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## **Case report**

# **Specific interpretation of hair concentrations in 2 fatal metformin intoxication cases**

**Nadia Arbouche<sup>1</sup>, Audrey Farrugia<sup>1</sup>, Alexis Walch<sup>1</sup>, Jean-Sébastien Raul<sup>1</sup>, Pascal Kintz<sup>1,2</sup>**

1. Institut de médecine légale, 11 rue Humann, 67000 Strasbourg, France
2. X-Pertise Consulting, 42 rue principale, 67206, Mittelhausbergen, France

Author for correspondance : Nadia Arbouche, Institut de médecine légale, 11 rue Humann, 67000 Strasbourg, France .Email : [nadia.arbouche@hotmail.it](mailto:nadia.arbouche@hotmail.it)

## **ABSTRACT**

Hair analysis is very useful for toxicological investigations since, by providing a wider detection window, it gives the possibility to perform a retrospective study on the historical consumption of a substance. Unfortunately, there are no data available for hair concentrations in metformin-related deaths. In this study, the authors present 2 cases of fatal metformin intoxication in which, for the first time, hair analysis was performed using a specific GC-MS/MS method. Metformin was tested positive in femoral blood (112.3 mg/L and 64.7 mg/L respectively) and cardiac blood (226.9 and 203.2 mg/L) of the two subjects. For case 1, other samples were also tested positive, including vitreous humor (31.1 mg/L) and gastric contents (773.5 mg/L). In case 2, metformin was measured at 844.9 mg/L in urine. Metformin hair concentrations were 28.3-44.8 and 22.5 ng/mg for both cases, respectively. The concentrations found in the 2 fatal cases are clearly higher than those obtained in a previous study with subjects under treatment (0.3 to 3.8 ng/mg) or those found in 3 post-mortem cases where metformin death was excluded (0.6 to 1.4 ng/mg). Excessive sweating during the agonal phase due to fatal hypoglycemia could explain these elevated concentrations as sweat can have contaminated the hair.

**Keywords:** metformin, hypoglycemic drug, hair, GC-MS/MS

## **1. INTRODUCTION**

Metformin (Glucophage™) is the most worldwide used hypoglycemic agent for the treatment of type 2 diabetes mellitus (T2DM). Half a century ago, various biguanides were used for the treatment of T2DM (metformin, phenformin, buformin). In the 70s they were all removed from the market due to a high association with lactic acidosis. However, the role of metformin has been reassessed in recent years and now represents the first-line therapy for type 2 diabetics [1]. Metformin is available as oral tablets in the immediate release form and in the extended release form at dosages of 500, 850 and 1000 mg to be taken two or three times a day [2].

Metformin is a small molecule (molecular weight of 129.2 daltons) with a bioavailability between 50-60%. It is eliminated unchanged by the kidneys. The peaks plasma concentrations

at therapeutic doses are between 1.5-3 mg/L [3]. The hypoglycemic action of metformin consists in inhibiting the production of glucose in the liver and in stimulating glucose uptake in peripheral tissues.

Although metformin is considered as one of the safest antidiabetics, fatal intoxications, whether accidental or intentional, are not rare events. Problems related to metformin are due to its hypoglycemic action and to the excessive production of lactic acid. In fact, metformin, by inhibiting liver gluconeogenesis, contributes to the increase of blood lactate level, which, under certain conditions and for certain patients, can be fatal. This side effect is known as the metformin-associated lactic acidosis (MALA). In subjects with normal renal function, metformin is eliminated as parent drug by glomerular filtration and tubular secretion. In case of kidney dysfunction, metformin will accumulate and produce increased lactate levels. Marked acidosis can cause significant vasodilation and depression of myocardial contractility. These mechanisms will lead to hypotension and hypo-perfusion, which in turn will cause acute renal failure followed by multisystem organ failure [4,5].

