What are the benefits of screening urine by means of LC-QTOF-MS/MS? A case report on colchicine

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Aims: GC-MS is usually applied in order to screen blood and urine for drugs and pharmaceuticals. Comprehensive mass spectral libraries facilitate this process until the entry "no match" is returned. This study investigates whether high-resolution mass spectrometry coupled with liquid chromatography (LC-HRMS) should then be the method of choice. **Methods:** Urine was screened using GC-MS and LC-QTOFMS. The resulting pharmaceuticals apparent in femoral blood were then quantified by LC-QTRAP®MS. In addition, colchicine was quantified in femoral blood, urine, gastric contents, bile fluid, and liver. **Results and Discussion**: A typical GC-MS-based screening workflow was undertaken to establish the cause of intoxication with the result that the only match caffeine did not contribute to the occurrence of death. The LC-HRMS analysis revealed two compounds (compound 1: $C_{22}H_{25}NO_6$ and compound 2: $C_{22}H_{25}F_2NO_4$), which were later identified as colchicine and nebivolol. In contrast to colchicine, nebivolol - quantified in femoral blood (0.001 µg/mL) - did not contribute to the onset of death. It may have been taken in the past. The highest concentration of colchicine was found in bile fluid (67 µg/mL), followed by liver (0.86 µg/g), urine (0.56 µg/mL), and gastric contents (0.14 µg/mL). The lowest concentration was found in femoral blood (0.1 µg/mL). From these values, we conclude that colchicine caused the onset of death and was subject to the enterohepatic cycle and post-mortem redistribution. **Conclusion**: High-resolution mass spectrometry coupled with liquid chromatography (LC-HRMS) should be the method of choice if the GC-MS screening did not come up with any match and an intoxication is assumed during autopsy. Then, LC-HRMS should be applied to identify new sources of intoxication, such as non-GC/MS amenable compounds. It is time to introduce a high-resolution mass spectral compendium across laboratories to easily find "new" intoxicants.

1. Introduction

Analysis of biological matrices obtained from autopsy by means of GC-MS has been a well-established technique in forensic toxicology since the 1980s [1]. Commercially available mass spectral libraries such as the one from Maurer/Pfleger/Weber or the Designer Drugs Library from Rösner [2] facilitate the downstream identification process, as both libraries continue to accumulate tens of thousands of mass spectra. As a result, sources of intoxication can be elucidated in a reasonable period of time as long as the requested compound is vaporizable and is represented by a reference spectrum. What can we do if the GC-MS analysis does not come up with any "match" and we suspect an intoxication based on the results of the autopsy?

In response to this problem, multi-targeted LC-MS/MS methods were developed to particularly cover low abundant drugs and pharmaceuticals as well as non-vaporizable compounds [3]. These methods allow for a qualitative and quantitative analysis of a broad range of substance classes. However, analytes not included in the target list cannot be detected. That is why non-targeted screening techniques emerged to cover additional targets, with high-resolution mass spectrometer coupled with a liquid chromatograph prevailing [4]. In particular, QTOF instruments allow the forensic investigator to collect MS and MS/MS information on every detectable

peak within the acquired mass range [5]. In the next step, these MS/MS spectra can be matched to a library in a targeted or non-targeted manner. For targeted data evaluation, four parameters are available for compound matching: the mass accuracy and isotope pattern of the protonated molecular ion, the confidence of the retention time to the reference substance, and the match of the MS/MS spectrum to the library [6].

We present a case study in which GC-MS did not detect any cause of intoxication. Only LC-HRMS was able to shed light on the matter. Finally, colchicine was quantified in various biological matrices to evaluate the intoxication.

2. Materials and Methods

2.1. Facts about the fatal case

A 59-year-old woman (height: 172 cm, body weight: 60 kg) was found dead by her husband in the morning. Her mouth was smeared with a bloody substance and superficial cutting injuries were observed on her left wrist and neck. According to witness statements, she suffered from depression and had previously attempted suicide. In addition, she suffered from various heart diseases. The evening before she was found, she complained about nausea and vomited in the presence of a visiting couple. Due to autopsy she presumably died upon sudden cardiac death. An intoxication cannot be excluded.

