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ORIGINAL ARTICLE

Determination of antidepressants in human postmortem blood, brain tissue, and hair using gas chromatography–mass spectrometry

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Abstract A gas chromatographic–mass spectrometric (GC–MS) method in positive ion chemical ionization mode in combination with a solid phase extraction was optimized for new-generation antidepressants and their metabolites in postmortem blood, brain tissue, and hair. Twelve antidepressants and their active metabolites (i.e., mirtazapine, viloxazine, venlafaxine, citalopram, mianserin, reboxetine, fluoxetine, fluvoxamine, sertraline, maprotiline, melitracen, paroxetine, desmethylfluoxetine, desmethylmianserin, desmethylmirtazapine, desmethylsertraline, desmethylmaprotiline, desmethylcitalopram, and didesmethylcitalopram) could be quantified. In this article, in addition to the validation of the GC–MS method, four postmortem cases are discussed to demonstrate the usefulness of the described method in forensic toxicology. In these cases, sertraline, fluoxetine, citalopram, and trazodone in combination with their active metabolites were quantified. Blood concentrations ranged from subtherapeutic to toxic concentrations, while brain to plasma ratios ranged from 0.8 to 17. Hair concentrations ranged from 0.4 to 2.5 ng/mg depending on the compound and hair segment.

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Introduction

The new generation of antidepressants (ADs) is known to have a low toxicity profile as acute intoxication with these ADs is rare. These highly prescribed drugs are, however, frequently used together with other legal or illegal drugs and can result in synergy of symptoms and intoxications [1–12]. Furthermore, severe life-threatening interactions such as the serotonin syndrome have been described [13–16]. Therefore, analytical methods for the detection of ADs in blood and tissues are of interest in the field of forensic toxicology.

In forensic toxicology, whole blood is the main postmortem matrix as it provides a direct link between the compound concentration and effect. Brain tissue has several advantages over blood as it is an isolated compartment in which putrefaction can be delayed. In addition, the metabolic activity is lower resulting in a more prominent presence of the original compounds as compared to degradation products [17]. Hair analysis is a complementary approach as it yields a picture of long-term exposure over a time window of several months. Moreover, the sample can be stored at room temperature for a long time without degradation [18–20].

Several chromatographic methods have been developed for the determination of ADs in biological matrices [21]. Gas chromatography–mass spectrometry (GC–MS) with electron ionization (EI) is the preferred technique for drug analysis in forensic toxicology because of the possibility of

compound identification through commercially available libraries. However, due to the extensive fragmentation of some ADs using EI, the positive ion chemical ionization mode (PICI) could provide more selectivity, especially in complex matrices such as brain tissue [22–24]. Most methods determining ADs in brain tissue or blood use liquid–liquid extraction for sample preparation due to clogging of sorbents during solid phase extraction (SPE). SPE, however, has several advantages such as high efficiency and decreased solvent volumes. While some publications deal with the extraction of (tricyclic) ADs from hair [25–28], it has not yet been described for brain tissue.

Therefore, the objective of this study was to develop and validate an SPE and a GC–MS method in PICI mode for simultaneous determination of several ADs and their active metabolites in human blood, brain tissue, and hair. Trazodone and its metabolite *m*-chlorophenylpiperazine (*m*-cpp) were analyzed using a high-performance liquid chromatography–diode array detection (HPLC–DAD) method due to chromatographic problems and irreproducible quantification results for trazodone using GC–MS analysis [22]. In addition, the implementation of the analytical techniques is discussed in postmortem forensic cases.

Materials and methods

Chemicals and reagents

The AD standards are described in Table S1 ("Electronic Supplementary Material"). The derivatization reagent, 1-(heptafluorobutyryl) imidazole (HFBI), was purchased from Sigma-Aldrich (Steinheim, Germany), while Promochem (Molsheim, France) delivered fluoxetine d_6 oxalate, mianserin d_3 (Md_3), and paroxetine d_6 maleate (100 μg/ml in MeOH). The following reagents were purchased from Merck (Darmstadt, Germany): ammonia solution (25%), orthophosphoric acid (85%), sodium dihydrogen phosphate monohydrate, potassium carbonate, sodium hydroxide, methanol, acetonitrile and water (HPLC grade), and toluene (Suprasolv). The strong cation exchanger (SCX) with 200-mg sorbent mass was obtained from Phenomenex (Bester, Amstelveen, The Netherlands). Drug-free blood was obtained from healthy volunteers. Drug-free postmortem brain tissue and case samples were obtained from the Department of Forensic Medicine (Ghent University, Belgium).

