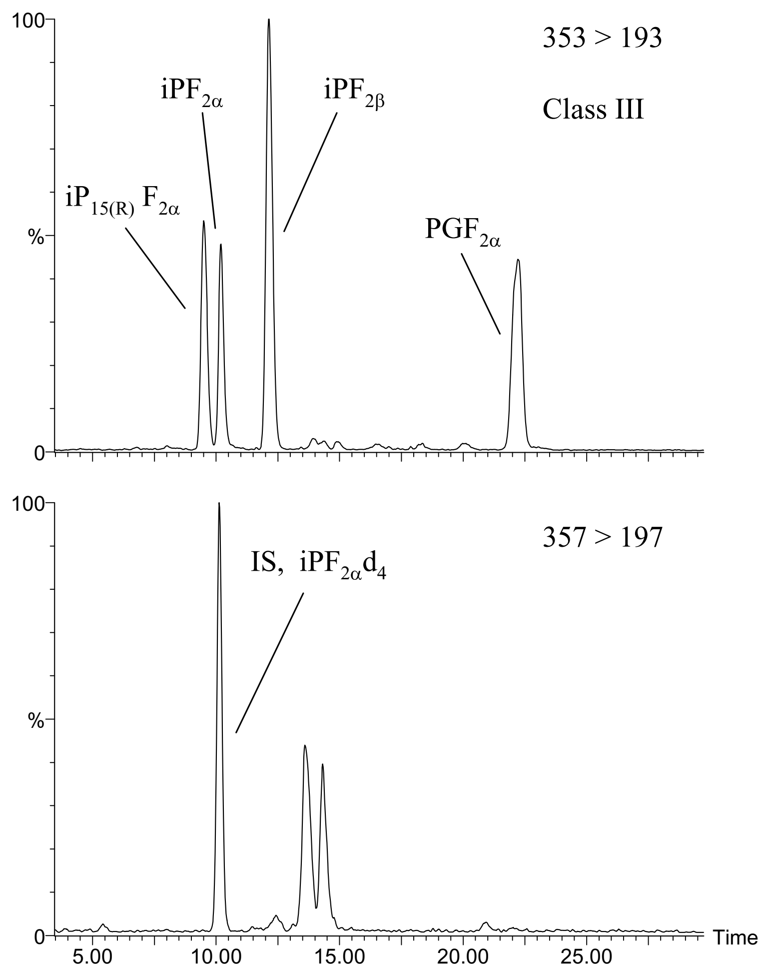

Fig. 4. HPLC/MS/MS analysis of a urinary sample spiked with the standards in Fig. 2.

discriminate between compounds according to steric factors. This gives it unique qualities that are useful in separating structurally related compounds. Both of these features were utilized in this investigation. Fig. 4 shows a chromatogram of urine spiked with all of the available standards. The isoprostanes were separated within 15 min, while the prostaglandin was retained to a greater degree, and eluted after 20 min. The retention and separation of the analytes are highly dependent on both the amount of ammonia added and on the ratio of ACN to methanol. The analytes are completely retained on the column if pure ACN is used as the mobile phase, they elute in a single peak in less than 5 min if ammonia is added to the ACN, but they are retained for more than an hour if pure methanol is used, even when ammonia is added. If ACN, methanol and ammonia are mixed together, both sufficient retention and good separation can be achieved. Fig. 5 shows the relationship between the retention of iPF2α and the composition of the mobile phase. The dotted line represents the conditions of mobile phase B (40% MeOH, 60% ACN and 0.5% NH_4) giving the best separation of the three standard isoprostanes. These results imply that ammonia promotes elution, but the effect does not seem to be exclusively pH dependent, as the separation shown in Fig. 4 can also be achieved when ammonia is exchanged for a relatively high concentration of ammonium acetate (40 mM, adjusted to