2.2. LC-HRMS analysis

Preparation of the urine sample: 10 µL urine were diluted to 1 mL with 4 mM ammonium formate buffer. *LC parameters:* see [7]. *MS parameters:* total scan time 1.7 s; source and gas parameters: ion source gas 1 40 psi, ion source gas 2 60 psi, curtain gas 35 psi, CAD gas 7 psi, temperature 600°C; experiment: SWATH, polarity positive, spray voltage 5500 V, TOF MS (*m/z* 105 – 700; accumulation time: 200 ms; declustering potential 60 V; DP spread 0 V; Collision energy 10 V; CE spread 0 V; time bins to sum 4), TOF MSMS (*m/z* 50 – 700; accumulation time: 70 ms; charge state 1; declustering potential 60 V; DP spread 0 V; Collision energy 35 V; CE spread 15 V; time bins to sum 8) with 20 windows overlapping by 1 Da. *Data evaluation:* Multi-targeted screening method with an in-house library consisting of 255 compounds (latest version: April 2019); integration: minimum peak width: 3 points, minimum peak height: 1000, XIC width: 0.02 Da, gaussian smooth width: 0 points, noise percentage: 40 %, baseline subtract window: 2 min, peak splitting: 2 points; retention time: expected RT, RT half window: 60 sec; perform library search: library search algorithm: smart confirmation search, results sorted by: fit, library spectra type: accurate mass only, libraries to search: in-house library; algorithm parameters: precursor mass tolerance: 0.01 Da, collision energy: 5eV; qualitative rules: mass error below 5 ppm (combined score weight 40 %), error in retention time below 1 % (combined score weight 20 %), difference in isotope ratio below 5 % (combined score weight 10 %), library hit score above 25 (combined score weight 30 %).

2.3. LC-QTRAP®MS analysis

Preparation of samples: For a detailed sample preparation procedure see [7]. In summary, urine, gastric content, and bile fluid were centrifuged, spiked with an aliquot nortriptylin-d$_3$ and diluted with 4 mM ammonium formate buffer. Femoral blood was mixed with nortriptylin-d$_3$, the existing protein precipitated using methanol and the supernatant reconstituted in buffer. The liver sample was treated in an ULTRA TURRAX® Tube Disperser, the suspension centrifuged, internal standard added and the supernatant diluted with buffer.

LC parameters: see [7]. *MS parameters:* Colchicine was quantified at transition 400 -> 358 (DP: 96 volts, EP: 10 volts; CE: 31 volts; CxP: 10 volts) and nebivolol at transition 406 -> 151 (DP: 91 volts, EP: 10 volts; CE: 43 volts; CxP: 10 volts). The values were checked by consideration of the second transition, namely 400 -> 326 (DP: 96 volts, EP: 10 volts; CE: 35 volts; CxP: 8 volts) and nebivolol at transition 406 -> 103 (DP: 91 volts, EP: 10 volts; CE: 77 volts; CxP: 6 volts). All other settings are summarized in [7]. *Reference substance:* Colchicine and nebivolol HCl (Sigma-Aldrich, min. 95 % purity).

3. Results and Discussion

The GC-MS screening of the urine sample only revealed caffeine. However, did the deceased also take any drug or pharmaceutical that contributed to the entrance of death?

