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ORIGINAL ARTICLE

Isomers of fluoroamphetamines detected in forensic cases in Denmark

Sys Stybe Johansen · Tina Maria Hansen

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Abstract A study was performed on the detection, separation and quantification of isomers from the new designer drugs named fluoroamphetamines (FAs) in forensic cases in eastern Denmark. The drugs were detected in whole blood extracts by ultraperformance liquid chromatography with time of flight mass spectrometer (UPLC-TOF-MS) and thereafter verified and quantified by UPLC tandem mass spectrometer (MS/MS). The quantitative method involved liquid–liquid extraction of FAs from whole blood, evaporation of organic solvent, and reconstitution with a mobile phase mixture. Identification of the FAs was achieved by the retention time, multiple reaction monitoring (MRM) traces [154>109 (quantifier); 154>137], and ion ratio of the two transitions. For all FAs, LOQ was 0.002 mg/kg with linear ranges from 0.002 to 1.0 mg/kg whole blood. Since 2008, a total of 15 forensic investigations, mainly driving under the influence of drugs (DUID) cases, involving 4-fluoroamphetamine (4-FA) have been observed with whole blood concentrations ranging from 0.006 to 0.58 mg/kg. One autopsy case involved 4-FA; however, it was determined to be a combined intoxication. In 2010, *ortho*-fluoroamphetamine (2-FA) was discovered in forensic samples by the same UPLC/MS/MS method and MRM functions because of variation in retention time and ion ratio. Up to now, three eastern Danish DUID cases have involved 2-FA. The whole blood concentrations of 2-FA were 0.028, 0.041 and 0.37 mg/kg, respectively. Thirteen cases with 4-FA and the three cases with 2-FA also contained amphetamine, but no correlation was observed between the amount of FA and amphetamine. So far, 3-FA has not been observed in any cases, and although it co-elutes with 4-FA, 3-FA will be identified by its variation in ion ratio. To our knowledge, this study has confirmed 2-FA in blood from DUID cases for the first time, and provides typical whole blood concentrations of FAs in forensic cases.

Keywords Fluoroamphetamines · Forensic blood samples · UPLC-MS/MS

S. S. Johansen (✉) · T. M. Hansen
Section of Forensic Chemistry, Department of Forensic Medicine,
Faculty of Health Sciences, University of Copenhagen,
Frederik V's vej 11,
2100 Copenhagen, Denmark
e-mail: SSJ@forensic.ku.dk

Introduction

Amphetamine and its derivatives are widely abused as central nervous system stimulants, and every year new amphetamine variants, closely related structurally, are observed on the illegal market and in forensic investigations [1, 2]. This development makes it difficult for forensic laboratories to detect, identify and quantify structurally related designer drugs such as fluoro-substituted amphetamines [3]. This work demands highly sensitive and specific instruments and therefore forensic analysis typically involves mass spectrometry. However, the ionization of regioisomeric ring substituted aromatic compounds such as fluoroamphetamines (FAs) by mass spectrometry is very difficult to use for identification purposes due to the similarity of the mass spectra and fragmentation. Furthermore, 2-, 3- and 4-substituted aromatic compounds can only be insufficiently separated by chromatography due to very similar retention abilities, making a distinction between the isomers difficult or impossible [3–5]. This trouble has been observed by others, for instance, Westphal et al. [5], who used gas chromatography with tandem mass spectrometry (GC-MS/MS) analysis and found chemical ionization useful in some differentiation between the regioisomers of FAs.

FAs belong to the group of phenylethylamines, just as amphetamine, and have similar stimulating effects by increased release of dopamine and norepinephrine, but FAs have also shown serotonin-releasing effects like 3,4-methylenedioxymethamphetamine (MDMA) [6, 7]. Since 2007, *para*-fluoroamphetamine (4-fluoroamphetamine [4-FA]), has been observed in seizures in the Netherlands and from 2009 in many other European countries [8]. It has been controlled in Denmark since March 2009. However, *ortho*- (2-) and *meta*- (3-) FA were not regulated as illegal drugs until June 2010. The first cases described in this paper initiated the regulation.

