Aim: New psychoactive substances (NPS) have become a serious problem since the late 2000s. In order to evaluate these substances with respect to mortality, forensic toxicological data are required. Methods: Immunoassay and GC-MS analysis of urine samples were conducted. Identified NPS-targets were quantified in femoral blood, heart blood, urine, gastric contents, bile fluid, liver, and liquor using a LC-MS/MS system. Results: Three autopsy cases are presented, in which NPS were abused. In two autopsy cases the fentanyl analogue fluorofentanyl was found and in another case the MDMA methylene homologue heliomethylamine and the synthetic cathinone 3,4-dimethylmethcathinone (3,4-DMMC). Quantitation analyses showed the highest concentration in gastric content. Discussion: Our results suggest that these substances were consumed orally. In each case the cause of death may be attributed to acute poisoning caused by one or more of these substances. Conclusion: The data presented are a valid resource for the evaluation of fatal NPS intoxications and a good starting point for further postmortem investigations.

1. Introduction

Hundreds of new psychoactive substances (NPS), also called “legal-highs” have emerged on the drug market over the last decade [1]. These substances are structural analogues of traditionally controlled substances and have similar psychotropic effects like their corresponding controlled drug or pharmaceutical [2,3]. They are sold in the internet or smart shops as incense, bath salt or standard not for human use. Often the substituent or the position of the substituent is slightly changed to avoid juridical consequences [3]. In order to control these substances by the government, new psychoactive substances were classified into chemical classes, such as synthetic cathinones, synthetic cannabinoids, phenethylamines, piperazines, and tryptamines [3]. These substance classes are known to be pharmacologically and toxicologically hazardous and have been reported in some cases of death [e. g. 4]. The targets of interest were obtained from LC-MS and GC-MS-based screening approaches [5-7].

2. Material and Methods

2.1. Material

All solvents were purchased from Sigma-Aldrich (St Louis, USA) and were of analytical grade. Acetic anhydride was obtained from Merck (Darmstadt, Germany) and distilled prior to use. Trimethylsulfonium hydroxide was used as obtained from Acros Organics (New Jersey, USA). Water was purified with a Pure Lab flex instrument from Elga (Celle, Germany). Heliomethylamine was obtained from Lipomed GmbH (Arlesheim, Switzerland) and 3,4-Dimethylcathinone (3,4-DMMC) from LGC GmbH (Wesel, Germany). Fluorofentanyl was used as collected from the site of crime and the purity verified by HPLC-DAD (220 nm) and GC-MS analyses. In contrast to De Boer et al. [8], we did not detect any
impurities. They reported 30-400 µg p-fluorofentanyl and caffeine as adulterant in confiscated capsules and tablets in an illicit production site in the Netherlands [8].

Autopsy case 1 and 2: Autopsy case 1 and 2 were of suicidal intent. A couple (male: 34 years and female: 33 years) was found dead lying on a couch in their apartment. Both suffered from psychological problems and were known to abuse narcotic substances. A screw-topped cream bottle with 0.2 g white powder was ensured by the police. According to autopsy report no beginning of decomposition was observed and no needle insertions were found.

Autopsy case 3: A 26-year old man was found dead by his brother. At the point of discovery, he was located in the bed of his apartment. Several pills and powder were found at the site of crime. According to autopsy report, absolutely no needle insertions were apparent and decomposition of the subject was already observed.

2.2. Methods

Immunoassay screening

Urine samples were centrifuged and analysed with the help of CEDIA® Drugs of Abuse Assays using the Indiko instrument from Thermo Fisher Scientific (Waltham, Massachusetts). Barbiturates, benzodiazepines, opiates, cocaine, cannabinoids, methadone, amphetamine, and salicylate were covered by this approach.