3.1. Qualitative LC-HRMS screening

In order to pursue this question, the urine sample was analysed by LC-QTOFMS/MS using the SWATH^TM acquisition method. This method allows for the almost simultaneous acquisition of MS and MS/MS spectra. The data can be evaluated in a targeted or non-targeted manner. We evaluated our data in a multi-targeted manner and found colchicine (retention time deviation: 0%, mass error: -1.8 ppm, library score: 27) and nebivolol (retention time deviation: 0.01 %, mass error: -0.9 ppm, library score: 81) (Fig. 1).
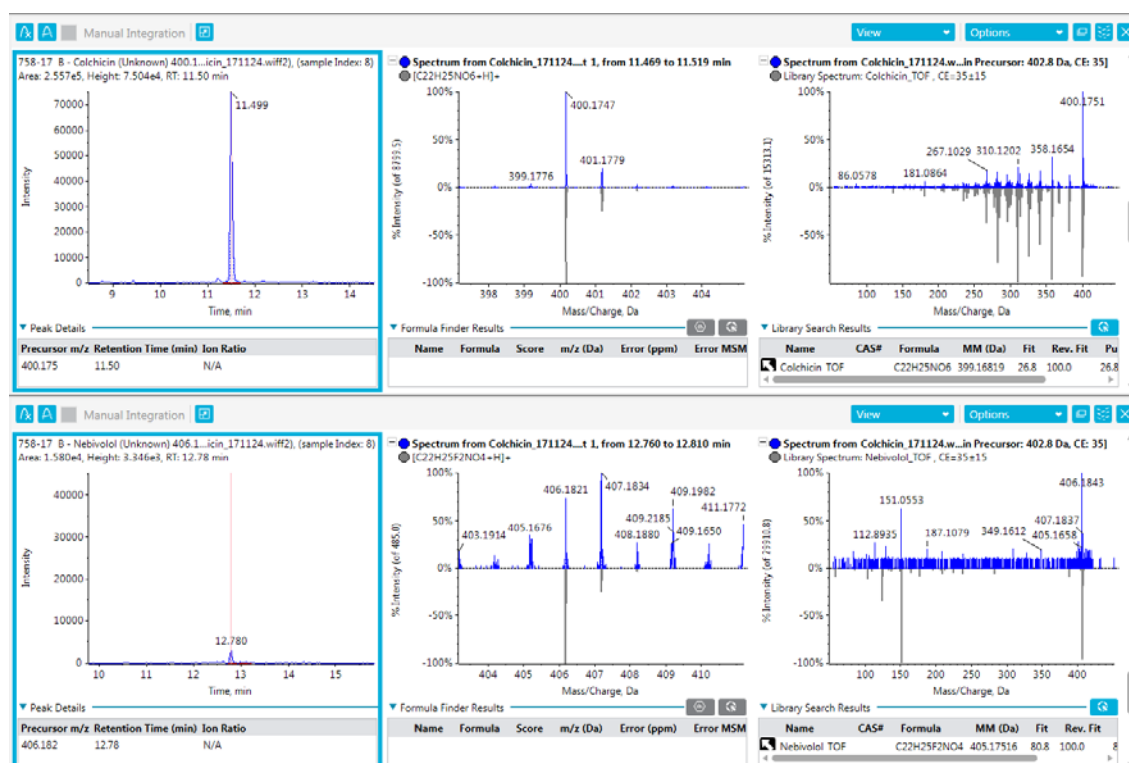

Fig. 1. Outcome of the LC-HRMS data analysis concerning extracted ion chromatogram (left), MS (middle) and MS/MS spectrum (right) after match with the in-house library (blue: acquired MS/MS, grey: library MS/MS). Upper panel: colchicine; lower panel: nebivolol.

Colchicine is a naturally-occurring alkaloid of the Autumn crocus (*Colchicum autumnale*) and is used for the treatment of acute gouty arthritis and familial Mediterranean fever. It is a potent inhibitor of cellular mitosis and affects organs with a high rate of cell turnover. According to

literature, it has a low therapeutic index; 0.5 – 0.8 mg/kg can already be poisonous [8-11]. Nebivolol is a cardioselective third-generation β-blocker and is used for the treatment of hypertension [12].

We proved the presence of both compounds while injecting an authenticated reference substance and confirmed their presence while comparing the retention time, the exact mass of the protonated molecular ion ($[M+H]^+$) and the MS/MS fragmentation pattern of the peak in the urine sample with the one in the reference substance. As expected, colchicine exhibited a protonated molecular ion at m/z 400.1747 corresponding to the elemental composition $C_{22}H_{26}NO_6^+$ (error: -1.9 ppm); the potassium adduct at m/z 438.1314 ($C_{22}H_{25}NO_6Na^+$, error: 0.1 ppm) with 17 % relative intensity additionally proves this elemental composition. Nebivolol only exhibited a protonated molecular ion at m/z 406.1824 ($C_{22}H_{26}F_2NO_4^+$, error: 0.2 ppm), but no further adducts.