Röhrich et al. [9] have recently published methods (cloned enzyme donor immunoassay [CEDIA] and GC/MS) for 4-FA in serum and urine that were used for two driving under the influence of drugs (DUID) cases. In comparison, this paper describes another analytical technique (ultraperformance liquid chromatography with tandem mass spectrometer [UPLC-MS/MS]) used to determine all three regioisomers of FAs, and presents the whole blood levels of 2-FA and 4-FA measured in forensic investigations, mainly DUID cases, in eastern Denmark. However, one fatal case involving 4-FA is described.

Materials and methods

Chemicals and reagents

2-FA and 4-FA from Alfa Aeser (Ward Hill, MA, USA) were used as reference materials, and late in the revision stage of this report, 3-FA was obtained from Chiron (Trondheim, Norway). Deuterated amphetamine-*d*5, which along with other common drugs of abuse was obtained from Cerilliant (Round Rock, TX, USA), was used as internal standard. 4-Methylamphetamine was purified from a solid seizure. All other reference standards were obtained from the pharmaceutical industry and were of high purity (>98%).

Methanol and acetonitrile of LC/MS grade was obtained from Fisher Scientific UK (Leicestershire, United Kingdom, UK), formic acid (98–100%) was acquired from Fluka (Buchs, Switzerland), and butyl acetate for analysis and sodium hydroxide were supplied by Merck (Darmstadt, Germany). Purified water was obtained from Millipore Synergy UV water purification system (Millipore A/S, Copenhagen, Denmark). Acidic water (0.05% formic acid in water) and acidic acetonitrile (0.05% formic acid in acetonitrile) were prepared and used as mobile phases. Blank human whole blood (BB) was purchased from the Blood Bank at Copenhagen University Hospital (Copenhagen, Denmark) and preserved by adding 1% sodium fluoride and 0.025% potassium oxalate. BB was investigated with in-house screening procedures, a general unknown screening using GC-MS, high pressure liquid chromatography with diode array detection (HPLC/DAD), and LC-MS/MS methods to confirm the absence of drugs.

Stock solutions (1,000 mg/l in methanol) of each FA were prepared and stored at −20°C. A working solution of all analytes was prepared in methanol at 50 mg/l, and an aqueous internal standard solution (IS) of amphetamine-*d*5 was made up at 0.25 mg/l. The stock solution was diluted in water, and six calibrators were produced by spiking BB with 20 μl aqueous dilutions corresponding to blood levels at 0.002, 0.010, 0.050, 0.20, 0.50, and 1.0 mg/kg, respectively.

This laboratory performs toxicological analysis of medicolegal autopsies, DUID, and other criminal investigations including seizures from eastern Denmark (Zealand, surrounding islands and Bornholm). This area covers nearly half of the population in Denmark (i.e., 2.4 million inhabitants). All authentic forensic samples of human whole blood were preserved as BB and stored at −20°C. Quality control samples at 0.020 and 0.30 mg/kg of 4-FA were prepared in BB and stored at −80°C. The controls were included in all analysis involving 4-FA.

For sample preparation, 0.100 g whole blood was mixed with 150 μl water and 20 μl IS solution. Then, 50 μl 2 M NaOH was added and extraction with 250 μl butyl acetate was carried out. After the mixture has been centrifuged for 10 min at 2,000× *g* and 5°C, the organic fraction was collected, acidified with 20 μl 1% formic acid in methanol and evaporated to dryness at 40°C under a stream of nitrogen. The samples were reconstituted in 100 μl mobile phase, which consisted of acetonitrile/methanol/0.05% formic acid in water (5:20:25, v/v/v). Five μl of each extract was injected into the LC-MS/MS system.

Analysis

Screening was performed according to the method described by Dalsgaard et al. [10] which involved a solid phase extraction of whole blood and detection by ultra performance liquid chromatography with a time of flight mass spectrometer (UPLC-TOF-MS). This is an effective screening method for basic drugs in blood. The compounds were detected by their exact masses with an accuracy of ±5 mDa and retention time of ±0.2 min. For further information, see Ref. [10].

