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Carbofuran self-poisoning: forensic and analytic investigations in twins and literature review

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The authors declare that there is no conflict of interest.

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Abstract

Carbofuran is a pesticide widely used in agricultural context to kill insects, mites and flies by ingestion or contact. Along with literature review, we aimed to (i) present the clinical, autopsy and toxicological findings of carbofuran self-poisonings in two 69-year-old twins, resulting in the death of one of them and (ii) assess carbofuran metabolites distribution using molecular networking. Quantitative analysis of carbofuran and its main metabolites (3-hydroxycarbofuran and 3-ketocarbofuran) was carried out using an original liquid chromatography-tandem mass spectrometry method on biological samples (cardiac or peripheral blood, urine, bile and gastric contents). Toxicological analysis of *postmortem* samples (twin 1) highlighted high concentrations of carbofuran and its metabolites in cardiac blood, bile, and gastric contents. These compounds were also quantified in blood and/or urine samples of the living brother (twin 2), confirming poisoning. Using molecular networking approach to facilitate visualization of mass spectrometry datasets and sample-to-sample comparisons, we detected two more metabolites (7-phenol-carbofuran and 3-hydroxycarbofuran glucuronide) in bile (twin 1) and urine (twin 2). These results highlight the value of (i) these compounds as carbofuran consumption markers and (ii) bile samples in *post-mortem* analysis to confirm poisoning. From an analytical point of view, molecular networking allowed the detection and interpretation of carbofuran metabolite ammonium adducts which helped to confirm their identification annotations, as well as their structural data. From a clinical point of view, the different outcomes between the two brothers are discussed. Overall, these cases provide novel information regarding the distribution of carbofuran and its metabolites in poisoning context.

Keywords: carbofuran, pesticide toxicity, metabolism, carbamate, literature review

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Fig. 1. Carbofuran molecule (a) and its macroscopic aspect in DIAFURAN 5G® commercial product (b)

Fig. 2 : Molecular networking of carbofuran and its metabolites from twins biological samples using a Q Exactive™ Orbitrap mass spectrometer. Nodes are labelled with the exact protonated mass (*m/z*) and the links are labelled with the exact mass shift. Each matrix is depicted in a specific color: cardiac blood in red, bile in green, gastric contents in blue for twin brother 1, and peripheral blood in pink, and urine in yellow for twin brother 2. In-source fragments are indicated in the blue and purple squares. Levels of confidence are defined according to Schymanski *et al.* (2014). RT = retention time

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1. Introduction

Carbofuran (2,3-dihydro-2,2-dimethyl-7-benzofuranylmethylcarbamate), sold notably as DIAFURAN 5G® (Fig. 1) is a carbamate insecticide widely used in gardening and agriculture for the control of soil-dwelling and flying insects, such as carrot flies, wireworms, thrips, codling moths, leafhoppers, and white grubs. Carbamates are classified into N-allylcarbamate herbicides and N-methylcarbamate insecticides in function of their chemical structures and biological effects. Carbofuran is a broad-spectrum N-methylcarbamate pesticide and is highly soluble in water making it a high potential pesticide for groundwater contamination but breaks down in sunlight, is metabolized in the soil and degraded in water. According to the World Health Organization recommended classification of pesticides by hazard and guidelines to classification, [1] carbofuran is considered as highly hazardous (class Ib) with an oral LD50 of 5 mg/kg in rats [2].

Carbamate pesticides are toxic due to their binding at the active site of the acetylcholinesterase which leads to the reversible inhibition of the enzyme and therefore an accumulation of acetylcholine at the nerve synapse [3]. The inhibition of the hydrolysis reaction of acetylcholine induces excessive cholinergic stimulation and poisoning symptoms, such as miosis, tremor of the extremities, hypersalivation, lacrimation, urinary incontinence, sweating, gastrointestinal cramping, diarrhea and emesis. More severe forms of poisoning may appear such as seizures, coma, bradycardia or tachycardia. Death may occur from severe bronchoconstriction and/or respiratory paralysis.

In the whole European Union, since 31 August 2008, the sale of plant protection products containing carbofuran is prohibited due to its toxicity, and utilization since 13 December 2008 [4]. However, carbofuran is still widely used in many countries as Korea, Japan, Sri Lanka [5]. Several carbofuran poisoning cases (suicide or accidental absorption with dermal exposure, ingestion or inhalation) leading to fatal issues have been reported [5, 6].

