# A Fatal Overdose of Cocaine Associated with Coingestion of Marijuana, Buprenorphine, and Fluoxetine. Body Fluid and Tissue Distribution of Cocaine and Its Metabolites Determined by Hydrophilic Interaction Chromatography–Mass Spectrometry (HILIC–MS)

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## Abstract

Chromatographic separation of highly polar basic drugs with ideal ionspray mass spectrometry volatile mobile phases is a difficult challenge. A new quantification procedure was developed using hydrophilic interaction chromatography–mass spectrometry with turbo-ionspray ionization in the positive mode. After addition of deuterated internal standards and simple clean-up liquid extraction, the dried extracts were reconstituted in 500 µL pure acetonitrile and 5 µL was directly injected onto a Waters Atlantis™ HILIC 150- × 2.1-mm, 3-µm column. Chromatographic separations of cocaine, seven metabolites, and anhydroecgonine were obtained by linear gradient-elution with decreasing high concentrations of acetonitrile (80–56% in 18 min). This high proportion of organic solvent makes it easier to be coupled with MS. The eluent was buffered with 2mM ammonium acetate at pH 4.5. Except for *m*-hydroxy-benzoylecgonine, the within-day and between-day precisions at 20, 100, and 500 ng/mL were below 7 and 19.1%, respectively. Accuracy was also below ± 13.5% at all tested concentrations. The limit of quantification was 5 ng/mL (%Diff < 16.1, %RSD < 4.3) and the limit of detection below 0.5 ng/mL. This method was successfully applied to a fatal overdose. In Switzerland, cocaine abuse has dramatically increased in the last few years. A 45-year-old man, a known HIV-positive drug user, was found dead at home. According to relatives, cocaine was self-injected about 10 times during the evening before death. A low amount of cocaine (0.45 mg) was detected in the bloody fluid taken from a syringe discovered near the corpse. Besides injection marks, no significant lesions were detected during the forensic autopsy. Toxicological investigations showed high cocaine concentrations in all body fluids and tissues. The peripheral blood concentrations of cocaine, benzoylecgonine, and methylecgonine were 5.0, 10.4, and 4.1 mg/L, respectively. The brain concentrations of cocaine, benzoylecgonine, and methylecgonine were 21.2, 3.8, and 3.3 mg/kg, respectively. The highest concentrations of norcocaine (about 1 mg/L) were measured in bile and urine. Very high levels of cocaine were determined in hair (160 ng/mg), indicating chronic cocaine use. A low concentration of anhydroecgonine methylester was also found in urine (0.65 mg/L) suggesting recent cocaine inhalation. Therapeutic blood concentrations of fluoxetine (0.15 mg/L) and buprenorphine (0.1 µg/L) were also discovered. A relatively high concentration of $\Delta^9$-THC was measured both in peripheral blood (8.2 µg/L) and brain cortex (13.5 µg/kg), suggesting that the victim was under the influence of cannabis at the time of death. In addition, fluoxetine might have enhanced the toxic effects of cocaine because of its weak pro-arrhythmogenic properties. Likewise, combination of cannabinoids and cocaine might have increase detrimental cardiovascular effects. Altogether, these results indicate a lethal cocaine overdose with a minor contribution of fluoxetine and cannabinoids.

## Introduction

According to the Swiss Federal Office of Public Health, 10% of the population between 15 and 39 had used illicit drugs at least once in 1997. Although most of these cases involved cannabis, about 30,000 people had a prevalence for cocaine [http://www.bag.admin.ch/e/]. In the same year, 7% of men between 25 and 39 admitted taking cocaine at least once during

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their lifetimes, up from 3.9% in 1992. Furthermore, there were approximately 250 drug-related deaths, with an additional 150 drug users dying of AIDS (1). Forensic investigations carried out at our institute show similar patterns in the French part of Switzerland. There was a threefold increase in the number of cocaine-related deaths in the last three years. These statistics are concordant with a price drop and an increase in accessibility, showing that cocaine has been becoming a popular recreational drug among teenagers and young adults. The routes of administration have also changed with more opiate addicts under methadone substitution therapy injecting or inhaling cocaine (2–5). The acute effects of cocaine are an intense euphoria, a heightened energy level, enhanced alertness, and self confidence. This euphoric phase is followed by depression and craving for the drug (6,7). Cocaine has two well-defined pharmacological effects: it is a local anesthetic and a monoamine blocker that interferes with catecholaminergic and serotonergic neurons. Serotonin is believed to be important in the euphoric effects of cocaine because administration of fluoxetine, a serotonin reuptake blocker, attenuates the positive reinforcing action of cocaine in rats (8). At high concentrations, cocaine also acts on cholinergic receptors, contributing to the hemodynamic responses and cardiovascular toxicity (9). Vasospasm and thrombus formation, rupture of cerebral aneurysms and intracranial hemorrhage, cardiac ischemia, stroke, seizures, and sudden death are the consequences of this toxicity (7,10,11). Intravenous intake or inhalation of cocaine results in a rapid onset because of fast increases in plasma levels often exceeding 1–2 mg/L (12). Symptoms of toxicity generally occur with some delay after peak concentrations of cocaine in the blood. However, the peak plasma values vary considerably between individuals. In addition, only poor dose-response relationship to toxicity exists, complicating the interpretation of blood levels (6). The elimination half-life of cocaine in plasma is in the range of 40 to 90 min in humans (13,14). While a small proportion of cocaine is excreted unchanged into urine, the majority is hydrolyzed by plasma and liver carboxylesterases, yielding benzoylecgonine (BE) and ecgonine methylester (EME). Spontaneous chemical hydrolysis of cocaine into benzoylecgonine may also occur (15,16). Pyrolysis of cocaine during smoking results in the formation of methylecgonidine or anhydroecgonine methylester (AEME) (17). Figure 1 depicts the main cocaine pyrolysis and metabolic pathways. Liver P-450 enzymes *N*-demethylate a small amount of cocaine to produce norcocaine (NCOC) which is hepatotoxic (18). If cocaine is used in conjunction with ethanol, a transesterification reaction occurs forming cocaethylene or ethylcocaine (CE) (19,20). CE is further metabolized into ecgonine ethylester (EEE) and BE. Aromatic hydroxylation of cocaine may also occur resulting in the formation of *meta-* (*m*-OH-COC) and *para-* substituted hydroxy-cocaine and hydroxy-benzoylecgonine (*m*-OH-BE). The latter has been shown to be present in significant concentrations in the meconium of cocaine-exposed babies (21).

