Bull. Environm. Contam. Toxicol. 24, 818-823 (1980)

Routine Analysis of 2,3,7,8-Tetrachlorodibenzo-*p*-dioxin in Biological Samples from the Contaminated Area of Seveso, Italy

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Among the first and most dramatic evidence of the severity and extension of the contamination caused by an explosion in a chemical plant near Seveso (ICMESA, owned by La Roche, Switzerland) (HAY 1976, 1977, SAPERE 1976, WHITESIDE 1978), was an outbreak of animal mortality in areas surrounding the factory; deaths occurred mostly among small domestic animals such as rabbits and poultry, grown in a wide populated area (BONACCORSI et al. 1978). The chemical cloud that had escaped from the plant contained, among other chemicals, the extremely toxic compound 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) (WORLD HEALTH ORGANIZATION 1977). Soon after the accident, the Regional Veterinary Service, in collaboration with the Institute of Veterinary Preventive Medicine of Lombardy and Emilia, set up a program to study the extent of the contamination of animal life and to keep under control the food chain.

Early assays on rabbit liver showed high TCDD levels up to hundreds of parts per billion (ABBRUZZI et al. 1978), but it soon became clear that an analytical method with a sensitivity limit in the high ppt range was required to monitor TCDD in animals living in areas with a wide range of contamination; moreover, the large number of samples to be analyzed called for a method suitable for routine use.

Development of the analytical procedure focused on liver in view of the high concentrations of TCDD found in this organ in the first samples analyzed; the choice of liver rather than adipose tissue, which is also known to accumulate TCDD (PIPER et al. 1973, VAN MILLER et al. 1976), was made considering the problems related to extraction and clean-up of biological samples with a high fat content (BAUGHMAN 1974). Liver was later confirmed as the right choice for monitoring TCDD in rabbits because in this animal the compound reaches higher levels in liver than in adipose tissue (FANELLI, personal communication). No efforts were made to seek tetrachlorodibenzo-p-dioxins other than the 2,3,7,8,-isomer in biological samples, because only traces of another unidentified isomer were sporadically found in soil samples, and generally only 2,3,7,8-TCDD was detected (BUSER 1978). Furthermore, this isomer is by far the most toxic dioxin known (WORLD HEALTH ORGANIZATION 1978).

Gas chromatography-mass fragmentography (GC-MF) was chosen as detection technique, because the serious problems connected with the

0007-4861/80/0024-0818 $01.20

pollution made it mandatory to use a highly specific and reliable method; in fact, where TCDD was detected, restrictions in behaviour and food consumption were imposed by local health authorities.

The analytical procedures for the extraction and clean-up of the samples described here are derived from methods reported by several authors (BAUGHMAN & MESELSON 1973a, BAUGHMAN & MESELSON 1973b, SHADOFF & HUMMEL, 1975) which were modified in order to develop a simple and reliable technique suitable for routine analysis of hundreds of samples.

EXPERIMENTAL

a) Safety precautions

TCDD standards and contaminated material were handled with caution in order to prevent exposure of laboratory personnel. The laboratory work area was confined and operators wore disposable protective gloves and coats. Glassware and all residual material from the analytical operations were stored in a special room and periodically transferred for disposal to the contaminated area (Zone A of Seveso). Concentrated standard solutions were kept in a safe and only diluted solutions (ppb range) were kept in the laboratory area. Periodic decontamination of the work area and smear tests were carried out to check for contamination of the laboratory. All operators underwent a complete clinical examination every 3 months.

b) Instrumentation

The instrument used was an LKB-2091-051 gas chromatograph-mass spectrometer operated in electron impact mode, equipped with an LKB-2130 computer for data acquisition and calculation. GC-MF operating conditions were as follows: glass column, 2 m x 2 mm i.d., packed with 3% silicone OV-1 on 100-120 mesh Gas Chrom Q; oven temperature, 280°C; flash heater, 290°C; separator, 250°C; ion source, 250°C; helium flow, 25 ml min^{-1}; electron energy, 70 eV; trap current, 100 μA; measuring time, 50 msec ion^{-1}; source slit, 0.16 mm; collector slit, 0.25 mm; resolution, 400.

