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Multi target analysis of putrefactive specimens by liquid chromatography – tandem mass spectrometry to prove multiple poisonings by hypnotics and muscle relaxants

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The muscle relaxant rocuronium and the hypnotics etomidate, diazepam and midazolam were candidate poisons in a case of a suspected series of fatal intoxications in a hospital. A robust, specific and sensitive multi-target screening procedure was established to identify unequivocally the parent compounds and diagnostic metabolites and artefacts in putrefied specimens, obtained from exhumed bodies. The analytical findings of relevant compounds could be traced partially back to authorised therapeutic measures, whereas the identification of rocuronium proved potentially lethal intoxications in 13 (of a total number of 42) cases.

Moreover, the detection of certain hypnotics revealed an improper administration of these compounds in another nine cases, which suggested manipulation but was not indicative of fatal intoxications.

Quantitative estimations of substance concentrations were highly correlated with the post-mortem time intervals and did not reveal any information on doses, initial serum concentrations or toxicological effects.

Keywords: exhumation; liquid chromatography-mass spectrometry; multi-target analyses; muscle relaxants; post mortem toxicology

Introduction

Fatal poisonings may be attributed to accidental mistreatment, active or indirect euthanasia, manslaughter or murder. These categories are mainly based on legal definitions and toxicological analyses alone are insufficient to establish whether they apply in any given instance. In common jurisdiction, the classification 'murder' requires, by definition, that a 'killing with intention' is related to certain characteristics of crimes and perpetrators (such as hostility against an unsuspecting and defenceless victim). Toxicological analyses may well prove the administration of harmful compounds, provide quantitative estimation of the presumptive lethal progress of a poisoning and may indicate potential maliciousness, but the final legal evaluation represents a rather complex process.

Murder by poisoning became rather infrequent due to the limited access to suitable compounds and the ease of identifying the substances at lethal concentrations levels. Opioids are likely to be the substance group that is most frequently associated with lethal poisonings, whether due to accidental administration of lethal doses (for example, drugs of abuse, in particular heroin), suicides, or active or indirect euthanasia.

In cases of legally approved killing or executions, substance combinations are used to avoid unnecessary pain and suffering. Typically the co-administration of narcotics/anaesthetics and muscle relaxants (whether or not in combination with local anaesthetics or potassium) is used to execute death penalties (subsequent injection of thiopental, pancuronium and potassium chloride) or euthanasia of animals (for example, combination of embrutramide, mebenzonium and tetracaine). Death is caused within a few minutes by central depression, circulatory collapse and asphyxia.

Muscle relaxants terminate the transmission at the neuromuscular junction, causing a paralysis of the affected skeletal muscles and are therapeutically applied prior to surgery. Because administration of respective substances may paralyse muscles required for breathing, access to mechanical ventilation is mandatory to maintain adequate respiration.

Several nondepolarising (such as rocuronium – see Figure 1) or polarizing (succamethonium) muscle relaxants differ considerably regarding typical onset times, duration of effects and side effects. Typically, relatively high dosages are injected in combination with anaesthetics. Short onset and elimination half-life are essential features for the selection of adequate muscle relaxants. Rocuronium is characterised by quick onset (0.6–8.2 min) and moderate duration of activity (~46 min).[1–3]

These pharmaceuticals are not used in ambulatory therapy or for self-therapy or as drugs of abuse. Consequently, the unexplainable shortage of several vials of injectable muscle relaxants in a hospital immediately raised severe concerns as to a potential crime. Furthermore, an association with a significant number of sudden