As shown, both compounds did not only exhibit a distinct molecular weight but also a unique fragmentation pattern (Fig. 1). The MS/MS spectrum of colchicine resulted in peaks at m/z 358.1621 ($C_{20}H_{24}NO_5^+$) and m/z 341.1371 ($C_{20}H_{21}O_5^+$), which were attributed to the loss of acetaldehyde and acetamide (Fig. 2). The fragment at m/z 341.1371 was either followed by the loss of a methyl radical to give m/z 326.1299 ($C_{19}H_{18}O_5^{\bullet+}$) or the loss of a methoxy radical at the C^{10} carbon atom to give m/z 310.1161 ($C_{19}H_{18}O_4^{\bullet+}$). This fragment likely lost CO to give m/z 282.1218 ($C_{18}H_{18}O_3^{\bullet+}$). The MS/MS spectrum of nebivolol showed less peaks. The most prominent peak at m/z 151.0539 ($C_9H_8FO^+$) resulted from the elimination of the fluorinated benzodihydropyran unit, which then lost CO to give m/z 123.0597 ($C_8H_8F^+$) and finally hydrogen fluoride to give m/z 103.054 ($C_8H_7^+$). Putative chemical structures of the most relevant fragments are summarized in Fig. 2.
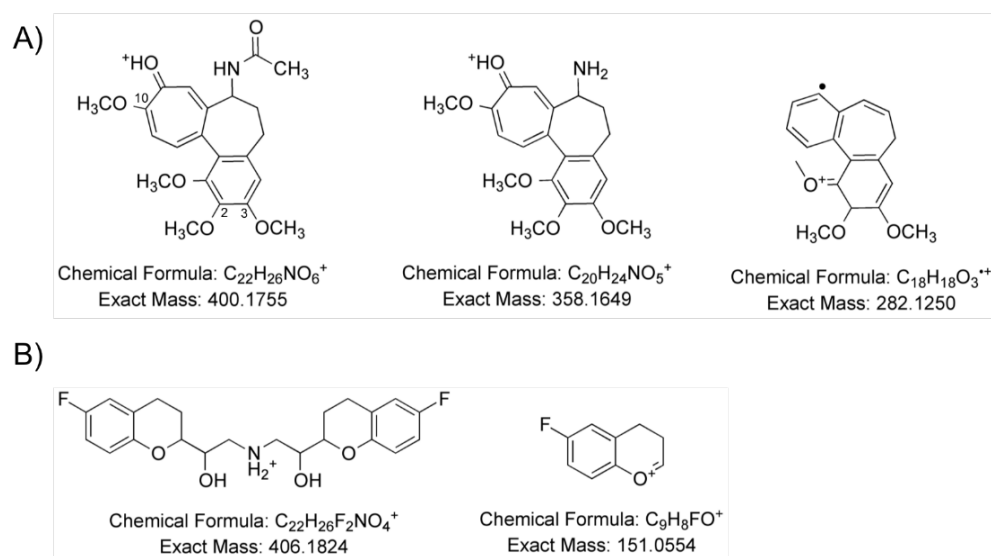

Fig. 2. Putative chemical structures of A) colchicine and B) nebivolol MS fragments.

3.2. Metabolites of colchicine and nebivolol

The detection of metabolites in different specimens is a further indication for the intake of the substance. In accordance with Schönharting et al. [13], we detected three peaks at m/z 386.1596 (Fig. 3) corresponding to the elemental composition $C_{21}H_{24}NO_6^+$. These demethylcolchicine isomers predominantly occur in the bile fluid, and to a minor extent in the urine and liver, and can be assigned due to their MS/MS fragmentation pattern (Fig. 4) and relative abundance of the protonated molecular ion (m/z 386.1596, Fig. 3).
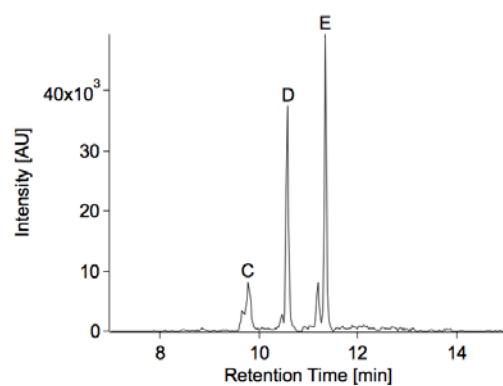
O^3-demethylcolchicine and O^{10}-demethylcolchicine were marked by the loss of C_2H_2O to give m/z 344.1495 ($C_{19}H_{22}NO_5^+$), onto which multiple losses of methanol followed. In between, ammonia (m/z 295.0971, $C_{18}H_{15}O_4^+$) and carbon monoxide (m/z 267.1018, $C_{17}H_{15}O_3^+$) were eliminated.