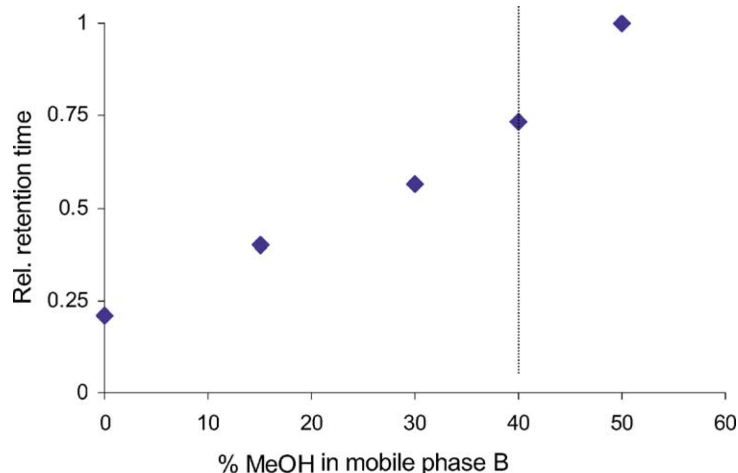

Fig. 5. Relationship between the retention of iPF2α on the PGC column and the composition of the mobile phase. The dotted line represents the solvent B composition (40% MeOH, 60% ACN with 0.5% NH_4) giving separation of the three standard isoprostanes.

pH 5.7), data not shown. The retention mechanism of PGC is complex and not completely understood. Current knowledge on the subject is reviewed elsewhere [25–27], and elucidating its fundamental basis was not a goal of this investigation. Our prime objective was to obtain the best separation and detection conditions for our specific analytes. The ESI process is favored when there are high levels of easily evaporated organic solvents in the mobile phase, and it is also advantageous to have the solutes highly ionized by the time they reach the interface. When using PGC, it is thus possible to achieve retention and separation of the weakly acidic isoprostanes at a pH of 9.5, where the acid is completely dissociated, while maintaining high organic contents in the mobile phase. The high amount of ammonia in the mobile phase did not reduce the ionization and detection sensitivity. This was tested by infusing IPF2α with 0, 0.05, 0.1, 0.2 and 0.5% ammonia in the mobile phase while simultaneously monitoring the response on the MS. The mobile phase containers need to be sealed to prevent the evaporation of ammonia. Loss of ammonia might otherwise generate prolonged retention times. All four groups of isoprostanes generate the same pseudo-molecular ion ($M - \mathrm{H}$) at m/z 353 in negative mode ESI conditions. Class-specific product ions were selected, as suggested in [18], i.e. class III, m/z 193; class IV, m/z 127; class V, m/z 151 and class VI, m/z 115.

3.1.3. Method evaluation

The recovery of the extraction procedure was determined at three concentration levels of 8-iso Prostaglandin F2α: 0.18, 0.88 and 2.65 ng/ml spiked to a reference urine sample from a healthy volunteer. The originally present level of 8-iso Prostaglandin F2α in this reference urine sample was determined separately and found to be 99 pg/ml. This value was subtracted from the found levels in the spiked samples. The recovery obtained was approximately 75% at all three investigated levels. The wide-range linearity of the assay was evaluated by spiking urine with all available isoprostane standards to concentrations ranging from 35 pg/ml to 350 ng/ml ($n = 8$); the working range linearity in a similar manner from 35 pg/ml to 35 ng/ml ($n = 8$). The levels in the real samples were expected to fall within this latter range. The assay was linear within the tested ranges, with a correlation coefficient, $r^2 > 0.99$, for all the available standards. The assay's accuracy and precision were estimated by spiking control samples at four levels: 0.30, 1.45, 2.30 and 4.16 ng/ml. Within-day and between-day precision data are presented in Table 2. The limit of detection (3S/N) was calculated for 8-iso Prostaglandin F2α to be 0.7 pg injected on column, corresponding to 35 pg/ml. After injecting 350 ng/ml of each of the available standards, the carry-over was less than 0.01%.

Table 2
Assay repeatability and reproducibility

Sample concentration (ng/ml)	Within-day[a]		Between-day[b]	
	Mean	% CV	Mean	% CV
0.30	0.28	7.0	0.28	7.8
1.45	–	–	1.45	5.2
2.30	2.29	5.2	2.24	6.0
4.16	–	–	4.21	3.8

[a] $n = 6$.
[b] $n = 5$ days, 10 samples.