GC-MS-based screening for suspects

Preparation of alkaline extracts and derivatization: Prior to adding 2 mL 0.1 M phosphate buffer (pH 6), the pH of the urine sample was adjusted to 5-7. Subsequently, the Bond Elut LRC cartridge equipped with 130 mg sorbent from Agilent Technologies (Santa Clara, USA) was washed (2 mL methanol) and conditioned with 2 mL phosphate buffer (pH 6). After sample load (2 mL), the cartridge was successively washed with 1 mL 1 M acetic acid and 5 mL methanol. Finally, the targets of interest were eluted with 2 mL solvent mixture consisting of methylene dichloride, iso-propanol, 25wt% ammonia (40:10:1, v/v). Subsequently, the eluate was dried at 40°C until 2/3 of the solution remained. 50 µL iso-propanol/0.1 M HCl were added and the solvent was evaporated. The remaining residue was reconstituted in 100 µL methanol, thoroughly vortexed and one half (50 µL) transferred to a glass vial and subjected to GC-MS analysis. The other half (50 µL) was evaporated until dryness and incubated with 100 µL acetic anhydride and 50 µL pyridine at 80°C for 30 min. At last, the solvent was evaporated under nitrogen, the residue reconstituted in 100 µL methanol and submitted to GC-MS analysis.

Preparation of an acidic extract and derivatization: All steps of the procedure as described above were maintained except for the elution step and the derivatization step. After sample load (2 mL), 1 mL phosphate buffer/methanol (4:1, v/v), 1 mL 1 M acetic acid, and 1 mL hexane were applied to the cartridge. Finally, the compounds were eluted with 2 mL acetone, the eluate dried under nitrogen, and the residue reconstituted in 100 µL methanol. Thereof, 50 µL were taken off, incubated with 50 µL methanol and 20 µL trimethylsulfonium hydroxide for 30 min at 80°C, and subjected to GC-MS analysis.

GC-MS analysis: All samples were analysed with a Finnigan Trace GC 2000 system from Thermo Quest (Manchester, UK). The capillary column used was called Zebron ZB-5MSi (Phenomenex, Torrance, USA) and exhibited the following quality parameters: 30 mL × 0.25 mm ID x df 0.25 µm. The temperature of the source and the transfer line were set to 200°C and 300°C during the entire analysis. Injections were made at 70°C with a split flow of 10 mL/min using a PTV in splitless mode and electron ionization. The column temperature
profile was as follows: 70°C hold for 3 min, then to 300°C for 20°C/min and finally hold at 300°C for 15.5 min. Helium was used as carrier gas. The column flow was set to 1 mL/min and constant flow. General MS parameters were as follows: filament/multiplier delay: 4.5 min, multiplier voltage: 350V, mass range: 50-500 Da, scan time: 0.5s, and electron energy: 70 eV. Data were evaluated with the Thermo XCalibur software 3.1.66.10 with the following parameters settings: Identification (peak detect: IC IS; smoothing points: 1; baseline window: 20; area noise factor: 2; peak noise factor: 10), Spectrum Enhancement (enabled; window size: 6 s; noise threshold: 2), Library Search Options (search type: identity, normal with 5 hits at maximum). All annotations were evaluated manually.

Quantitation of NPS in different specimens by means of LC-MS

Preparation of femoral blood and heart blood samples: 10 µL internal standard containing 0.1 ng/µL MBDB-d5 and 0.1 ng/µL fentanyl-d5 were mixed with 570 µL methanol before adding 20 µL blood sample. After protein precipitation, the spiked blood sample was centrifuged at 13000 rpm for 5 min and 300 µL of the upper phase were transferred to a glass vial. Next, 10 µL iso propanol/HCl were added, and the solution evaporated to dryness under a gentle stream of nitrogen. The residue was dissolved in 500 µL 4 mM ammoniumformate/methanol (pH 3.5; 25:1, v/v) and 20 µL injected onto the LC-MS/MS system.

Preparation of urine, gastric content, bile fluid, and liquor samples: 10 µL internal standard were added to 10 µL sample and filled up to 1 mL with 4 mM ammoniumformate. Beforehand, gastric content and bile fluid were diluted 1:10 (v/v) and 1:5 (v/v) with 4 mM ammoniumformate, respectively.