In contrast, O^2-demethylcolchicine did not show a peak at m/z 344.1495 due to the lack of adjacent methoxy groups. Instead a fragment at m/z 341.1397 ($C_{20}H_{21}O_5^+$) was observed followed by the loss of a methoxy radical to yield m/z 310.1179 ($C_{19}H_{18}O_4^{\bullet+}$). This then lost carbon monoxide and a methyl group to give m/z 282.1243 ($C_{18}H_{18}O_3^{\bullet+}$) and m/z 267.1008 ($C_{17}H_{15}O_3^+$), respectively. If the abundance of O^3-demethylcolchicine is compared with that of O^{10}-demethylcolchicine (Fig. 3), then O^3-demethylcolchicine can be distinguished from O^{10}-demethylcolchicine. In accordance with Schönharting et al., O^{10}-demethylcolchicine showed a lower intensity than O^3-demethylcolchicine and eluted before O^3-demethylcolchicine [13].
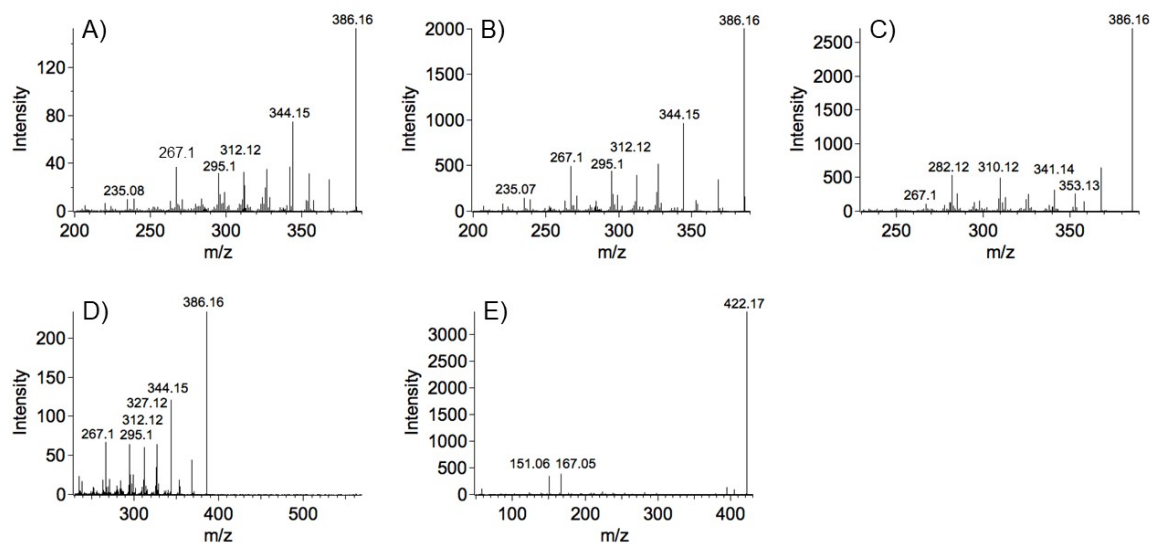

Fig. 4. MS/MS spectra of A) O^{10}-demethylcolchicine, B) O^3-demethylcolchicine; C) O^2-demethylcolchicine, D) O^3-demethylcolchicine glucuronide and E) 4-OH-nebivolol. Mass spectra were extracted from bile fluid.

Besides the demethylcolchicine isomers, we also detected the glucuronide of O^3-demethylcolchicine (retention time: 8.8 min, m/z 562.1922, $C_{27}H_{32}NO_{12}^+$, error: 0.5 ppm). The MS/MS spectrum mainly showed one peak at m/z 386.1606 ($C_{21}H_{24}NO_6^+$) resulting from the loss of glucuronic acid ($C_6H_8O_6$).

