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Case Report

Analysis of Clothing and Urine from Moscow Theatre Siege Casualties Reveals Carfentanil and Remifentanil Use

James R. Riches, Robert W. Read, Robin M. Black, Nicholas J. Cooper and Christopher M. Timperley*

Detection Department, Defence Science and Technology Laboratory (Dstl), Porton Down, Salisbury, Wiltshire, SP4 0JQ, UK

*Author to whom correspondence should be addressed. E-mail: cmtimperley@dstl.gov.uk

On October 26, 2002, Russian Special Forces deployed a chemical aerosol against Chechen terrorists to rescue hostages in the Dubrovka theatre. Its use confirmed Russian military interest in chemicals with effects on personnel and caused 125 deaths through a combination of the aerosol and inadequate medical care. This study provides evidence from liquid chromatography–tandem mass spectrometry analysis of extracts of clothing from two British survivors, and urine from a third survivor, that the aerosol comprised a mixture of two anaesthetics—carfentanil and remifentanil—whose relative proportions this study was unable to identify. Carfentanil and remifentanil were found on a shirt sample and a metabolite called norcarfentanil was found in a urine sample. This metabolite probably originated from carfentanil.

Introduction

On October 23, 2002, Chechen terrorists seized the Melnikov Street Theatre in Moscow during a sell-out performance of the musical "Nord-Ost," taking over 800 hostages and demanding immediate and unconditional withdrawal of Russian troops from Chechnya. The siege ended in the early morning of October 26 after a Special Forces unit belonging to the Russian Federal Security Service (FSB) pumped a chemical aerosol into the building and stormed it. At least 33 terrorists and 129 hostages died during or shortly after the raid. The terrorists were shot dead after falling unconscious to the effects of the aerosol, explosives strapped to them were removed, and a bomb in the auditorium was deactivated. Two hostages were shot by the terrorists, while 125 died through a combination of the aerosol and inadequate medical treatment following the rescue. Medical treatment of the casualties was complicated because the Russian government did not disclose the composition of the aerosol. The head of the Moscow Public Health Department announced that all but one of the hostages killed in the raid had died of the effects of the gas, which was surmised to comprise an anaesthetic or chemical warfare agent. Foreign embassies in Moscow issued official requests for more information on the aerosol to aid treatment, but were ignored. Armed guards were posted at Moscow hospitals and doctors were ordered not to release any of the casualties. Refusing to disclose the content of the aerosol used, the Russian government informed the United States Embassy on October 28 about some of its effects. Based on this information and examination of some of the casualties, doctors concluded that the aerosol had contained a morphine derivative. On October 30, Russia responded to increasing domestic and international pressure with a statement by its Health Minister, Yuri Shevchenko (1), who identified the aerosol as that of a fentanyl derivative, although the precise composition was not disclosed. Shevchenko stated that fentanyl was an anaesthetic that fell into the category of non-lethal medical preparations and that the troops had not used any substances prohibited by the Chemical Weapons Convention.

After the siege, various hypotheses were proposed to account for the Russian explanation that an aerosolised form of fentanyl had been used: a mixture of fentanyl and the anaesthetic gas halothane (2), or fentanyl alone, for example, but these ideas were soon discredited based on the insufficient potency of these chemicals (2–4). A recent European Court of Human Rights report (5) of a legal case against Russia, lodged by 64 Russians that survived or lost relatives in the siege, provides an authoritative account of events. Therein the Russian Government revealed that the aerosol was a "mixture based on derivatives of fentanyl" and "a composite chemical compound of general narcotic action," suggesting more than one component. However, the composition was not disclosed to the court. This case report provides evidence from the analysis of clothing from two British survivors, and urine from a third survivor with a Russian name, that the aerosol comprised a mixture of two fentanyls, carfentanil and remifentanil.

Case History

Clothing and blood samples from British survivors held in the theatre throughout the siege were received at the Defence Science and Technology Laboratory (Dstl) Porton Down on the afternoon of October 28, 2002, for analysis. A jumper and leather jacket from Casualty 1 (male), a shirt from Casualty 2 (female), and two blood samples from each casualty, were provided. The blood samples had been taken approximately 19 h (Casualty 1) and 12 h (Casualty 2) after the chemical aerosol had been released into the theatre. The casualties had been near an exit door, which appears to have been a major factor in their survival. They were among the first casualties evacuated from the theatre after it was assaulted.

Both casualties were interviewed separately by British police on November 12 and 13, 2002. The following information is taken from these interviews (6): During the morning of October 26, the hostage-takers pointed to a white aerosol that appeared to be emerging silently from the balcony wall. The aerosol—dense, white and cloudy—spread evenly and descended slowly. Casualty 1 claimed it had an indescribable smell, Casualty 2 remembered it as odourless; people were not coughing or spluttering, even on the balcony. Both casualties knelt on the floor and covered their faces with their clothing. From first spotting the aerosol to being overcome by it took "10–30 seconds" for Casualty 1 and "at least 30 seconds" for

Casualty 2. Neither casualty saw the assault team enter the theatre.

Upon awakening in the hospital, Casualty 1 recalled vomiting and seeing blood, which concerned him, but strangely, he felt no pain in his stomach. Nurses told him not to sleep, but eventually he dozed off. Two bottles of fluid were administered by intravenous drip; he was told they contained sugar. He received at least two injections. One was supposed to make him go to the toilet. He did not know what the others were for. He was given pyjamas to wear because his clothes were wet. He noticed them drying on a radiator and put them on to go home.

Casualty 2 was led down steps and placed in a vehicle. On the way to the hospital, she drifted in and out of consciousness, and in the hospital a drip was inserted into her arm: she was surprised to feel no pain. Doctors appeared worried by the blood in her hair. She thought it must have been blood from a terrorist. Later, she found blood on her clothes and realized she had probably been dragged from the theatre.

A single urine specimen was received from a 56-year-old man (Casualty 3) who had survived the siege. The specimen was provided on October 31, 2002, five days after the man had inhaled the chemical aerosol, and arrived at Dstl Porton Down one day later. According to the accompanying medical notes, the man had been exposed to the aerosol for "maybe 1 hour" and had fallen unconscious for "at least 2-3 hours." After being rescued, he had been moved to a hospital in Moscow, where he had received: a mucolytic, corticosteroids, frusemide (a diuretic), amikacin and ofloxacin (both antibiotics), ipratropium (an anticholinergic drug) and propranolol (a beta-blocker). He was later discharged and had not received any medication the day before he donated the urine sample.