Carbofuran is described to be fastly and completely metabolized by oxidation in two main toxic metabolites, 3-hydroxycarbofuran and 3-ketocarbofuran and metabolized by hydrolysis in 7-phenolcarbofuran and in minor metabolites, 3-hydroxy-7-phenolcarbofuran, 3-keto-7-phenolcarbofuran, N-hydroxymethylcarbofuran and 3-hydroxy-N-hydroxymethylcarbofuran [7–9]. Glucuronides and sulfates conjugates forms of carbofuran are excreted into urine [10]. Cytochrome P450 (CYP) 3A4 is the major isoform responsible for carbofuran oxidation, although CYP1A2 and CYP2C19 are also involved to a much lesser extent [11].

We report a case of carbofuran self-poisoning in 69-year-old male twins resulting in one fatal issue and one admission to an intensive care unit (ICU). The toxicological analyses were performed by a liquid chromatography coupled tandem mass spectrometry (LC-MS/MS) method. We aimed to assess (i) carbofuran metabolism and toxicological profile on twins (*post-mortem* and *in vivo*) samples and (ii) carbofuran metabolites distribution using molecular networking. Since this self-poisoning case of twin men with a fatal issue for one of them and not for the other is unique, we also aimed to discuss the individual susceptibility in intoxication cases, namely why the twins reacted differently to carbofuran ingestion.

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2. Case report

2.1. Twin brother 1

The twin brother 1 (69-year-old man, 1.79 m, 112 kg, BMI 34.95 kg/m^2) was found dead on a bed, in right lateral decubitus position, in his closed and locked home. The *post-mortem* interval was evaluated between 6 and 12 hours using rigor mortis (rigidity) and livor mortis (lividity), compared with the survey data. Alcohol bottles were found in the habitation but not drug packaging. The subject's medical history and possible drug treatment are unknown. According to pre hospital emergency medical team, the atmosphere analysis for the detection of carbon monoxide was negative. The autopsy was performed four days after the body was found. An external inspection of the body did not reveal any specific lesions that could testify a violent intervention by a third party or explain the death. The autopsy showed a heart weighing 535 g for an expected average of 438 g [353-523 g] [12] and macroscopic examination of the liver (1561 g) showed mainly a doubtful appearance of steatosis. Samples of bile, gastric contents (estimated at 150 mL) and cardiac blood were collected for toxicological analysis. COHb measured on peripheral blood samples was found to be at 0.7%. The medical examiner concluded to an asphyxiation syndrome and a possible previous cardiac condition, which are not incompatible with a notion of intoxication. Histological examination showed hypertrophic cardiomyopathy and moderate monovessel atherosclerosis without acute or old infarction, edematous and congestive asphyxia, in a context of acute infectious bronchopathy, and a massive macro- and microvacuolar hepatic steatosis.

2.2. Twin brother 2

At the foot on this same bed, the twin brother 2 (69-year-old-man, 1.80 m, 97.5 kg, BMI 30 kg/m^2) was found in a confused mental state. On admission to the ICU, the body temperature was 35°C, and SpO_2 was 98% with 9L/min of oxygen, with a respiratory rate of 24 per minute. Neurologic examination found a Glasgow coma scale of 12 and a bilateral miosis. Electrocardiogram showed a sinus rhythm at 93 bpm, a PR interval at 120 ms, a QTc interval at 410 ms, fine QRS and no repolarization disorder. The patient presented a dyspnea with no crackles, a dry mouth and stigmata of a prolonged position on the floor with erythema of the left hemithorax and left lower limbs. Because of the stabilization of vital functions in the resuscitation unit, the patient did not require atropine and/or pralidoxime.

Initial laboratory tests at ICU admission are summarize in Table 1.

Table 1: Initial laboratory test results of twin brother 2

Test	Value	Normal range
Creatinine	92 µM	59 – 104 µM
Urea	11.5 mM	2.8 – 8.1 mM
Natremia	146 mM	136 – 145 mM
Potassium	3.8 mM	3.4 – 4.5 mM
Creatine phosphokinase	5,714 U/L	20 – 200 UI/L
Leukocytes	28.1 G/L	4 – 10 G/L
Polynuclear neutrophils	25.6 G/L	1.8 – 7.5 G/L
Hemoglobin	16.5 g/dL	13 – 17 g/dL
Mean corpuscular volume	107 fL	79 – 97 fL
AST	136 UI/L	0 – 35 UI/L