With respect to its hypoglycemic effect, unlike other antidiabetic agents such as sulfonylureas, which act directly by stimulating the release of insulin, metformin has a more complex effect, which allows maintaining a balance in blood glucose levels without causing significant decrease in concentration. Nevertheless, fatal hypoglycemia cases have already been reported, especially when metformin is used in combination with other antidiabetics [6].

Until now, in metformin-related death cases published in the medical literature, hair analysis has never been reported. Hair analysis has gained increasing importance in recent years both in the forensic and clinical practice, as it allows to establish the pattern of exposure to a drug and to evaluate, in some controlled circumstances the frequency and dosage of drug use during the previous months. The drug detection window in hair is extended to weeks, months or even years based on the length of the hair, in comparison to the standard hours/days for both blood and urine. For some drugs, if a correlation between the administered doses and the concentrations found in hair is established, hair test results can be useful in therapeutic drug monitoring. In a fatal case, segmental hair tests allow to verify treatment observance, which can be critical with hypoglycemic drugs [7].

The abuse of oral hypoglycemic agents for other purposes than therapeutic ones, has emerged in the more recent times [8-10]. In order to better explain unclear hypoglycemic events such as in the context of Münchhausen or Münchhausen by proxy syndromes or in situations of incapacitation, hair analysis seems to be a very useful to complement blood and urine since it gives retrospective data on drug exposure.

In this study, metformin was tested for the first time in the hair of two subjects deceased from fatal metformin overdose, using a new GC-MS/MS specific method.

## **2. CASE REPORT**

### **2.1 Case 1**

A 35-year-old woman with a history of diabetes, hypertension and depression was found dead by her partner in the bathroom of her home. It has been stated that the woman had experienced symptoms of extreme tiredness since the previous day. The woman was under treatment with metformin (1000 mg), extended-release insulin detemir (100 units/mL), paracetamol (500 mg), atenolol (50 mg), ramipril (10 mg) and mirtazapine (15 mg). External examination and medicolegal autopsy operations revealed an asphyxial syndrome associated to cyanosis of the extremities and multi-visceral congestion. Histological examination revealed pulmonary edema and hepatic steatosis. Since drug poisoning was suspected, samples of femoral and cardiac blood, vitreous humor, gastric content, urine and hair were taken for toxicological tests. The samples were stored at +4° C until analysis. Head hair specimen (23 cm, brown) was stored in an envelope at room temperature.

### **2.2 Case 2**

A 57-year-old man was found dead in his bed. Based on the drugs found close to the corpse, it would appear that the subject was diabetic and depressed. In fact, the subject seemed to have been under treatment with drugs such as insulin lantus, metformin (1000 mg), zopiclone (7.5 mg), Prozac (20 mg) and pregabalin (200 mg). The body examination and the post-mortem body scanner revealed a marked asphyxiation syndrome with nail cyanosis. In order to perform toxicological tests, femoral and cardiac blood, urine and hair samples were collected. Samples of biological fluids were stored at +4 °C until analysis. Head hair specimen (3 cm, dark brown) was stored in an envelope at room temperature.

## **3. MATERIAL AND METHODS**

### **3.1 Chemical and reagents**

Metformin hydrochloride (1,1-dimethylbiguanide hydrochloride) at a concentration of 1 mg/mL in methanol was purchased from LGC (Middlesex, UK). Metformin-d6 hydrochloride (1 mg) was supplied by Toronto Research Chemicals (Toronto, Canada). N-methyl-bis-(trifluoroacetamide) (MBTFA) was purchased from Sigma-Aldrich (St. Louis, MO, USA). Methanol and acetonitrile for HPLC Isocratic Grade were obtained from V.W.R. Chemicals ProLabo (Fontenay-sous-Bois, France).

Metformin-d6 was used as internal standard (IS) at a concentration of 1 mg/L in methanol. For calibration standards, adequate dilutions were made in methanol from the initial solution of metformin at 1mg/mL.