Other eyewitness accounts suggest that the Russian Special Forces pumped the chemical aerosol through the ventilation system (5) at approximately 5:00 a.m. Other hostages inside the theatre also saw and smelt the aerosol, were rapidly overwhelmed by it, and after 30–60 min were evacuated to hospitals in Moscow. Unconscious, they had inhibited tendon, pupil and corneal reflexes, respiratory depression and cyanosis. The Russian Government (5) commented that hospital care for those most intoxicated involved oxygen administration, mechanical ventilation and injection of naloxone (a standard antidote for treating opioid overdose) (7). Less affected individuals were torpid, disorientated and vomiting, had pinpoint pupils, bradycardia and hypotension, and received symptomatic treatment.

Experimental

Materials and methods

Fentanyl and its analogues are highly physiologically active: analytical standards should be handled with care, wearing gloves and eye protection, and working in an efficient fume cupboard.

Sample storage

Separately packaged clothing items that arrived at Dstl Porton Down were stored in a sealed container in a cold room (4°C). Two blood samples were obtained from each survivor (Casualties 1 and 2). They were supplied in ethylenediaminetetraacetic acid (EDTA)-treated (4 mL) and Z-serum sep-clot activator (8 mL) Vacutainer tubes (BD, Franklin Lakes, NJ). EDTA-treated tubes were centrifuged for 5 min at 5,000 rpm and stored at 4°C. The Z-serum clot-activated samples were similarly stored.

Standards

Fentanyl hydrochloride, *cis*-3-methylfentanyl free base, carfentanil oxalate, sufentanil citrate, lofentanil oxalate, remifentanil hydrochloride, norcarfentanil and remifentanil acid were synthesised in-house and were >98% pure by ^{1}H and ^{13}C nuclear magnetic resonance (NMR) spectroscopy. Stock solutions of the standards were prepared in Milli-Q water (0.5 mg/mL), except for *cis*-3-methylfentanyl free base, which was dissolved in acetonitrile (Distol grade, BDH Ltd., Poole, Dorset, UK), and subsequently diluted.

Clothing samples

Cutting and placing fabric swatches into sample bottles was conducted with new equipment in a room separate from the laboratory in which the solvent extraction and analysis was performed. Three swab samples using methanol-soaked 10 cm^2 sections of lint cloth (RS Components Ltd., Oxford, UK) that had been pre-cleaned with dichloromethane (CH_2Cl_2) were taken from different areas of the leather jacket of Casualty 1: front left-hand side (including the sleeve), front right-hand side (including the sleeve) and the back (excluding the sleeves). The surface area of each swabbed section was approximately 2,500 cm^2. The samples were extracted with water (10 mL) for 30 min with occasional shaking. Aliquots of each extract (3 mL) were then treated with 1 M aqueous NaOH (200 μL) and extracted with CH_2Cl_2. The samples were vortexed and centrifuged, and the CH_2Cl_2 layer was separated, concentrated to dryness (nitrogen stream at 40°C) and redissolved in CH_2Cl_2 (100 μL) for gas chromatography–mass spectrometry (GC–MS) analysis.

Samples of the front sections of the jumper and shirt from Casualties 1 and 2, respectively, of area 10 × 10 cm, were extracted with CH_2Cl_2 (200 mL). Extracts were decanted into clean sample bottles. Excess solvent was removed under a nitrogen stream and the residues were extracted with water (200 mL). This procedure allowed fentanyls to be extracted as free base (organic extract) or salt form (aqueous extract). The dichloromethane extracts were filtered. Aliquots of the filtered extracts were evaporated to dryness and redissolved in CH_2Cl_2 (100 μL) for analysis by GC–MS. Aliquots (10 mL) of the aqueous extract of each item of clothing were treated with 1 M aqueous NaOH (500 μL) and extracted with CH_2Cl_2 (1 mL), as described for the leather jacket extracts. Aliquots of the organic extracts were extracted with water (10 mL). The water extracts were treated with 1 M aqueous NaOH (500 μL) and extracted with CH_2Cl_2, as already described. Fresh portions of the jumper and shirt were also extracted with water only and aliquots of these samples (10 mL) were basified and extracted with CH_2Cl_2. To investigate the detection limit, two samples of the shirt were spiked with 1 μg of the fentanyl salt mixture and 9 μg of sufentanil (internal standard). Samples were extracted with CH_2Cl_2, then water, and with water only, as already described.

Phase extraction

Solid-phase extraction (SPE) was used to clean samples before analysis by liquid chromatography–tandem mass spectrometry (LC–MS-MS) based on the method of Shou (8), but scaled up to reflect the larger sorbent bed volume of the SPE cartridges used (130 mg versus 25 mg) instead of the SPE plates described in the published method. Plasma (1 mL) or aqueous extracts (up to 5 mL) were loaded onto 130 mg Bond Elut Certify SPE cartridges (Varian Ltd., Oxfordshire, UK) preconditioned with 2 mL of methanol–water–5% acetic acid solution. Cartridges were then washed with 5% aqueous acetic acid (1 mL) and methanol (1 mL) and dried under gentle vacuum. Cartridges were eluted with two 0.75 mL aliquots of 2% aqueous NH_4OH in 4:1 v/v chloroform–isopropanol. Samples were concentrated to dryness under a stream of nitrogen and dissolved in 100 μL of 95:5:0.05 v/v/v acetonitrile–water–trifluoroacetic acid (TFA).

LC–MS-MS method

A Surveyor LC system (Thermo-Finnigan, Hemel Hempstead, UK) comprising a quaternary pump (but using two channels) and an autosampler was used. A Betasil Silica-100 (50 × 3 mm) column (Thermo Hypersil Keystone) was used with a flow rate of 200 μL/min. The isocratic mobile phase comprised 7% 0.05% TFA in water (v/v) and 93% 0.05% TFA in acetonitrile (v/v). The injection volume was 10 μL. A TSQ Quantum triple quadrupole mass spectrometer (Thermo-Finnigan) was operated in positive ion electrospray mode with the electrospray needle maintained at 3 kV. The instrument was tuned by infusing a 100 ng/mL solution of fentanyl in 1:1 v/v water–acetonitrile at a flow rate of 10 μL/min. Sheath gas and auxiliary flow rates were set to 30 and 10, respectively, and capillary temperature to 350°C. Both quadrupoles were maintained at unit resolution (0.7 at half-peak height). Fentanyl and analogues produced abundant $[M + H]^+$ ions. The tube lens and capillary offset settings were optimised for the transitions m/z 337 → 188 and m/z 337 → 105 (optimum collision energy for these transitions was 28 and 44 V, respectively). For *cis*-3-methylfentanyl and carfentanil, selected reaction monitoring (SRM) transitions were selected by acquiring the product ion spectra of the parent ions m/z 351 and 395, respectively. Carfentanil gave a wider range of fragment ions than fentanyl and *cis*-3-methylfentanyl; thus, the overall sensitivity of the SRM method was lower.