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INR	1.13	-
pH	7.37 mmHg	7.38 – 7.42 mmHg
pCO_2	37 mmHg	37 – 42 mmHg
pO_2	67 mmHg	80 – 105 mmHg
Bicarbonate	17 mM	22 - 31 mM
Lactates	1 mM	0.5 – 1.6 mM
COHb	1.4%	< 2.5%
Plasma cholinesterase concentration	2,625 U/L	5,320 – 12,920 U/L
Erythrocyte cholinesterase concentration	13,835 U/L	11,000 and 16,700 U/L

On day-1, the patient presented a hemodynamic failure treated by high-dose vasopressors and an acute respiratory distress syndrome (ARDS). He required invasive mechanical ventilation, sedation by midazolam and morphine for 8 days, neuromuscular blockade for 2 days, and antimicrobial treatment with amoxicillin/clavulanic acid. After resolution of the ARDS, the patient was extubated and discharged from the ICU.
He confirmed the twins plan to end their lives through the ingestion of a pesticide, type carbofuran 5% (DIAFURAN® 5G) presented as purplish granules of 0.5 to 2 mm in diameter (Fig. 1). The twins were farmers and had easy access to insecticides and phytosanitary products.

3. Materials and methods

3.1. Literature review

Google scholar and PubMed databases were searched for relevant papers published in English using the following search terms: carbofuran, poisoning, intoxication, blood, urine, detection, human. Studies providing information on the concentrations of carbofuran and its metabolites found in human matrices during exposure events were included for review.

3.2. Chemicals and reagents

Carbofuran, 3-hydroxycarbofuran and 3-ketocarbofuran were purchased from Sigma-Aldrich (Saint Louis, MO, USA), 7-phenolcarbofuran and ketamine-D4 (selected as the internal standard) from LGC Standard (Molsheim, France). Methanol, water, acetonitrile and formic acid were purchased from Carlo Erba Reagents (Val de Reuil, France). Ammonium acetate and zinc sulphate were supplied from Merck (Darmstadt, Germany).

3.3. Sample preparation

3.3.1. Extraction of carbofuran from commercial product

Granules seized from the twins' home was dissolved in LC grade water at 10 g/L and diluted into mobile phase. Dilutions were analyzed and quantified by LC-HRMS using a Q Exactive™ Orbitrap mass spectrometer (Thermo Scientific, San Jose, CA, USA).

3.3.2. Biological samples

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Cardiac blood, bile and gastric contents (estimated at 150 mL) samples were *post-mortem* collected in the fatal intoxication case (twin brother 1). Biological samples of twin brother 2 (alive) were collected at ICU admission (peripheral blood H+2 and urine H+4 after the patient was discovered).

3.3.3. Extraction of carbofuran and its metabolites from biological samples

The blood, bile, urine and gastric content samples (200 µL) were transferred into 5 mL glass tubes for protein precipitation. Samples were extracted with 300 µL of zinc sulphate solution at 0.1 M and additional 500 µL of methanol containing the internal standard (ketamine-D4) were supplemented as already reported [13–16]. Supernatants were evaporated and residues dissolved in 200 µL of water/acetonitrile/formic acid (90/10/0.09, v/v/v). The final extracts were transferred into vials for LC-MS/MS analysis and quantitation.

3.4. Toxicological analysis

3.4.1. Standard Toxicological analysis

The biological samples (*post-mortem* and *in vivo*) were tested for various analytes (i.e. alcohol, psychotropic agents, illegal drugs and opioid withdrawal medications). Volatile compounds were quantified on a Thermo Scientific TRACE 2000 GC including a TriPlus Headspace autosampler (Thermo Scientific) by headspace gas chromatography – flame ionization detector (HS-GC-FID). Drugs were analyzed by LC-HR-MS using a Q Exactive™ Orbitrap mass spectrometer (Thermo Scientific, San Jose, CA, USA) coupled to an Accela 1250 pump (Thermo Scientific) as already described [17, 18]. Toxicological screening was performed using LC-HR-MS/MS, as previously reported [13, 14, 16]. COHb measurement was performed using a blood gas analyzer (ABL80 FLEX-CO-OX; Radiometer S.A.S. Neuilly-Plaisance, France).

