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Article

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Comparison of Comprehensive Screening Results in Postmortem Blood and Brain Tissue by UHPLC–QTOF-MS

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Abstract

Alternative specimens collected during autopsies can be valuable in postmortem toxicology in cases where peripheral blood is not available. The applicability of brain tissue as an alternative matrix for drug screening by ultra-high-performance liquid chromatography–quadrupole time-of-flight mass spectrometry was investigated in this study. Results of the 50 most frequently detected drugs and metabolites of toxicological interest in blood and brain tissue samples from 1,719 autopsy cases were compared. Examination of the results in paired blood and brain tissue samples revealed that the two matrices were in general comparable, as the majority of the 50 analytes were observed in a high number of the examined cases in both blood and brain tissue. This demonstrates the potential of brain tissue as an alternative matrix for drug screening in postmortem toxicology or as a secondary matrix for confirmation.

Introduction

In postmortem toxicology, a variety of specimens are collected as autopsy material, where peripheral blood is the most frequently used (1). However, drug concentrations in blood may change compared with the antemortem situation due to postmortem redistribution and/or decomposition and thus interpretation may be difficult (2–5). Investigation of an alternative matrix on which to base a conclusion is therefore beneficial. Brain tissue has been suggested as a suitable alternative to blood (6, 7). The brain's position relative to other organs in the body is secluded and protected to some extent, consequently delaying putrefaction and/or postmortem redistribution from other tissues. The entry of drugs to the brain is dependent on diffusion across the blood–brain barrier, which can occur for lipophilic compounds with a low molecular weight (8). As such, measurements in brain tissue may give lower pre-analytical sampling variation compared with blood (9–11). Positron emission tomography scans of the brain revealed that distributions of drugs are more homogenous within the brain compared with the remaining body (12). The brain is the primary site of action for a number of drugs, and measurements in the target organ can therefore be preferable (10, 13).

Comprehensive screening that encompasses detection, identification and confirmation of various drugs of toxicological interest is of high importance in forensic toxicology. Targeted screening of known compounds using mass spectrometry techniques is often used in routine analysis to facilitate unequivocal identification. The use of an ultra-high-performance liquid chromatography–quadrupole time-of-flight mass spectrometry (UHPLC–QTOF-MS) instrument has proven to be a suitable tool when screening for a large number of drugs of toxicological interest in complex biological matrices (14–19). It relies on the development of a spectral library through the acquisition of compound information such as exact mass, isotopic pattern, retention time (RT) and fragmentation data using certified reference standards.

In this study, the use of brain tissue as an alternative matrix for drug screening in postmortem toxicology using UHPLC–QTOF-MS is examined. Results obtained from 1,719 paired blood and brain tissue samples from forensic autopsy cases from a 7-year period were compared for the 50 most frequently detected drugs and metabolites of toxicological interest.

Materials and Methods

Chemicals and reagents

Reference standards including the internal standards diazepam-d_5, morphine-d_6, methadone-d_3, mianserin-d_3 and amphetamine-d_5 or MDMA-d_5 were purchased from Lipomed GmbH (Arlesheim, Switzerland), Cerilliant (Round Rock, TX, USA) or Toronto Research Chemicals (Toronto, ON, Canada). All reference standards were of ≥98% purity, as stated in the certificates. Acetonitrile (≥99.9%), methanol (≥99.9%), purified water, 2-propanol (≥99.5%) and ammonium formate (≥99.9%) were obtained from Fisher Scientific (Loughborough, UK) and were all LC–MS grade. Formic acid (98–100%) was purchased from Merck

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(Damstadt, Germany). Leucine enkephalin acetate was purchased from Sigma-Aldrich (St. Louis, MO, USA).

Blood and brain tissue samples

At the Department of Forensic Medicine, decedents were stored at 5°C prior to autopsy, which is generally performed within 1–4 days after decedent delivery. A total of 1,719 routine forensic autopsy cases performed from 2014 to 2021 formed the basis for the study. They include cases where screening was performed in both blood and brain homogenate as an ordinary part of routine toxicological investigations. Femoral whole-blood samples taken during autopsy without proximal ligation of the iliac vein. All blood samples (typically 4–8 mL) were preserved with 100 mg sodium fluoride and 22.50 mg potassium oxalate. Brain tissue samples of 5–20 g were collected from the frontal cerebral cortex and stored without preservatives. All samples were stored at –20°C prior to extraction and analysis.