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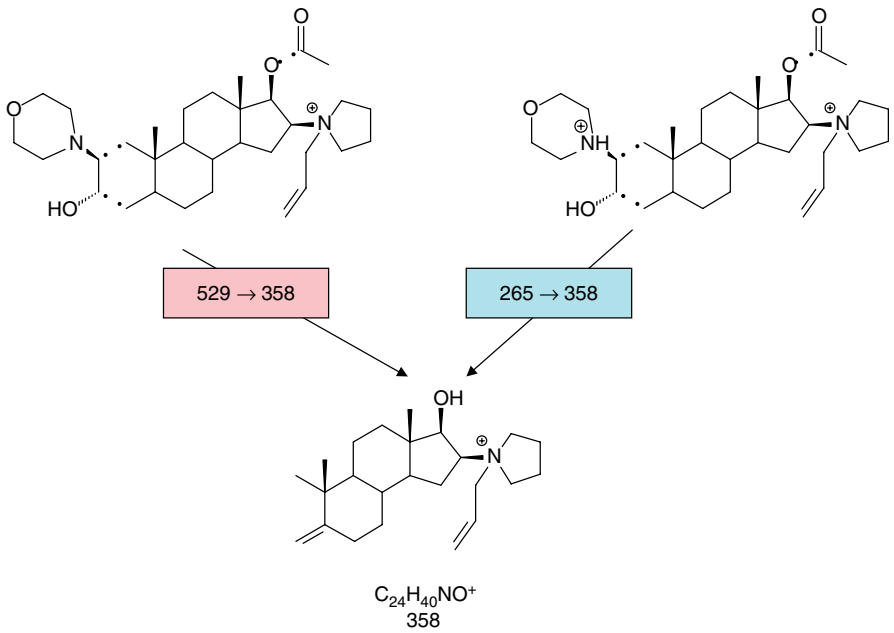

Figure 1. Fragmentation reactions of the permanently charged rocuronium and the corresponding protonated (doubly charged) cations. Characteristic product ions are formed, for example, by deacetylation (m/z = 488) and fragmentation of the A ring (m/z = 358).

deaths by respiratory arrest was suggested. As a consequence, 42 of the suspicious cases were selected for exhumation and toxicological analysis.

The patients suffered from potential life-threatening diseases, for example apoplectic stroke, cancer or chronic obstructive pulmonary disease, with loss of expectation of life. In some cases the doctors certified an unexpected death at that time; nevertheless no autopsies were requested by the physicians.

The lengths of stay in the graves ranged from 26 to 578 days. The only exhumation material that was consistently available was putrefactive fluid and could mostly be assigned to the abdominal or thoracic cavity and was therefore used for systematic screening analyses. Other specimens (liver, blood, lung, kidney) were applied for counter analysis, in case of low concentrations.

Material and Methods

Methanolic stock solutions of diazepam, nordazepam, oxazepam, midazolam and α-hydroxymidazolam were purchased from LGC Promochem (Germany). Rocuronium was obtained by dilution of a 10 mg/ml injection solution of Esmeron® (Organon, Switzerland).

The samples were pretreated by initial centrifugation of all specimens after initial addition of atracurium as internal standard. Proteins were precipitated by the addition of 0.5 mL of acetonitrile to 0.1 mL of putrefaction liquid, followed by vortexing and centrifugation of the mixture. The resulting supernatand was cleaned using Captiva columns (0.45 µm polypropylene filter, Varian). The alternative approach of a liquid-liquid-extraction of the samples with chlorobutane proved to result in significant higher amount of matrix and was not maintained.

Table 1. Mass spectrometric parameters of relevant substances included into the screening procedure

Substance	Precursor ion (Da)	Product ion (Da)	Declustering potential (V)	Collision Energy (eV)
Rocuronium	529	488	106	45
Rocuronium (doubly charged)	265	235	51	27
		244		23
Midazolam	326	291	76	37
		209		47
Hydroxymidazolam	342	203	76	37
		324		29
Diazepam	285	154	66	37
		193		43
Nordazepam	271	208	66	39
Oxazepam	287	241	41	37
		269		19
Etomidate	245	105	36	35
		141		15
Laudanosine (int. Std.)	358	206	66	27