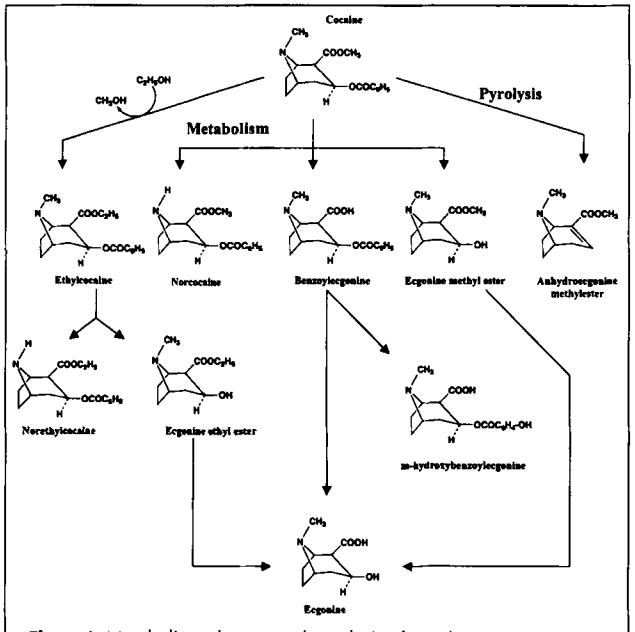


**Figure 1.** Metabolic pathways and pyrolysis of cocaine.

Gas chromatography–mass spectrometry (GC–MS) operating in the SIM mode is the method most frequently used for the analysis of cocaine and metabolites in biological samples (6). To achieve acceptable sensitivity, metabolites of cocaine must be derivatized prior to analysis. An alternative method to GC–MS is liquid chromatography–mass spectrometry (LC–MS) with the advantages of no required derivatization step and negligible thermal degradation during injection and artifactual formation of AEME (22). The method has been successfully applied to urine samples after solid-phase extraction (23). Chromatography is generally carried out on ODS reversed-phase columns interfaced with either ES or APCI sources (24). Flow-injection ionspray-MS–MS is also possible (25).

We present a new quantification procedure using hydrophilic-interaction chromatography–mass spectrometry (HILIC–MS) with turbo ionspray ionization in the positive mode. A silica-packed Atlantis HILIC column (Waters, Rupperswil, Switzerland) is well suited for LC–MS analysis because separation of cocaine and its metabolites is obtained with high concentrations of acetonitrile (26,27). The method was successfully applied to a death case after multiple cocaine injections. The cocaine and metabolite distribution was determined in several biological fluids and tissues.

## Methods for Cocaine Analysis

### Materials and solvents

Cocaine standards, metabolites as well as their deuterated homologues were purchased from Lipomed (Lipomed AG, Arlesheim, Switzerland) and CIL (Cambridge Isotope Laboratories, Innerberg, Switzerland). A 100 µg/10 mL stock solution of deuterated compounds [cocaine-(*N*-methyl-$d_3$), benzoylecgonine-(*N*-methyl-$d_3$), ecgonine methylester-(*N*-methyl-$d_3$), anhydroecgonine methylester-(*N*-methyl-$d_3$), and norcocaine-($d_3$-methylester)] in acetonitrile was prepared and stored at –20°C. Four working solutions were prepared in acetonitrile. The first two contained cocaine, benzoylecgonine, ecgonine methylester, and cocaethylene in 10 and 1 µg/mL, and the

two others contained anhydroecgonine methylester, *m*-hydroxybenzoylecgonine, norcocaine, norcocaethylene, and ecgonine ethylester in the same concentrations. All standard solutions were prepared and used with glass Hamilton syringes (Hamilton, Bonaduz, Switzerland). Acetonitrile (HPLC grade, quality gradient) was obtained from Riedel-de Haën (Fluka, Buchs, Switzerland). Ammonium acetate and acetic acid (Biochemika grade), potassium chloride, chloroform, and isopropanol (puriss grade) were purchased from Fluka. Boric acid and potassium carbonate were purchased from VWR Merck Eurolab (Dietikon, Switzerland). Deionized water was purified by a Milli-Q system (Millipore). The borate buffer was prepared by mixing 700 mL of a solution containing 61.8 g boric acid and 74.6 g potassium chloride in 1 L of distilled water and 300 mL of another solution containing 106 g potassium carbonate to reach a pH of 8.4. The borate buffer and the extraction solution of chloroform/isopropanol (3:1, v/v) were stored at room temperature.

### Biological specimens

The blank blood samples were obtained from the local hospital blood bank. Biological fluids and tissues specimens from the victim were taken during the autopsy. Blood samples were collected in 5.5 mL S-Monovettes containing 1 mg fluoride/mL and 1.2 mg EDTA/mL blood as preservatives (Sarstedt, Sevelen, Switzerland). Tissues were manually homogenized with a Teflon pestle and a Potter-Elvehjem glass homogenizer in 5 mL 0.9% saline.

### Instrumentation

The LC–MS system consisted of two high-pressure Perkin Elmer series 200 micro pumps, a series 200 autosampler, and a Lee-Visco-Jet micro-Mixer with 10 µL internal volume connected to an Applied Biosystems MDS Sciex API 150EX single-quadrupole system (Applera Europe, Rotkreuz, Switzerland). For atmospheric pressure ionization, a turbo-ionspray interface was used. The modules were controlled by a Macintosh computer running OS 8.1, and data collection was performed using MassChrom 1.4 software. Quantitative results were processed with TurboQuan 1.0 software. The calibration curves were obtained by weighted ($1/x$) least-squares linear through zero or quadratic regression analysis.

### Extraction procedure

The internal standards (10 µg/mL acetonitrile) and aliquots of both working solutions of standards for the calibration curve (10 or 1 µg/mL acetonitrile) were added to 10 mL Pyrex SVL tubes (GlasKeller, Basel, Switzerland) and evaporated under $N_2$ at 37°C. Then 1 mL blank blood, biological fluid, or tissue homogenate; 2 mL borate buffer (pH 8.4); and 3 mL of chloroform/isopropanol (3:1, v/v) (added with a digital dispenser Calibrex 520, Socorex, ReactoLab, Servion, Switzerland) were vortex mixed for 1 min and extracted for 30 min on a horizontal shaker at 180 motions/min (Edmund Bühler, GlasKeller, Basel). After centrifugation (30 min, 3000 rpm), the lower organic phase was collected and evaporated under $N_2$, and the dried extract was reconstituted into 500 µL of acetonitrile prior to LC–MS analysis. Biological samples were appropriately diluted (generally 2 or 10 times) in order to yield results inside the quantification range.

