Dioxins and Furans Determination in Postmortem Blood by Gas Chromatography–High-Resolution Mass Spectrometry

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Abstract

Dioxins and related compounds (furans) are persistent environmental contaminants that cause adverse biological effects. Their influence on humans is still unclear, except for accidental high-dose exposure. However, chronic exposure to these compounds seems to be involved in cancer, endocrine disruption, and neurobehavioral effects. For several years, a large concern about the potential health risks of dioxins is emerging in Europe and United States. Dioxin levels in biological specimens are extremely low and require very sensitive and specific methods of analysis. In this study, gas chromatography coupled to high-resolution mass spectrometry was used to evaluate dioxin body burden of two women deceased from generalized cancer. Fat fraction of blood specimens was obtained after precipitation with ethanol and extraction of both liquid and solid phases spiked with labeled $^{13}$C$_{12}$-dioxin analogues. Organic phases were grouped, washed, and evaporated to weigh the lipid content. Lipids were dissolved in hexane, hydrolyzed with concentrated sulfuric acid, and discarded during water washes. Dioxins purification was achieved using three successive columns: silica, alumina/sodium sulfate, and carbon/Celite. Finally, the toluene eluent was evaporated and the extract injected in the analytical system. After chromatographic separation, detection was achieved in single ion monitoring mode using a high-resolution mass spectrometer operating in electron impact ionization mode (40 eV, minimal resolution of 10,000). Dioxin levels were expressed in pg TEQ/g of fat as defined by the World Health Organization. Quantification limits for each dioxin congener ranged from 2.5 to 12.0 pg/g fat with a relative extraction recovery always higher than 60%. Dioxin concentrations in the blood of the two deceased women were 35.0 and 42.7 pg TEQ/g fat, respectively. These concentrations are largely lower than those observed after accidental releases, but in the range of those observed in the general European population. Therefore, it was not possible to correlate dioxin body burden of the two women as a potential contributor of their cancer pathology. Nevertheless, knowledge of dioxin body burden in the French population would be of interest for an accurate interpretation of these results.

Introduction

Polychlorinated dibenzo-para-dioxins (PCDDs) and polychlorinated dibenzofurans (PCDFs) are two groups of tricyclic aromatic compounds (Figure 1). These compounds are planar, poorly soluble in water, lipophilic, and stable. PCDDs are formed as inadvertent by-products, sometimes in combination with PCDFs, during the production of halogenated pesticides (1). They also may be produced in thermal processes such as incineration or metal-processing and in the bleaching of paper pulp with free chlorine (2). Because of their physicochemical properties (thermal stability and low natural degradation leading to a half-life of several years in the environment), dioxins and furans (PCDD/Fs) are ubiquitous in soils, sediments, and air (3). Excluding occupational or accidental expo-

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Figure 1. Structure of polychlorinated dibenzo-para-dioxins (PCDDs), polychlorinated dibenzofurans (PCDFs), and 2,3,7,8-tetrachlorodibenzo-para-dioxin (2,3,7,8-TCDD)
sures, most human exposure to dioxins occurs as a result of eating (meat, milk, eggs, fish, and related products), as dioxins are persistent in the environment and accumulate in animal fat (4,5).

The toxicity of the 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) and related 2,3,7,8-substituted PCDD/Fs has been demonstrated in many animal species (4). Human exposure due to background contamination and accidental release has caused particular concerns for the public health because these compounds seem to be involved in cancer, endocrine disruption, and neurobehavioral effects (6-8). In 1997, TCDD or "Seveso dioxin" was classified as carcinogenic to humans by the International Agency for Research on Cancer (4).