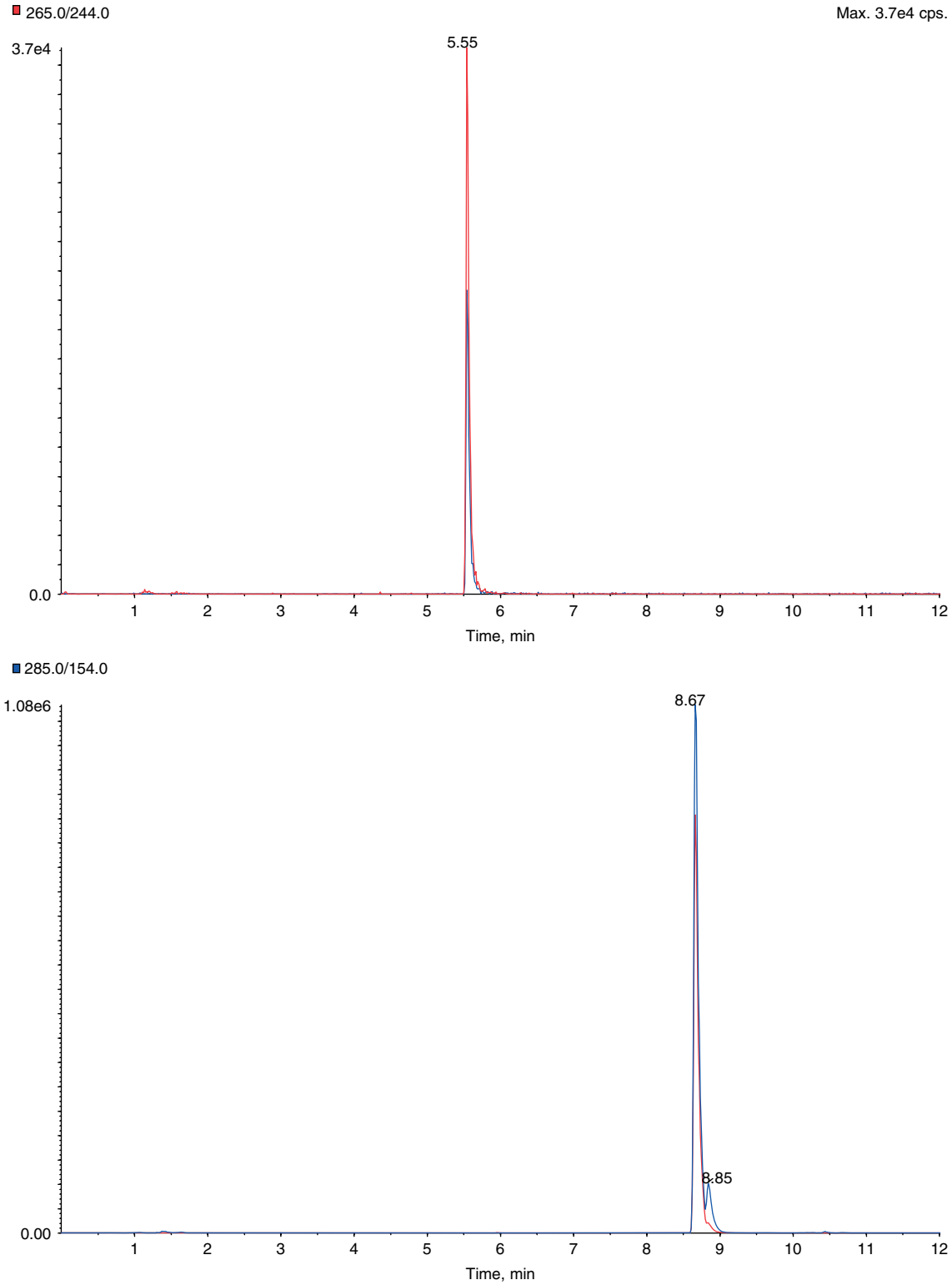

Figure 2. Detection of ROC (120 ng/mL) and DIA (~16 ng/mL) in putrefactive fluid. The presence of the metabolite nordazepam at concentrations greater than 0.1 ng/mL was excluded.