### Chromatography

A Waters Atlantis HILIC silica column (150 × 2.1-mm i.d., 3-µm particle size) and guard columns (10 × 2.1 mm, 3 µm) packed with the same material were purchased from Waters. Cocaine derivatives were separated at room temperature (23°C) by gradient elution of an acetonitrile/2mM ammonium acetate buffer eluent at a flow-rate of 250 µL/min. The pH of the eluent was adjusted to 4.5 with acetic acid. Before injection, the column was prewashed for 10 min with the starting eluent. After a 5-µL injection, an isocratic elution was performed with 80% acetonitrile for 1 min. From then on, a gradient elution of acetonitrile decreased linearly to 56% in 18 min. This latter concentration was maintained for 1 min. Subsequently, the system was returned to its initial conditions in 1 min and equilibrated for 10 min before injection of the next sample.

### MS conditions

The MS was operated in the positive mode. Nitrogen was used as a nebulizing, heating, and curtain gas. The gas flow rates were set to about 0.9 L/min (nebulizer gas setting = 9), 7 L/min, and 0.9 L/min (curtain gas setting = 9) respectively. The Turbo probe temperature was 475°C and the Ionspray voltage was set at 3600 V. The voltages of the orifice and of the

**Table I. LC–MS Parameters for Cocaine, Cocaine Metabolites, and Anhydroecgonine Methylester**

| Analyte | Retention Time (min) | Retention Time (%RSD, $n = 12$) | Selected Ion ($m/z$) | Internal Standard (IS) | IS Selected Ion ($m/z$) | Fit $1/x$ Weighted | LOD (ng/mL) |
|---|---|---|---|---|---|---|---|
| Cocaine | 10.21 | 0.84 | 304.1 | Cocaine-$d_3$ | 185.2 | linear through 0 | 0.1 |
| Benzoylecgonine | 5.49 | 1.89 | 290.3 | Benzoylecgonine-$d_3$ | 293.22 | linear through 0 | 0.3 |
| Ecgonine methylester | 16.87 | 0.44 | 200.2 | Ecgonine methylester-$d_3$ | 203.2 | linear through 0 | 0.5 |
| Cocaethylene | 9.89 | 0.78 | 318.2 | Cocaine-$d_3$ | 185.2 | linear through 0 | 0.1 |
| *m*-Hydroxybenzoylecgonine | 4.68 | 1.21 | 306.1 | Benzoylecgonine-$d_3$ | 293.22 | quadratic | 1.5 |
| Ecgonine ethylester | 15.22 | 0.48 | 214.1 | Ecgonine methylester-$d_3$ | 203.2 | quadratic | 0.3 |
| Anhydroecgonine methylester | 13.82 | 0.64 | 182.1 | Anhydroecgonine methylester-$d_3$ | 185.2 | quadratic | 0.2 |
| Norcocaine | 9.32 | 0.90 | 290.1 | Norcocaine-$d_3$ | 293.22 | quadratic | 0.3 |
| Norcocaethylene | 9.10 | 0.94 | 304.1 | Norcocaine-$d_3$ | 293.22 | quadratic | 0.1 |

focusing ring were optimized by flow injection at a flow-rate of 250 µL/min and found to be similar for all cocaine derivatives, that is, 10 and 130 V for the orifice and ring, respectively. The composition of the eluent was 70% acetonitrile and 30% ammonium acetate buffer. Twelve ions corresponding to the protonated molecules or ion fragments, monitored in the SIM mode for quantification are listed in Table I along with the retention time of the corresponding substance. The dwell-time was set at 200 ms. Calibration of the mass analyzer was performed by infusion of a PPG (polypropylene glycol) standard mixture using a Harvard syringe pump at a flow-rate of 0.6 mL/h.

### Validation

The validation procedure was adapted from (28). The evaluation of linearity was carried out with blood. Blank matrix was spiked with the internal standards mix (500 ng/mL) and with cocaine derivatives at nine different levels, yielding calibration points at 2, 5, 10, 20, 50, 100, 200, 500, and 1000 ng/mL. The recoveries were determined by spiking a blood-blank matrix with 500 ng/mL before and after sample preparation ($n$ = 5). Matrix suppression was studied by extracting a blank matrix and spiking the extract with both cocaine derivatives and the internal standards at concentrations of 20, 100, and 500 ng/mL into the organic layer ($n$ = 5) (29). These samples were compared to reference samples that were prepared by spiking the same substances into the organic layer. The ion suppression effect was then assessed by calculating the area ratio between the peak areas obtained in the presence and in the absence of co-extracted potential interferences. In order to determine the limit of detection (LOD: signal-to-noise ratio > 3), samples containing low concentrations of cocaine derivatives (down to 0.1 ng/mL) were prepared and analyzed between two blank probes. The limit of quantification was set at 5 ng/mL, accuracy and precision values (< |20%|) were checked by carrying out five determinations. The selectivity of the method was studied by including one blank sample containing no internal standard and one specimen spiked with the deutarated internal standards mix only in each batch. Furthermore, quality control samples (Medidrug BTMF 1/03-A S-plus or BTMF 2/01-A S-plus) known to contain cocaine, ecgonine methylester, and benzoylecgonine were monitored and checked for the absence of other derivatives and acceptable quantitative levels.

Because of the high temperature of the turbo-ionspray (475°C), the conversion of cocaine into AEME during LC–MS analysis was assessed by injecting high concentrations of cocaine (500 and 100 ng/mL) and measuring AEME concentration down to 0.1 ng/mL.