Sample preparation

In all brain tissue samples, gray and white matter were separated manually with a scalpel, and only the gray matter was analyzed. Approximately 0.50 g of brain tissue was diluted with LC–MS-grade water in a ratio of 1:3 in M tubes using a gentleMACS dissociator from Miltenyi Biotec (Bergisch Gladbach, Germany) with a 50-second program applying up to 4,000 rpm. The samples were then centrifuged at 2,000 *g* and 20°C for 10 minutes, and the supernatant was transferred to other tubes. The whole-blood samples were used without dilution.

Preparation of whole-blood and brain homogenate samples was performed using an automated protein precipitation method on a Tecan Freedom EVO 200 robotic platform (Tecan, Männedorf, Switzerland), as described previously for blood analysis (20, 21). From each sample of either whole blood or brain homogenate, an aliquot of 0.100 g was transferred to a 96-well plate. All samples were spiked with 20 µL of internal standard solution followed by 700 µL acetonitrile for protein precipitation and shaken. The deep-well plate was then centrifuged at 1,000 *g* for 10 minutes. Fifty microliters of 10% formic acid in acetonitrile was added to the supernatants, which were evaporated to dryness under a gentle flow of nitrogen heated to 35°C. The dried eluate was then reconstituted in 100 µL water:methanol:formic acid (74:25:1, v/v/v) and shaken. Afterward, the samples were centrifuged at 1,000 *g* for 10 minutes, and 45 µL of the supernatants was transferred to individual wells of a new 96-well plate and analyzed on the UHPLC–QTOF-MS system.

Instrumentation

The screening was performed using an UHPLC–QTOF-MS system consisting of an ACQUITY UPLC I-Class coupled to a Xevo G2-S QTOF (Waters, Milford, MA, USA). Chromatographic separation was achieved using an ACQUITY UPLC® HSS C_{18} (1.8 µm 2.1 × 150 mm) column (Waters) maintained at 50°C and a flow rate of 0.4 mL/min. The mobile phases consisted of 5 mM aqueous ammonium formate buffer adjusted to pH 3 with formic acid (A) and 0.1% formic acid in acetonitrile (v/v) (B). The gradient was 13% solvent B (0–0.5 minutes), 13–50% solvent B (0.5–10.0 minutes), 50–95% solvent B (10.0–10.75 minutes), 95% solvent B (10.75–12.25 minutes), 95–13% solvent B (12.25–12.5 minutes) and 13% solvent B (12.5–15 minutes). The injection volume was 3 µL.

The mass spectrometer was operated with a Z-spray in positive electrospray ionization mode (ESI+) using the following source conditions: desolvation gas flow 800 L/h, desolvation temperature of 400°C, cone gas flow 20 L/h, source temperature 150°C, capillary voltage 0.80 kV, cone voltage 25 V and argon as the collision gas. Data were acquired using data-independent acquisition mode with elevated collision energy. The low collision energy was set at 4 eV, while the high collision energy was set to a collision energy ramp from 10 to 40 eV. The acquisition time was the entire run (15 minutes), with a scan time of 0.200 seconds. The range of mass-to-charge (*m/z*) was 50–950. Mass calibration was performed weekly with 5 mM sodium formate solution in 2-propanol:water (90:10, v/v). Lock mass correction was performed with leucine enkephalin as reference mass at *m/z* 556.2766.

Data processing

All samples were processed using UNIFI Scientific Information System (Waters) software as described by Mollerup et al. (16). The targeted screening was achieved using a library. All targets in the library consisted of a molecular formula and structure, expected RT, and the exact masses of precursor and product ions. The targeted screening library used was an in-house expansion of the Forensic Toxicology Screening Application Solution with UNIFI from Waters Corporation. Targeted identification was performed using mass tolerance of 3 mDa for precursor and product ion and observed RT within ±0.5 min.

The 50 analytes that were identified as positive in either blood, brain tissue or both in the highest number of samples were further investigated. Only one metabolite per parent drug was included in the investigated data.

Results and Discussion

The analysis of the paired samples selected from 1,719 autopsies revealed that 1,473 of the cases were positive for at least one analyte from the screening library. A case was overall considered positive for a given analyte if it was detected in either the blood sample or brain tissue sample or both. Results from all samples assessed as positive were included in this study, where the 50 most frequently detected analytes, which included 18 metabolites and 2 adulterants, were further investigated. A summary of the positive cases in blood and brain tissue for each analyte can be seen in Table I. Of the 1,473 positive cases in total, 1,411 cases contained at least one of the 50 investigated analytes. Here, a total of 6,859 and 6,323 positive hits were observed in blood and brain homogenate, respectively.