Similar to nebivolol, the MS/MS spectrum of the active first pass metabolite, 4-OH nebivolol [12], showed two representative fragments at m/z 151.0559 ($C_9H_8FO^+$) and m/z 167.0509 ($C_9H_8FO_2^+$). These peaks resulted from the loss of the hydroxylated and non-hydroxylated fluorinated benzodihydropyran unit. The metabolite, 4-OH nebivolol, was exclusively detected in the urine sample, indicating that nebivolol had been taken in the past.

3.3. Quantitation of colchicine and nebivolol in femoral blood

To evaluate whether colchicine or nebivolol or even a mixture of both led to the occurrence of death, both compounds were quantified in femoral blood by means of LC-QTRAP®MS. 0.1 µg/mL colchicine and 0.001 µg/mL nebivolol were found in femoral blood. The concentration of colchicine was in the range of previous investigations with a lethal outcome. They reported concentrations ranging between 0.04 to 0.062 µg/mL in blood samples [10,14]. In contrast, the concentration of nebivolol could be ranked at the sub-therapeutic level [15].

3.4. What concentration can we expect in the other samples taken during autopsy?

The concentration of colchicine in all the other samples is of interest to evaluate the colchicine intoxication. The highest concentration of colchicine was found in the bile fluid (67 µg/mL), followed by liver (0.86 µg/g), urine (0.56 µg/mL), and gastric contents (0.14 µg/mL). The lowest amount of colchicine was found in femoral blood (0.1 µg/mL) (Tab. 1).

Tab. 1. Colchicine concentrations in various samples taken during autopsy.

Sample	Colchicine concentration
Bile fluid	67 µg/mL
Liver	0.86 µg/g
Urine	0.56 µg/mL
Gastric contents	0.14 µg/mL
Femoral blood	0.1 µg/mL

The high concentration of colchicine found in the bile fluid indicates that colchicine was subject to enterohepatic circulation. Indeed, Kintz et al. found 20-fold less in case of a fatal poisoning [14]. Hence, we conclude that the deceased was already on colchicine therapy before death.

The gastric contents were used to estimate the amount of colchicine ingested. By multiplying the concentration of the gastric contents by its volume, this results in 32 µg colchicine, indicating that the deceased had not taken any tablet. A Colchicum tablet usually contains 0.5 mg colchicine [9]. The high enterohepatic circulation rate may have caused this distorted value. The enterohepatic cycle obviously has an impact on the amount detected in the liver as well. A higher concentration of colchicine was found in the liver sample compared to the urine and femoral blood sample.

Post-mortem redistribution is an additional factor which should be considered if a "new" compound is investigated. From these values, we conclude that colchicine is subject to post-mortem redistribution, as the liver/peripheral blood (L/P) ratio expressed as liver/femoral blood amounted 9 L/kg [16]. Consequently, the postmortem colchicine concentration in blood may not precisely reflect the ante mortem colchicine concentration.

4. Conclusion

GC-MS-based screening approaches failed to detect substances in urine samples due to e. g. their high limit of detection. Consequently, further sensitive analytical methods were required to detect these substances as well. LC-HRMS, by means of a QTOF instrument, may represent one approach. In the following, colchicine and nebivolol were detected quite easily by LC-QTOFMS. However, only colchicine contributed to the occurrence of death, as shown by the concentration found in femoral blood and bile fluid.

According to literature, colchicine has a narrow therapeutic index. For this reason, there is no clear-cut line between the toxic and non-toxic dosage. Survival seems to depend on the total

amount of colchicine ingested and the time between intake and therapeutic intervention at the hospital. This study provides post-mortem concentration values for colchicine in various samples taken during autopsy.

In view of this case report, we recommend analysing selective urine samples by means of GC-MS and also LC-HRMS in future in order to make statements about further compounds and potential causes of intoxication. If we had only analysed the urine sample by GC-MS, the conclusion of the autopsy would have been sudden cardiac death. The intake of colchicine would have remained entirely undetected.

5. References

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