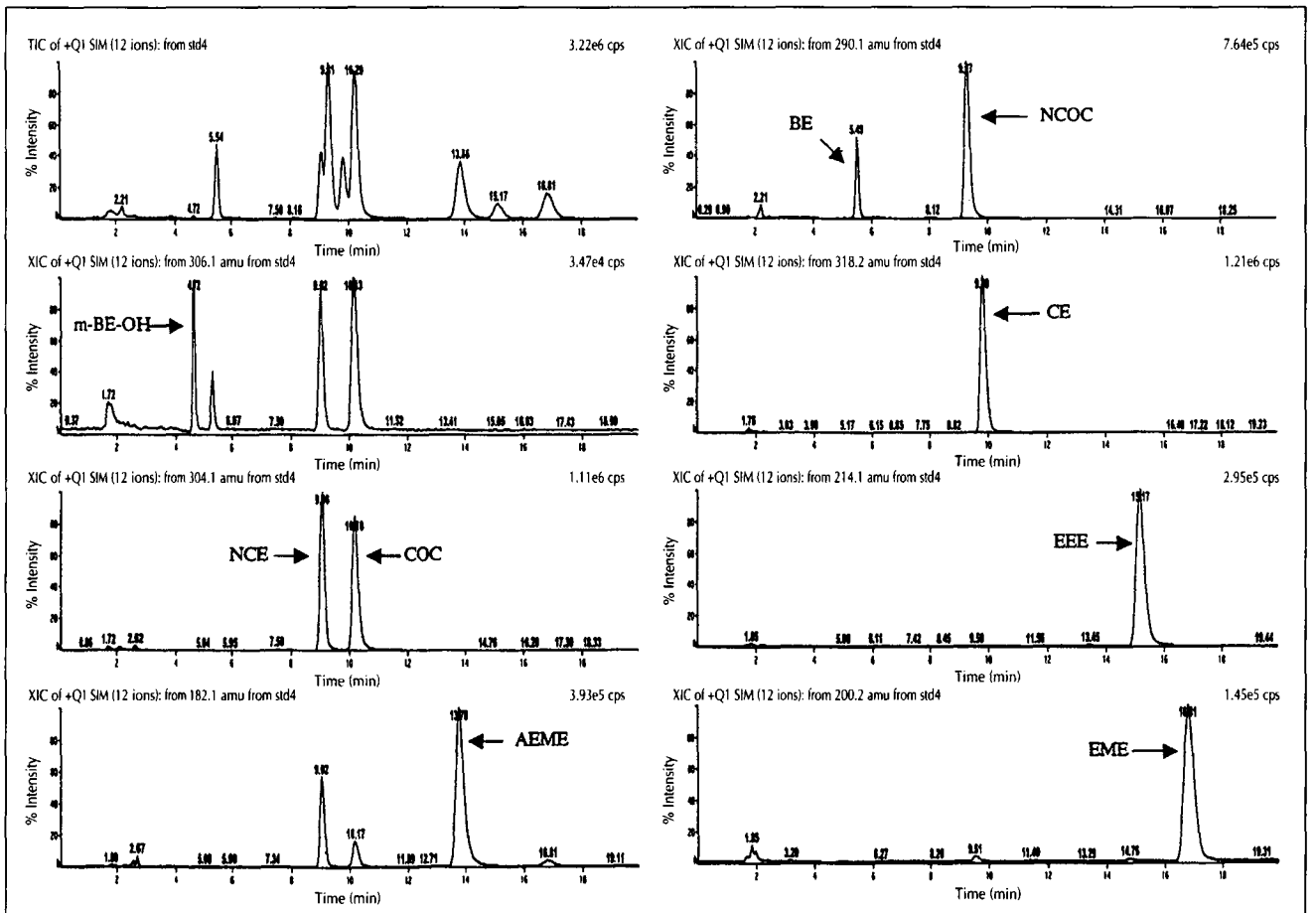


**Figure 2.** Total ion (TIC) and extracted ion (XIC) chromatograms of an extract of blood spiked with 50 ng/mL of cocaine derivatives. Left: *m*-BE-OH = *meta*-hydroxy-benzoylecgonine, NCE = norcocaethylene, COC = cocaine, AEME = anhydroecgonine methylester; right: BE = benzoylecgonine, NCOC = norcocaine, CE = cocaethylene, EEE = ecgonine ethylester, and EME = ecgonine methylester.

## Additional Methods for Case Report Investigations

### Screening methods

A comprehensive drug screening was carried out according to known procedures (30). Urine was screened by immunoassays and GC–MS methods. Basic extracts as well as hydrolyzed and acetylated basic extracts were analyzed. Volatile compounds were screened by headspace GC–flame-ionization detection (FID). Blood analysis was performed by high-performance liquid chromatography–diode-array detection (HPLC–DAD), and GC–MS.

### Hair analysis

The method described by Girod and Staub (31) was used with some modifications: the whole hair sample (6 cm-long) was washed twice with 10 mL dichloromethane, dried in a folded filter paper, and cut in pieces of 1–3 mm. Twenty milligrams of hair fragments was added to 1 mL of methanol, 1 mL of phosphate buffer (0.1M, pH 6.0), and 50 µL of a deuterated standard solution (1 µg/mL). After overnight incubation at 50°C, the methanol/buffer extraction solution was diluted with 5 mL of phosphate buffer (pH 6.0). It was then transferred to a preconditioned mixed-phase extraction column (ChromaBond Drug, 200 mg, 3 mL) (Macherey-Nagel, Oensingen, Switzerland). The column was washed with 2 mL 1.0M acetic acid and 3 mL methanol. After 5 min air-drying, the drugs were eluted with 3 mL of a dichloromethane/2-propanol/ammonium hydroxide (80:20:2, v/v/v) mixture. After evaporation under $N_2$, the dried residues were derivatized for 30 min at 80°C with trifluoroacetic anhydride, hexafluoro-2-propanol and ethyl acetate. One microliter was injected into the GC–MS system operating in the SIM mode. The following ions were monitored: *m/z* 182, 272, 303 (cocaine); *m/z* 318, 334, 439 (benzoylecgonine); and *m/z* 182, 264, 295 (ecgonine methyl ester), and the ions for the $d_3$ homologues were *m/z* 185, 275, 306; *m/z* 321, 337, 442; and *m/z* 185, 267, 298, respectively.

### Buprenorphine analysis

Buprenorphine and norbuprenorphine in peripheral blood and urine were determined by turbo-ionspray LC–MS operating in the positive mode according to a procedure adapted from Tracqui et al. (32). Buprenorphine-$d_4$ and norbuprenorphine-$d_3$ (CIL, Innerberg, Switzerland) were used as internal standards at a concentration of 10 and 20 ng/mL, respectively. Urine was hydrolyzed with *E. coli* β-glucuronidase prior to purification through three successive base-acid-base extraction steps. A calibration curve was obtained with 7 standard concentrations between 0.1 and 10.0 ng/mL. The dried extracts were reconstituted in 100 µL of the mobile phase made of 20% acetonitrile and 80% ammonium formate (1mM, pH 3.8). HPLC separations were performed on a Waters XTerra MS C8 3.5-µm, 150- × 2.1-mm column fitted with a XTerra MS C8 10- × 2.1-mm precolumn. Isocratic elution at a flow rate of 250 µL/min was kept for the first 5 min following a 10-µL injection. The acetonitrile was then increased up to 80% in 2 steps: from 20 to 60% in 10 min and then from 60 to 80% in 5 min. The following ions were monitored: *m/z* 468.3 (buprenorphine), 472.22 (buprenorphine-$d_4$), 414.25 (norbuprenorphine), and 417.25 (norbuprenorphine-$d_3$). The nebulizer, curtain gas, and ionspray voltage were set at 12, 10, and 3500 V, respectively. The turbo-ionspray temperature was 480°C. The orifice and ring voltages for buprenorphine were of 66 and 260 V; those for norbuprenorphine were 56 and 230 V, respectively. Ten microliters was then injected onto the column.