Preparation of standard solutions and calibrators

Primary stock solutions of each individual AD, the standard mixture, and the working solutions were prepared in methanol and stored at −20°C (Table S1). Primary stock solutions of each internal standard (IS) were prepared in methanol at a concentration of 10 μg/ml and were stored protected from light at 4°C. For the preparation of sample calibrators, working solutions were spiked to 1 ml of blood or 1 g of brain tissue. Calibrators ranged from the sub (10 ng/ml) to high therapeutic concentrations (500 ng/ml) of the individual ADs in blood. For brain tissue, calibration ranged from 50 to 1,000 ng/g. A 50-μl Hamilton injection needle was used to spike the compounds directly into the brain tissue. Samples were equilibrated at −4°C overnight after spiking.

Sample preparation

IS (200 ng) was added to the samples before the SPE procedure published previously [29]. Before SPE, blood samples were diluted with 4 ml of phosphate buffer (pH 2.5; 25 mM), sonicated for 15 min, and submitted to the SPE tube. Brain tissue was mixed after addition of 2 ml of acetonitrile and 0.5 ml of potassium carbonate buffer (1 M pH 9.5) and centrifuged for 15 min at 1,850×*g*. The supernatant was removed, diluted with phosphate buffer (pH 2.5; 25 mM), and adapted to pH 2–3 with orthophosphoric acid before loading onto the sorbent bed. Hair samples were washed in HPLC-grade water (5 min) and rinsed three times with 1 ml methanol. Thereafter, they were cut in segments of approximately 2 cm. The hair fragments were digested in sodium hydroxide solution (1 M, 1 ml) for 10 min at 100°C and diluted with phosphate buffer, and the pH was adapted to 2–3 with orthophosphoric acid. If compounds were not stable in the sodium hydroxide solution, hair fragments were soaked in 4 ml of phosphate buffer (pH 2.5, 25 mM) for 18 h at 55°C and sonicated for 1 h before SPE.

After evaporation of the solid phase extracts under nitrogen at 40°C, 50 μl of HFBI was added at 85°C for 30 min. Thereafter, an extraction with 0.5 ml of HPLC-grade water and 2 ml of toluene occurred. After centrifuging the sample at 1,121×*g* for 5 min, the toluene layer was separated and evaporated at 40°C. The residue was resolved in 50 μl of toluene and 1 μl was injected onto the GC–MS. For HPLC analysis, the extracts were not derivatized but directly resolved in 140 μl starting eluent of the HPLC method and 50 μl was injected on the column.

Gas chromatography–mass spectrometry

An HP 6890 GC system was used equipped with an HP 5973 mass-selective detector and an HP 7683 split–splitless autoinjector (Agilent Technologies, Avondale, PA, USA). The chromatographic as well as the mass-selective parameters using PICI were previously described by our research group [22].

High-pressure liquid chromatography–diode array detection

A LaChrom HPLC (Merck-Hitachi, Darmstadt, Germany), consisting of an L1700 pump, an L7200 autosampler, an L7360 column oven, and an L7455 DAD, was used. A PurospherStar RP-8 end-capped 4×4-mm guard column combined with a C8 end-capped PurospherStar (Merck, Darmstadt, Germany) LiChroCART 125–4 mm ID (5 μm) column was applied. The gradient run started at 95% A (860 ml water–40 ml phosphate buffer, 250 mM, pH 2.3–100 ml methanol) and 5% B (40 ml phosphate buffer–210 ml water–750 ml methanol). At 8 min, the B phase contribution was 25%, and at 16 min 55%. Then, during 8 min, the gradient was switched to 95% B. After 5 min, the run was switched to the starting conditions and equilibrated for 12 min before the next injection (total run time, 30 min). The DAD measured from 220 to 350 nm and chromatograms were integrated at 230 nm.