Liquid chromatography - electrospray ionisation - mass spectrometry

All LC-MS analyses were carried out using a 1100 LC system (binary pump and autosampler, Agilent, CA, USA) coupled to an API 4000 mass spectrometer (Applied Biosystems, CA, USA), equipped with a Turbo-Ion-Spray (ESI) source. The instrument software, Analyst (version 1.4.1), was used for data processing. Optimum ionisation and fragmentation conditions are summarised in Table 1. The LC was equipped with an Agilent Zorbax XDB-C18 analytical column (3 mm × 150 mm, 5 µm particle size).

The mobile phase consisted of (A) 0.1% (v/v) formic acid (AppliChem, Germany) with 5 mM ammonium formate (Fluka, Germany) and (B) acetonitrile (gradient grade) + water + formic acid (90 + 10 + 0.01, v/v/v) containing 5 mM ammonium formate. A linear gradient from 0% B (at t = 0–1 min) to 100% B (t = 9–12 min) was employed at a flow rate of 700 µL/min, compatible with a source temperature of 550 °C and source gas flow settings (nitrogen as sprayer and heater gas) of 50 psi. The injection volumes were 5 µl.

Screening concepts

Due to the heavy putrefaction of all specimens, presumptive post-mortem redistribution, dilution, microbial and chemical decompositions, none of the analytical results could be considered as quantitative data, suitable for comparisons with clinical reference data.

Instead, the qualitative identification of the compounds and collection of comprehensive analytical information (in particular the presence of biotransformation products and/or artefacts) was of primary importance. First attempts to carry out general unknown screening analyses by immunological, or liquid chromatography-diode array detection and gas chromatography-mass spectrometric techniques did not succeed owing to the large number of interfering matrix compounds. Instead, a multitarget analysis based on the pharmacological potential and the access to drugs (pharmaceuticals used in the respective hospital) was created. The muscle-relaxant rocuronium and the hypnotics ethomidat, midazolam, diazepam, nordazepam and oxazepam were included in the initial screening analysis according to the results of the initial criminal investigations. Later, the procedure was extended to other compounds with muscle-relaxant (atracurium, pancuronium) or potential sedative, narcotic or hypnotic effects (morphine, fentanyl, tramadol, haloperidol, propofol, ketamine and piritramid).

Results

Rocuronium

The unexplained presence of rocuronium (its detection without corresponding documented surgical operation) was the most significant analytical finding, indicating unauthorised and life-threatening administration of the drug. The substance could be identified in 16 cases. Two of them could clearly be traced back to authorised medical procedures a few days prior to death. Owing to the availability of a multitude of characteristic fragmentation reactions (Figure 1), a sensitive analytical screening procedure could be established (Figure 2). In all positive cases, 17-desacetylrocuronium was identified as an additional marker.

The maximum rocuronium concentration estimated in putrefied liquid of one of the cases was lower than 420 ng/mL (Figure 3). This is significantly lower than typical therapeutic serum concentrations, which are considered to be potentially lethal if mechanical ventilation is not provided. The low concentrations are likely to be due to post-mortem tissue redistribution, physical dissolution or chemical and microbial degradation. There is a clear tendency for concentrations to decrease with post-mortem time intervals (Figure 3). On the other hand, an elevated amount of the 17-desacetyl artefact (relative to ROC) was increased with increasing putrefaction intervals. This is consistent with the assumption[1] that 17-desacetyl-ROC represents mainly a degradation product rather than a metabolite.