### **3.2 Metformin analysis**

#### **3.2.1 Blood analysis**

For the extraction of metformin from blood samples, a method developed by Uçaktürk in 2013 was used [11]. A sample volume of 0.2 mL of human whole blood was subjected to protein precipitation for the extraction of metformin. The specimen was spiked with 20 µL of 100 mg/L deuterated internal standard (metformin-d6) and 3 mL of acetonitrile. The specimen was vortexed for 1 min, then centrifuged at 3500 rpm for 5 minutes. The supernatant was transferred to another tube and evaporated to dryness under a stream of nitrogen at 50°C. Following dryness, the specimen was derivatized with 40 µL of MBTFA at 80 °C for 60 min and then transferred to injection vials and 1 µL was injected in the GC-MS/MS system.

A 7-point calibration curve was prepared using the following concentrations: 0, 1, 2, 5, 10, 20, 50, 250 mg/L of metformin in blood. Calibration, achieved in blood, was used for the other liquid specimens.

#### **3.2.2 Hair analysis**

Both hair specimens were brown in color. Hair of the first case was oriented root-tip, with a length of 23 cm. The first 6 cm were analyzed after being divided into 3 segments. In acute poisoning deaths, we do not find it interesting to search for a molecule beyond the first 6 cm. Hair of the second one was not oriented, with a length of 3 cm. In both cases, hair sampling was carried out following the guidelines of the SOHT (Society of Hair Testing) [12]. For metformin hair analysis, a method previously developed and validated by the authors was used [13]. Hair specimens were decontaminated twice with 5 mL of dichloromethane for 2

minutes. Once dry, they were cut into small pieces and 20 mg were incubated in the presence of 20 ng of metformin-d6 (20  $\mu$ L of a 1 mg/L solution in methanol) in 1 mL of methanol and placed in an ultrasound bath for 2 h at room temperature. After centrifugation at 3000 rpm for 15 minutes, the supernatant was collected and evaporated to dryness at 50 °C under a nitrogen flow. The residues were derivatized with 40  $\mu$ L of MBTFA at 80°C for 60 min and 2  $\mu$ L was injected into the GC-MS/MS system for analysis.

A 6-point calibration curve was prepared using the following concentrations: 0, 0.1, 1, 2, 5, 10, 50 ng/mg of metformin in hair.

### **3.2.3 Instrument (GC-MS/MS) conditions**

GC separation was performed on an Agilent GC 8890 using an Agilent 30 m x 0.25 mm, 0.25  $\mu$ m HP-5MS UI column for separation. A 1  $\mu$ L split injection was made with a split ratio of 10:1 for blood analysis method and a 2  $\mu$ L splitless injection was made for hair analysis. The flow rate of the carrier gas (helium) was set at 1.2 mL/min. The gradient temperature was as follows: the initial oven temperature was set at 100 °C, and then the temperature was increased to 270° C at a rate of 20° C/min. The total run was 7.5 min. A 7010B triple-quadrupole mass selective detector was used for analysis of the compounds. The tandem mass spectrometer was operated in electron ionization mode (EI) and multiple reaction monitoring mode (MRM) was used for the identification of metformin and metformin-d6. The following conditions were found to be optimal for the analysis of metformin and the IS: electron energy at 70 eV, inlet temperature at 280 °C, ion source at 230 °C and interface temperature at 280 °C. The solvent delay was set at 4 min. Collision energy was adjusted to optimize the signal of the 2 most abundant products ions of the derivatized metformin: m/z 303>288.2, 303>125.1. For the internal standard the following transitions were used: m/z 309.1>291.1 and 309>128. The ions m/z 303>288.2 for metformin and 309>291.1 for IS were selected for quantification. MassHunter 10.0 was used for quantification [13].