Method validation

We recently published the validation of this GC–MS method for ADs in plasma in EI, PICI, and negative ion chemical ionization mode [22] based on the Food and Drug Administration guidelines [30]. This method was revalidated for postmortem blood and brain tissue. For hair samples, only selectivity was checked, as spiking hair samples does not reflect reality (no drug incorporation) and would lead to false recovery and sensitivity results.

Results

Method development

Our previously published SPE procedure for ADs and their metabolites from human plasma was adapted for blood, brain tissue, and hair [29]. For the *blood samples*, dilution of the sample with phosphate buffer resulted in disruption of the ADs' protein binding and in positively charged ADs interacting with the SCX. However, in contrast to plasma samples, diluted blood samples were not centrifuged as this leads to irreproducible and lower extraction efficiencies. Coprecipitation of ADs with the red blood cell fragments probably leads to this recovery loss, as amphiphilic ADs are bound to proteins attached to the red blood cell membranes [31, 32]. For *brain tissue*, the sample preparation had to be modified due to the solid nature and the high fat content of the matrix. ADs were extracted from the mixed brain sample using acetonitrile and potassium carbonate. After centrifugation, the pH was adjusted to 2.5 using orthophosphoric acid to assure ideal SPE conditions. *Hair samples* were first washed to remove possible external contamination. As compounds are incorporated in the hair through diffusion from blood, sweat, or sebum, the solid hair matrix should be destroyed or the compounds should be extracted from that matrix. Digestion of hair using 1 M sodium hydroxide is a commonly used method for basic drugs [19, 25, 26]. A temperature of 100°C was chosen as the hair segments were digested in only 10 min, leading to a shorter contact in the alkaline medium. However, venlafaxine (30% loss), citalopram (CIT), desmethylcitalopram (DMC), and didesmethylcitalopram (DDMC; 60–93% loss) were unstable in this medium and therefore, for these compounds, the hair segments were not digested but soaked in phosphate buffer (25 mM, pH 2.5). Under these conditions, the basic ADs will be protonated and diffused from the hair. No degradation was observed in this acid environment, except for fluvoxamine (30% loss).

Validation

Stability of heptafluorobutyryl derivatives and stock solutions was already described by our research group [22]. Compound stability in blood and brain samples was reevaluated. Freeze–thaw stability of *m*-cpp, viloxazine, desmethylfluoxetine, melitracen, desmethylsertraline (DMSer), reboxetine, citalopram, maprotiline, and desmethylmaprotiline (58–83% of the initial value) did not fulfill the acceptance criteria in blood. However, this instability was not seen at medium and high AD blood levels. For brain tissue, freeze–thaw instability was seen for *m*-cpp and citalopram, while short-term instability was only seen for *m*-cpp. Overall, the stability is acceptable for most ADs in blood and brain tissue.

The SCX extraction leads to reproducible and high recoveries for most compounds in blood (Table S2; "Electronic Supplementary Material"), ranging between 73% and 106%, except for venlafaxine (51%). The recovery from brain tissue was slightly lower than for blood, but reproducible. Especially, venlafaxine and fluvoxamine show low extraction efficiencies.

Blank blood samples ($n=5$) were analyzed for endogenous interference. As shown in Fig. 1a, no significant endogenous interferences were seen, except for venlafaxine. Eighteen blank brain tissue samples were checked for interference and thus selectivity of the method. These samples were obtained from three different individuals at six different locations in the brain (Fig. 1b). Two blank hair samples were analyzed (Fig. 1c) and no interference was seen.

Limits of quantitation (LOQs; Table S2) gave a signal to noise >10, a precision less than 20%, and acceptable accuracy. For brain tissue, citalopram and reboxetine had a variation in precision at LOQ level slightly above 20% at the indicated spiked concentrations (25–62.5 ng/g).