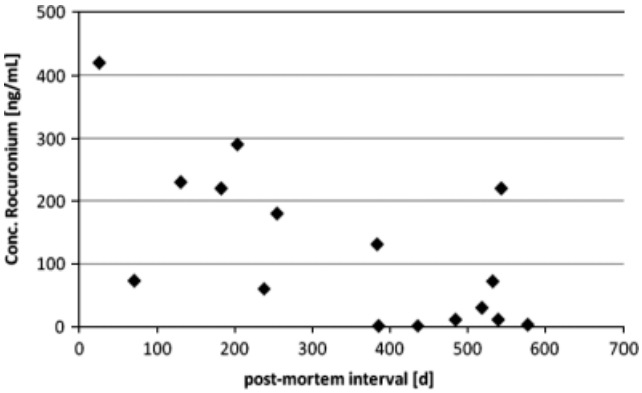

Figure 3. Correlation of rocuronium concentrations in putrefactive fluid and corresponding time intervals of decomposition in the grave.

Anaesthetics/hypnotics

The most interesting target substances with hypnotic effects, suitable to introduce anaesthesia and available in the relevant ward of the hospital were etomidate, midazolam and diazepam. These were identified in conjunction with its biotransformation products, nordazepam, oxazepam and a-hydroxy-midazolam. Among the 42 cases investigated in total there were three findings of etomidate (two of them iatrogenic), ten cases of midazolam detection (four iatrogenic) and 16 administrations of diazepam (two iatrogenic). The use of etomidate is strictly restricted to introduction of general anaesthesia and an ambulant therapeutic use of midazolam is rather infrequent in Germany. In contrast, diazepam is widespread and self-medication has to be carefully considered as an alternative origin of the drug.

The absence of adequate amounts of metabolites (such as nordazepam/diazepam) might be indicative of an acute poisoning, next to hepatic failures or genetic polymorphisms.[4–6]

Furthermore, the iatrogenic administration of other benzodiazepines (prazepam) and opioides (morphine, fentanyl and tramadol) could be confirmed. There were no other cases of undeclared substance administration. Interestingly, the identification of morphine (eight cases in total) was possible as long as 18 months post mortem.

Matrix effects

Matrix effects were a major concern in this particular case and it was not possible to compensate for them adequately because of the lack of deuterated standards and matrix heterogeneity. Special precautions were taken to proof the identity of rocuronium and to check the relevance of matrix effects (ion suppression). In each presumptive rocuronium case the identity was confirmed by monitoring the singly and doubly charged precursor ions (see Table 1). For verification purposes, the desacetyl artefact was detected by identification of a total of six product ions of its singly and doubly charged precursor ions.

In four cases exhibiting low concentrations of rocuronium (estimated amounts <20 ng/mL) the results were counter-checked in alternative specimens (lung and/or kidney).

The coincidental presence of the permanently charged quarternary ammonium ion and its protonated, doubly charged counterpart provided the opportunity to check the matrix-induced suppression of protonation by direct comparison of both ions.

A chromatogram of a blank matrix sample is enriched by a continuous post-column infusion of rocuronium (100 ng/min).