3.4.2. Carbofuran and metabolites quantitation

Stock solutions of carbofuran, 3-hydroxycarbofuran, 3-ketocarbofuran were prepared at 1.0 g/L in methanol. The latter were appropriately diluted with methanol to obtain working standard solutions. The stock solutions were stored at -20°C. Linear concentration ranges from 0.05 to 500 µg/L for carbofuran, from 0.1 to 500 µg/L for 3-hydroxycarbofuran and from 1 to 500 µg/L for 3-ketocarbofuran were used for quantitation.

Carbofuran and its metabolites were measured by liquid chromatography-tandem mass spectrometry (UPLC-MS/MS) using a Xevo TQ-XS quadrupole mass spectrometer with an ESI ionization source. The instrument was coupled to a Waters Acquity Class-H Plus equipped with a quaternary pump system (Waters, MA, USA). The column was held at 40°C and the autosampler was set at 15°C. Two µL of sample were injected and LC separation was performed on an Acquity UPLC BEH C18 column (2.1 x 100 mm, 1.7 µm; Waters Inc., MA) with a binary gradient of elution at a flow rate of 0.5 mL/min, consisting of ammonium acetate 10 mM/0.1% formic acid (solvent A) and 0.1% formic acid in acetonitrile (solvent B). The gradient started with 5% solvent B for 1.5 min and then ramped up to 30% solvent B over a period of 3.5 min, and up to 40% solvent B over another period of 3 min and finally up to 95% solvent B within 0.5 min. The gradient was then maintained at these conditions for 0.5 min before recycling back to 5% solvent B in 1 min and then held at 5% solvent B for 1 min.

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The MS/MS data were acquired in multiple reaction ion monitoring (MRM) mode using positive electrospray ionization. Two MRM transitions were set up for each analyte: one for quantitation and one for confirmation; only one MRM transition for the IS. The following conditions were used: capillary 3 kV; source temperature 150°C, desolvatation temperature 550°C, cone gas flow 150 L/h, desolvatation gas flow 1000 L/h, nebulizer 7 bars. Declustering potential and collision energy were optimized in order to maximize the signal of each reaction monitored. Parameters for each transition are listed in Table 2.

Table 2. Optimized parameters and MRM transitions for quantitation of carbofuran, its metabolites and internal standard (IS).

Compound	Formula	RT (min)	Precursor ion (*m/z*)	Product ion (*m/z*)	Declustering potential (V)	Collision energy (V)
Carbofuran	$C_{12}H_{15}NO_3$	7.0	222.1	123.1* 165.1	23	22 12
3-hydroxycarbofuran	$C_{12}H_{15}NO_4$	4.8	238.1	163.1* 181.1	25	14 10
3-ketocarbofuran	$C_{12}H_{13}NO_4$	5.9	236.1	179.0* 161.0	8	11 16
7-phenolcarbofuran	$C_{10}H_{12}O_2$	6.9	165.1	123.1* 54.8	29	13 19
Ketamine-D4 (IS)	$C_{13}2H_4H_{12}ClNO$	4.4	242	129.0	30	26

* Product ion (*m/z*) used for quantitation

3.4.3. Molecular networking generation

Molecular networking (MN) applied to untargeted screening is an efficient tool for visualization and organization of MS data of unknown compounds in a graphical form and is based on spectral similarity. The generation of a molecular network requires an analysis by LC-HRMS/MS, then a reprocessing of the data by various software accessible in open source.

Concerning the analytical part, LC was performed on a Accucore Phenyl Hexyl column (100 x 2.1 mm, 2.6 µm) (Thermo Scientific, San Jose, CA), MS analyses were performed using Orbitrap Q Exactive™ mass spectrometer coupled to an UltiMate 3000 pump (Thermo Scientific, San Jose, CA) and a heated electrospray ionization source (HESI-II) was used for the positive ionization of the target compounds as already described [13, 14, 16, 19].

Concerning the reprocessing of data by open source bioinformatics software, the complete MN approach is already described elsewhere [20]. Briefly, raw spectral data converted in a .mzXML (ProteoWizard's MSConvert module) open mass format are preprocessed using a MZmine 2 software [21]. These preprocessing steps are required upstream of multi-sample MN generation, and include deconvolution, de-isotoping, alignment, gap-filling and annotations. The ensuing spectra file are saved as a single .mgf output file and loaded on the Global Natural Products social Molecular Networking (GNPS) web platform creating the multimatrix MN [22]. Lastly, the visualization of the obtained molecular network is done with the Cytoscape 3.8.0 software [23], thus simplifying interpretation of the results. Following settings were selected for this study: mass tolerance of precursor and fragments ions used for MS/MS spectral library searching set to 0.02 *m/z*; mass tolerance of MS/MS ions used for MN set to 0.02 *m/z;* minimum cluster size set to 1,