For mass fragmentography the magnet current and accelerating voltage alternator were adjusted to monitor the molecular ions of TCDD at m/e 320-322. In these experimental conditions, the retention time of TCDD was about 1.5 minutes.

c) Quantitative analysis

The linearity of the mass fragmentographic response was periodically tested by injecting different amounts of TCDD (50,100,300, 500 pg). The typical response of the mass spectrometer to injection of 50 pg of TCDD gave a signal-to-noise ratio better than 3.

Calibration curves were obtained by plotting peak height of m/e 322 versus injected amounts. A linear response was obtained in the range from 50 to 500 pg injected into the gas chromatographic column.

The limit of sensitivity of the method was established as 0.25 ng/g of liver tissue on the basis of the minimum amount of TCDD added to 10 g of liver tissue capable of giving a signal-to-noise ratio better than 3 on mass 322 in normal routine conditions. Figure 1 shows typical fragmentograms obtained after injecting standard TCDD or biological samples. Positive samples were identified on the basis of the retention time of the peak registered on masses 320 and 322 and of the isotopic ratio between the two ions.

TCDD was quantitatively analyzed by peak height comparison between samples and known amounts of standard TCDD. For routine analysis standard TCDD was injected every four samples.

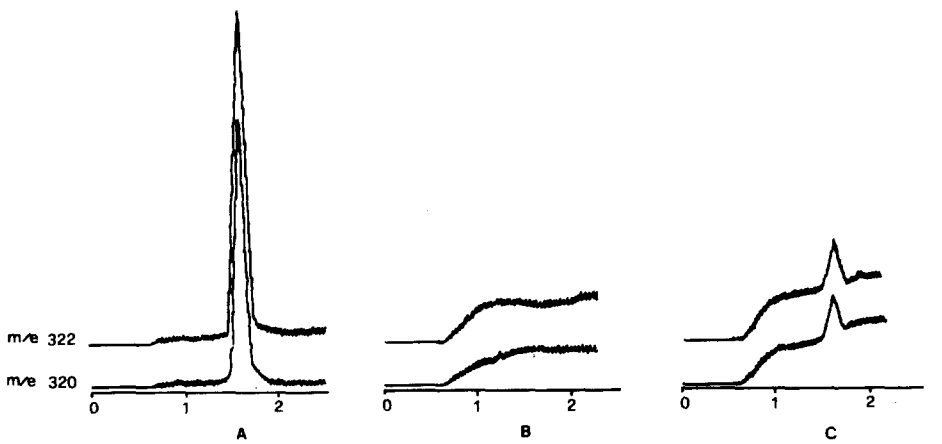

Figure 1. Typical mass fragmentograms (time scale: minutes) showing: A) standard TCDD (500 pg), B) tissue blank (10 g of liver), C) TCDD recovered from tissue (10 g of liver spiked with 2.5 ng TCDD).

d) Reagents and standards

The following reagents and standards were used:

- n-hexane, carbon tetrachloride, methylene chloride, diethylene dioxide, absolute ethyl alcohol, sodium hydroxide pellets, anhydrous sodium sulphate (RPE grade, C.ERBA, Milan, Italy).
- Extrelut columns for extraction of lipophilic compounds and aluminium oxide 90 active, neutral (grade I) for column chromatography (70-230 mesh), were purchased from MERCK (Dormstadt, Germany).
- antibumping granules (BDH Italia, Milan, Italy).
- H_2SO_4, 95-97% (HOECHST, Frankfurt am Main, Germany)
- 2,3,7,8-tetrachlorodibenzo-p-dioxin, purity 94% and 2,3,7,8 - tetrachlorodibenzo-p-dioxin-$^{37}Cl_4$, purity 67%, $^{37}Cl_4$ purity 94%, were obtained from KOR ISOTOPES, (Cambridge, Ma., USA).