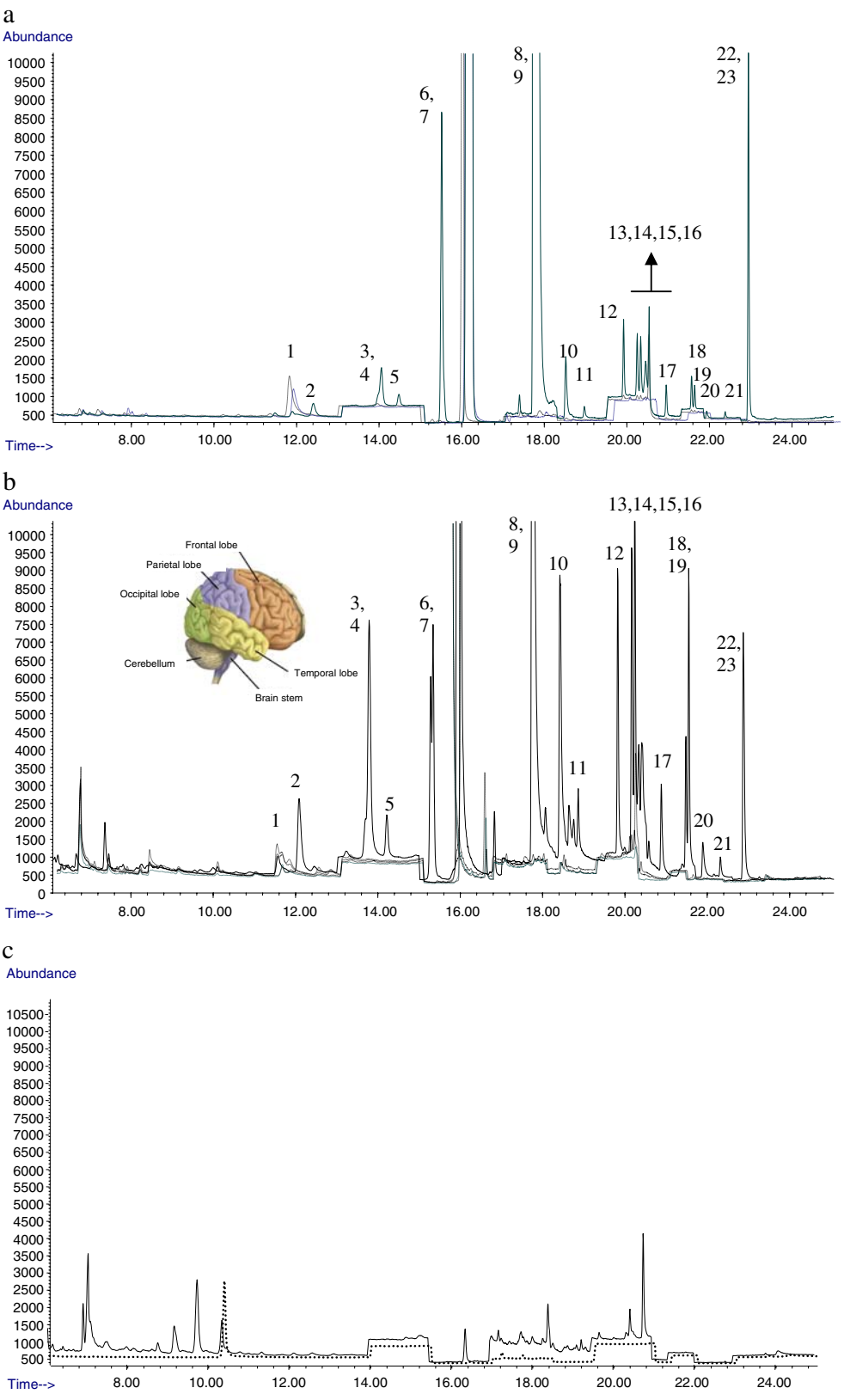

Fig. 1 GC–MS chromatograms of blank blood (**a**), brain tissue (**b**), and hair (**c**) combined with a low-concentration sample of 20 ng/ml in blood and 200 ng/g for brain tissue. **c** *Full line*: blank hair using sodium hydroxide; *dotted line*, blank hair using phosphate buffer. *1*, venlafaxine; *2*, *m*-chlorophenylpiperazine; *3*, desmethylfluoxetine; *4*, viloxazine; *5*, fluvoxamine; *6*, fluoxetine; *7*, fluoxetine d_6; *8*, mianserin; *9*, mianserin d_3; *10*, mirtazapine; *11*, melitracen; *12*, desmethylmianserin; *13*, desmethylsertraline; *14*, desmethylmirtazapine; *15*, reboxetine; *16*, citalopram; *17*, desmethylmaprotiline; *18*, maprotiline; *19*, sertraline; *20*, didesmethylcitalopram; *21*, desmethylcitalopram; *22*, paroxetine; *23*, paroxetine d_6

In blood, the intrabatch and interbatch precisions were acceptable for all compounds, except for sertraline at high concentration (19%). Intrabatch precision for brain tissue samples was acceptable, except the intrabatch precision for citalopram at low concentrations (16%).