Quantitative analysis Separation was performed on an Acquity UPLC ethylene bridged hybrid (BEH) C18, 1.7 μm, 2.1×100 mm column from Waters (Manchester, UK) by gradient elution at a flow rate of 0.6 ml/min, using acidic water and acetonitrile containing 0.05% formic acid as described in Table 1. The FAs were eluted by this system within 2 min as illustrated in the ion chromatogram of the quantifier trace in Fig. 1. To detect the analytes a tandem mass spectrometer, Acquity TQD was coupled to the UPLC system all from Waters (Manchester, UK). The detection

Table 1 List of UPLC conditions

UPLC conditions		Gradient		
System	Aquity UPLC-TQD (Waters)	Time	A (%)	B (%)
Column	Aquity BEH C18 1.7 µm, 2.1×100 mm	0.00	95	5
Column temperature	50°C	2.00	85	15
Mobile phase A	0.05% formic acid in water	9.00	70	30
Mobile phase B	0.05% formic acid in acetonitrile	9.10	20	80
Flow rate	0.6 ml/min	10.00	10	90
Run time	15 min	10.50	10	90
Injection volume	5 µl	11.10	95	5
Injection mode	Partial loop with needle overfill	15.00	95	5

was performed by positive electrospray ionization (ESI+), operating in multiple reaction monitoring (MRM) mode. The determination was done by two MRM transitions of the FAs (154>109 (quantifier), 154>137) in order to gain a secondary identification parameter (see Table 2). The conditions of the mass spectrometer were optimized by infusing standard solution through a T-piece, and specific settings for the analytes are listed in Table 2. Argon was used as collision gas at 0.45 Pa, and desolvation and cone gasflow were fixed at 1,100 and 50 l/h, respectively. The source temperature was set at 120°C and desolvation temperature at 450°C. Data was acquired and processed with MassLynx 4.1 software (Waters).

The LC-MS/MS method for quantitative determination of FAs in whole blood was validated according to current standard with small modifications [11, 12]. Ion suppression study was performed by infusion of a solution (0.05 mg/l) containing the 2-FA and 4-FA in concentrations corresponding to the second lowest calibration standard through a T-piece (infusion rate: 0.02 ml/min) while injecting blank spiked human whole blood extracts and extracts of negative forensic cases (N=4). The matrix effects were investigated to estimate the potential interferences with endogenous substances. Six negative forensic samples were extracted, then spiked and analyzed. The spiking level for the analytes (2-FA and 4-FA) was 0.010 mg/kg, and for the internal standard (amphetamine-*d5*), it was 0.050 mg/kg. The responses of the analytes in extracts were compared with that of solvent standard solutions at a similar concentration. Selectivity was further investigated by injecting standard solutions about 1 mg/l of other frequent drugs and drugs of abuse such as other stimulants (cocaine, methamphetamine, 3,4-methylenedioxyamphetamine [MDA], MDMA), benzodiazepines, antidepressants and antipsychotics. The overall extraction efficiencies were determined in six repetitions at two spiked whole blood levels (0.010 and 0.50 mg/kg). Inaccuracy and imprecision were determined from the results of the quality controls analyzed on nine different days. These quality controls were used to evaluate the stability of the analyte in whole blood.

