patients suffering from coronary heart disease, from cerebrovascular occlusive disease, or from peripheral arterial occlusive disease.

As shown in Table 1, there were distinctly higher homocysteine concentrations for seropositive subjects in subgroups of age, renal function, smoking status, and MTHFR gene mutations. These variables influencing serum homocysteine, as well as vitamin B<sub>6</sub>, vitamin B<sub>12</sub>, and folic acid, are moreover homogeneously (not significantly different) distributed between the C. pneumoniae-seropositive and -seronegative groups. Drugs possibly influencing serum homocysteine were either well balanced between the two groups or not prescribed (antagonists of folic acid, cyclosporine, anticonvulsants).

Possible confounding effects of these variables and inflammatory markers were considered by multiple regression analysis. However, the difference in serum homocysteine observed between the C. pneumoniae-seropositive and -seronegative groups remained significant (P < 0.005). The regression analysis also revealed considerable effects on serum homocysteine by age (P = 0.012), creatinine concentration (P < 0.005), smoking (P = 0.023), MTHFR gene mutations (P = 0.082), and tumor necrosis factor-α (P = 0.038). Vitamin B<sub>6</sub>, vitamin B<sub>12</sub>, folic acid, C-reactive protein, and interleukin 6 were only weakly associated with serum homocysteine (P > 0.1) and, therefore, were excluded from analysis.

Our results indicate that C. pneumoniae seropositivity is linked with hyperhomocysteinemia in male patients with established atherosclerosis. This correlation was statistically significant no matter whether IgG (as shown above) or IgA was used as the marker for seropositivity. Understanding the relationship between the two variables requires further investigations, which are likely to be facilitated by the recent sequencing of the C. pneumoniae genome (5). Hyperhomocysteinemia is a predictor of morbidity and mortality in patients with atherosclerosis. If the association of hyperhomocysteinemia and C. pneumoniae seropositivity can be confirmed in further studies, C. pneumoniae seropositivity may be associated with impaired prognosis in these patients.

Physiologic, pathologic, genetic, and nutritional factors may increase serum homocysteine (6). Some of these variables were used as exclusion criteria (diabetes mellitus, malignant neoplasia, chronic inflammatory disorders, severe renal insufficiency). Other variables (age, renal function, smoking, MTHFR gene mutations, vitamins, concomitant medication) were found not to be responsible for the difference in homocysteine between C. pneumoniae-seropositive and -seronegative patients. This was concluded from multiple regression analysis and the observation that the possibly confounding variables were homogeneously distributed between the C. pneumoniae-seropositive and -seronegative groups. The validity of the study is further supported by the finding that distinctly higher homocysteine concentrations for seropositive subjects were measured not only in the whole study population, but also independently in various subgroups investigated. The generally high serum concentrations of homocysteine found in this study may be explained by our selection of older patients (69.9 ± 10.1 years), with a high prevalence of smoking (>80%) and suffering from advanced atherosclerosis.

We thank Dr. G. Hinz, MEDIDATA, and Dr. P. Suter, University Hospital of Zurich, for valuable discussion. The project was financially supported by Aventis Pharma AG, Switzerland.

References


One-Step Solid-Phase Extraction Procedure for F<sub>2</sub>-Isoprostanes, Zheng Zhao, N. Magus Hjelm, Christopher W.K. Lam, and Chung S. Ho* (Department of Chemical Pathology, The Chinese University of Hong Kong, Prince of Wales Hospital, Shatin, New Territories, Hong Kong; * author for correspondence: fax 852-2636-5090, e-mail chungshunho@cuhk.edu.hk)

F<sub>2</sub>-isoprostanes (F<sub>2</sub>-iPs) are a group of prostaglandin-like isomers produced from peroxidized arachidonic acid (AA)-containing phospholipids (1). They are new and important markers of lipid peroxidation in vivo (1). However, their introduction into clinical practice has been limited by the need to extract the compounds from their biologic matrices before analysis by immunologic methods or mass spectrometry. Morrow et al. (2) and others (3, 4) used multiple steps, including thin-layer chromatography and HPLC, in their purification methods. Apart from being time-consuming, these procedures also led to substantial losses of the target compound. In one study (5), the recovery of a urinary metabolite of iP2<sub>2</sub>-III was only 20–30%. Recently, Nourooz-Zadeh et al. (3), using a reversed-phase C<sub>18</sub> cartridge followed by a normal-phase NH<sub>2</sub> cartridge, reported a recovery of 75% for F<sub>2</sub>-iPs (6). However, this procedure is still time-consuming.

In this report, we present a simplified and rapid procedure for extracting F<sub>2</sub>-iPs from biologic samples using an Oasis HLB extraction cartridge (Waters Corporation). This cartridge contains a unique copolymer sorbent with hydrophilic and lipophilic groups in proportions that allow
high and reproducible recoveries of acidic, basic, and neutral compounds, whether polar or nonpolar (7).

To study the performance of the Oasis cartridge, we prepared two F$_2$-iP samples. The first sample contained F$_2$-iP isomers generated by the free radical oxidation of AA, mainly described by Waugh et al. (8). We placed 300 μg of AA in 30 μL of ethanol in a 12-mL polypropylene tube. The fatty acid was immediately suspended in 2.91 mL of 10 mmol/L phosphate buffer saline by vortex-mixing. We added 30 μL of 300 g/L hydrogen peroxide and 30 μL of 1 mmol/L ferrous chloride. The resulting solution was mixed gently and incubated at 4 °C for 12 h.