3.2. Analysis of clinical samples

Urinary levels of all four classes of F2-isoprostanes were examined in both control and AD patient samples. Fig. 6 shows the results of HPLC/MS/MS analysis of F2-isoprostanes in a urinary sample from a male AD patient in which selected peaks were identified by comparison with synthetic standards. In all individuals, peaks originating from isoprostanes could be detected in three of the four classes (III, V and VI). In class III, 8-iso Prostaglandin F2α, 8-iso-15(*R*)-Prostaglandin F2α and Prostaglandin F2α could be identified and quantified (Fig. 6). No 8-iso Prostaglandin F2β was detected in any of the samples. The average level of iPF2α was 241 ± 163 pg/mg creatinine in the urine samples from AD patients (average ± standard deviation). The corresponding control value was 216 ± 101 pg/mg creatinine; so no statistically significant difference was observed. Similar results were obtained for iP15R; namely, 346 ± 271 pg/mg creatinine for AD patients and 342 ± 259 pg/mg creatinine for the control group. Paired analysis was also performed for the identified isoprostane peaks in the other classes for which standards were not available. No significant difference in relative area could be established for any of these peaks. The urine samples from some of the individuals, both in AD and control groups, showed peaks that did not occur in all samples. However, these samples did not give any information regarding AD. In order to determine whether or not a correlation pattern could be disguised within the large amount of data, a principal component analysis was performed, in which quantitative data for isoprostane peaks (i.e. factors) occurring in classes III, V and VI were used. The criteria for selecting a factor in the PCA were that the peak should appear in all samples and that it should be easily integrated. There were eight peaks that met these criteria. The columns of data were mean-centered and scaled to unit variance prior to analysis. The resulting score plot of the two first principal components (PC), was unable to separate the AD and control groups. The same was true for score

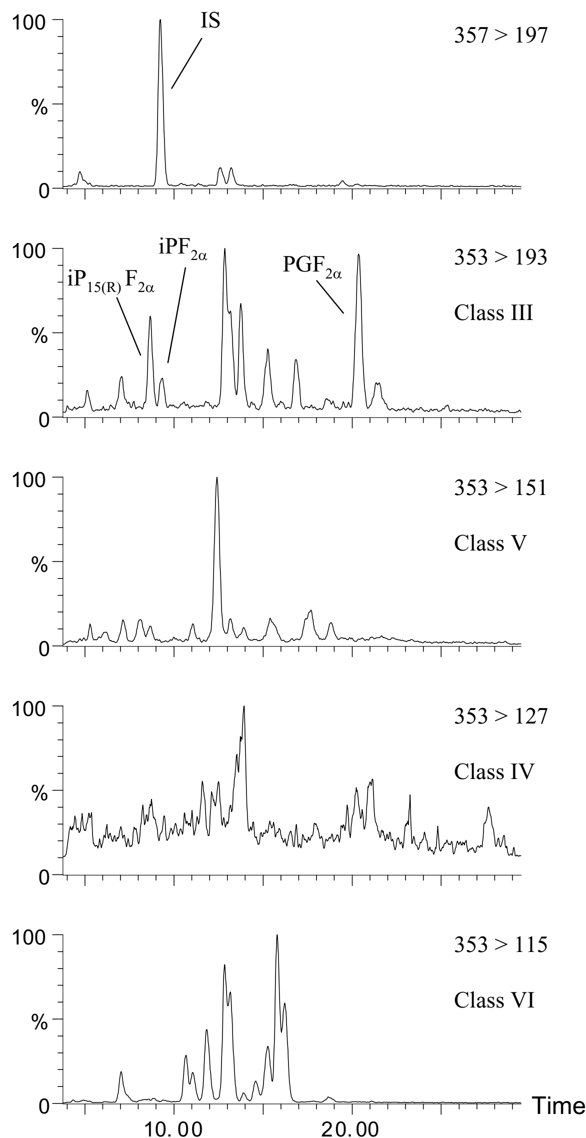

Fig. 6. HPLC/MS/MS analysis of a urinary sample from an AD patient.

plots based on the higher PCs. In order to verify that the PCA approach really had the power to resolve the two groups from each other a simple test was performed. An extra factor indicative of the true diagnosis of the patients was added to the PCA, and then the two groups constituted two easily distinguishable clusters in the score plot of the two first PCs.

4. Conclusions

The strengths of this study, in terms of the clinical aspects, are the thorough diagnosis of the patients and the consecutive analyses of the blindly analyzed urine samples with no selection of the AD patients. In terms of progress in analytical chemistry, we have shown that porous graphitic carbon is a very suitable stationary phase material for the difficult separation of the diastereoisomeric isoprostanes. The ionic analytes could be separated using a high pH and a mobile phase with high organic content, without the addition of an ion-pairing agent, thereby enabling low detection limits in ESI-MS detection. The selected analytical system is also suitable for the rapid and repeatable determination of isoprostanes in a complex matrix, such as urine, with good precision and recovery. The methodology should also be applicable to cerebrospinal fluid instead of urine and we have already initiated work with cerebrospinal fluid from the same patient group.