## **4. RESULTS AND DISCUSSION**

Metformin was found in femoral blood, cardiac blood, vitreous humour and gastric contents of the subject 1 at the following concentrations: 112.3 mg/L, 226.9 mg/L, 31.1 mg/L, 773.5 mg/L. For subject 2 analyses revealed a femoral blood concentration of 64.7 mg/L, a cardiac

blood concentration of 203.2 mg/L and a urine concentration of 844.9 mg/L. Metformin concentration in hair of the case 1 were: 28.3 ng/mg (root to 2 cm), 39.4 ng/mg (2-4 cm) and 44.8 ng/mg (4-6 cm), while for the case 2 we found a concentration of 22.5 ng/mg in the whole strand. (Table 1) Figure 1 and figure 2 show the chromatograms obtained after extraction of subject's 1 head hair (root to 2 cm) and subject's 2 hair.

Further analyses, achieved during a comprehensive toxicological screening, revealed in femoral blood the presence of ethanol at a concentration of 2.4 g/L in subject 1 (concentration mainly linked to post-mortem bio-transformation) and zopiclone (448 ng/mL), clobazam (324 ng/mL), amitriptyline (1.51 mg/L), pregabalin (81.4 mg/L) and gliclazide ( 1.2 mg/L) in subject 2.

In both cases, an experienced pathologist attributed the cause of death to acute metformin intoxication. These findings are in agreement with those reported in the medical literature. According to a publication presented by Walz et al. [14] in 2019, the average concentration of metformin in fatal intoxications by single metformin is 48.5 mg/L (range 13-210 mg/L) and in multiple intoxications (death caused by metformin and other drugs) is 21 mg/L (4.40-95.0 mg/L). In 2018, Hess et al. [15] have indicated, in a study on 95 diabetic patients under chronic metformin treatment, that therapeutic blood concentrations have an average of 1.846 mg/L. The same authors analysed a case of metformin-related death, where they found a concentration of 119 mg/L in femoral blood and 166 mg/L in cardiac blood [15].

As it was previously noticed by others, higher concentrations of metformin were found in cardiac blood when compared to femoral blood, suggesting a possible post-mortem redistribution. Although the analysis of various biological samples has been extensively treated in the literature, metformin has never been tested in hair collected after death.

Hair test results are now recognized to be very useful in forensic toxicology as it allows to obtain information on drug consumption history. Furthermore, when performing hair segmentation, and using an average growth rate of 1 cm/month, it is possible to establish whether exposure occurred on single or multiple occasions.

Surreptitious drug administration in the context of drug-facilitated crimes has been well documented since the last 20 years, particularly with the introduction of bench-top LC-MS/MS tools. Especially, in the recent years, cases of hypoglycaemia induced deliberately in healthy subjects using antidiabetic drugs have been described. For example, Villain et al. [10] have demonstrated repeated exposure to glibenclamide in a non-diabetic subject. Hair analysis, in this case, was the only retrospective evidence of drug exposure. Given that metformin is the most prescribed antidiabetic in the world and considering its widespread use, it seems



necessary to test it in cases of death or intoxication in which exposure to this drug is suspected. Hence, given the importance of hair analysis for retrospective information, in addition to test metformin in the standard samples, one should promote such investigations.

Since there is no reference in the literature on metformin in hair in cases of subjects deceased from acute metformin overdose, the toxicological significance of the findings remains to be discussed. In a previous study [13] involving 9 diabetic patients under treatment (1000 to 3000 mg per day), hair concentrations ranging from 0.3 to 3.8 ng/mg were measured. Additionally, hair specimens from two cases where metformin was found at therapeutic concentrations in post-mortem blood and who died from a different cause, were analysed and concentrations varied from 0.6 to 1.4 ng/mg. The method used has been fully validated in this previous work [13]. LOD and LOQ were respectively 1 pg/mg and 0.1 ng/mg. Having found concentrations not below 0.3 ng/mg at therapeutic doses, we found no interest in going below 0.1 ng/mg as the lowest concentration of our calibration curve. Nothing has been published on the dosage of metformin in post-mortem hair. Only Binz et al. [16] measured metformin in the hair of subjects under treatment. The authors found concentrations ranging from 10.7 to 100.7 pg/mg in 9 patients treated with daily doses ranging from 780 to 2652 mg/day. As already well discussed in a previous work [13], if we compare our results with those obtained by Binz et al., it must be specified that we rely on self-reported consumption and that an incorrect adherence to therapy cannot be excluded. Furthermore, we have no information about the presence or absence of cosmetic treatments and the incorporation rate of metformin in the keratin matrix is unknown.