Paracetamol (acetaminophen), a widely used over-the-counter analgesic and antipyretic, exhibited the highest frequency in both blood and brain tissue. It was present in a total of 745 cases, corresponding to 43% of all analyzed cases. It was detected in both matrices in the majority of the cases, with 716 (96%) positive cases in blood and 673 (90%) positive cases in brain tissue. However, in a few cases, paracetamol was only detected in either blood or brain tissue. Overall, this

Table I. Occurrence of the 50 Most Frequently Detected Drugs of Toxicological Interest in Paired Whole Blood and Brain Tissue Samples from 1,719 Autopsy Cases

Analyte	Cases positive in blood and/or brain tissue	Cases positive in blood (%)	Cases positive in brain tissue (%)
7-Aminoclonazepam	241	240 (100%)	199 (83%)
7-Hydroxyquetiapine	109	109 (100%)	78 (72%)
Amphetamine	85	79 (93%)	83 (98%)
Amiodarone	71	69 (97%)	59 (83%)
Amlodipine	106	104 (98%)	100 (94%)
Benzoylecgonine	279	258 (92%)	252 (90%)
Cetirizine	59	59 (100%)	39 (66%)
Chlordiazepoxide	104	103 (99%)	96 (92%)
Chlorprothixene	69	68 (99%)	62 (90%)
Citalopram	126	109 (87%)	126 (100%)
Cocaine	259	222 (86%)	221 (85%)
Codeine	182	180 (99%)	127 (70%)
Demoxepam	136	136 (100%)	122 (90%)
Diazepam	172	171 (99%)	149 (87%)
EDDP[a]	345	341 (99%)	314 (91%)
Fentanyl	94	90 (96%)	77 (82%)
Gabapentin	116	111 (96%)	110 (95%)
Lamotrigine	60	47 (78%)	59 (98%)
Levamisole	173	164 (95%)	141 (82%)
Lidocaine	238	215 (90%)	190 (80%)
Losartan	64	64 (100%)	7 (11%)
Methadone	415	360 (87%)	401 (97%)
Metoprolol	123	117 (95%)	121 (98%)
Metronidazole	59	57 (97%)	35 (59%)
Mirtazapine	112	107 (96%)	112 (100%)
Monoethylglycinexylidide	135	125 (93%)	117 (87%)
Morphine	188	181 (96%)	132 (70%)
N-Desethylamiodarone	60	59 (98%)	25 (42%)
N-Desmethylchlorprothixene	66	66 (100%)	62 (94%)
N-Desmethylcitalopram	119	110 (92%)	117 (98%)
N-Desmethylmirtazapine	111	108 (97%)	109 (98%)
N-Desmethylolanzapine	110	110 (100%)	98 (89%)
N-Desmethylzopiclone	108	108 (100%)	35 (32%)
Nordiazepam	256	252 (98%)	230 (90%)
Noroxycodone	110	110 (100%)	85 (77%)
O-Desmethyltramadol	155	148 (95%)	145 (94%)
O-Desmethylvenlafaxine	73	61 (84%)	70 (96%)
Olanzapine	114	109 (96%)	109 (96%)
Ondansetron	53	48 (91%)	48 (91%)
Oxazepam	74	74 (100%)	56 (76%)
Oxycodone	112	108 (96%)	108 (96%)
Paracetamol	745	716 (96%)	673 (90%)
Pregabalin	59	58 (98%)	57 (97%)
Promethazine	71	69 (97%)	71 (100%)
Promethazine sulfoxide	68	62 (91%)	63 (93%)
Quetiapine	164	144 (88%)	161 (98%)
Sertraline	90	89 (99%)	89 (99%)
Tramadol	211	172 (82%)	194 (92%)
Venlafaxine	76	60 (79%)	74 (97%)
Zopiclone	132	132 (100%)	115 (87%)

[a]EDDP: 2-ethylidene-1,5-dimethyl-3,3-diphenylpyrrolidine.

demonstrates that a brain tissue sample was often identified as positive in cases where the corresponding blood sample was also positive with regard to paracetamol. Likewise, the same tendency was observed for the majority of the investigated 50 analytes.