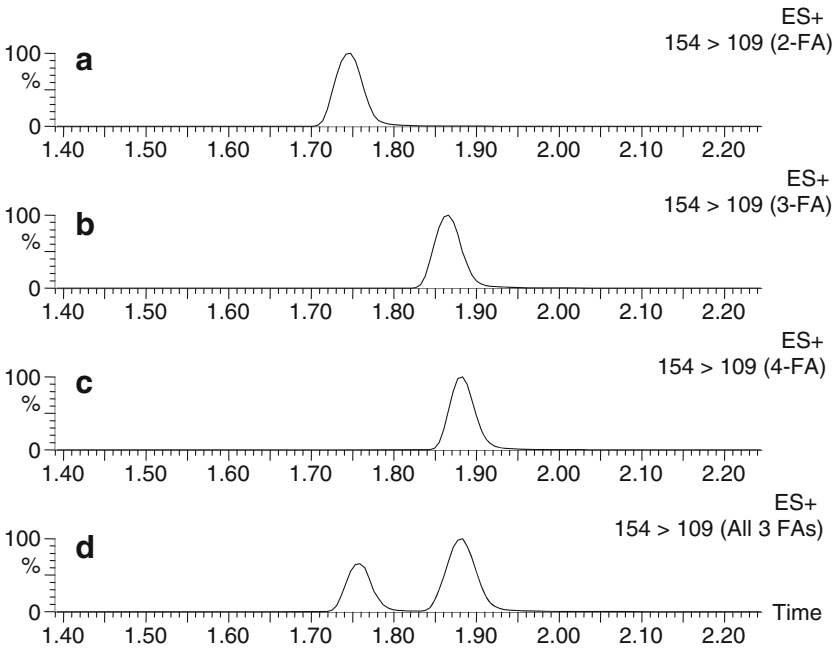

Fig. 1 Separation of 2-FA, 3-FA and 4-FA in a standard solution at 1 mg/l by the UPLC-MS/MS system (Tables 1 and 2) shown with the quantifier trace (154>109) injected separately (**a**–**c**). Furthermore, an ion chromatogram showing the analysis of a mixture of all three FAs at 0.33 mg/l where 3-FA and 4-FA co-elute (**d**)

Table 2 List of MS/MS conditions

Compound	Parent ion (*m/z*)	Product ion (*m/z*)	Cone voltage (V)	Collision energy (eV)	Retention time (min)	RRT	Ion ratio
2-Fluoroamphetamine	154	109 137	22	19 9	1.77	1.09	1.7
3-Fluoroamphetamine	154	109 137	22	19 9	1.92	1.17	1.6
4-Fluoroamphetamine	154	109 137	22	19 9	1.92	1.17	1.1
Internal standard: Amphetamine-*d*5	141	96	20	17	1.66		

RRT relative retention time

Results and discussion

Analysis

FAs were detected in blood by UPLC-TOF-MS screening, but on this system all three isomers co-eluted [10], so positive findings required another analysis for confirmation and quantification. A simple sample preparation using liquid–liquid extraction was applied for the identification and quantification of each isomer on the UPLC-MS/MS method. Using the liquid–liquid extraction, the overall extraction efficiency of FAs was about 65%, and for the internal standard it was 60%. Regarding detection of the analytes, two transitions were applied: one as a quantifier and one as a qualifier. Since the compounds are regioisomers, the sizes of the main fragments are identical and therefore detected with the same transitions as shown in Table 2. The significant fragment at *m/z* 109 corresponds to loss of ethylamine by α-cleavage of the benzylbond to a fluoro-substituted tropylium cation [4], while the *m/z* 137 corresponds to the typical loss of NH_3 from amphetamine [13].

The identification criteria of each FA included retention time (RT), the relative retention time (RRT), MRM traces, and ion ratio between the two transitions. Criteria of the maximum deviation of the RRT were ±0.01 and ±0.03 min of the absolute RT against the average of the standards in the run, while the maximum deviation of ion ratio (MRM1 against MRM2) was ±5% for the FAs within the measuring range. As illustrated in Fig. 1, 2-FA and 4-FA were separated by 0.15 min and nearly baseline separated. 3-FA became commercially available within the period of paper revision and was therefore included very late in the study. This delay also explains the small deviation in RT for 2-FA and 4-FA between the two figures and Table 2, as the analysis shown were performed with a year in between, but the RRT were identical. Unfortunately, it was discovered that 3-FA and 4-FA co-eluted and could, with this chromatographic system, not be separated better, not even if isocratic elution were used in the beginning of gradient elution. However, with the applied transitions and collision energies, the fragmentation pattern differed significantly and made it possible to distinguish between 3-FA and 4-FA due to different ion ratios, i.e., 1.6 and 1.1, respectively (see Table 2). When 3-FA and 4-FA were injected together the observed common ion ratio were somewhere in between 1.1 and 1.6. Therefore, requirements for small variation (±5%) of ion ratio compared to standards were important. To ensure that the quantification of each FA in the cases was correct, each FA was extracted and analyzed in separate analysis series with relevant samples. Consequently, if both 3-FA and 4-FA were present in a sample quantification of each isomer would not be possible.