Acknowledgements

Carlo Crescenzi is acknowledged for introducing us to porous graphitic carbon separations and Carina Norsten-Höög for letting us use the Ultima mass spectrometer.

References

[1] K.T. Akama, C. Albanese, R.G. Pestell, L.J. Van Eldik, Proc. Natl. Acad. Sci. U.S.A. 95 (1998) 5795.
[2] M.F. Beal, Ann. Neurol. 38 (1995) 357.
[3] W.R. Markesbery, Arch. Neurol. 56 (1999) 1449.
[4] A.J. Sinclair, A.J. Bayer, J. Johnston, C. Warner, S.R. Maxwell, Int. J. Geriatr. Psychiatr. 13 (1998) 840.
[5] J.D. Morrow, K.E. Hill, R.F. Burk, T.M. Nammour, K.F. Badr, L.J. Roberts II, Proc. Natl. Acad. Sci. U.S.A. 87 (1990) 9383.
[6] J.D. Morrow, J.A. Awad, H.J. Boss, I.A. Blair, L.J. Roberts II, Proc. Natl. Acad. Sci. U.S.A. 89 (1992) 10721.
[7] R.J. Waugh, R.C. Murphy, J. Am. Soc. Mass Spectrom. 7 (1996) 490.
[8] L.J. Roberts II, J.D. Morrow, in: Molecular and Cellular Basis of Inflammation, 1999, p. 141.
[9] A. Oguogho, H. Sinzinger, J. Physiol. Pharmacol. 51 (2000) 673.
[10] A. Greco, L. Minghetti, G. Levi, Neurochem. Res. 25 (2000) 1357.
[11] J.-L. Cracowski, T. Durand, G. Bessard, Trends Pharmacol. Sci. 23 (2002) 360.
[12] C. Feillet-Coudray, R. Tourtauchaux, M. Niculescu, E. Rock, I. Tauveron, M.C. Alexandre-Gouabau, Y. Rayssiguier, I. Jalenques, A. Mazur, Free Radic. Biol. Med. 27 (1999) 463.
[13] E. Waddington, K. Croft, R. Clarnette, T. Mori, R. Martins, Alzheimers Rep. 2 (1999) 277.
[14] D. Pratico, C.M. Clark, V.M.Y. Lee, J.Q. Trojanowski, J. Rokach, G.A. FitzGerald, Ann. Neurol. 48 (2000) 809.
[15] E.E. Tuppo, L.J. Forman, B.W. Spur, R.E. Chan-Ting, A. Chopra, T.A. Cavalieri, Brain Res. Bull. 54 (2001) 565.
[16] T.J. Montine, J.F. Quinn, D. Milatovic, L.C. Silbert, T. Dang, S. Sanchez, E. Terry, L.J. Roberts, J.A. Kaye, J.D. Morrow, Ann. Neurol. 52 (2002) 175.
[17] J.D. Morrow, L.J. Roberts II, Methods Enzymol. 300 (1999) 3.
[18] 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.
[19] Y. Murai, T. Hishinuma, N. Suzuki, J. Satoh, T. Toyota, M. Mizugaki, Prostaglandins Other Lipid Mediat. 62 (2000) 173.
[20] N. Ohashi, M. Yoshikawa, J. Chromatogr. B 746 (2000) 17.
[21] M.T. Gilbert, J.H. Knox, B. Kaur, Chromatographia 16 (1982) 138.
[22] J.H. Knox, B. Kaur, G.R. Millward, J. Chromatogr. 352 (1986) 3.
[23] American Psychiatric Association, Diagnostic Criteria from DSM-IV, Washington, DC, 1994.
[24] M.F. Folstein, S.E. Folstein, P.R. McHugh, J. Psychiatr. Res. 12 (1975) 189.
[25] P. Ross, J.H. Knox, Adv. Chromatogr. (New York) 37 (1997) 121.
[26] P. Ross, J.H. Knox, Adv. Chromatogr. (New York) 37 (1997) 73.
[27] P. Ross, R.E. Majors, LC–GC 18 (2000) 14.