As a measure of how well the results of each analyte in blood and brain tissue correlate, the percentages of positive cases in each matrix were compared. The results of each analyte were considered highly positive, when ≥85% of the cases in the given matrix were positive. An illustration of the relation can be seen in Figure 1. High percentages (≥85%) were observed in both blood and brain tissue. For 30 of the 50 analytes, the number of positive cases was ≥85% in both matrices, as seen in the upper right corner of Figure 1. For 16 analytes, the percentages of positive cases were ≥85% in blood and <85% in brain tissue (bottom left corner of Figure 1), whereas four analytes had results ≥85% in brain tissue and <85% in blood (upper right corner of Figure 1). These numbers show that the two matrices correlate well since most results of the measurements were observed in a high number of the examined cases in both blood and brain tissue with very few exceptions. Overall, this indicates that brain tissue is a good secondary choice of matrix for drug screening and can be useful in cases where whole blood is lacking or as a second matrix for confirmation.

A few of the analytes were not observed in both blood and brain tissue in the majority of the positive cases. Two analytes, lamotrigine and venlafaxine, were only detected in <80% of the cases in blood, where the corresponding brain tissue samples tested positive. However, some analytes were not detected as frequently in brain tissue samples compared with blood. Losartan, *N*-desethylamiodarone and *N*-demethylzopiclone were detected in <50% of the cases in brain tissue, where blood samples were tested positive (Figure 1). Likewise, the parent drugs, amiodarone and zopiclone, were more often detected in blood but not to the same extent as in their metabolites. A possible explanation for this could be the dilution factor, since the brain tissue samples are diluted by a factor of 4. The presence of an analyte in blood but its absence in the corresponding brain tissue sample could therefore be due to insensitivity because of the lower sample volume of brain tissue (100 mg of blood analyzed vs. 100 mg brain homogenate containing 25 mg brain tissue analyzed). In addition, other explanations could be limited crossing of the blood–brain barrier, either because of physicochemical characteristics or active transport out of the brain by P-glycoprotein or other transporters (4, 22).

The total ion chromatogram (TIC) for both matrices were investigated, in a case where no analytes were detected (Figure 2) and in a case where 13 analytes were detected (Figure 3). The results showed that the TIC for brain homogenates (bottom) was visually similar compared with that of blood (top), which also supports the assumption that screening in blood and brain tissue can often provide comparable results.

Hubbard et al. (23) and Metushi et al. (24) compared screening results in whole blood with vitreous humor for seven acidic drugs (89 cases) and 71 basic drugs (51 cases), respectively. Both studies concluded that vitreous humor can be an alternative matrix for qualitative drug screening, since most of the detected compounds in blood could be identified in vitreous humor as well. There is no definitive second choice of matrix when it comes to drug screening; nevertheless, results indicate that both vitreous humor and brain tissue are applicable.