e) Clean-up of biological samples

1. Saponification: Tissue sample of about 10 g were hydrolyzed with 10 ml of NaOH 10N and 20 ml of ethanol at 90°C in a reflux condenser for 1 h; Teflon tape was used on the ground glass joint.
2. Extraction: after cooling, the samples were extracted twice with 20 ml of n-hexane.
3. Kieselguhr chromatography: the combined organic phases were percolated through an Extrelut column on which 20 ml of H_2SO_4 95-97% had been previously adsorbed and equilibrated for 4-14 h. The column was then washed with 20 ml of n-hexane and the organic eluate was dried under a gentle N_2 or air flux at 25°C.
4. Alumina chromatography: the residue was chromatographed on a column (45 mm x 5 mm) of neutral alumina with a top layer (5 mm) of anhydrous sodium sulphate prewashed with 3 ml of CH_2Cl_2, and activated at 270°C for 12 h or at 400°C for 4 h. The sample was dissolved in 3 ml of n-hexane and transferred to the top of the column. The column was eluted with 6 ml of CCl_4 and then with 4 ml of CH_2Cl_2; this fraction was collected and evaporated to dryness. The residue was dissolved in 100 µl of diethylene dioxide and 5 µl aliquots were injected in GC-MF.

f) Recovery studies

An attempt to use 2,3,7,8-TCDD-$^{37}Cl_4$ for evaluation of recovery was unsuccessful because of the presence in the tissue extracts of interfering peaks at m/e 328; the efficiency and linearity of recovery were therefore examined by analyzing samples spiked with known amounts of TCDD (0.25, 1, 2, 4 ng TCDD/g of tissue) before extraction and clean-up. With each batch of samples, two samples spiked with TCDD (1 and 2 ng TCDD/g of tissue) were analyzed for recovery evaluation; blank samples were also processed to check for false positive results due to external contamination.

Recovery averaged 88.5 ± 5.3% (S.D.) and was found to be constant over the whole concentration range considered.

RESULTS AND DISCUSSION

The most significant modification made in the analytical procedure outlined here, as compared with methods described by other authors (14-16), is the use of a kieselguhr column saturated with concentrated H_2SO_4 for sample clean-up. This step was found to be faster and more efficient for destroying organic material, than time-consuming multiple washings of the organic extracts with concentrated sulphuric acid. Considering more than one thousand samples analyzed in a three-year period, the method described gave consistent and reproducible results even when applied by different operators. Only 5% of the samples showed interfering peaks on one or both the registered masses and these were, in most cases, completely eliminated by further purification on an alumina microcolumn. The method was

successfully applied to tissues other than liver, with minor modifications. The sensitivity of the method was found to be suitable for measuring TCDD in most of the samples collected in the contaminated areas. Table 1 shows TCDD concentrations in rabbit liver samples collected in areas surrounding the chemical plant within six months of the accident. Most of the positive samples show TCDD concentrations between 1 and 64 ng/g, the average concentration being 31 ng/g. This analytical procedure is routinely used in our laboratory for monitoring TCDD levels in animals living in the contaminated area; studies are in progress to increase the sensitivity of the method, keeping its simplicity and rapidity unchanged.

TABLE 1

TCDD distribution in rabbit liver samples collected from July to December 1976.

TCDD ng/g	% total positive samples (n = 341)
$\geq 0.25 < 1$	17.1
$\geq 1 < 4$	27.0
$\geq 4 < 16$	23.2
$\geq 16 < 64$	21.2
$\geq 64 < 256$	9.9
$\geq 256 < 1024$	1.6

ACKNOWLEDGEMENTS

We thank the Regione Lombardia, Ufficio Speciale di Seveso, for providing financial support for this project.

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