Although it cannot be generalized from 2 cases, there is evidence that the hair metformin concentrations are largely higher in case of acute fatal intoxication when compared to therapeutic use. This phenomenon can be explained through 2 hypotheses. First, one can suggest that the two subjects did not follow correctly their treatment but the difference between the 2 sets of concentrations is too high to account for this hypothesis. It must be considered that excessive sweating during the agonal phase due to fatal hypoglycemia can have contaminated the hair. The increase of sweating represents one of the neurogenic symptoms characteristic of hypoglycemia caused by an activation of the autonomic nervous system [17].

Drug incorporation into hair can take place not only through the bloodstream but also through sweat or other sebaceous secretions and sweat contamination is known to be difficult to eliminate by hair decontamination procedures. Molecules and metabolites present in sweat can be incorporated into hair and then become resistant not only to hygiene practices such as

the use of shampoos but also to laboratory decontamination procedures [7]. Cone et al. [18] reported that codeine was found in hair after only 24 hours post administration. The time between the administration of the substance and its appearance in the growing hair was too short to be caused by blood incorporation and suggested sweat implication. Henderson et al. [19] found cocaine in multiple hair segments after a single dose, supporting that sweat or other secretions are pathways for drug deposition in hair.

Sweat excretion depends on many factors, including molecular mass, pKa and hydrophilicity of the compound [20,21]. Since metformin is a highly hydrophilic, basic and small molecule, it is probably well excreted into sweat and this could explain hair contamination following situations of excessive sweating.

When analysing hair results of the first case reported, we can see that metformin concentrations in the hair increase going from root to tip (28.3-44.8 ng/mg). Since a possible contamination from sweat is assumed, a higher concentration is expected in the segment closest to the skin.

However, an important factor that must be considered is the porosity of the shaft. Today, it is accepted that the amount of a drug deposited from external contamination into the hair is depended on the porosity of the hair shaft. Very porous specimen absorbs high amounts of substances. [22]. Moving away from the root to the tip, we find segments that have been more exposed over time to cosmetic treatments (routine shampooing, conditioning, thermal and chemical treatments). These cosmetic procedures can alter hair texture and, especially, disrupt the cuticular sheath resulting in increased porosity of the hair [23].

Analyzing the washing solutions obtained during the decontamination process, could help in explain the presence of high concentrations of metformin in overdose cases.

Unfortunately, the analysis of the washing solutions has proved to be of interest only later. Metformin quantification in hair was done only after washing and extracting the molecule from the hair, and the surprising result we found, allowed the authors to elaborate the hypothesis of sweat contamination, so the interest of the washing solutions was revealed only later. It would also be interesting for future studies, in case of metformin deaths, to analyze the washing solutions to be able or not to prove external contamination by sweat.

Sweat contamination of the hair have already been described for other molecules such as methadone. Methadone tends to cause excessive sweating in patients under treatment and in fact in many cases of exposure to methadone by children whom the drug was found in the hair, a possible contamination through the sweat of the parents has been suggested in some studies [24-26]. As there is no other element of comparison, it is scientifically acceptable, at this

stage, that excessive sweating during the agonal phase of hypoglycaemia is responsible of high metformin concentrations found in the hair after acute fatal overdose.

## 5. CONCLUSION

Oral antidiabetic agents, despite the advantages in reducing morbidity and mortality of diabetic patients, represent a health problem due to their misuse, particularly in some situations of drug-facilitated crimes. In such cases, hair analysis can be very useful and sometimes can be the only evidence of exposure to a particular drug.