The UPLC method was used to determine many basic drugs in whole blood, and the gradient was therefore not optimized only for the FAs. Consequently, the LC run time could be reduced if a high throughput analysis is required. No interferences were observed from common pharmaceuticals or drugs of abuse. Very similar drugs such as methamphetamine, ephedrine, norephedrine, MDA and 4-methylamphetamine eluted at other retention times or deviated in MRM trace from the FAs, so no interference were observed. The selectivity was acceptable as the isomers of FA were separated (either by RT or by ion ratio), and it was therefore possible to determine which isomer was on the market (Table 2). Ion suppression was investigated in order to ensure the quantitative results. No enhancements or suppressions of the signal were observed in the time period where the FAs and the internal standard eluted in human whole blood extracts from BB and negative forensic samples. The matrix effects were also studied and for all three analytes (2-FA and 4-FA and amphetamine-*d*5) no interferences were observed. A small enhancement of less than 3% was eliminated by the IS.

The calibration curve was linear from 0.002 to 1.0 mg/kg of all isomers with correlation coefficients above 0.995 and residuals below 20%. An ion chromatogram of a whole blood extract containing 4-FA at 0.002 mg/kg (limit of quantification, LOQ) is shown in Fig. 2. At this level, the signal/noise ratio (S/N) was high (peak to peak >500) for the

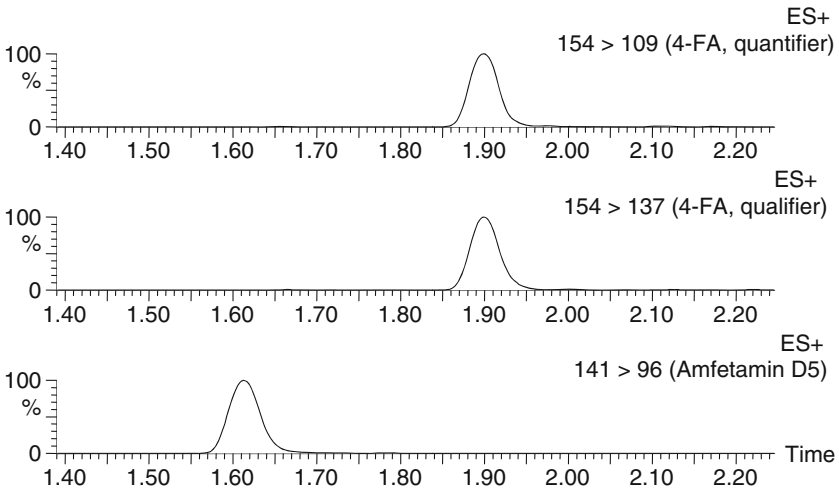

Fig. 2 Ion chromatograms (quantifier and qualifier traces) of a whole blood extract containing 4-FA at low measuring level (0.002 mg/kg) (**a** and **b**) and trace of the internal standard (amphetamine-*d*5) (**c**)

quantifier trace of all FAs, and the ratio for the qualifier trace was acceptable (S/N >50). This indicated that the limit of detection (LOD) for FAs was 1/10 of 0.002 mg/kg with regard to the qualifier trace. The imprecision of 4-FA was determined by RSD of the QCs to be 11.8% at level 0.02 mg/kg and 12.7% at 0.30 mg/kg, while the trueness was determined to be 111% and 106% at 0.02 and 0.30 mg/kg, respectively (N=9). The stability of the preserved quality controls in whole blood stored at −80°C was evaluated by these measurements and found stable for at least 1 year as no degradation in the levels were observed.