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minimum number of common MS/MS ions shared set to 6, links between nodes kept if nodes in top 10 most similar nodes. In a multisample network, a node is under a pie chart colored form where a color corresponds to one biological matrix and where size of pie section is proportional to compound abundance in the matrix. This semi-quantitative bioinformatics approach gathers data in linked nodes with a cosine score higher than 0.70. Compounds were identified according to three levels of confidence [24] : level 1 for structure confirmed by reference standard; level 2b for probable structure by diagnostic evidence and spectrum matching; level 3 for probable structure by diagnostic evidence. Therefore, MN is a visual tool in drug metabolism exploration forming clusters of parent molecule and associated metabolites according to their MS/MS spectra similarities.

4. Results

LC-MS/MS analyses confirmed that the product contained in the DIAFURAN 5G® boxes found near the twins was indeed carbofuran (purity 5%) and that this molecule was also found in the biological matrices of both twins. Table 3 shows carbofuran and its metabolites detection and/or quantitation in the different matrices in the two poisoning cases considered in our study.

In twin brother 1 (dead), only low levels of alcohol and alprazolam were detected in cardiac blood and gastric contents, ruling out fatal intoxication by these compounds at the time of death. Bile was shown to be the matrix containing the most carbofuran and its metabolites (carbofuran, 3-OH- carbofuran, 3-keto-carbofuran, 7-phenol-carbofuran, 3-OH-carbofuran glucuronide), and in the highest concentration. As a result, metabolites 7-phenol-carbofuran and 3-OH-carbofuran glucuronide were only found in bile. Gastric contents also contained a significant concentration of carbofuran, 3-OH-carbofuran and 3-keto-carbofuran in comparison with cardiac blood (Table 3).

In twin brother 2 (alive), neither alcohol nor drugs were detected in biological samples, excepted alprazolam at a low concentration and midazolam, which was given during hospital treatment. Carbofuran and its metabolites were predominantly found in urine, with 3-hydroxycarbofuran having the highest concentration. In addition, only carbofuran and 3-OH- carbofuran were found in peripheral blood (Table 3).

Table 3. Toxicological analyses in each biological matrix

Case	Biological matrix	Concentration (µg/L)							
		carbofuran	3-OH-carbofuran	3-keto-carbofuran	7-phenol-carbofuran	3-OH-carbofuran glucuronide	alprazolam	midazolam	ethanol (g/L)
Twin brother 1 (dead)	Cardiac blood	1.8	34.0	1.3	ND	ND	3.0	ND	0.38
	Bile	327.8	156.5	20.8	D/NQ	D/NQ	D/NQ	ND	-
	Gastric contents (i.e 0.15 L)	155.3 (i.e 23.3 µg)	67 (i.e 10.0 µg)	5.7 (i.e 0.85 µg)	ND	ND	4.5 (i.e 0.67 µg)	ND	0.56 (i.e 84 mg)
Twin brother 2 (alive)	Peripheral blood	0.7	1.3	ND	ND	ND	4.0	811.0	ND
	Urine	110.2	398.0	10.0	D/NQ	D/NQ	6.0	ND	-

ND : not detected, under limit of detection - D/NQ: detected but not quantified – in brackets : volume and amount present in gastric contents

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Among carbofuran metabolites, only 3-OH-carbofuran and 3-keto-carbofuran were quantified. Thus, we used MN as a bioinformatics data reprocessing approach to analyze the unquantified metabolites. By displaying MS/MS data acquired during analysis, MN showed clusters containing nodes corresponding to carbofuran (*m/z* 222.113) and structurally related compound including 7-phenol-carbofuran (*m/z* 165.091), 3-OH-carbofuran (*m/z* 238.107), and 3-OH-carbofuran glucuronide (*m/z* 414.140) (Fig. 2). This approach allowed us to perform sample-to-sample comparison as well as semi-quantitative analysis, thus going further in data analysis. Although we do not have a quantitative method for 7-phenol-carbofuran and 3-OH-carbofuran –glucuronide, we showed here that these two metabolites are mainly found in urine, but also in the bile to a lesser extent.