### Fluoxetine analysis

Fluoxetine and norfluoxetine were analyzed according to a procedure adapted from Crifasi et al. (33). Blank blood samples were fortified with fluoxetine and norfluoxetine (Sigma-RBI) at a concentration of 0, 0.05, 0.1, 0.2, 0.5, 1.0, and 5.0 µg/mL. After addition of the internal standard paroxetine (5.0 µg/mL) and of a saturated ammonium buffer (pH 9.5), the antidepressants were extracted into a chloroform/isopropanol (9:1, v/v) solution. After solvent evaporation under $N_2$, they were acetylated and analyzed by GC–MS operating in the SIM mode. The following ions were monitored for acetylated fluoxetine and acetylated norfluoxetine, respectively: *m/z* 86, 117, 190 and *m/z* 72, 117, 176.

### Cannabinoids analysis

The method of quantification of $\Delta^9$-tetrahydrocannabinol (THC), 11-hydroxy-$\Delta^9$-tetrahydrocannabinol (11-OH-THC) and 11-nor-$\Delta^9$-tetrahydrocannabinol carboxylic acid (THCCOOH) in blood by GC–MS after solid-phase extraction has been described elsewhere (34). Total THCCOOH was determined by GC–MS in urine after basic hydrolysis, extraction on SPEC column (Ansys purchased from Varian, Stehelin AG) and silylation with MSTFA (Macherey-Nagel, Oensingen, Switzerland) according to a procedure adapted from Wu et al. (35). THCCOOH-$d_9$ (CIL, Innerberg, Switzerland) was used as internal standard.

### Case history

A 45-year-old man, a known HIV-positive drug user, was found dead at home. According to relatives, the previous day, he injected himself with 10 cocaine doses. Cardiopulmonary reanimation was attempted without success. Several syringes were found nearby, one containing about half a milliliter of a bloody fluid. No evidence of violence was found. Forensic autopsy was carried out the following day. Biological specimens were taken for toxicological investigations.

## Results and Discussion

### Validation of HILIC–MS procedure for cocaine determination

A chromatographic profile of total (TIC) and extracted (XIC) cocaine ions, its main metabolites, and anhydroecgonine methylester at a concentration of 50 ng/mL of blood are shown in Figure 2. All XIC peaks are symmetric and well resolved. Very clean XIC chromatograms were obtained without interfering molecules. The elution times and their variation (%RSD) are indicated in Table I. Only cocaine, CE, and their *N*-demethyl-

ated metabolites eluted as a single cluster of partially overlapping peaks which could be resolved when specific ions were monitored (Figure 2). Matrix ion suppression was found to be less than 15% ($n$ = 5) for all molecules at the three tested concentrations (20, 100, and 500 ng/mL blood). Thermoconversion of cocaine into AEME during the LC–MS analysis did not occur because no trace of AEME could be detected down to a concentration of 0.1 ng/mL. The extraction scheme corresponds to a procedure which has been used for routine analysis of cocaine and metabolites by GC–MS in our laboratory for many years. The extraction yields were assessed at 500 ng/mL. They were excellent for cocaine and cocaethylene (83%); satisfactory for norcocaine, norcocaethylene, and benzoylecgonine (about 50%); and poor for the other molecules (< 15%). The limit of detection (LOD) was estimated by injecting decreasing concentrations of cocaine and derivatives down to a signal-to-noise ratio of 3. Except for $m$-hydroxybenzoylecgonine (LOD = 1.5 ng/mL), the LODs of all cocaine derivates were between 0.1 and 0.5 ng/mL of blood, indicating a good sensitivity despite a poor extraction yield (Table I). With the exception of $m$-BE-OH, a very good limit of quantitation (LOQ) of 5 ng/mL was achieved for all molecules with an accuracy and %RSD precision lower than 16.1. As shown in Table I, an acceptable curve fitting in the 2–1000 ng/mL range for COC, BE, EME, NCOC, and AEME was achieved with a $1/x$ weighted linear through zero regression ($R$ > 0.998). The other cocaine derivatives for which no corresponding deuterated internal standard were used were better fitted with $1/x$ weighted quadratic regression ($R$ > 0.997). Method accuracy ranged between –14.6% to +16.1% of the target value. The within day (WD) and between-day precisions (BD) were determined at 20, 100, and 500 ng/mL of blood ($n$ = 5) and found to be satisfactory and less than 15.3 and 19.1%, respectively. The main statistical parameters of the validation procedure are listed in Table II. For the limit of quantification (LOQ), the second lowest point of the calibration curve (i.e., 5 ng/mL) was adopted. At this concentration, the accuracy expressed as the percentage difference to the target value was less than ± 16.1 for all cocaine derivatives. The precision remained lower than RSD% = 4.3, with the exception of $m$-OH-BE which was 25.5 for 5 measurements.

**Table II. Accuracy (% Error) and Within-Day and Between-Day Precision (% RSD) at Three Concentrations (20, 100, and 500 ng/mL) and Accuracy and Within-Day Precision at LOQ (5 ng/mL)**

| Drug | Concentration (ng/mL) | Accuracy (±% Diff, $n$ = 5) | WD Precision (%RSD, $n$ = 5) | BD Precision (%RSD, $n$ = 25) |
|---|---|---|---|---|
| Cocaine | 5 | 0.6 | 3.7 | – |
| | 20 | -9.7 | 1.6 | 10.4 |
| | 100 | -1.4 | 1.9 | 7.3 |
| | 500 | -2.4 | 2.6 | 7.4 |
| Benzoylecgonine | 5 | 13.8 | 1.2 | – |
| | 20 | 5.9 | 1.7 | 14.5 |
| | 100 | 1.1 | 1.4 | 3.8 |
| | 500 | 2.8 | 2.3 | 2.5 |
| Ecgonine methylester | 5 | 7.9 | 4.3 | – |
| | 20 | 0.5 | 3.2 | 3.4 |
| | 100 | 0.3 | 1.1 | 3.6 |
| | 500 | 2.5 | 0.6 | 2.9 |
| Cocaethylene | 5 | 16.1 | 3.4 | – |
| | 20 | 13.5 | 2.7 | 18.2 |
| | 100 | -0.8 | 7.0 | 18.8 |
| | 500 | -12.9 | 3.5 | 15.0 |
| $m$-Hydroxybenzoylecgonine | 5 | -14.6 | 25.5 | – |
| | 20 | -7.3 | 15.3 | 12.0 |
| | 100 | 1.5 | 9.0 | 15.2 |
| | 500 | -5.4 | 4.9 | 12.2 |
| Ecgonine ethylester | 5 | 6.5 | 1.0 | – |
| | 20 | 7.2 | 3.1 | 7.3 |
| | 100 | 7.1 | 6.7 | 8.0 |
| | 500 | -2.1 | 1.1 | 3.9 |
| Anhydroecgonine methylester | 5 | -2.2 | 2.9 | – |
| | 20 | -2.4 | 2.0 | 12.1 |
| | 100 | 6.8 | 2.6 | 3.5 |
| | 500 | 2.3 | 2.4 | 3.4 |
| Norcocaine | 5 | 0.4 | 2.4 | – |
| | 20 | 0.0 | 1.1 | 3.5 |
| | 100 | 4.4 | 2.4 | 2.0 |
| | 500 | 3.3 | 1.5 | 3.8 |
| Norcocaethylene | 5 | 8.2 | 2.8 | – |
| | 20 | 9.0 | 2.2 | 19.1 |
| | 100 | -2.3 | 2.8 | 7.4 |
| | 500 | 1.6 | 2.1 | 4.3 |