Applications

Since 2008, a total of 15 forensic investigations involving 4-FA have been observed in eastern Denmark from January 2009 to July 2011, and these are listed in Table 3. All case determinations were performed in replicate measurements of two within the analytical run, although only the calculated mean is given in Table 3. The difference between these determinations was below 25%. In 2010, 2-FA was detected in a case as a result of a minor variation from 4-FA with regard to retention time and ion ratio in the quantitative analysis. We noticed that it was not possible to distinguish between 2-FA and 4-FA with our UPLC-TOF-MS screening due to co-elution, but it was possible with the quantitative UPLC-MS/MS method. Up to now (summer 2011), three 2-FA cases have been observed all in 2010, and they are shown in Table 3. Sixteen of the 18 FA cases were DUID cases involving mainly men at the ages of 19–38 years. A 21-year-old woman was observed in one DUID case. The last two cases concerned a female rape victim and a fatal poisoning of a female drug addict, respectively.

The detected whole blood concentrations of 4-FA in DUID cases ranged from 0.006 to 0.43 mg/kg with a mean at 0.087 mg/kg and a median at 0.021 mg/kg. The whole blood concentrations of 2-FA were determined at 0.028, 0.041 and 0.37 mg/kg in DUID cases (Table 3). Amphetamine was found in almost all cases except for two cases. The concentration level of amphetamine varied from low abuse level (0.049 mg/kg) to toxic level (0.70 mg/kg). One very recent DUID case from late spring 2011 also involved other designer amphetamine analogues: 4-methyl-amphetamine (0.002 mg/kg) and methamphetamine (0.038 mg/kg). In two cases, the persons involved were also driving under the influence of alcohol, exceeding the legal blood alcohol content (BAC), which is 0.50 ‰ in Denmark. Furthermore, in ten cases, other drugs than amphetamines were detected such as several benzodiazepines, tetrahydrocannabinol (THC), methadone, ketamine, and lidocaine as shown in Table 3. Several of the latest cases involved 4-FA and clonazepam, but no seizures with that mixture has been observed on the Danish illegal market.

In one of the DUID cases (no. 4 in Table 3), a high level of 4-FA (0.43 mg/kg) was detected and amphetamine in 1/10 of 4-FA. Unfortunately, no case histories or information about impairment and/or driving performance is recorded in DUID cases. However, with this high level of FA in the blood, influence on the driving is expected. The person was convicted by the Danish Traffic Act of 2007 included per se legislation of illegal drugs in DUID cases. The per se limit for amphetamine is 0.020 mg/kg in Denmark, although 50% is added to the limit as compensation for analytic uncertainty and the real limit is hence 0.030 mg/kg. The applied limit for the FAs at 0.030 mg/kg is based on their similarities with amphetamine [1]. Eight of the 16 DUID cases had concentrations above the limit of FAs, and 14 of the DUID cases had amphetamine levels above the real limit. Although amphetamine and FAs were observed together in most of the cases mentioned here, no correlation between the amounts in blood was observed. Nevertheless, the seizures observed in Denmark were typically powder mixtures containing 4-FA and 1% amphetamine (impurity) [8]. The findings of a high amphetamine

Table 3 Forensic cases in eastern Denmark involving fluoroamphetamines (FAs)

Case/ year	Type of case	Dead/living	Gender	Age years	Whole blood concentration (mg/kg)					
					2-FA	4-FA	Amph.	BAC	THC	Others
1-09	Drug addict	D	F	44		0.58	0.30			Benzodiazepines [a], Methadone (0.65)
2-09	DUID	L	M	23		0.010		0.75	0.032	
3-09	DUID	L	M	29		0.046	0.061	1.28		(Ketamine 0.003)[b]
4-09	DUID	L	M	29		0.43	0.049			Lidocaine (0.04)
5-09	Rape	L	F	17		0.061	0.21			Lidocaine (0.02)
6-09	DUID	L	M	35		0.10	0.17			
7-09	DUID	L	M	23		0.009			0.0038	Alprazolam (0.069), Cocaine trace, Ketamine (0.049)
8-09	DUID	L	M	21		0.093	0.14			
9-10	DUID	L	M	24	0.041		0.19		0.0072	
10-10	DUID	L	M	29		0.021	0.39			Diazepam (0.21), Methamphetamine (0.19)
11-10	DUID	L	M	33		0.32	0.060			
12-10	DUID	L	M	38	0.37		0.60			
13-10	DUID	L	M	38	0.028		1.9			
14-11	DUID	L	M	29		0.018	0.086			Clonazepam (0.019/0.030)
15-11	DUID	L	M	19		0.050	0.70		0.0017	Clonazepam (0.14/0.094)
16-11	DUID	L	M	21		0.019	0.22			Clonazepam (0.066/0.041), Metamphetamine (0.038), 4-Methylamphetamine (0.002)
17-11	DUID	L	M	29		0.010	0.28			Clonazepam (0.008/0.018)
18-11	DUID	L	F	21		0.006	0.18		0.0035	