LC–MS-MS analysis of clothing extracts

The LC–MS-MS method was very sensitive for fentanyl and *cis*-3-methylfentanyl. Its linearity determined via a six-point calibration curve, evaluated over the concentration range 0.025 to 0.5 ng/mL, gave a line of regression with a correlation coefficient (r^2) of 0.999 (Supplementary Figure S1). The lowest concentration detected for each analyte was 0.01 ng/mL, close to the detection limit of the instrument. Samples used for the GC–MS study were dried and redissolved in 95:5 acetonitrile–water (containing 0.05% TFA). They were screened for fentanyl and *cis*-3-methylfentanyl using a method that monitored for two transitions (negative for all samples) and for a carfentanil standard in full scan mode. Appropriate transitions were added to the SRM method for carfentanil and a strong signal was obtained for the water extract of the shirt at the correct retention time for carfentanil. A portion (5 mL) of the original water extract of the shirt was processed using SPE and analyzed for carfentanil using a method containing four transitions for carfentanil, and a positive signal was obtained for all four transitions. To confirm this, a fresh sample of the shirt was processed by SPE with a full glassware blank. Again, a positive result was obtained. The jumper extract and leather jacket swab samples were analyzed under identical conditions. The shirt was also extracted with dichloromethane and methanol, but carfentanil was not detected in either extract.

Shirt sample extracts were examined for remifentanil based on the transitions m/z 377 to 228, 261, 285 and 317. The transition m/z 377 → 285 was adversely affected by chemical noise, so the transition m/z 377 → 116 (40 eV) was selected instead. Remifentanil was less sensitive than fentanyl, but a 0.1 ng/mL standard offered good signal-to-noise. Analysis of this sample resulted in a strong signal for four and three SRM transitions. A remifentanil standard was analyzed by SRM in positive ion chemical ionization mode on the benchtop GC–MS system (Finnigan MAT GCQ ion trap mass spectrometer). The limit of detection was ~10 ng/mL, approximately 100 times higher than from the LC–MS-MS method.

SPE efficiency

Recovery of the fentanyls was studied using the SPE protocol used for the clothing extracts. A water sample was spiked with all analytes at a concentration of 10 ng/mL. This sample was processed as normal. A second clean water sample was processed and spiked at the same concentration after elution from an SPE cartridge. The ratio of the peak areas for the cartridge spiked before the SPE procedure with that spiked afterwards was calculated as a percentage (recovery) and offered the following results: fentanyl (71%), *cis*-3-methylfentanyl (62%), carfentanil (67%), remifentanil (102%) and lofentanil (67%). These data confirmed that the SPE method provided good recoveries of all the analytes.

Analysis of blood samples

Aliquots of plasma (500 μL) from the EDTA-treated blood were pipetted into 2 mL Aquasil-treated vials, aqueous NaOH (4 M, 50 μL) and then 1-chlorobutane (1 mL) were added to each vial, and the samples were vortexed and left for 10 min, and vortexed again and left for a further 5 min. The organic layer was removed and placed in another 2 mL Aquasil-treated vial and the solvent was removed under a gentle stream of nitrogen at 40°C. The residue was dissolved in toluene (20 μL). Plasma for control and spiking experiments was obtained with consent from one of the authors.

Plasma samples were spiked with low concentrations of fentanyl salts, cleaned up as described earlier, and analyzed by GC–MS-MS. Again, good linearity was obtained for the fentanyl standards ($r^2 \geq 0.99$). The samples spiked with the lowest concentration standard (0.2 ng/mL) showed a detectable peak for all the analytes, except carfentanil (which was identified in the sample spiked at the 0.4 ng/mL level).

Plasma samples spiked with fentanyl and *cis*-3-methylfentanyl were processed by the SPE method and analyzed by LC–MS-MS ($r^2 \geq 0.99$). The lower limit of detection achieved for both analytes was 0.05 ng/mL, which is in agreement with that of the study by Shou (8) on which this method was based. Because of the limited sample available, the samples used for the GC–MS-MS analysis were evaporated to dryness, dissolved in acetonitrile–water, and analyzed by LC–MS-MS using a method incorporating transitions for fentanyl, *cis*-3-methylfentanyl and carfentanil.

LC–MS-MS analysis of urine

All extractions were performed manually. Control urine was a commercial freeze-dried product (Randox Laboratories Ltd, Crumlin, County Antrim, UK). Blank and spiked control samples were analyzed together with aliquots of urine from Casualty 3. Briefly, the urine (1 mL) was diluted with 0.2 M ammonium acetate buffer (pH 4, 1 mL) and loaded onto an Oasis HLB (60 mg/3 mL) cartridge (Waters Ltd, Elstree, UK) pre-conditioned with methanol and water. The cartridge was washed with water and 20:80 methanol–water. Excess solvent was expelled and the cartridge was eluted with methanol. The eluate was evaporated to dryness in a centrifugal evaporator (SPD 121P Speedvac, ThermoSavant, Holbrook, NY) at 50°C. Residues were dissolved in 0.05% TFA in water and transferred to glass autosampler vials with 250 μL glass inserts.

LC–MS-MS was performed using a Surveyor MS pump and autosampler (Thermo Scientific) with a Halo C8, 50 × 2.1 mm, 2.7 μm particle size column (Hichrom, Reading, UK), interfaced to a Deca XP Plus ion trap mass spectrometer (Thermo Scientific). The mass spectrometer was equipped with an electrospray ionization source. Mobile phase consisted of 0.05% TFA in water and acetonitrile at a flow rate of 0.25 mL/min. Elution used a gradient of 0.05% TFA in 5% acetonitrile–95% water for 2 min, which was increased to 75% acetonitrile–25% water at 24 min and held for 2 min. The column was fitted with a C8 SecurityGuard guard cartridge (Phenomenex, Macclesfield, Cheshire, UK) and maintained at 25°C. The injection volume was 10 μL.

The mass spectrometer was operated in positive ion SRM mode. Source conditions were: spray voltage, 5.5 kV; sheath gas (nitrogen) flow, 50; sweep gas (nitrogen) flow, 20; heated capillary temperature, 250°C. The monitored SRM transitions were m/z 291.0 → 259.0 for norcarfentanil and m/z 363.0 → 330.9 for remifentanil acid. The limit of detection was approximately 0.02 ng/mL for both norcarfentanil and remifentanil acid.