In contrast to reversed-phase analysis, for which a very high proportion of water is often required to get enough retention of polar metabolites (e.g., EME), HILIC of a broad family of xenobiotic derivatives with a broad range of polarity indexes can be carried out with a relatively low amount of the water eluent. The advantages of Atlantis HILIC silica are several: organic eluents immiscible with water (e.g., hexane) are not needed, acetonitrile constitutes a weak solvent and can be injected directly onto the column. Furthermore, mobile phases containing highly organic solutions (in this case acetonitrile) lead to favorable spraying conditions at the LC–MS interface necessary for adequate sensitivity (26,27,36).

## Case results

### *Paraphernalia*

The analysis of the bloody fluid found in the syringe by LC–MS revealed the presence of cocaine at a concentration of 906 mg/L corresponding to a total amount of about 0.45 mg cocaine in 0.5 mL (Table III).

### *Forensic autopsy*

Multiple old and recent injection traces were discovered. The deceased weighed 57 kg and was 171 cm tall. The heart weight was 330 g. Its macroscopic examination revealed a discrete arteriosclerosis of the coronary arteries

only. The other organs were macroscopically normal.

The histological examination detected a small fibrous patch in the lateral wall of the left ventricle. The microscopic examination of the other organs showed a discrete thickening of the small renal arteries associated with a chronic inflammatory infiltration. No lesions could be detected in the brain and in the liver. The presence of multiple injection wounds, the detection of a relatively high cocaine concentration in the syringe discovered near the corpse, and the story of the relatives confirms the injection route. Drug addicts under substitution therapy are known to inject cocaine up to 30–40 times per day. This compulsive use of cocaine has grown to reach epidemic proportion in Switzerland, challenging drug rehabilitation treatment centers.

*Toxicological results*

*Screening results.* A comprehensive drug screening of blood and urine was carried out by immunoassays, headspace GC–FID, HPLC–DAD, and GC–MS. Immunoassays were positive for cocaine, cannabinoids, and buprenorphine. Neither ethanol nor other volatile compounds could be detected in whole blood by headspace GC–FID. The chromatographic analyses performed by GC–MS operating in the scan mode and by HPLC–DAD and LC–MS revealed the presence of a large array of xenobiotics which are listed in Table IV. A few compounds (cannabinoids, buprenorphine, and *m*-OH-BE) which could be detected by dedicated quantitative methods only are also included in this table. Cocaine and its two main metabolites and degradation products, benzoylecgonine and ecgonine methylester (15,16), were detected in all biological specimens submitted to toxicological investigations (Table V). Other metabolites, AEME, *m*-OH-BE, and NCOC were also detected (Table VI).

**Table III. Concentrations and Total Amounts of Cocaine and Cocaine Metabolites or Cocaine degradation Products Determined by HILIC LC–MS**

| Syringe | Cocaine | Benzoylecgonine | Ecgonine Methylester |
|---|---|---|---|
| Total amount (mg) | 0.45 | 0.12 | 0.04 |
| Concentration (mg/L) | 906 | 230 | 81 |

**Table IV. Qualitative Results of Toxicological Investigations Obtained by Chromatographic Methods**

| Specimen | Xenobiotics | |
|---|---|---|
| Blood | Cocaine | Fluoxetine |
| | Benzoylecgonine | Norfluoxetine |
| | Methylecgonine | THC |
| | Anhydroecgonine methylester | 11-OH-THC |
| | Norcocaine | THCCOOH |
| | Hydroxy-benzoylecgonine | Acetaminophen |
| | Buprenorphine | Phenacetin |
| | Norbuprenorphine | Cotinine |
| | | Caffeine |
| Urine | Cocaine | Fluoxetine |
| | Benzoylecgonine | Norfluoxetine |
| | Methylecgonine | THCCOOH |
| | Anhydroecgonine methylester | Acetaminophen |
| | Norcocaine | Phenacetin |
| | Hydroxy-benzoylecgonine | Trimethoprim |
| | Cinnamoylcocaine | Lidocaine metabolites |
| | Buprenorphine | Cotinine |
| | Norbuprenorphine | Theobromine |
| | | Caffeine |

*Cocaine.* When cocaine is smoked in a glass pipe as a free base, it is thermally degraded to benzoic acid, AEME and other degradation products (17). In the present case, according to relatives, cocaine was administered by injection. This route of administration means that no AEME should be found. Nevertheless, AEME was detected! Several hypotheses can be formed to explain its presence. First, it could result from previous cocaine inhalation because free-base smoking is also common among drug addicts. We can also assume that AEME was the consequence of thermal degradation of cocaine during volatilization in the injection port of the GC (37). In this second case, AEME should be considered as an artifactual contaminant. This hypothesis could be ruled out because AEME was also detected by LC–MS. Finally, AEME could already be present in the powder as a manufacturing byproduct. AEME as well as norcocaine and hydroxy-cocaine have been indeed detected in refined illicit cocaine (38). Because no cocaine sample was found near the body, this latter hypothesis could not be tested. The analysis of the syringe revealed the presence of a low amount of AEME that could originate from the powder or from the blood. The presence of AEME must therefore be interpreted with caution. In our opinion, inhalation of free-base cocaine followed by several injections is the most likely scenario.