In order to evaluate the dose-response relationship to support a reliable health risk assessment, the knowledge of the internal dose or body burden of the exposed population is essential. The degree of dioxin exposure in humans has been evaluated by analyzing the dioxin level in biological samples such as blood, breast milk, fat, and organs (9,10). In regards to biological specimens, only blood can be collected from a wide population of age groups, regardless of gender. Moreover, human blood has the advantage of being relatively easier to collect than other samples. However, the concentration of those compounds in blood is quite low. Because of its specificity and sensitivity, high-resolution gas chromatography (GC) coupled to high-resolution mass spectrometry (MS) has become the reference method in this field (11).

The aim of this study is to provide an analytical method using GC–high-resolution MS to quantify the 17 highly toxic PCDD/F congeners in postmortem blood specimens and its application to 2 forensic cases.

### Experimental

#### Case histories

Two women (67 and 70 years old, respectively) expired in the hospital from generalized cancer. During the autopsies, their bodies showed no signs of violence and manner of death (cancer) was confirmed. In the two cases, blood and urine samples were collected in 50-mL plastic tubes containing no preservatives and stored at +4°C until the analyses were performed.

As the two subjects lived in the same area, near a solid waste incinerator, a judge in charge of the case regarding potential malfunction of the incinerator leading to possible contamination required the measurement of dioxin body burden of the two women along with a more classical toxicological screening.

#### Toxicological analysis

An initial screening on blood and urine was performed by immunoassay for the generally targeted medications and drugs of abuse. Complementary and confirmatory toxicological analyses were performed on biological fluids after liquid–liquid extraction using high-pressure liquid chromatography coupled to a photodiode-array detector (HPLC–DAD) and GC–MS techniques (12,13).

Clean-up procedures and analyses were carried out according to U.S. EPA 1613, NF EN 1948-2 and -3 (June 1997), 2002/69/CE, and 2002/70/CE. For the determination of the lipophilic dioxins, an original procedure was developed to extract fat fraction. Briefly, blood (35 mL) was precipitated with 60 mL of ethanol and filtered on Whatmann paper N°2. Both liquid and solid phases were spiked with 50 pg of labeled 13C12-PCDD/F analogues (13C12-2,3,7,8-TCDD, 13C12-1,2,3,7,8-PeCDD, 13C12-1,2,3,4,7,8-HxCDD, 13C12-1,2,3,6,7,8-HxCDD, 13C12-1,2,3,4,6,7,8-HpCDD, 13C12-1,2,3,4,6,7,8,9-OCDD, 13C12-1,2,3,7,8-TCDF, 13C12-1,2,3,7,8-PeCDF, 13C12-1,2,3,4,7,8-PeCDF, 13C12-1,2,3,4,7,8-HxCDF, 13C12-1,2,3,6,7,8-HxCDF, 13C12-1,2,3,6,7,8-HpCDF, 13C12-1,2,3,4,6,7,8-HpCDF, and 13C12-1,2,3,4,7,8,9-HpCDF) used as extraction standards (BCP, Lyon, France). The liquid fraction was extracted 3 times with a total volume of 150 mL of diethylther/hexane (2:1, v/v), and the protein fraction was extracted with acetone/hexane (1:1, v/v) using the Accelerating Solvent Extraction technology from Dionex (140°C, 2000 psi). The different organic phases of the same sample were grouped, washed with one volume of water, dried on anhydrous sodium sulfate (10 g), and evaporated to dryness for gravimetric determination of fat content. Before the PCDD/Fs purification step, the fat portion was dissolved in 25 mL of hexane and digested with concentrated sulfuric acid (several steps with 30 mL of acid). After neutralization of the organic phase by several washes with water (50 mL each), solvent was dried with anhydrous sodium sulfate (10 g) and reduced to an approximate volume of 2 mL. Purification was achieved using three successive “homemade” columns after addition of 100 pg 37Cl1-2,3,7,8-TCDD used as purification standard. The first column was filled with silica (2 g neutral, 8 g acidic, and 4 g basic forms) and the second one with alumina (6 g) and sodium sulfate (2 g). After elution of PCDD/Fs with 30 mL hexane/methylen chloride (1:1, v/v), the extract was finally put on a carbon/Celite (1:11.5, w/w) column and back-eluted with 7 mL of toluene. Before injection, the eluent was spiked with 250 pg 13C12-1,2,3,4-TCDD and 13C12-1,2,3,7,8,9-