Results

Fentanyl, first synthesized over 45 years ago (9), is a short-acting analgesic used clinically in humans to treat acute and chronic pain (10). One of a family of related compounds (11, 12) it binds like morphine to μ-opioid receptors (13). It is 80–100 times more potent than morphine and some of its relatives are more powerful still. The fentanyls that ranked highest among contenders used to end the siege were fentanyl, *cis*-3-methylfentanyl and carfentanil (Figure 1A), which various media spokespeople speculated had been used. *Cis*-3-methylfentanyl (14) had appeared on the

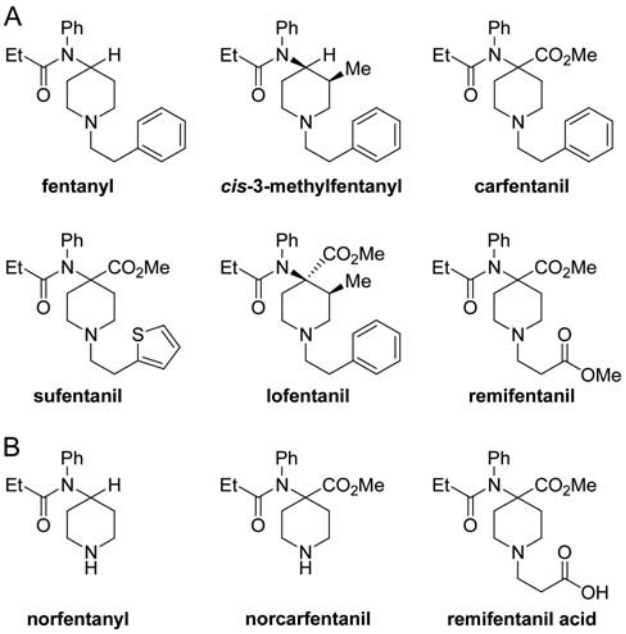

Figure 1. Structures of fentanyl compounds: fentanyl, sufentanil and remifentanil are analgesic-anaesthetics used in humans, and carfentanil is used in veterinary medicine to tranquillise large mammals (A); fentanyl and carfentanil are metabolized by the liver to nor compounds, and remifentanil reacts with blood enzymes to produce remifentanil acid; the metabolites are not especially biologically active and are excreted unchanged in urine (B).

Russian black market (15) under the name of "Krokodil" (crocodile) and the Russian press had already commented that a trimethylfentanyl (allegedly investigated under the codename "Kolokol-1" by the KGB) had been used. Other candidate fentanyls included sufentanil (16), lofentanil and remifentanil (17), a metabolically-labile variant that had been introduced into clinical practice by GlaxoSmithKline in the 1990s.

The GC–MS and GC–MS-MS results after analysis for fentanyl, *cis*-3-methylfentanyl, carfentanil, sufentanil, lofentanil and remifentanil were negative for the clothing and blood samples. Further analyses were undertaken after development of a very sensitive analytical method using LC–MS-MS; analyte concentrations approximately 50 times lower could be detected in comparison to the less sensitive GC–MS-MS protocol. Extracts were specifically analyzed for fentanyl, *cis*-3-methylfentanyl, carfentanil and lofentanil. A subsequent analysis was undertaken for remifentanil (clothing only). None of the analytes were detected in extracts of the jacket of Casualty 1. A suggestion of carfentanil was observed in the jumper extracts. However, the chromatographic peaks were too obscured by interfering components to allow a positive identification to be made (Supplementary Figure S2). Low concentrations (< 0.5 ng/mL) of carfentanil and remifentanil were detected in the concentrated aqueous extracts of the shirt of Casualty 2. No fentanyls were found in the blood samples.

In a confirmatory analysis, monitoring four structurally specific fragmentations of the protonated molecule, carfentanil and remifentanil were detected in fresh shirt extracts. Good signal-to-noise ratios were obtained for each at the correct retention time. Relative peak areas agreed closely with those of a carfentanil standard (Table I). Those for remifentanil showed a

Table I
LC–MS-MS MRM Detection of Carfentanil and Remifentanil in Shirt Extract*

Production	Relative peak areas (%)†	
	Sample	Standard
Carfentanil	—	—
335.1	100	100
279.2	40	36
246.2	58	50
134.1	37	35
Remifentanil	—	—
228.1	100	100
116.0	41	57
261.0	82	77

*Note: Relative peak areas of product ions m/z 395.3 for carfentanil and m/z 377.1 for remifentanil in an extract of shirt sample are shown versus those measured for the pure analytical standards dissolved in ultra-high-purity water (0.5 ng/mL).
†Reference ion ratios were from standards selected to provide an equivalent signal to that from the sample as far as possible.

greater variability due to a lower concentration and greater background interference. Closer agreement with a standard was obtained by monitoring three fragmentations (Table I). No carfentanil or remifentanil was detected in the glassware blanks. The total amounts of carfentanil and remifentanil extracted were estimated as <5 ng, with carfentanil present at the higher concentration. In fact, comparison of the signal from the shirt sample with standard solutions indicated that the concentration of carfentanil in the concentrated shirt extract was approximately 0.3 ng/mL. This corresponded to a concentration of 10 pg/mL in the original extract. Examples of chromatograms derived from the shirt extracts, and from positive and negative controls, appear in Figures 2 and 3. Following these results, more concentrated extracts of the shirt after SPE clean-up were specifically analyzed by GC–MS-MS for carfentanil and remifentanil (data not shown). These analyses were negative, but the detection limits were not as low as those obtained by LC–MS-MS and the chromatograms revealed many more extraneous components, which adversely affected detection limits and degraded column performance.

The urine from Casualty 3 was analyzed for the major metabolites of carfentanil and remifentanil. Although the main metabolite of carfentanil had not been identified unequivocally in humans before, based on its resemblance to fentanyl, which is metabolized to norfentanyl (18, 19), it was possible to predict a likely candidate: norcarfentanil (20) (Figure 1B). Remifentanil, characterized by very short action and potency similar to fentanyl, is metabolized quickly in blood to remifentanil acid (17, 21). The metabolites of fentanyls generally lack appreciable physiological action (17).

A known procedure for automated SPE of fentanyls and metabolites from urine was used (22), modified slightly to allow for a larger sample volume. An initial analysis for norcarfentanil and remifentanil acid showed a peak corresponding to norcarfentanil in chromatograms from the urine sample. The peak was comparable (~75% height) to that from a sample of control urine spiked with 0.1 ng/mL norcarfentanil. No remifentanil acid was detected (Figure 4A). Repeat analysis for norcarfentanil alone confirmed the presence of this metabolite (Figure 4B). Although it may be argued that the identification of norcarfentanil in the urine sample is not definitive because only a single transition was monitored, it is entirely consistent with the other results of these analyses and provides additional evidence for the use of carfentanil. Analyses including SRM transitions for intact remifentanil and carfentanil provided no evidence for their presence.