Norcocaine has been found in all screened biological specimens. NCOC is a *N*-desmethyl cocaine metabolite produced by liver cytochrome P-450 with hepatotoxic properties (18,39). Reports of hepatotoxicity attributed to cocaine use are rare (6) because NCOC is a minor metabolite (Table VI). In the present

**Table V. Body Fluid and Tissue Distribution of Cocaine, Benzoylecgonine, and Ecgonine Methylester Determined by HILIC–MS**

| Specimen | Cocaine | Benzoylecgonine | Ecgonine Methylester |
|---|---|---|---|
| Peripheral blood | 5.0 mg/L | 10.4 mg/L | 4.1 mg/L |
| Cardiac blood | 9.0 mg/L | 20.1 mg/L | 14.4 mg/L |
| Pericardic liquid | 9.9 mg/L | 12.0 mg/L | 8.4 mg/L |
| CSF | 4.1 mg/L | 4.5 mg/L | 2.6 mg/L |
| Vitreous humor | 5.3 mg/L | 5.6 mg/L | 2.6 mg/L |
| Urine | 47.9 mg/L | 929.8 mg/L | 283.3 mg/L |
| Bile | 5.3 mg/L | 19.1 mg/L | 6.2 mg/L |
| Gastric contents | 209.4 mg/kg | 16.7 mg/kg | 28.3 mg/kg |
| Hair | 160 ng/mg | 37 ng/mg | 19 ng/mg |
| Brain cortex | 21.2 mg/kg | 3.8 mg/kg | 3.3 mg/kg |
| Skeletal muscle | 9.6 mg/kg | 7.1 mg/kg | 3.7 mg/kg |
| Liver | 8.5 mg/kg | 86.4 mg/kg | 49.3 mg/kg |

case, no significant hepatic lesions could be detected during autopsy or by histology suggesting a minor involvement of NCOC in the death. NCOC was also found in the brain (0.76 mg/kg) where it could contribute to oxidative stress, promoting neuronal toxicity (40). Liver esterases transform cocaine and alcohol into cocaethylene which is further metabolized into ecgonine ethylester (19,20). None of these metabolites could be detected in significant amounts demonstrating that alcohol and cocaine were not taken simultaneously.

According to Isenschmid (6), the most common clinical manifestations following acute cocaine intoxication include profound CNS stimulation with psychosis and repeated grand-mal convulsions, ventricular arrhythmias and respiratory dysfunction with Cheyne-Stoke breathing and ultimately respiratory paralysis. Excited delirium accounts for another 10% of cocaine related deaths.

An average blood level of 3.0 mg/L was observed for intravenous fatalities (41), a concentration which is slightly lower than the peripheral blood level which was measured in our case (5.0 mg/L). Intravenous administration of 200 mg of cocaine in humans result in a similar mean plasma concentration of 3.2 mg/L (range: 2.53–3.87 mg/L) (13). Therefore, the blood levels which are measured in living and dead people overlap to some extent. Moreover, the interpretation of cocaine blood levels is much more complicated because it is prone to chemical and enzymatic degradation (15) and to postmortem redistribution (42,43). To minimize drug degradation, biological specimens were collected rapidly and stored at –20°C until analysis. In addition, blood was preserved with fluoride and EDTA. Postmortem redistribution is exemplified by the cardiac to peripheral blood concentration ratio of 1.8 (see Table V). A similar value has been found by others (44). Pericardial fluid cocaine concentration was similar to cardiac blood levels, whereas cerebrospinal fluid and vitreous humor (VH) levels were in the range of the peripheral blood concentration. These latter values probably reflect more accurately perimortem concentrations because these specimens are relatively protected and less subjected to drug degradation and redistribution (43). Perimortem VH cocaine concentrations were significantly lower than perimortem femoral concentrations in a administration study carried out with 9 swines (45). By the end of an 8-h postmortem interval, however, VH cocaine concentrations increased to reach the cocaine concentration measured in perimortem femoral blood. This administration study demonstrates that VH concentrations change after death. It shows also that VH should not be considered as the specimen of choice to evaluate peripheral blood concentration at the time of death. The skeletal muscle has also been investigated as an alternative to peripheral blood for intoxication diagnosis. Unfortunately, it has been shown that large site-to-site variability exists (46). Cocaine concentration of skeletal muscle was similar to cardiac blood, pericardial fluid, and liver values and about twice that of peripheral blood (Table V). It has been claimed that brain tissue is a useful specimen for the determination of cocaine and metabolites because they are relatively stable in brain and not subjected to postmortem redistribution. Spiehler and Reed (47) have shown that cocaine is evenly distributed throughout the brain and remains stable for a long period in refrigerated tissue. More recently, Kalasinsky et al. (48) have also shown that concentrations of cocaine and of its major metabolites show little regional heterogeneity in postmortem brain of chronic users of cocaine. The mean blood concentrations were 4.6 mg/L of cocaine and 7.8 mg benzoylecgonine/L (this case: 5.0 and 10.4 mg/L, respectively, Table V), the mean brain concentrations were 13.3 mg/kg of cocaine (this case: 21.2 mg/kg) and 2.9 mg/kg of benzoylecgonine (this case: 3.8 mg/kg) (44). They also reported that in cocaine overdose cases, the mean brain to blood ratio was 9.6 (range: 0.65–155) for cocaine and 0.36 (range: 0.04–1.0) for benzoylecgonine. The same ratios calculated for the present case are 4.2 and 0.36, respectively. Animal studies have demonstrated that cocaine readily crosses the blood-brain barrier and that brain concentrations are about four times those of plasma at peak plasma concentrations (49). All these data indicate an acute cocaine overdose.

*Buprenorphine.* LC–MS only was sensitive enough to confirm the presumptive positive result of the buprenorphine radioimmunoassay. Buprenorphine, an opioid mixed agonist-antagonist, is a potent analgesic widely used in the treatment of opiate abuse. In Switzerland, methadone is preferred to buprenorphine for long lasting substitution therapy. Buprenorphine (Subutex®) is generally prescribed for short-term substitution therapy to patients enrolled in a detoxification process (50). As far as we know, buprenorphine is not prescribed to diminish the craving for cocaine. A trace amount of 6-monoacetylmorphine could be detected in hair only. Morphine was not detected in biological fluids suggesting that the buprenorphine substitution therapy was successful, at least as far as opiate abuse is concerned.