The low concentrations were underestimated for most compounds in PICI mode in blood. Accuracy ranged from 71% to 117% at low concentrations (mean 88%). This phenomenon was not seen in brain tissue. Accuracies of ADs at medium and high concentrations were all in line with the validation guidelines.

Case studies

Urine, stomach contents, and blood of the postmortem cases described were screened using our laboratory systematical toxicological analysis system. Blood, brain, and hair samples (if available) were analyzed by GC–MS after SPE. Trazodone and *m*-cpp (cases 3 and 4) were analyzed with the HPLC system after the described SPE. An overview of the most important autopsy findings is presented in Table 1. A summary of the toxicological results can be found in Table 2.

Case 1

A 92-year-old depressed woman died unexpectedly during admission in hospital. In urine, 315 ng/ml CIT was detected during screening. Analyses of the blood sample with the developed GC–MS method resulted in a CIT concentration of 14.1 ng/ml and a DMC concentration of 18.3 ng/ml. The mean brain concentration was 104 ng CIT per gram.

Case 2

The cause of death in this case was a central nervous system suppression, with a resulting lethal cardiorespiratory depression due to a polydrug intoxication; a combination of bromazepam (160 ng/ml), lorazepam (50 ng/ml), morphine (38 ng/ml), acetaminophen (1,430 ng/ml), ethanol (1.36 g/l), clotiapine (600 ng/ml), and fluoxetine (1,640 ng/ml; all blood values). The urinary level of fluoxetine was 4,750 ng/ml, while in the stomach contents fluoxetine reached a level of 260 ng/ml. Fluoxetine and desmethylfluoxetine were homogenously distributed in the brain with mean concentrations of 4,532 and 3,862 ng/g, respectively.

Case 3

The cause of death of this person was a polydrug intoxication, with high morphine levels (76.4 ng/ml). Thus, death was due to a suppression of the central nervous system, with cardiorespiratory depression. Sertraline and DMSer were found in blood at levels of 93 and 185 ng/ml, respectively. The mean brain concentration was 1,635 ng/g for sertraline and 3,717 ng/g for DMSer. Trazodone (93 ng/g) and *m*-cpp

Table 1 Overview of the autopsy findings

Case number	1	2	3	4
Age	92	40	27	43
Sex	Female	Female	Male	Male
Length (cm)	142	160	171	182
Weight (kg)	44	73	70	82
Body mass index	21.8	28.5	23.9	24.8
Cause of death	Sudden cardiac death (cf. ischemic heart disease)	Polydrug intoxication: opiates, alcohol, benzodiazepines, ADs	Polydrug intoxication: opiates (high morphine blood level; 6-MAM and papaverine pointing to heroin), trazodone quetiapine	Polydrug intoxication: high cocaine and high morphine blood level; methamphetamine and ethyl-amphetamine
Manner of death	Natural	Accidental overdose? Suicide not excluded	Accidental overdose	Accidental overdose
Mechanism of death	Sudden (sub)acute cardiopulmonary failure	(Sub)acute cardiopulmonary failure, MOF	(Sub)acute cardiopulmonary failure	(Sub)acute cardiopulmonary failure
PMI (days)	2.73	3.96	3.48	±3.50
Putrefaction signs	None	None	None	Skin marbling, flyctenes
Brain	1,135 g; atherosclerotic arteries (±20%); pronounced dilatation lateral ventricles	1,220 g; acute vascular congestion, multiple, scattered microthrombi, slight edema	1,550 g; obvious congestion and edema	1,700 g; pronounced brain edema; obvious congestion

PMI postmortem interval (days), *ADs* antidepressants, *MOF* multiple-organ failure, *6-MAM* 6-monoacetylmorphine, *ATN* acute tubular necrosis