#### Table 1. Ions and Isotopic Ratios for Each PCDD/F Congeners Monitored During the Acquisition in High-Resolution Detection Mode

<table>
<thead>
<tr>
<th>Native Congeners</th>
<th>13C12 Analогues</th>
<th>m/z</th>
<th>m/z</th>
<th>Ratio</th>
<th>m/z</th>
<th>m/z</th>
<th>Ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>TCDF</td>
<td></td>
<td>303.9016</td>
<td>305.8986</td>
<td>0.77</td>
<td>315.9417</td>
<td>317.9387</td>
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<td></td>
<td>319.8965</td>
<td>321.8936</td>
<td>0.77</td>
<td>331.9368</td>
<td>333.9339</td>
<td>0.77</td>
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<tr>
<td>PeCDD</td>
<td></td>
<td>339.8979</td>
<td>341.8958</td>
<td>1.55</td>
<td>351.9000</td>
<td>353.8970</td>
<td>1.55</td>
</tr>
<tr>
<td>PeCDF</td>
<td></td>
<td>355.8546</td>
<td>357.8517</td>
<td>1.55</td>
<td>367.8949</td>
<td>369.8919</td>
<td>1.55</td>
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<tr>
<td>HxCDD</td>
<td></td>
<td>373.8207</td>
<td>375.8178</td>
<td>1.24</td>
<td>383.8639</td>
<td>385.8610</td>
<td>0.51</td>
</tr>
<tr>
<td>HxCDF</td>
<td></td>
<td>389.8156</td>
<td>391.8127</td>
<td>1.24</td>
<td>401.8557</td>
<td>403.8527</td>
<td>1.24</td>
</tr>
<tr>
<td>HPCDF</td>
<td></td>
<td>407.7818</td>
<td>409.7788</td>
<td>1.04</td>
<td>417.8253</td>
<td>419.8220</td>
<td>0.44</td>
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<tr>
<td>HPCDD</td>
<td></td>
<td>423.7767</td>
<td>425.7737</td>
<td>1.04</td>
<td>435.8169</td>
<td>437.8140</td>
<td>1.04</td>
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<tr>
<td>OCDF</td>
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<td>441.7428</td>
<td>443.7398</td>
<td>0.89</td>
<td>469.7780</td>
<td>471.7750</td>
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</tr>
<tr>
<td>OCDD</td>
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<td>459.7348</td>
<td>0.89</td>
<td>469.7780</td>
<td>471.7750</td>
<td>0.89</td>
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were estimated for each sample by comparing the ratio between the extraction and purification standard (labeled laC12-PCDD/F analogues) with those of the laCla-PCDD/F analogues with respect of the isotopic ratios according a tolerance of 15%. Quantitative results were obtained by comparison of relative retention times of the target dioxins with those of the l3C12-PCDD/F analogues in the standards added just before the extraction and purification. Congener recoveries were calculated for a signal-to-noise ratio of 10 for each congener.

Results and Discussion

In most vertebrate species, the 2,3,7,8-substituted dioxins are the congeners predominantly retained. If chlorine atoms are present on all 2,3,7,8 positions, the biotransformation rate of dioxins is strongly reduced, resulting in significant bioaccumulation. The toxicity of 2,3,7,8-TCDD and related 2,3,7,8-substituted dioxins has been demonstrated in many animal species. The lethal dose of 2,3,7,8-TCDD, however, varies more than 5000-fold between the most sensitive species, the guinea pig (LD50 = 0.6 pg/kg), and the least sensitive species, the hamster (LD50 = 5050 pg/kg). Others signs of 2,3,7,8-TCDD intoxication include thymic atrophy, hypertrophy/hyperplasia of hepatic, gastrointestinal, urogenital and cutaneous epithelia, atrophy of the gonads, subcutaneous edema, and systemic hemorrhage (4). In human studies after in vivo exposure, there have been no unequivocal reports of effects of dioxins upon the frequencies of chromosomal aberrations (4). If adverse biological effects of heavy dioxin exposure in humans are well documented (chloracne, alterations in liver enzyme levels, changes in the immune system), there is a limited evidence for their carcinogenicity following chronic low exposure (4).