**Table VI. Body Fluid and Tissue Distribution of Minor Metabolites of Cocaine Determined by HILIC LC–MS**

| Specimen | Cocaethylene | Norcocaine | Nor-Cocaethylene | Ecgonine Ethylester | *m*-Hydroxy-Benzoylecgonine | Anydroecgonine Methylester |
|---|---|---|---|---|---|---|
| Peripheral blood | nd* | 0.08 mg/L | nd | nd | 0.089 mg/L | 0.02 mg/L |
| Brain cortex | nd | 0.76 mg/kg | nd | nd | nd | 0.20 mg/kg |
| Liver | nd | 0.25 mg/kg | nd | traces | 0.73 mg/kg | 0.10 mg/kg |
| Muscle | nd | 0.24 mg/kg | nd | nd | nd | 0.12 mg/kg |
| Urine | nd | 0.96 mg/L | nd | traces | 13.6 mg/L | 0.65 mg/L |
| Bile | nd | 0.99 mg/L | nd | nd | 1.2 mg/L | 0.18 mg/L |

* nd, not detected.

According to Tracqui et al. (32), fatalities involving buprenorphine alone seem very unusual. Therapeutic concentrations of buprenorphine are in the range 0.5–5.0 µg/L (51). Blood levels for 20 fatalities ranged from 1.1 to 29.0 µg buprenorphine/L and 0.2 to 12.6 µg norbuprenorphine/L (32). The concentrations found in the present case (Table VII) are below or in the low therapeutic range. The unusually high norbuprenorphine to buprenorphine concentration ratio suggests that death occurred late in the elimination curve.

*Fluoxetine.* Fluoxetine is an antidepressant often prescribed to addicts. Its clinical efficacy in combination with buprenorphine as a anti-craving medication has been shown to be less efficient than tricyclics (desipramine) and amantadine (52). To cure depression of cocaine addicts, Swiss practitioners prefer to administrate sedative antidepressants (e.g., mirtazapine). Fluoxetine, which is known to be a strong P-450 enzyme inhibitor should be administred with caution because of the high risk of pharmacokinetic interactions with other drugs metabolised through the P-450 pathways. For instance, norfluoxetine is known to inhibit the metabolism of both methadone and buprenorphine in vitro (53). Fluoxetine toxicity appears to be relatively benign (54). The concentrations determined in the blood from our case (Table VII) are within the therapeutic range (51). Symptoms of toxicity have been correlated with blood concentrations higher than 1 mg/L. In the absence of other risk factors, the lowest concentration determined to have resulted in death was 0.63 mg/L (55). Nevertheless, fluoxetine intake may present a significant risk factor for patients with atherosclerotic cardiovascular disease. Cardiovascular toxic effects, such as ventricular torsade, conduction abnormality, and ventricular tachyarrhythmia, and seizures have been occasionally reported (56–58). These unwanted toxic effects again indicate that fluoxetine must be prescribed to cocaine addicts with caution. On the other hand, no adverse physiological interactions between the two drugs have been observed in cardiovascular tests of patients challenged with ascending doses of cocaine (20–40 mg) and fluoxetine (10–40 mg) (59).

*Cannabinoids.* Cannabinoids were measured in relatively high concentrations in blood, urine, and brain cortex (Table VII). The blood concentrations of THC and 11-OH-THC suggest that the victim was under the influence of cannabis at the time of death (60). Mathematical models have been proposed to evaluate the time of administration, and in a roundabout way the time-period of influence from THC and THCCOOH concentrations in blood. However, these models have been validated with living people only (61) and are therefore difficult to implement in death cases.

**Table VII. Buprenorphine, Fluoxetine, and Cannabinoid Concentrations in Biological Samples**

| Xenobiotic | Peripheral Blood (µg/L) | Urine (µg/L) | Brain Cortex (µg/kg) |
|---|---|---|---|
| Buprenorphine | 0.1 | 16.3* | n/m† |
| Norbuprenorphine | 0.6 | 24.3* | n/m |
| Fluoxetine | 150 | 180 | n/m |
| Norfluoxetine | 330 | 280 | n/m |
| THC | 8.2 | n/m | 13.5 |
| 11-OH-THC | 2.1 | n/m | 11.5 |
| THCCOOH | 18.6 | 696.4* | 23.5 |

* After hydrolysis.
† n/m, not measured.

The process of postmortem redistribution can affect the concentration of all analytes in body fluids and tissues. Cell lysis causes changes in drug concentrations along gradients of diffusion (42,43). The extent of postmortem redistribution is particulary significant for drugs with high lipid solubility or large volume of distribution (e.g., cannabinoids). In addition, the extent of postmortem degradation of cannabinoids is not yet known (62). It is thought that brain concentrations are less influenced by drugs released from fat compartments and that brain concentrations reflect direct influence of the drug on the behavior (43). Nevertheless, in that case, brain cannabinoids levels were in a similar range as those measured in the blood, suggesting that massive postmortem redistribution did not occur. It is also worth mentioning that in vitro experiments with the aim of studying the neuroactive and neurotoxic effects of THC are often carried out with very high concentrations of cannabinoids, typically 3–10µM THC (63). These values are at least two orders of magnitude higher than that which was found here (0.043µM), suggesting that pharmacological studies with brain tissues must be carried out with lower, more realistic cannabinoid concentrations.

Like cocaine, cannabinoids also affect the cardiovascular system. THC can induce tachycardia, orthostatic hypotension, and decreased platelet aggregation. Exposure to cannabinoids may also aggravate pre-existing cardiovascular illness (64). Although cannabis toxicity is considered to be low, acute cardiovascular fatalities following cannabis use have been reported (65), suggesting that cannabinoids might have enhanced the toxic effects of cocaine. Marijuana and cocaine have been shown to interact to increase heart rate above normal levels compared to the effects of each drug alone (66).

*Suicide or accidental overdose?* A significant proportion of cocaine-related deaths are probably intentional but are not detected as such because no notes expressing intent are found at the scene of death (67). Compulsive self-administration of cocaine by injection may also result in accidental death. It has been suggested that a lethal overdosage is more likely to occur with injection than with other routes of administration (68).

## Conclusions

Unlike other HPLC methods based on reversed-phase separation, Atlantis silica-packed HILIC columns allow the analysis of basic molecules characterized by a broad range of polarity (e.g., cocaine and metabolites). Furthermore, acetonitrile constitutes a weak solvent and can be injected directly into the LC–MS. The proportion of acetonitrile is kept high during the whole experiment improving the spraying conditions and coupling with the MS. LC–MS presents the additional advantage of no AEME formation and no required derivatization step.

The method was successfully applied to a death case after

multiple cocaine injections. Altogether, the toxicological results indicate a lethal cocaine overdose. In addition, fluoxetine might have enhanced the toxic effects of cocaine because of its weak pro-arrhythmogenic properties. Likewise, combination of cannabinoids and cocaine might have increased detrimental cardiovascular effects.

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