Interestingly, metabolites 3-OH-carbofuran and 3-OH-carbofuran glucuronide were also found in the form of ammonium adduct, namely *m/z* 255.134 ([3-OH-carbofuran + NH4]$^+$) and *m/z* 431.166 ([3-OH-carbofuran glucuronide + NH4]$^+$), respectively. These compounds were identified through a mass shift of 17.0265 and their fragmentation spectra, and because they exhibited the same retention time as their respective compound (Fig. 2).

Lastly, we identified in-source fragments from [3-OH-carbofuran glucuronide + NH4]$^+$ (*m/z* 220.097, *m/z* 163.075) at RT = 5.0 min and [3-OH-carbofuran + NH4]$^+$ (*m/z* 181.086, *m/z* 220.097, *m/z* 163.075) at RT = 5.6 min based on MS/MS scans (Fig. 2). The identification of in-source fragments with respect to their parent molecule was performed by comparing their same retention time, fragmentation spectrum and ionization intensities between samples.

5. Discussion

We reported on two-twin brothers carbofuran severe intoxication in the context of a voluntary ingestion and resulting in one death. In view of (i) the results of the autopsy (concluding to an asphyxia syndrome) and the conclusions of the judicial investigations concerning twin brother 1, (ii) toxicological analysis concerning both twins and (iii) the story described by the surviving brother, the most probable explanation was self-poisoning by ingestion. The initial toxicological results ruled out massive intoxication by alcohol or illegal drugs at the time of death (twin brother 1) or medical management (twin brother 2). Although the hypothesis of alprazolam intoxication in brother 1 can be ruled out because of the therapeutic concentrations found postmortem, this hypothesis (or at least a much higher concentration at the time of poisoning) cannot be ruled out in brother 2 because of the delay in collecting the samples.

Carbofuran is known to have three main metabolites (3-hydroxycarbofuran, 3-ketocarbofuran, and 7-phenolcarbofuran)[7–9]. In the literature, numerous cases of carbofuran poisoning have been described, whether from occupational exposure, voluntary or accidental ingestion. Table 4 summarizes data from the literature on human cases of exposure to carbofuran and reporting the concentrations found (carbofuran and/or metabolites). In this study, carbofuran and two metabolites (3-OH- carbofuran and 3-keto- carbofuran) were quantified. Low concentrations of carbofuran were found in twin brother 1 matrices compared with other fatal cases reported in the literature, as exemplified by carbofuran concentrations in cardiac blood (1.8 µg/L in this study *versus* 400 – 27,300 µg/L in literature data), in bile (327.8 µg/L in this study *versus* 400 – 60,000 µg/L in literature data), and in gastric contents (155.3 µg/L in this study *versus* 3,840 µg/L in the literature) (Table 4). Interestingly, no data are available in the literature on *post-mortem*

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concentrations of 3-OH-carbofuran and 3-keto-carbofuran. Here, our results concerning concentration of carbofuran and its metabolites in gastric contents were consistent with an intake of carbofuran few hours before death, although postmortem redistribution phenomena may have overestimated the concentrations found [25]. In twin brother 2 (alive), carbofuran and its metabolites concentrations in blood were consistent with literature data while concentrations in urine were higher than those already reported (Table 4).

A MN approach enabled us to efficiently compare datasets by highlighting the distribution of carbofuran and its metabolites in the different matrices in a semi-quantitative manner. In addition to quantitative results, we found that 7-phenol-carbofuran and 3-OH-carbofuran glucuronide were predominantly excreted in urine, but also in bile. Overall, these results confirm the available pharmacokinetic data in humans highlighting high urinary elimination of carbofuran metabolites and show the particular interest of 3-OH-carbofuran, 7-phenol-carbofuran and 3-OH-carbofuran glucuronide as relevant consumption markers in urine [10]. In addition, we show that bile is a very interesting matrix for demonstrating carbofuran consumption in *postmortem* settings due to the high intensity of metabolites in this matrix.
MN also allowed us to highlight the in-source fragmentation of carbofuran metabolites, which produces nodes with fragment masses, but also $[M+NH_4]^+$ adducts as reported in the literature for other molecules [26]. Adducts production make automatic annotation and data interpretation difficult and are often poorly taken into account by existing annotation tools [27]. Here, the new molecular ion $[M+NH_4]^+$ adducts observed (*m/z* 431.166 and *m/z* 255.134) can lead to wrong identifications if they are not known or considered, while they can support correct annotation and help resolve misidentification of compounds of the same mass when identified. Interestingly, oxygen atoms are known to stabilize $[M+NH_4]^+$ adducts and $[M+NH_4]^+$ adduct formation has been characterized as a hydrogen-bonding interaction. Therefore, our data suggest that $[M+NH_4]^+$ adducts are bound to the ketone group in this study, which helped us to support our structure identification data within the molecular network [28]. These important data should be taken into account in case of toxicological analysis by high-resolution mass spectrometry following exposure to carbofuran. To achieve a precise quantitation, it is preferable to use MS/MS with determined and optimized transitions with the help of a standard, in order not to distort the concentrations.