Discussion

The case histories of Casualties 1 to 3 agree with those of other Moscow Theatre Siege survivors (5). The rapid unconsciousness upon inhalation of the aerosol and loss of pain sensation upon awakening experienced by Casualties 1 and 2 are consistent with exposure to fentanyl/s. The effects of such compounds are well known: in moderate doses, pain suppression, and at higher doses, a sleep-like state that can progress to coma. Fentanyls depress the urge to breathe in a dose-dependent manner, and when unconscious, breathing may slow to the point that sufficient oxygen cannot be maintained in the blood to sustain normal function. Even if breathing continues at a lowered rate, fentanyls can cause the neck and tongue to become limp, which can lead to airway obstruction. Additionally, when administered rapidly, fentanyls can cause muscular rigidity, which can result in cessation of breathing. It is uncertain whether the three casualties were treated with naloxone post-aerosol exposure, as evidence suggests for some survivors (4, 5). Naloxone was not among the drugs given to Casualty 3 while he was hospitalized. However, he did receive ipratropium, which is used for treating chronic obstructive lung disease, and this may have helped him to breathe normally. While in the hospital, Casualties 1 and 3 appear to have received diuretic drugs, presumably to help excrete any fentanyls.

The LC–MS-MS results for the shirt and urine sample resolve the mystery of whether the Russian Special Forces used a single chemical or a combination of chemicals. Positive identification of carfentanil and remifentanil on the shirt of Casualty 2 suggests that the aerosol contained these compounds. Norcarfentanil, identified in the urine sample from Casualty 3 after he donated it five days after he had been exposed, implies that carfentanil was used. Remifentanil is not appreciably metabolized by the lung (23), but has a methyl ester linkage that is hydrolyzed by blood and tissue enzymes to give remifentanil acid. Piperidine *N*-dealkylation of remifentanil to produce norcarfentanil is not a significant metabolic pathway in humans (23). The detection of norcarfentanil in urine donated so many days after inhalation of the aerosol is presumably due to metabolic *N*-dealkylation of carfentanil (20) and the high fat-solubility of carfentanil; elimination of lipophilic fentanyls is limited by their redistribution in the body (17). An absence of remifentanil acid in the urine was unsurprising: remifentanil is rapidly metabolized with a mean half-life in humans of ~2 h (23), and this casualty had undergone forced diuresis in hospital. The blood samples were collected 19 h (Casualty 1) and 12 h (Casualty 2) after exposure to the aerosol, and detection of any agent in the samples was considered unlikely.

Carfentanil is more potent than fentanyl (24). Its sole approved use is by veterinarians to incapacitate wildlife for examination and procedures; it is not approved for use in humans. The first documented human exposure to carfentanil in the open literature became available only recently (24).

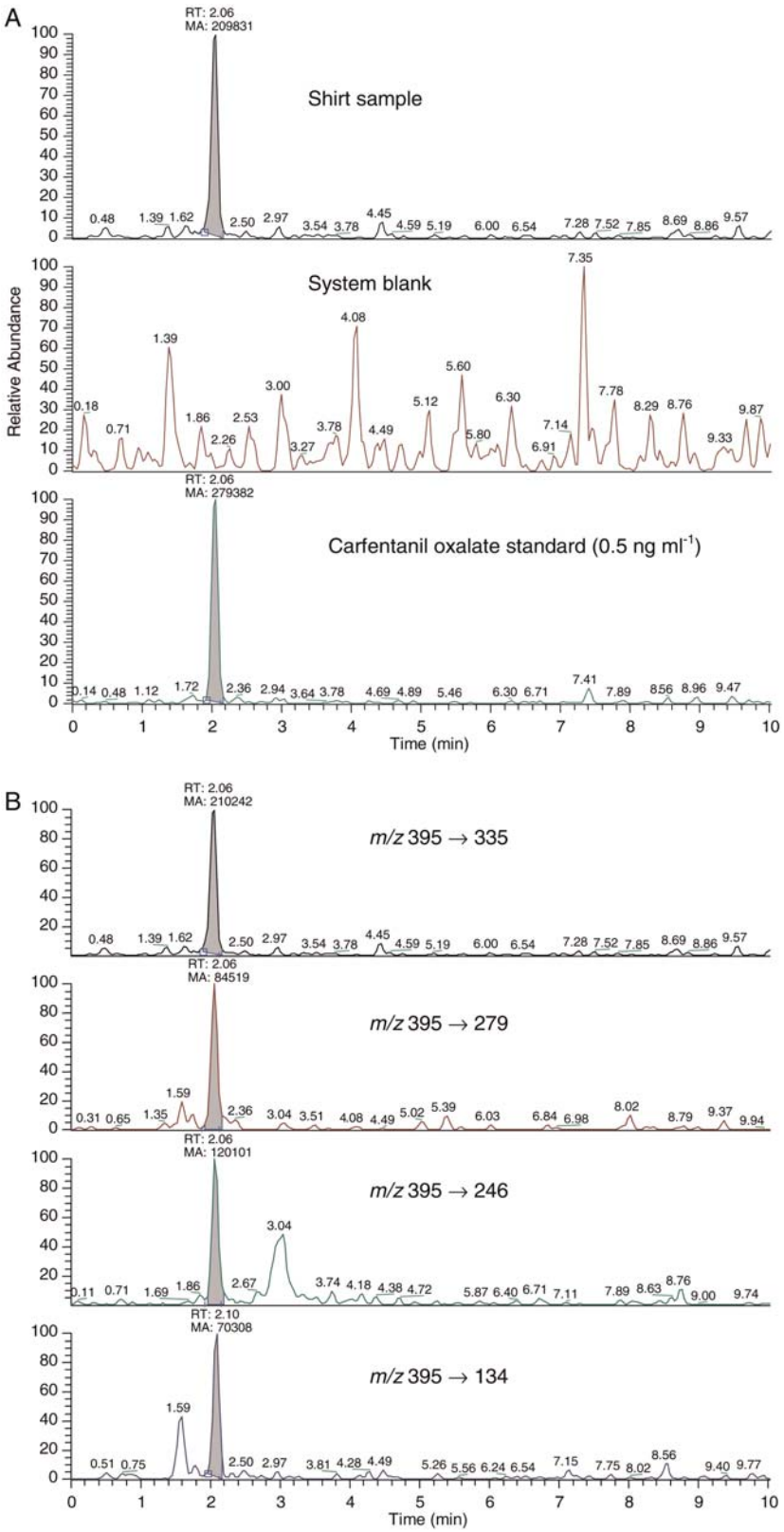

Figure 2. LC–MS-MS MRM detection of carfentanil on the shirt of Casualty 2: detection of carfentanil, monitoring fragmentation m/z 395.3 → 335.1 (top), glassware control (middle, baseline magnified ~10 times) and a carfentanil standard (bottom) (A); detection of carfentanil in the shirt extract monitoring four fragmentations of the protonated molecule m/z 395.3 (B).