Table 2 Antidepressant concentrations for the forensic autopsy cases

		Case					
		1	2	3		4	
Compound		Cit/DMC	Fluox/DMF	Traz/*m*-cpp	Ser/DMSer	Traz/*m*-cpp	CIT/DMC
Blood conc. (ng/ml)		14/18	1,640/	nd	93/185	nd	191/104
Brain conc. (ng/g)	Temporal lobe	27/24	4,454/3,762	75/26	1,466/3,624	492/112	53/64
	Parietal lobe	187/43	4,611/3,800	85/50	1,924/4,517	119/nd	95/59
	Occipital lobe	148/43	4,673/4,228	115/34	2,008/4,280	661/138	251/72
	Frontal lobe	30/22	4,979/4,312	90/21	1,750/4,392	556/139	196/54
	Stem	107/35	4,822/4,515	82/nd	1,671/3,172	77/nd	174/62
	Cerebellum	125/31	3,656/2,556	108/24	993/2,319	85/nd	162/155
Hair conc. (ng/mg)	Segment 1			nd/nd	0.6/0.5	nd/nd	2.5/1.9
	Segment 2			nd/0.4	0.8/1.4		
	Segment 3			nd/0.8	1.6/2.6		

(31 ng/g) were also detected in brain tissue. In urine, a trazodone concentration of 142.7 ng/ml was monitored, while trazodone was not detected in blood. Hair samples were also analyzed for this case. A hair sample with a length of 5.5 cm was taken from the vertex and cut into two fragments of 2 cm and one of 1.5 cm, giving a time window of approximately 2 months per segment. The first fragment (20 mg), closest to the scalp, contained 0.6 ng sertraline per milligram and 0.5 ng DMSer per milligram. The second fragment (61.2 mg) contained 0.8 ng sertraline per milligram, 1.4 ng DMSer per milligram, and 0.4 ng *m*-cpp per milligram. The third fragment contained 1.6, 2.6, and 0.8 ng/mg of sertraline, DMSer, and *m*-cpp, respectively.

Case 4

In this case, large amounts of cocaine (3.43 μg/ml), amphetamine (4.5 μg/ml), and morphine (167 ng/ml) were found in blood which could induce death due to cardiac arrhythmia (cf. stimulants) and/or respiratory depression (cf. opiates). The urine contained CIT (5.38 μg/ml) and trazodone metabolites (6.95 μg/ml), while the concentration in stomach contents was 1.12 μg/ml CIT and 115 μg/ml trazodone. Trazodone and *m*-cpp were detected in brain tissue although they were not found in blood. A mean concentration of 332 ng/g was found for trazodone, while a mean of 130 ng/g was found for *m*-cpp in the frontal, occipital, and temporal lobes. CIT and DMC were found in brain tissue with mean concentrations of 155 and 61 ng/g, respectively. Blood concentrations as determined by GC–MS were 194 and 104 ng/ml, respectively. Dark brown hair with a length of 6 cm was taken from the vertex and cut into two fragments of 3 cm because of the limited amount available. The first fragment (closest to the scalp; 23.2 mg) contained 2.5 ng CIT per milligram and 1.9 ng DMC per milligram. The second fragment (27.3 mg) did not contain any AD.

Discussion

Referring to Table 1, we want to indicate that not only obvious lethal intoxications should be considered.

Case 1

In this sudden cardiac death (see Table 1), the blood level of CIT was subtherapeutic as therapeutic concentrations range from 20 to 200 ng/ml (The International Association of Forensic Toxicologists, http://www.tiaft.org/). The brain concentrations of CIT and DMC were sample dependent, with the highest concentrations in the parietal and occipital lobe and in the cerebellum. The DMC to CIT ratio ranged from 0.3 to 0.9 with a mean of 0.45. The same ratio was seen in case 4 where DMC–CIT ranged from 0.3 to 1.2 with a mean of 0.51. In both cases, the highest ratio was observed in the temporal lobe. Referring to the brain to blood ratio of 7, it can be concluded that CIT penetrates the brain rather easily.