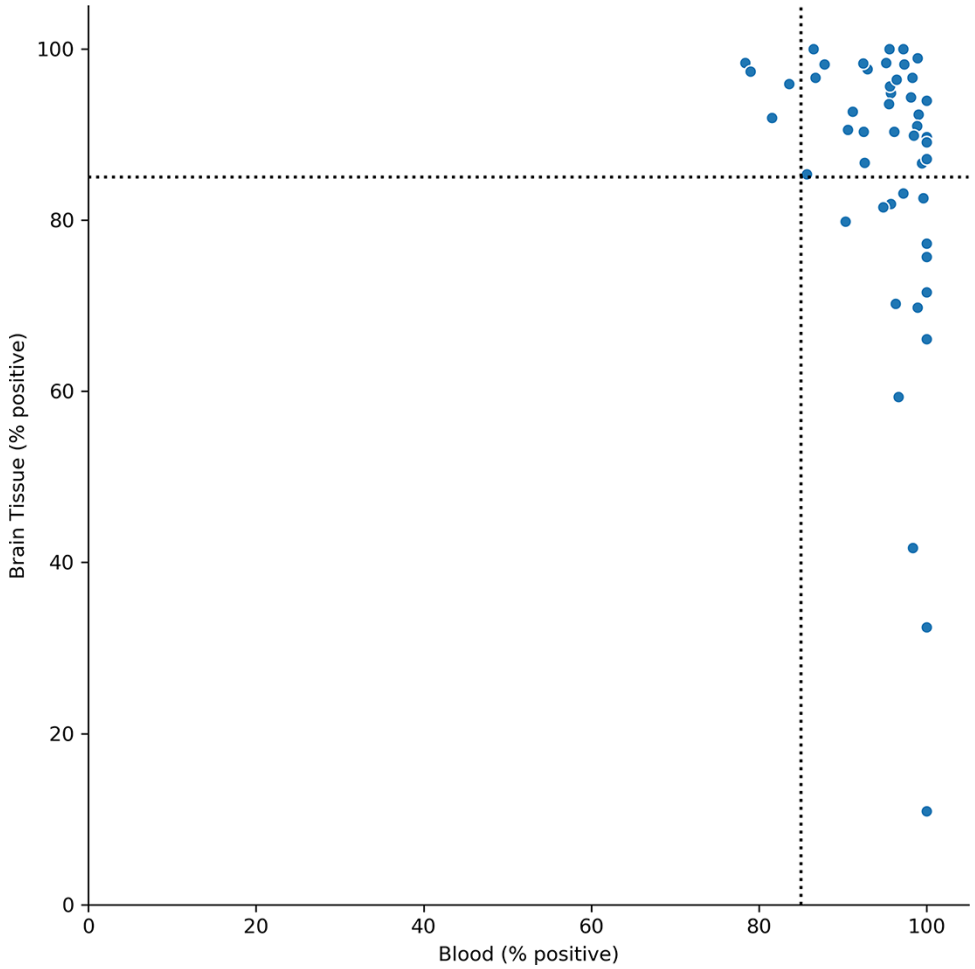

Figure 1. Paired percentages of all positive cases for whole blood (x-axis) and brain tissue (y-axis) for the 50 most frequently detected analytes. The black dotted lines (vertical and horizontal) represent 85%.

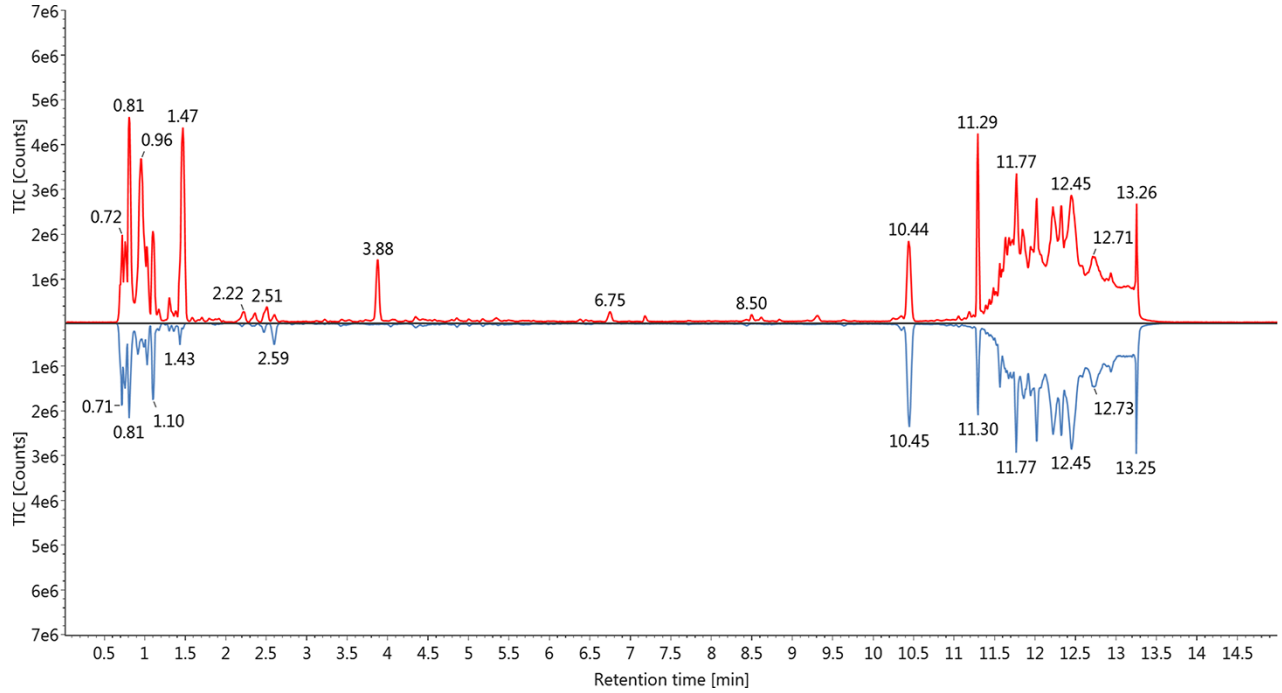

Figure 2. TIC of an authentic case in blood (top) and brain homogenate (bottom) where no analytes were detected.