In the first forensic case, immunological screening of urine samples revealed the presence of opiates and paracetamol. Other medications or drugs of abuse were not detected. Paracetamol (14.9 mg/L) and morphine (210 mg/L) were identified by HPLC–DAD and GC–MS, respectively, and confirmed pain treatment. For the second case, paracetamol (9.5 mg/L), propranolol (95 ng/mL), fluoxetine (289 ng/mL), and oxazepam (169 ng/mL) were detected, in accordance with a therapeutic treatment.

After a sophisticated preparation technique, highly specific and sensitive determination of dioxins by GC–HRMS allowed us to identify many congeners in both cases. A typical chromatogram is shown in Figure 2. The method was able to detect all the congeners with quantification limits ranging from 2.5 to 12.0 pg/g and a relative extraction recovery always higher than 60%. In the first postmortem blood sample, 4 out of 7 and 7 out of 10 target dibenzo-para-dioxins and dibenzofurans, respectively, were detected. Five dibenzo-para-dioxins and 7 dibenzofurans were found in the second case (Table II). Figure 3 shows the patterns of PCDDs and PCDFs in the blood of the two women. Total congener concentrations in blood were 35.0 and 42.7 pg TEQ/g fat, respectively. Dioxin concentrations in these cases were largely lower than those observed after accidental releases (15–18). For example, 2,3,7,8-TCDD concentrations reported by Mocarelli and co-workers (15) ranged from 828 to 56,000 pg TEQ/g of fat in serum of Seveso residents.

Blood and tissue distribution data were also reported in postmortem cases (10,19). The first one published by Montagna et al. (19) concerned a 55-year-old Seveso woman who died from pancreatic cancer 7 months after the dioxin incident. The concentration of 2,3,7,8-TCDD measured in her blood was 6 pg/g
of sample. Of the six cases published by Kitamura et al. (10), a spinal tumor, cerebral palsy, and pneumonia were at the origin of three deaths. Dioxin concentrations were in the range of 15 to 60 pg TEQ/g fat.

Epidemiological studies conducted to assess the dioxin body burden of the general population in Europe have also been published (20-23). In Finland, dioxin concentrations of control subjects living in unpolluted areas ranged from 12 to 81 (mean 33) pg TEQ/g fat (20) and those observed in Germany ranged from 11 to 112 (mean 40.8) pg TEQ/g fat (21). In Belgium, these concentrations ranged from 5 to 71 (mean 23.9) pg TEQ/g fat (22). In the general population, including people living in industrial area or near waste incinerators, it was observed that dioxin body burden was not always significantly higher than in rural location; except for geographic location, several other parameters can contribute significantly to the dioxin body burden such as gender, age, body mass index, or eating habits (22-24).

In regard to these observations, the dioxin body burden of the two women deceased from cancer appear in the range of the one observed in the general European population. Therefore, it was not possible to correlate dioxin body burden of the two women as a potential contributor of their cancer pathology. Nevertheless, it would be interesting to know the dioxin body burden of the French population for an accurate interpretation of these results.

Conclusions

The sensitive and specific method developed seems to be suitable for the detection and quantification of 7 polychlorodibenzofurans and 10 polychlorodibenzofurans in postmortem blood specimens. An extensive purification process and mass spectrometry in high resolution mode of detection are necessary to measure the extremely low dioxin levels in biological fluids.

References