For Clonazepam, the concentration of 7-Aminoclonazepam is given afterwards

Amph. amphetamine, *BAC* blood alcohol content (in ‰), *DUID* driving under the influence of drugs

[a] Benzodiazepines: Diazepam (0.029 mg/kg) and Oxazepam (0.043 mg/kg)

[b] Ketamine level is below limit per se (0.010 mg/kg)

level suggest separate abuse of this compound. In general among the DUID cases the levels were for the majority lower than the two cases reported by Röhrich et al. [9], who found levels at 0.35 and 0.48 mg/l serum. The blood/plasma ratio varies from 0.6 to 1.0 for amphetamine depending on the concentration [14]. A similar level is expected for FAs, and therefore this will influence on the comparison. Furthermore, the duration from the traffic stop to the sampling is unknown for the DUID cases, but these delays may influence on the measured level due to the relatively short half-life of amphetamines (7–34 h) [14] although it might be of minor importance. Also, the mixtures with other stimulating drugs such as amphetamines will enhance the effects and thereby the impairment.

One of the two cases where the case history was available concerned a 17-year-old woman who was raped and sexually abused by a 42-year-old man while sleeping (case no. 5 in Table 3). She had been drinking alcohol and taking cocaine prior to the rape incident. No cocaine was found in her blood, but amphetamine at 0.20 mg/kg, 4-FA at 0.061 mg/kg and traces of lidocaine were detected in her blood. Lidocaine may occur from the cocaine intake since it is a typical adulterant in the preparation of cocaine [1].

The autopsy case shown (no. 1 in Table 3) involved a 44-year-old HIV-positive female drug addict. The deceased was found dead on the floor in her mother's apartment where she had been living due to weakness and sickness during the last month of her life. She had been a drug addict for 18 years and was undergoing methadone treatment. The mother informed the police that she had been taking amphetamine prior to her death. High level of methadone was found in her blood (0.65 mg/kg), and high levels of 4-FA (0.58 mg/kg) and amphetamine (0.30 mg/kg) were also detected. The death was evaluated to be caused by the high level of methadone in combination with 4-FA and amphetamine.

By studying the measured ion ratio of our 4-FA cases, it was possible to clarify that 3-FA was not involved in any of those cases. Consequently, 3-FA has not been observed in any biological samples among our forensic cases, and it has never to our knowledge been reported by others either. It is also noticeable that none of the cases have contained a mixture of 2-FA and 4-FA, or a mixture of 3-FA and 4-FA as it would have given diverging ion ratio. This could indicate that clandestine laboratories use precursors which are fluorinated from the beginning. Different methods for synthesis of commonly known amphetamine analogues are

described in the literature. Some of the more frequently mentioned, which also have proved applicable for synthesis of FAs, are the aluminium foil method [3] and the Leuckart reaction [15], in which the commercially available corresponding fluorophenylacetones are used as starting materials. These compounds are non-controlled substances in comparison to the non-fluoronated version, phenylacetone (BMK), which is a schedule II controlled substance as it is a precursor for amphetamine [3]. To determine the most frequent synthetic routes, identification of route-specific by-products in seizures would be required, but it is outside the scope of this study.

Conclusion

The importance of separation and detection of drugs with very similar structures such as the isomers of FAs are illustrated here. The new designer drugs 2-FA and 4-FA were observed in 18 forensic cases in eastern Denmark, mainly DUID cases. The whole blood level ranged from 0.006 to 0.43 mg/kg FA among DUID cases. 4-FA (0.58 mg/kg) was involved in one fatal intoxication.

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Conflict of interest The authors declare that they have no conflict of interest.

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