In this study the authors presented 2 cases of fatal metformin intoxication in which hair analysis was also performed. Metformin has never been tested in hair of subjects deceased after acute metformin overdose. Based on data collected, the concentrations found in fatal cases are clearly higher than concentrations found in subjects under treatment. Probably, excessive sweating caused by hypoglycemia is responsible for these findings.

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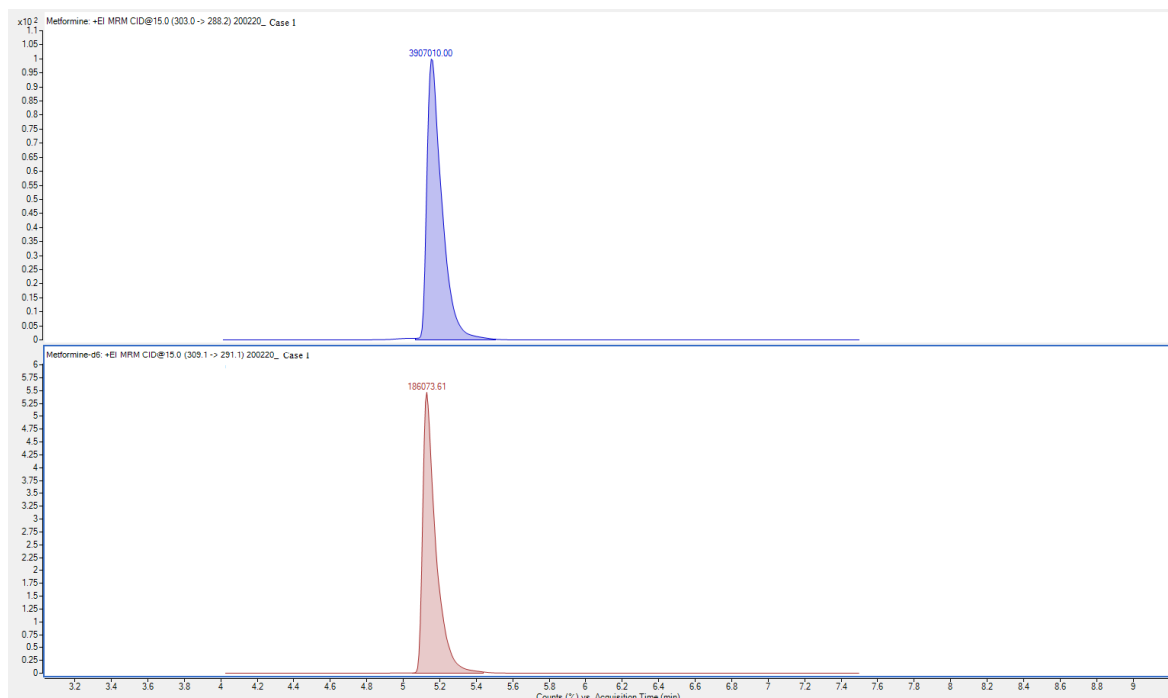
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**Table 1. Results of femoral blood, cardiac blood, vitreous humor, gastric contents, urine and hair analysis in the 2 reported cases**

	<b>Femoral blood (mg/L)</b>	<b>Cardiac blood (mg/L)</b>	<b>Vitreous Humor (mg/L)</b>	<b>Gastric contents (mg/L)</b>	<b>Urine (mg/L)</b>	<b>Head hair (ng/mg)</b>
<b>Case 1</b>	112.3	226.9	31.1	773.5	/	28.3 (root to 2 cm) 39.4 (2-4 cm) 44.8 (4-6 cm)
<b>Case 2</b>	64.7	203.2	/	/	884.9	22.5

**Figure 1. Chromatogram of Subject's 1 hair, 0-2 cm (from top to the bottom: 1 transition of metformin and 1 transition for IS metformin-d6). Concentration was 28.3 ng/mg**



**Figure 2. Chromatogram of Subject's 2 hair (from top to the bottom: 1 transition of metformin and 1 transition for IS metformin-d6). Concentration was 22.5 ng/mg**