Lastly, the different outcome between the two twin brothers raises many questions about the fact that one of two twins died. Carbofuran ingested doses are not known, not allowing to conclude about the interpretation of carbofuran and its metabolites concentrations between the different matrices. The blood and urine of the living twin were collected a few days after the carbofuran ingestion, leading to concentrations that do not reflect acute intoxication. Since we do not know whether the twins were homo or heterozygous, a difference in CYP polymorphism explaining a difference in susceptibility to carbofuran toxicity between twins cannot be excluded. A different metabolic associated fatty liver disease state between the two individuals, leading to a different metabolism of carbofuran, may have explained a difference in toxicity [29, 30]. These data are supported by the appearance of hepatic steatosis found at autopsy in twin brother 1, although we do not have similar data in twin brother 2. The different pathologies accumulated throughout life could also have predisposed twin brother 1 to a susceptibility to carbofuran intoxications. This hypothesis is supported by (i) literature data reporting cardiotoxicity of carbofuran [31] and (ii) a pathological aspect of the heart (hypertrophic cardiomyopathy) of twin brother 1 observed during autopsy, although we have no similar data for twin brother 2.

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6. Conclusion

We describe here an original case of intoxication of two twins by the same product Diafuran 5G®, which we quantified as containing 5% carbofuran. Concentrations of carbofuran and its main metabolites were measured with an original LC-MS/MS method in biological samples and analyzed regarding the literature. Using a MN approach, we brought new data regarding carbofuran metabolite distribution especially in *postmortem* settings. As a result, we confirmed in this original case the particular interest of bile samples in *post-mortem* analysis when urine is not available and interest of metabolites identification/quantitation in context of carbofuran intoxication. From an analytical point of view, we highlight that the detection and interpretation of ammonium adducts allows to confirm the identification annotations of the metabolites, as well as their structural data.

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Table 1 4. Review of the literature on human cases of exposure to carbofuran and reporting the concentrations found (carbofuran and/or metabolites)

Profile	Exposure mode	Clinical symptoms	Outcome	Detection method	Molecules detected and quantified	References
8 cases (6 male / 2 woman) Age: 3.5-59 year-old	5 suicides / 3 homicides	Burning sensation, bitter taste, irritation in the mouth and vomiting	Fatal	Stomach contents/liver/beverage: TLC, GC-MS, and HPLC analysis Blood, bile: TLC, GC–MS and HPLC analysis.	Carbofuran • Bile : 400-60,000 µg/L • Stomach/stomach and contents : 0.3-300 µg/g • Liver : 2.2 µg/g • Suspected drink: 2.1-3.8 g/L • Blood : 400-18,000 µg/L Metabolites not quantified	Sakunthala *et al.* (2013) [6]
4 cases (3 woman/1 male) Age : 24-41 year-old	Unknown	Unknown	Fatal	Gastric contents: TLC, GC-MS Blood: GC-NPD	Carbofuran: 320-11,600 µg/L in blood	Ameno *et al.* (2001) [5]
50 year-old woman 55-year-old man	Ingestion of carbofuran added to yoghurt		Fatal (woman) Rescued (man)	GC-MS	Carbofuran • Venous blood : 2,530 – 3,230 µg/L • Gastric lavage liquid: 69,700-100,600 µg/L • Gastric contents: 3,840 µg/L • Heart blood: 10 µg/L • Urine: 10 µg/L 7-phenol-carbofuran • Venous blood : 430-600 µg/L • Gastric lavage liquid: 8,410-12,440 µg/L • Gastric contents: 590 µg/L • Heart blood: 20 µg/L • Urine: 590 µg/L	Liu *et al.* (2016) [32]
2 woman Age: 17 and 20 year-old	Unknown	Fatal death	Unknown	Unknown	Carbofuran in blood : 9.71 µg/g	Sancewicz-Pach K *et al.* (1997) [33]
72-year-old man	Volontary ingestion	Coma, cardiac arrest	Rescued	LC-MS	Carbofuran in blood : 1,500 µg/L	Lacassie *et al.* 2001 [34]
51-year-old man	Ingestion of "chumbinho", an illegal rodenticide sold in Brazilian street markets that contains carbofuran	Unknown	Fatal	GC-MS	Carbofuran in blood : 27,300 µg/L	Ferrari Junior *et al.* (2018) [35]