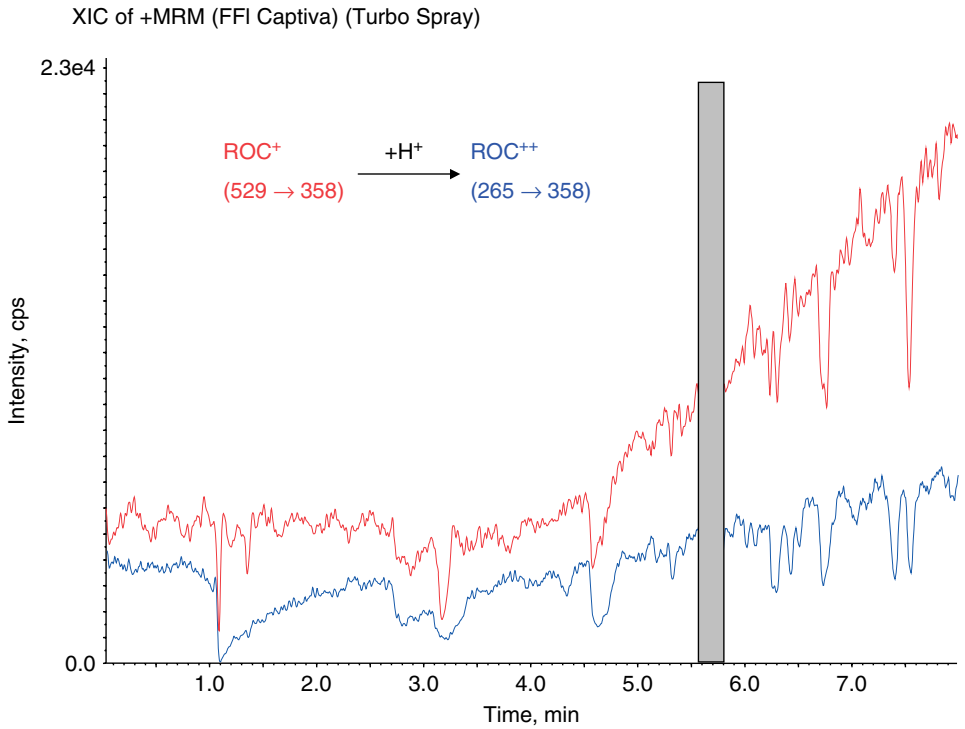

Figure 4. LC-MS/MS chromatograms of a blank matrix sample, overlaid by a post column infusion of rocuronium at a rate of 100 ng/min. Both fragmentation reactions are randomly affected by matrix compounds whereas the singly charged precursor ion (529 → 358, top) is apparently more robust than its protonated counterpart (265 → 358).

Figure 4 shows a comparison between singly and doubly charged precursor ions. Any variation in the signal abundance reflects interference by matrix constituents (ion suppression or enhancement). According to common consensus, ion suppression may be caused by a deterioration of spray formation and/or a reduction of ionisation efficacy.[7,8]

Logically, both ions are sporadically affected by coeluting matrix compounds while the permanently charged quarternary ammonium ion is more robust than the protonated precursor, in particular at low retention times. Matrix effects proved to be comparatively low near the eluting time of rocuroinium (5.55 min, compare Figure 2).

Therefore, the respective values were expected to be appropriate estimations of substance concentrations, present in the decomposed matrices.

Succamethonium

An analytical proof of the administration of succamethonium (Lysthenon ®), which was another highly suspicious candidate for poisoning in this case, could not be enabled. This is due to the rapid degradation to succinylmonocholine[9] and further to succinic acid.[10] Both decomposition products are (at least partially) endogenous and accepted to be no indicator for external substance administration in post mortem cases.

Case evaluation

Following analytical procedures, the cases were divided into four categories:

- All substances detected (if any) could be allocated to therapeutic measures according to clinical records, no indication of illegal manipulations (14 cases).
- Hypnotics, which could not be traced back to therapeutic measures, were detected. The presence of these compounds indicated unauthorised substance administration but could not explain respective fatalities. A co-administration of succamethonium is suspected in these nine cases.
- Thirteen cases of detection of rocuronium as potential cause of deaths, mostly in combination with hypnotics.
- Six cases with suspicious analytical findings, which could not be sufficiently clarified due to incomplete documentation of medications.

Discussion

The unequivocal identification of the muscle-relaxant rocuronium and various hypnotics in putrefactive liquids was able to clarify of a series of poisonings in a hospital. Liquid chromatography-mass spectrometry proved to be adequate for the detection of trace amounts of the target compounds despite severe alterations and heterogeneity of specimens. Estimated concentrations are significantly affected by time-dependant decomposition and redistribution and do not permit quantitative conclusions to initial serum concentrations, doses, or pharmacological effects.

A male nurse was convicted of 12 cases of murder, 15 cases of manslaughter and one killing 'by request' (active euthanasia).

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