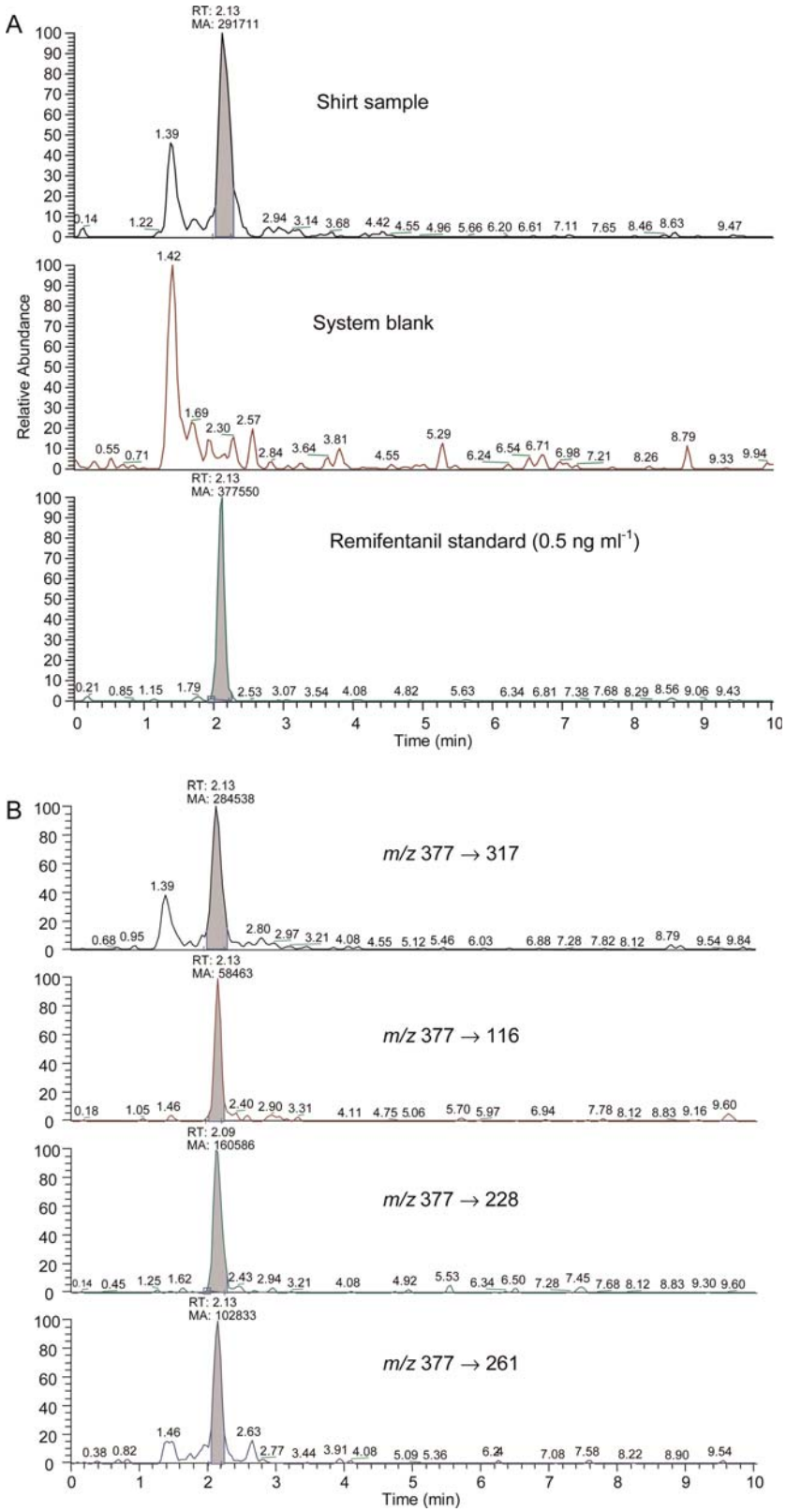

Figure 3. LC–MS-MS MRM detection of remifentanil on the shirt of Casualty 2: detection of remifentanil, monitoring the fragmentation m/z 377.1 → 317.1 (top), the glassware control (middle) and a remifentanil standard (bottom) (A); detection of remifentanil in a shirt extract monitoring four fragmentations of the protonated molecule m/z 377.1 (B).