Case 2

The fluoxetine blood concentration was toxic, but not lethal, as toxic concentrations range from 1,500 to 2,000 ng/ml (The International Association of Forensic Toxicologists, http://www.tiaft.org/). According to Bolo et al. [33], the steady-state brain concentration of the sum of fluoxetine and its active metabolite desmethylfluoxetine ranges from 1,800 to 6,000 ng/g, and the sum of 8,394 ng/g reported in this case was higher. In addition, the brain concentration of desmethylfluoxetine was almost as high as the fluoxetine concentration which might be explained by the elimination half-life increase from 4–6 days for the parent drug to 4–16 days for the metabolite [34]. The brain to blood fluoxetine ratio of 2.8 in our case is comparable to the brain–plasma correlation of 2.6 for the sum of fluoxetine and desmethyl-

fluoxetine found by Renshaw et al. [35]. However, this ratio is much lower than the ratio of 10 described by Bolo et al. [33]. These authors examined the plasma–brain concentration relationship for other selective serotonin reuptake inhibitors (SSRIs; fluoxetine and fluvoxamine) in vivo through ^{19}F magnetic resonance spectroscopy. However, we must keep in mind that the comparison between brain–blood and brain–plasma results is not obvious as amphiphilic ADs bind to red blood cell membranes [31, 32].

Case 3

The therapeutic range of sertraline in plasma ranges from 50 to 250 ng/ml (The International Association of Forensic Toxicologists, http://www.tiaft.org/). The observed sertraline concentration in this case is thus within the lower therapeutic range. Sertraline concentrations were determined in six different locations in the brain and it is clear that in this case it was homogeneously distributed over the brain tissue as shown in Table 1. Calculation of the brain to blood sertraline ratio provided a value of 17.6. This value is higher but in the range of the proposed ratio of 10 for SSRIs by Bolo et al. [33]. The ratio of sertraline to DMSer in hair was 1.2 in the first segment, while it was 0.5 and 0.6 for segments 2 and 3. Sertraline was not detected in urine. It is clear from the hair and urine analysis that there was a regular but not daily intake of sertraline during the past 6 months and, therefore, a noncompliant therapy must be suspected.

In this case, neither trazodone nor its metabolite was found in blood; however, they were determined in brain tissue. The level of trazodone in brain tissue was 200–500 times lower than concentrations found by Martin and Pounder [36]. However, these authors described intoxications in which about 700 mg of trazodone was ingested. This case demonstrates that ADs can still be determined in brain tissue, even when they are no longer present in blood, providing information about the treatment and administration of AD drugs before death. The urinary concentration of trazodone and the presence of its metabolite *m*-cpp in hair confirm this conclusion.

Case 4

CIT was detected in blood, brain, urine, and stomach contents. The brain to blood ratio was quite low (0.8) compared to case 1, which could be explained by the recent and irregular intake, while for case 1 a steady-state AD therapy can be presumed. The presence of CIT in the brain could be due to rapid migration from the stomach contents and storage in this compartment or more likely be an indication of previously consumed CIT. Referring to the CIT concentrations substantiated in the hair fragments, we can conclude that the use of CIT occurred during the past 3 months. Trazodone was also found in the stomach contents and brain tissue, but not in blood, leading to the conclusion of the longer detection window for brain tissue.

Conclusions

This study should be of interest for forensic pathologists and toxicologists and enhance the collaboration between these disciplines. The developed SPE GC–MS method for several new-generation ADs and their active metabolites in postmortem matrices was validated and tested on real postmortem cases. While blood is still the preferred matrix to link concentration and effect, analysis of brain tissue and hair can provide additional information, not only in pure overdose cases (see Table 1). A possible advantage of postmortem toxicological brain analysis is the fact that ADs can still be determined in brain tissue, even when they are no longer present in blood, providing information about earlier treatment and administration of AD drugs. In addition, although ADs selectively bind to receptors located in specific brain regions, it was clear that the ADs spread rather homogeneously over the total brain content in most cases. It cannot be excluded that this distribution is increased due to postmortem redistribution of the ADs, following liberation from their specific binding sites. Therefore, in postmortem analysis, a detailed location of a brain sample is in fact of no importance for the quantitative result. Hair samples were used as a positive control for the brain results in this work. However, it is also clear that this matrix is of interest to increase the detection window, certainly in decayed corpses. The brain tissue is prone to autolysis, but our technique can be used in postmortem intervals up to about—at least—4 days (see Table 1).

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