The second sample contained total F$_2$-iPs present in the whole-blood sample collected from a healthy Chinese male subject. Blood (4 mL) was collected into a lithium-heparin tube containing indomethacin, an inhibitor of cyclooxygenase, at a final concentration of 15 μmol/L. The blood tube was centrifuged at 2400 g for 10 min at 4 °C to separate plasma and erythrocytes. Aliquots of plasma (1 mL) were transferred to Eppendorf tubes containing butylated hydroxytoluene, a scavenger of free radicals, at a final concentration of 20 μmol/L. One milliliter of 1 mol/L KOH was added to the Eppendorf tube containing the plasma sample. The plasma sample was hydrolyzed at 40 °C for 30 min to release bound (esterified) lipids. At the end of the hydrolysis, 1 mL of 1 mol/L HCl and 2 mL of 100 mmol/L formate buffer (pH 3.0) were added. The sample was centrifuged at 2400g for 10 min; the supernatant was then ready for solid-phase extraction.

Table 1. Analytical performance of the one-step method.

<table>
<thead>
<tr>
<th></th>
<th>Present one-step method</th>
<th>Method of Nourooz-Zadeh et al. (3)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Recovery, %</td>
<td>89.5 ± 4.8 (n = 12)</td>
<td>70.2 ± 7.7 (n = 12)</td>
</tr>
<tr>
<td>Intraassay CV, %</td>
<td>5.3 (n = 16)</td>
<td>5.5 (n = 16)</td>
</tr>
<tr>
<td>Interassay CV, %</td>
<td>7.8 (n = 16)</td>
<td>12 (n = 16)</td>
</tr>
<tr>
<td>Linearity (range, 0–1000 ng/L)</td>
<td>y = 0.001x + 0.016</td>
<td>y = 9.38 × 10^{-4}x + 0.008</td>
</tr>
<tr>
<td></td>
<td>$r^2 = 0.996$</td>
<td>$r^2 = 0.994$</td>
</tr>
<tr>
<td>Limit of detection</td>
<td>10 ng/L</td>
<td>10 ng/L</td>
</tr>
</tbody>
</table>

Fig. 1. Representative GC-NICI-MS chromatograms of a selected ion (m/z 569) of F$_2$-iPs (iPF$_{2\alpha}$-III) from human plasma (A and C) and generated from free radical oxidation of AA (B and D) after purification by the present one-step method (A and B) and by the method of Nourooz-Zadeh et al. (3) (C and D).
water (15:85 by volume). F2-iPs were eluted by washing the cartridge with 2 mL of hexane–ethanol–propan-2-ol (30:65:5 by volume). For comparing the analytical performance, the samples were also purified by the method described by Nourooz-Zadeh et al. (3) using two solid-phase cartridges.

F2-iPs obtained by the purification procedures were analyzed by gas chromatography–negative ion chemical ionization–mass spectrometry (GC-NICI-MS). The eluting solution was evaporated under nitrogen before the derivatization. The extracted F2-iPs in the samples were incubated with 25 μL of pentafluorobenzyl bromide (100 mL/L in acetone) and 25 μL of N,N-diisopropylethylamine (200 mL/L in acetone) at 60 °C for 10 min. Excess reagents were evaporated under nitrogen. The resulting pentafluorobenzyl ester was incubated with 50 μL of N,O-bis-(trimethylsilyl)triﬂuoroacetamide and 5 μL of N,N-diisopropylethylamine (200 mL/L in acetone) at 60 °C for 5 min. Excess reagents were removed under nitrogen. The pentafluorobenzyl–trimethylsilyl derivatives were reconstituted in 40 μL of isoctane and the samples analyzed on a GC8000 series gas chromatograph (in NICI mode) coupled to a MD800 mass spectrometer (Fisons Instruments) using methane as the reagent gas. Chromatography was carried out on an SPB-1701 column (30 m × 0.25 mm; film thickness, 0.25 μm; Supelco Inc.) using helium as the carrier gas. Injections (1 μL) were performed by an AS800 autosampler (Fisons Instruments). The temperature of the injector was kept at 250 °C. The oven temperature was increased from 150 °C to 235 °C at a rate of 15 °C/min, increased from 235 °C to 280 °C at 10.3 °C/min, and then maintained at 280 °C for 10 min. The MD800 was programmed for optimal detection with the following settings: electron energy, 70 eV; emission energy, 250 μA; ion energy, 1.0 eV; and ion energy ramp, 1.0 mV per atomic mass unit. Selected ion monitoring was performed to monitor the carbonyl anion (M−181; loss of CH3C=CH2) at ions m/z 569 and 573 for F2-iPs and the internal standard, respectively. Peak identification was based on the comparison of the relative retention indices with the internal standard. The concentration of F2-iP isomers in the samples was calculated using the ratio of the peak height of m/z 569 to that of m/z 573.

To evaluate the analytical performance of the present method, one of the F2-iP isomers, iPFe2α-III, was used in the quantification and subsequent calculation of recovery and precision performance. The results and the comparison with the method of Nourooz-Zadeh et al. (3) are presented in Table 1. A recovery of ~90% was achieved with our method compared with ~70% with the purification method of Nourooz-Zadeh et al. (3) with comparable data for intraassay and interassay imprecision and linearity. As shown in Fig. 1, the Oasis purification procedure led to identical GC-NICI-MS chromatograms for F2-iPs in calibration solutions and plasma.

In summary, a substantially improved recovery for isolating F2-iPs from biologic matrices can be achieved by our one-step purification procedure compared with previous methods. It is also simpler and takes only one-half the extraction time needed by the two-cartridge method (3). The procedure is also well suited as a preparatory step to isolate F2-iP isomers in complex tissue matrices before applying specific purification procedures, e.g., immunoaffinity chromatography of iPFe2α-III.

References