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140 women and their newborns (Exposure survey)	Unknown	Unknown	Alive	GC-MS	Maternal serum: 0.007-17.63 ng/g (n=140) Cord serum: 0.007-13.97 ng/g (n=77)	Barr *et al.* 2010 [36]
27-year-old man 2-year-old child	Man : suicide Child: had ingested grains of carbofuran from the flower pots of his balcony	Man : NA Child: drowsiness, vomiting, abdominal pain, dysarthria, and loss of balance	Fatal (man) Rescued (child)	GC-MS	Man : carbofuran at 466 µg/L in blood Child : carbofuran 91 µg/L in blood	Papoutsis *et al.* 2012 [37]
38-year-old man 44-year-old woman	Suicide to benfuracarb (Main metabolite : carbofuran)	Unknown	Fatal	GC-NPD	Carbofuran: Blood: 1,450-1,4700 µg/L Urine: 530-2,660 µg/L	Lee *et al.* 1999 [38]
51-year-old man	Suicide	Unknown	Fatal		In blood: Carbofuran : 27,300 µg/L 3-OH-carbofuran : detected but not quantified	Ferrari Junior *et al.* 2021 [39]
158 plasma samples of human with acute carbamate self-poisoning	Unknown	Unknown	Unknown	LC-MS/MS	Plasma : 20.7 – 62,200 µg/L	Mostafa *et al.* 2011 [40]
5 farmers Age: 23-68 year-old	Occupational exposure	Unknown	Alive	GC-MS/MS	In plasma: Carbofuran: < LOQ-3.04 µg/L 7-phenol-carbofuran: 14.1-22.9 µg/L 3-OH-carbofuran : 1.38-5.67 µg/L 3-keto-carbofuran : < LOQ 3-keto-carbofuran-7-phenol : 1.07-1.17 µg/L	Petropoulou *et al.* (2006) [41]
5 farmers Age : 35-71 year-old	Occupational exposure	Nausea, headache and respiratory problems	Alive	GC-MS/MS	In urine: Carbofuran: < LOQ 7-phenol-carbofuran: 39.7-113 µg/L 3-OH-carbofuran : <LOQ-2.88 µg/L 3-keto-carbofuran : < LOQ-0.96 µg/L 3-keto-carbofuran-7-phenol : 1.22-2.53 µg/L	Petropoulou *et al.* (2006) [42]
2 twin brothers	Suicide attempt by ingestion	Unknown	Fatal (twin brother 1) Alive (twin brother 2)	LC-HRMS/MS	Cardiac blood: • Carbofuran: 1.8 µg/L • 3-OH-carbofuran : 34 µg/L • 3-keto-carbofuran : 1.3 µg/L Bile: • Carbofuran: 327.8 µg/L • 3-OH-carbofuran : 156.5 µg/L	This study

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		• 3-keto-carbofuran : 20.8 µg/L • 7-phenol-carbofuran: detected but not quantified • 3-OH-carbofuran glucuronide: detected but not quantified Gastric contents: • Carbofuran: 155.3 µg/L (i.e 23.3 µg) • 3-OH-carbofuran : 67 µg/L (i.e 10.0 µg) • 3-keto-carbofuran : 5.7 µg/L (i.e 0.85 µg) Peripheral blood: • Carbofuran: 0.7 µg/L • 3-OH-carbofuran : 1.3 µg/L Urine: • Carbofuran: 110.2 µg/L • 3-OH-carbofuran : 398 µg/L • 3-keto-carbofuran : 10 µg/L • 7-phenol-carbofuran: detected but not quantified • 3-OH-carbofuran glucuronide: detected but not quantified

Abbreviation: TLC: Thin layer chromatography; GC-MS: gas-chromatography coupled with mass spectrometry; GC-MS/MS: gas-chromatography coupled with tandem mass spectrometry; HPLC: high performance liquid chromatography; GC-NPD: Gas Chromatography – Nitrogen Phosphorous Detector.

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