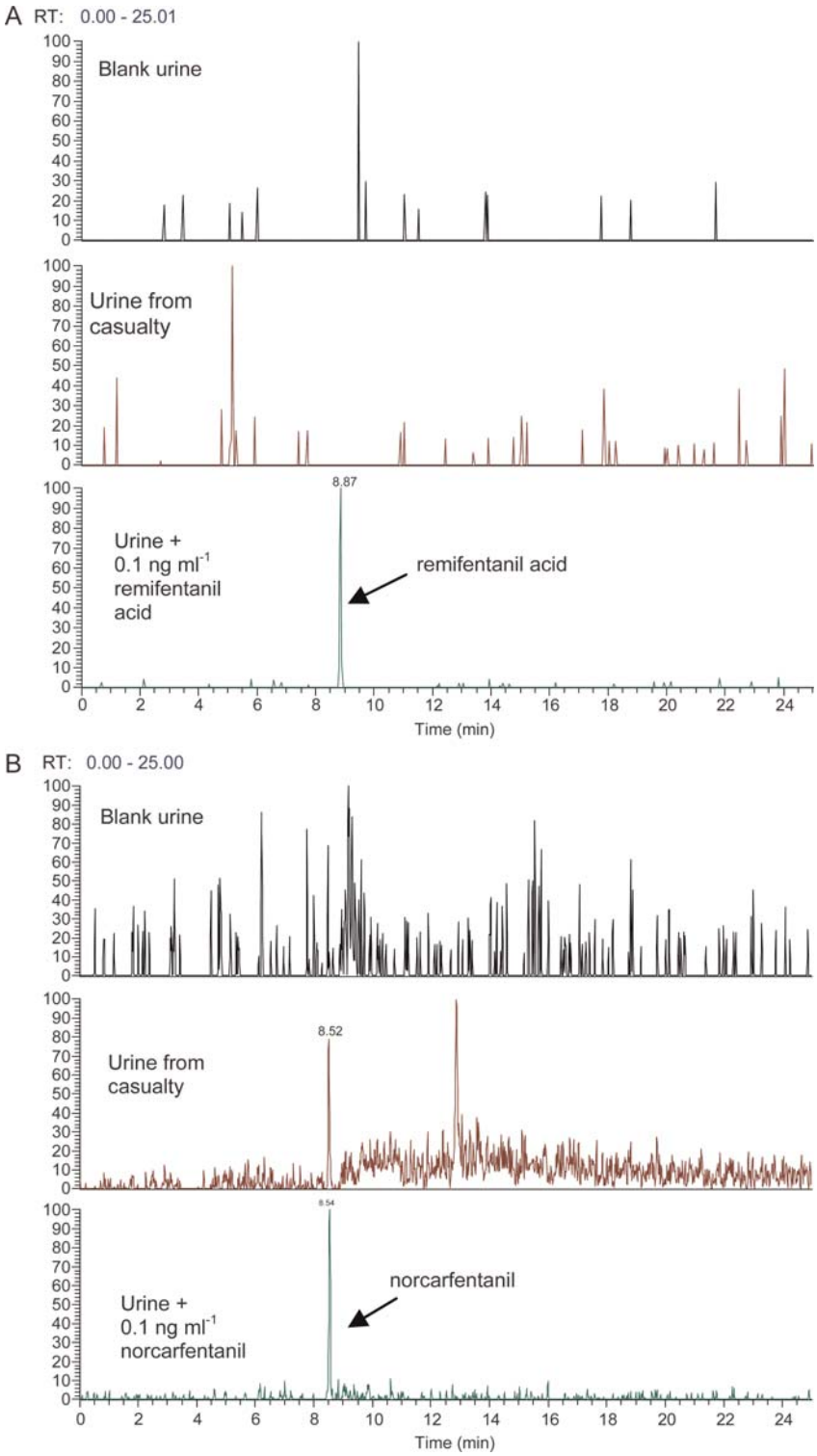

Figure 4. LC–MS-MS SRM chromatograms for the urine sample from Casualty 3: transition m/z 363.0 → 330.9 from an extract of blank urine (top), urine from Casualty 3 (middle) and spiked urine (bottom) showing the absence of remifentanil acid (A); transition m/z 291.0 → 259.0 from an extract of blank urine (top), urine from Casualty 3 (middle) and spiked urine (bottom) showing the presence of norcarfentanil (B).

A 47-year-old male splashed his eyes and mouth accidently with the contents of a tranquillizer dart containing 1.5 mg carfentanil citrate. He washed his face immediately with water, but became drowsy two minutes later. His colleagues, recognizing that he had been poisoned, administered 100 mg of the reversal agent naltrexone (whose antagonistic potency is approximately twice that of naloxone). This presumably saved his life: the man complained an hour later, while in the

hospital, of mild and transient chest discomfort, but this disappeared and he was discharged in a stable condition a day later. This account suggests that carfentanil exposures may cause severe intoxication or fatality in the absence of prompt and appropriate medical treatment. The same conclusion can be drawn for remifentanil. Both carfentanil and remifentanil have narrow safety margins, meaning that potentially fatal side effects, including respiratory depression, can occur at doses only slightly higher than those that impart medical benefits.

Carfentanil may have been mixed with the less potent, shorter-acting remifentanil in an attempt to lessen the number of fatalities that may have otherwise resulted from exposure to carfentanil alone. If the remifentanil was used to dilute the carfentanil, then presumably it was disseminated in higher concentration (this was not reflected by the analysis results, possibly due to its lower stability). An advantage of remifentanil for surgical use is that the depth of narcosis can be titrated so that the dose rate approximates the metabolism rate. Its use by the Russian Special Forces may partially explain why casualties survived an estimated aerosol exposure of 30–45 min. Human data for other fentanyls suggest that once patients lose consciousness, life-threatening respiratory depression can occur.

Scientific papers published by Russian military officers indicate an interest in fentanyls extending back 12 years: opioid receptor studies (7, 25), fentanyl analysis (26) and synthesis of fentanyl precursors (27, 28). That Russian military research on fentanyls occurred before 1994 is evident from a passage in a book by General Antonov (29), a former director of the Military Chemical Institute in Shikhany, which states that "the action of analgesics is a knock-out blow—personnel subject to an attack of forces only a few minutes after the beginning of a chemical attack will lose their capacity to stand, not to mention move about. In severe cases people will enter an 'unconscious state' and 'carfentanil is one of the most active substances of the entire group of the studied derivatives of fentanyl. It manifests its activity for different pathways of entry into the organism, including inhalation of vapours or aerosol.'" To the authors' knowledge, this is the only information on carfentanil aerosolization in the public domain. One further use of an incapacitant has been reported since the theatre siege (30). On October 13, 2005, armed Chechen separatists attacked the Russian town of Nalchik. In response, Russian Special Forces employed a "narcotic agent" against the separatists who were holding two women hostage in a shop. No information about the narcotic agent has emerged, although the affected hostages were rescued and revived successfully by an unidentified antidote.

Conclusions

The detection of carfentanil and remifentanil by LC–MS-MS on the shirt of Casualty 2 worn throughout the siege, monitoring four and three specific fragmentations, respectively, provides high confidence in the identification. Differences between the relative peak areas for the fentanyls identified in extracts of the sample and those of analytical standards were between 2–8% for carfentanil and 5–16% for remifentanil (Table I). In other areas of trace analysis, monitoring of two fragmentations at the correct retention time, with relative peak areas within 20% of those of a standard, is usually considered sufficiently specific for unequivocal identification. Ideally, identification of this mixture by two independent techniques would have been desirable, but complementary GC–MS-MS results were not achieved due to the lower sensitivity of the GC instrument coupled with high levels of chemical noise. The identification of the metabolite norcarfentanil by LC–MS-MS analysis of urine donated by a separate casualty, considered alongside the clothing results and the expected metabolic differences between carfentanil and remifentanil, suggest that this metabolite most likely originated from carfentanil. Similar searches in the urine sample for intact carfentanil and remifentanil, and its metabolite remifentanil acid, were unsuccessful, as were analogous searches among the blood samples.

The finding that the aerosol comprised carfentanil and remifentanil is consistent with the outcome arising from its use, for literature data suggest little margin of safety between their therapeutic and lethal doses in humans, and a high lethality in the absence of prompt and appropriate medical intervention. It is highly improbable that a chemical agent exists for which a dose can be calibrated in a tactical environment to incapacitate opponents reliably and without substantial mortality. Mass spectrometric techniques such as those described are sufficiently powerful to detect the presence of fentanyls in a range of matrices at trace levels and allow their use to be verified and attributed to individuals or states.

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References

1. (2002) "Russian Health Minister says substance used in raid is not prohibited by CWC." Russian News Agency (ITAR-TASS), Moscow, news bulletin in English, 30 October, 1540 GMT.
2. Schiermeier, Q. (2002) Hostage deaths put gas weapons in spotlight. *Nature*, **420**, 7.
3. Rieder, J., Keller, C., Hoffmann, G., Lirk, P. (2003) Moscow theatre siege and anaesthetic drugs. *The Lancet*, **361**, 1131.
4. Wax, P.M., Becker, C.E., Curry, S.C. (2003) Unexpected "gas" casualties in Moscow: A medical toxicology perspective. *Annals of Emergency Medicine*, **41**, 700–705.
5. Finogenov and Others v. Russia (2011) Judgment of the European Court of Human Rights (First Section), Applications Nos. 18299/03 and 27311/03, Strasbourg. http://cmiskp.echr.coe.int/tkp197/viewhbkm.asp?sessionId=83685113&skin=hudoc-en&action=html&table=F69A27FD8FB86142BF01C1166DEA398649&key=95152&highlight= (accessed 16 January 2012).
6. New Scotland Yard (NSY) (2002) British hostage debrief reports: Casualties 1 and 2 interviewed by a detective inspector from the NSY Hostage & Crisis Negotiation Unit and a clinical psychologist from the British National Crime Faculty, London, UK.
7. Kuzmina, N.E., Kuzmin, V.S. (2011) Development of concepts on the interaction of drugs with opioid receptors. *Russian Chemistry Reviews*, **80**, 145–169.
8. Shou, W.Z. (2001) A highly automated 96-well solid phase extraction and LC tandem MS method for the determination of fentanyl in human plasma. *Rapid Communications in Mass Spectrometry*, **15**, 466–476.