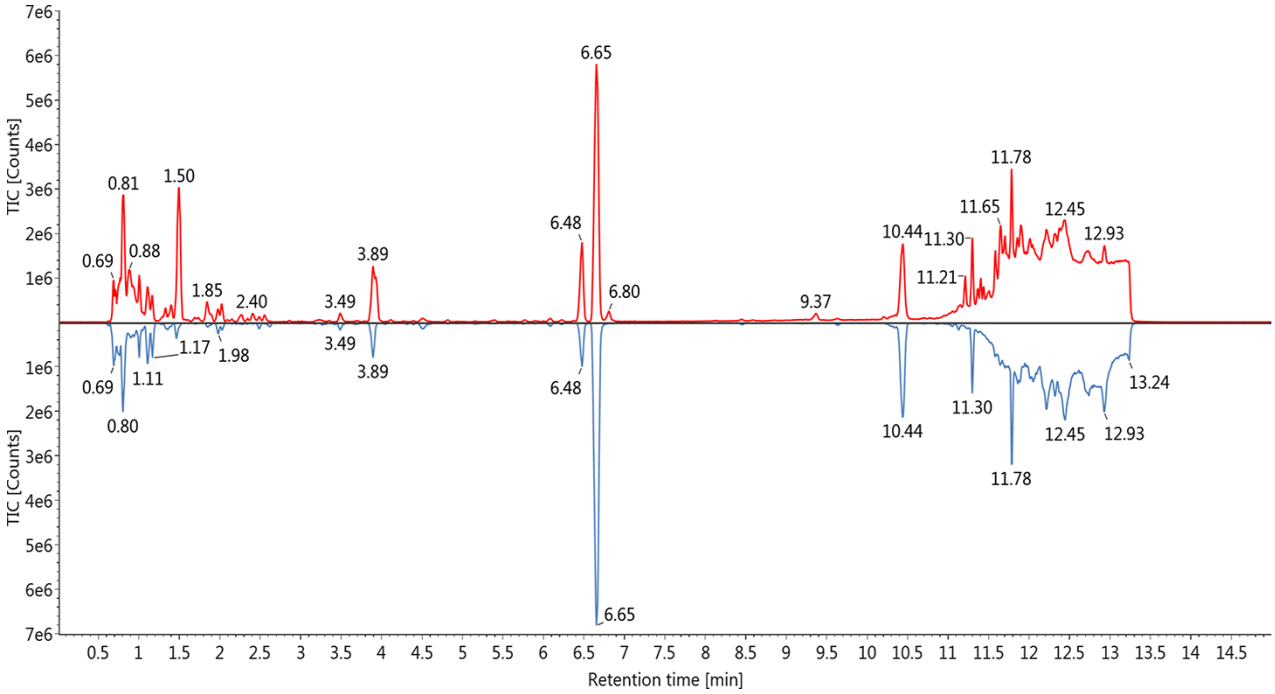

Figure 3. TIC of an authentic case in blood (top) and brain homogenate (bottom) where paracetamol (RT: 1.45), codeine (RT: 1.69), *N*-desmethylolanzapine (RT: 1.83), pregabalin (RT: 1.85), olanzapine (RT: 1.98), *N*-desmethylzopiclone (RT: 3.36), zopiclone (RT: 3.49), metoprolol (RT: 3.89), *N*-desmethylcitalopram (RT: 6.47), citalopram (RT: 6.66), demoxepam (RT: 6.86), nordiazepam (RT: 8.85) and diazepam (RT: 10.36) were detected.

Conclusion

Comparison of the results obtained in paired blood and brain tissue samples showed that screening in the two matrices can often provide comparable results, since most of the 50 most frequently detected analytes often were identified as positive in brain tissue samples where the corresponding blood sample was also positive. Brain tissue therefore appears to be a good alternative matrix for drug screening in postmortem toxicology, which can be useful in cases where blood is not available, as well as a suitable second matrix for confirmation.

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