Preparation of liver samples: 1 g liver sample was treated for 3 min in an ultratorax twice using 5 mL 4 mM ammonium formate. Then, the suspension was centrifuged at 4000 rpm for 5 min. Finally, 10 µL internal standard were added to 100 µL supernatant and the resulting solution filled up to 1 mL with buffer.

LC-MS/MS analysis: LC separations were carried out on a Shimadzu system equipped with a LC-20AD binary pump, a SIL-20AC-HT autosampler, and a CTO-10ASvp column oven (Shimadzu, Duisburg, Germany). Separations were performed with a Phenomenex Kinetex C18 column (100 mm x 3 mm, particle size 2.6 mm,) and a C18 4 x 2.0 mm security guardTM® cartridge (Phenomenex). The analytical column was stabilized at 40°C during the entire analysis. Water supplemented with 4 mM ammonium formate was used as solvent A and methanol supplemented with 4 mM ammonium formate as solvent B. The mobile-phase gradient used was as follows: solvent B was held at 5% for 1 min. Then, solvent B was increased to 100% within 15 min, held at 100% for additional 10 min, spaced back to 5% within 4 min, and held at 5% for additional 10 min to clean the column and adjust the LC to its initial conditions. The total flow rate through the column was 0.2 mL/min. Injection volume was 20 µL. Mass spectrometric analysis was performed on a Sciex 4000 QTrap LC–MS/MS (Sciex, Darmstadt, Germany) instrument in multiple reaction monitoring mode, equipped with a Turbo VTM Source. Total eluent flow from the LC was directed to the turbo ion spray source without splitting. Needle voltage was 5000V, turbo ion spray heater temperature 500°C, nebulizer gas (nitrogen) 40 psi, and turbo heater gas (nitrogen) 60 psi. Curtain gas (nitrogen) was set to 25 psi and collision gas (CAD, nitrogen) pressure in the collision cell to medium. The optimum values for declustering potential, entrance potential, collision energy, and collision cell exit potential were optimized individually for each compound and are as follows: fluoroctantynl (81V, 10V, 37V, 16V), heliometrilmamine (81V, 10V, 53V, 8V), and 3,4-DMMC (21V, 10V, 29V, 2V). All data were evaluated with Analyst® 1.6.2 and quantified with the IntelliQuan Algorithm by means of linear fit without any weighting. For this purpose, the area was used and the smoothing width set to 0 points.
3. Results and Discussion

New Psychoactive Substances have become a serious problem in forensic toxicology [9-11]. In Germany, 39 fatal cases have been reported for the year 2015. Compared to 2014 the number of deceased persons has increased by 14 [12]. Due to the difficulties in analysis the estimated number of unreported cases should even be higher.

3.1. Non-targeted screening for suspects

With respect to the following autopsy cases, routine immunoassay analysis was applied. Positive results were found for benzodiazepines in case of the first and second autopsy case, cannabinoids and salicylates were detected with respect to the third autopsy case (Tab. 1).

Tab. 1. Suspects found in the immunoassay analysis for the three autopsy cases. Positive results appear in bold.

<table>
<thead>
<tr>
<th>Suspect</th>
<th>Cut-Off [ng/mL]</th>
<th>Autopsy case 1 [ng/mL]</th>
<th>Autopsy case 2 [ng/mL]</th>
<th>Autopsy case 3 [ng/mL]</th>
</tr>
</thead>
<tbody>
<tr>
<td>barbiturates</td>
<td>200</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>benzodiazepine</td>
<td>200</td>
<td>3455</td>
<td>180</td>
<td>0</td>
</tr>
<tr>
<td>benzodiazepine after hydrolysis</td>
<td>200</td>
<td>&gt; 5000</td>
<td>319</td>
<td>0</td>
</tr>
<tr>
<td>opiates</td>
<td>300</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>cocaine</td>
<td>150</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>cannabinoids</td>
<td>50</td>
<td>47</td>
<td>0</td>
<td>73</td>
</tr>
<tr>
<td>methadone</td>
<td>300</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>amphetamine</td>
<td>500</td>
<td>239</td>
<td>331</td>
<td>0</td>
</tr>
<tr>
<td>salicylate</td>
<td>100</td>
<td>44</td>
<td>14</td>
<td>212</td>
</tr>
</tbody>
</table>