9. Janssen, P.A.J. (1964) Method for producing analgesia. *US Patent 3,141,823*.
10. Drdla-Schutting, R., Benrath, J., Wunderbaldinger, G., Sandkühler, J. (2012) Erasure of a spinal memory trace of pain by a brief, high-dose opioid administration. *Science*, **335**, 235–238.
11. Van Daele, P.G.H., De Bruyn, M.F.L., Boey, J.M., Sanczuk, S., Agten, J.T.M., Janssen, P.A.J. (1976) Synthetic analgesics: N-(1-[2-arylethyl]-4-substituted 4-piperidinyl) *N*-arylalkanamides. *Arzneimittel Forschung*, **26**, 1521–1531.
12. Van Bever, W.F.M., Niemegeers, C.J.E., Schellekens, K.H.L., Janssen, P.A. J. (1976) *N*-4-Substituted 1-(2-arylethyl)-4-piperidinyl-*N*-phenylpropanamides, a novel series of extremely potent analgesics with unusually high safety margin. *Arzneimittel Forschung*, **26**, 1548–1551.
13. Dosen-Micovic, L., Ivanovic, M., Micovic, V. (2006) Steric interactions and the activity of fentanyl analogs at the μ-opioid receptor. *Bioorganic and Medicinal Chemistry*, **14**, 2887–2895.
14. Gergov, M., Nokua, P., Vuori, E., Ojanperä, I. (2009). Simultaneous screening and quantification of 25 opioid drugs in post-mortem blood and urine by liquid chromatography-tandem mass spectrometry. *Forensic Science International*, **186**, 36–43.
15. Godunov, A.V., Kireeva, A.V., Volchenko, S.V. (2007). Determination of 3-methylfentanyl by GC/MS in biological and non-biological objects. *Forensic Science International*, **169S**, S29–S35.
16. Liang, L., Wan, S., Xiao, J., Zhang, J., Gu, M. (2011) Rapid UPLC-MS/MS method for the determination of sufentanil in human plasma and its application in target-controlled infusion system. *Journal of Pharmaceutical and Biomedical Analysis*, **54**, 838–844.
17. Feldman, P.L., James, M.K., Brackeen, M.F., Bilotta, J.M., Schuster, S.V., Lahey, A.P. *et al.* (1991) Design, synthesis, and pharmacological evaluation of ultrashort- to long-acting opioid analgetics. *Journal of Medicinal Chemistry*, **34**, 2202–2208.
18. Labroo, R.B., Paine, M.F., Thummel, K.E., Kharasch, E.D. (1997). Fentanyl metabolism by human hepatic and intestinal cytochrome P450 3A4: Implications for interindividual variability in disposition, efficacy, and drug interactions. *Drug Metabolism and Disposal*, **25**, 1072–1080.
19. Coopman, V., Cordonnier, J., Pien, K., Van Varenbergh, D. (2007) LC-MS/MS analysis of fentanyl and norfentanyl in a fatality due to application of multiple Durogesic® transdermal therapeutic systems. *Forensic Science International*, **169**, 223–227.
20. Endres, C.J., Bencherif, B., Hilton, J., Madar, I., Frost, J.J. (2003) Quantification of brain μ-opioid receptors with [^{11}C]carfentanil: Reference-tissue methods. *Nuclear Medicine and Biology*, **30**, 177–186.
21. Said, R., Pohanka, A., Andersson, M., Beck, O., Abdel-Rehim, M. (2011) Determination of remifentanil in human plasma by liquid chromatography-tandem mass spectrometry utilizing micro extraction in packed syringe (MEPS) as sample preparation. *Journal of Chromatography B*, **879**, 815–818.
22. Wang, L., Bernert, J.T. (2006) Analysis of 13 fentanils, including sufentanil and carfentanil, in human urine by liquid chromatography-atmospheric-pressure ionization-tandem mass spectrometry. *Journal of Analytical Toxicology*, **30**, 335–341.
23. Navapurkar, V.U., Archer, S., Gupta, S.K., Muir, K.T., Frazer, N., Park, G.R. (1998) Metabolism of remifentanil during liver transplantation. *British Journal of Anaesthesia*, **81**, 881–886.
24. George, A.V., Lu, J.J., Pisano, M.V., Metz, J., Erickson, T.B. (2010) Carfentanil—An ultra potent opioid. *American Journal of Emergency Medicine*, **28**, 530–532.
25. Dukhovich, F.S., Darkhovskii, M.B., Gorbatova, E.N., Polyakov, V.S. (2005) The agonist paradox: Agonists and antagonists of acetylcholine receptors and opioid receptors. *Chemistry & Biodiversity*, **2**, 354–366.
26. Zlobin, V.A., Bukreeva, L.P., Kuznetsov, P.E., Panfilov, A.V., Nazarov, G.V., Kirsanov, A.T. (2000) Analysis of opiates by HPLC with indirect spectrophotometric detection. *Pharmaceutical Chemistry Journal*, **34**, 279–280.
27. Panfilov, A.V., Markovich, Y.D., Ivashev, I.P., Zhirov, A.A., Eleev, A.F., Kurochkin, V.K. *et al.* (2000) Sodium borohydride in reductive amination reactions. *Pharmaceutical Chemistry Journal*, **34**, 76–78.
28. Panfilov, A.V., Markovich, Y.D., Zhirov, A.A., Ivashev, I.P., Kirsanov, A.T., Kondrat'ev, V.B. (2000) Reactions of sodium borohydride in acetic acid: Reductive amination of carbonyl compounds. *Pharmaceutical Chemistry Journal*, **34**, 371–373.
29. Antonov, N.S. (1994) Chemical weapons at the turn of the century. *English Translation LN72-96*, Progress Publisher, Moscow, Russia.
30. Crowley, M. (2009) Dangerous ambiguities: Regulation of riot control agents and incapacitants under the Chemical Weapons Convention. *Bradford Non-Lethal Weapons Research Project*, University of Bradford, UK, p. 76.