According to these findings, we did not expect any NPS. However, GC-MS analysis revealed nicotine, caffeine, amitriptyline, opipramol, nordiazepam, amphetamine, fluorofentanyl, and THC-COOH for the first autopsy case and nicotine, caffeine, amitriptyline, opipramol, and fluorofentanyl for the second autopsy case. With respect to the third autopsy case, nicotine, caffeine, THC-COOH, MDMA, heliomethylamine, and 3,4-DMMC were annotated. The latter two compounds as well as fluorofentanyl are part of the drug library (www.swgdrug.org/ms.htm; download 02-2015) and yielded a reverse match factor above 900 for 3,4-DMMC and heliomethylamine, and 748 as well as 787 for fluorofentanyl. Relevant mass spectra extracted from urine samples are summarized in Fig. 1.

![Fig. 1. Mass spectra of investigated suspects A) 3,4-DMMC, B) fluorofentanyl, C) heliomethylamine. Characteristic fragments are highlighted in the spectrum.]
Fluorofentanyl shows characteristic peaks at \( m/z \) 164, 207, and 263, which are in accordance to fragments reported in literature [13]. Heliomethylamine yielded peaks at \( m/z \) 135, 161, and 176, which result from the formation of the tropylium ion and the loss of the amino group [14]. 3,4-DMMC showed only one peak at \( m/z \) 58, which is indicative for the \( N \)-methyl phenethylamine unit [15]. As expected, 3,4-DMMC was also found after acetylation.

3.2. Quantitation of NPS in different specimens

To evaluate the toxicological importance of the NPS found, knowledge on the concentration among the different specimens is required. Consequently, the amount of all three targets was determined by LC-MS/MS analysis.

The designer amphetamines 3,4-DMMC and heliomethylamine are structurally very close related to mephedrone and MDMA, respectively. Consequently, they most likely act stimulating and euphorigenic like their structural analogue. An oral intake of a typical dose of 100 mg MDMA leads to 200 ng/mL in femoral blood [16]. Assuming similar physiochemical properties, the combination of an increased amount of 3,4-DMMC (540 ng/mL) and a common amount of heliomethylamine (130 ng/mL) can be considered as toxic. For comparison, Usui et al. reported 27000 ng/mL 3,4-DMMC for iliac vein and 7600 ng/mL for urine after syringe injection [4]. We found a minor amount of both drugs in femoral blood due to an assumed oral intake.

According to Fig. 2, the maximum amount of NPS was determined in gastric contents for all three targets (1300-14600 ng/mL; corresponds to 0.3-2.5 mg absolute) assuming an oral administration. Fluorofentanyl is a highly potent opioid analgesic agent. Due to its structural similarity compared to fentanyl, similar pharmacological properties are expected. Corresponding to practical experience, the therapeutic fentanyl concentration does not exceed 3 ng/mL after buccal intake [16]. Compared to fentanyl we assume an enhanced potency for fluorofentanyl. For mice, Higashikawa et al. [2] reported a lethal dose (LD50) of 9.3 mg/kg for \( p-\)
fluorofentanyl and 62 mg/kg for fentanyl if the drug was administered perorally. In regard to our results, we conclude that the concentration of 25-35 ng/mL fluorofentanyl in femoral blood can be considered as highly toxic.

For bile fluid, the amount of heliomethylamine (900 ng/mL) was three times higher than for fluorofentanyl (300 ng/mL) and the concentration of 3,4-DMMC was near to the limit of quantification (10 ng/mL). Comparable amounts were found in the liver (20 – 80 ng/mg) for all three targets.

4. Conclusion

We present three case reports on new psychoactive substances. In each of the autopsy cases the cause of death may be attributed to acute poisoning caused by one or more NPS. All other analyte concentrations stayed in the subtherapeutic range and were of no further interest. The data presented are a valid resource to evaluate the toxicity of fluorofentanyl, heliomethylamine, and 3,4-DMMC. More autopsy cases are required to estimate the toxic concentration range of each substance.

5. References