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Ionic Liquid-Based Liquid–Liquid Microextraction for Benzodiazepine Analysis in Postmortem Blood Samples*

ABSTRACT: Sample preparation is rapidly improving to fulfill the need for faster and more environmentally friendly alternatives. In this respect, ionic liquid-based dispersive liquid–liquid microextraction (IL-DLLME) is an interesting technique. However, it has not yet been evaluated for the analysis of postmortem samples, which are frequently analyzed in forensic toxicology. This study investigates the applicability of IL-DLLME coupled to liquid chromatography—tandem mass spectrometry (LC-MS/MS), for the analysis of benzodiazepines in postmortem blood of 11 forensic cases. The method was compared with a validated solid-phase extraction (SPE) method. Bland–Altman analysis was performed on 24 benzodiazepine measurements. Both methods gave comparable results, except for flurazepam and temazepam (>55% difference). A feasible explanation is high postmortem matrix variability that was not considered during IL-DLLME validation experiments. Another issue could be the use of a single nondeuterated SPE internal standard. Overall, IL-DLLME has proven its usability for the analysis of postmortem blood.

KEYWORDS: forensic science, forensic toxicology, ionic liquid-based liquid-liquid microextraction, postmortem cases, Bland-Altman, LC-MS/MS

Presently, a thorough sample clean-up step is still indispensable in quantitative bioanalysis. Matrix components should be eliminated, to avoid ion suppression that can lead to quantitation errors, and improve the robustness of a method. In addition, analyte enrichment is desired to enhance sensitivity and enable detection of low-dose drugs (1,2). Analytical instruments are becoming more efficient over time and sample preparation should keep up pace. Therefore, the constant need for fast and simple sample preparation techniques should be investigated. Two established techniques are solid-phase extraction (SPE) and liquid-liquid extraction (LLE). The first is frequently used as a result of thorough matrix elimination and high recoveries. However, lengthy, complex procedures and column blockage are problematic. LLE offers a more user-friendly alternative. LLE protocols used to consume large solvent volumes, however, the current microextraction method seems to alleviate this issue (1,3-5). This trend focuses on the use of only small volumes of extraction solvent (µL range), resulting in high concentration factors and more environmentally friendly procedures (1,4,5).

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Several liquid–liquid microextraction procedures have been developed, among which dispersive liquid–liquid microextractions (DLLMEs) are an alternative choice, due to their fast, simple, and inexpensive protocols (4–6). DLLME was introduced in 2006 (7) and has been well integrated into current sample preparation methods. The technique consists in adding a small volume of organic extraction solvent to the aqueous sample. The organic solvent is dispersed into the aqueous phase by means of a disperser solvent. From an environmental perspective, the use of a ternary solvent system to obtain a dispersion is not desirable. Alternative dispersion techniques have been reported that focus on physical agitation, such as vortex-assisted and ultrasound-assisted approaches (1,4,5,8).

Another trend in sample preparation is the search for novel extraction solvents, as conventional volatile organic solvents are not considered safe or environmentally sound, due to their flammable and volatile nature. The potential of deep eutectic solvents and ionic liquids (ILs) has been investigated as favorable alternatives (9-11). ILs have received notable attention from different research fields (electrochemistry, organic catalysis, analytical chemistry, etc [12,13]), as a result of their low vapor pressures at room temperature, good thermal and chemical stability, and good solubility for a wide variety of compounds. ILs are also named "liquid salts," as they are ionic compounds that occur in the liquid state below 100°C. As anions and cations can easily be exchanged and chemically altered, ILs can be modified into task-specific solvents, which is possibly their most useful advantage (10-12,14,15).

ILs have been applied as extraction solvents in DLLME protocols, in a technique called ionic liquid-based dispersive liquidliquid microextraction (IL-DLLME). The sample preparation

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method has been reported for the extraction of metal ions and small molecules from diverse sample matrices, such as water or even more complex biological samples (6,8,11). Despite the potential of ILs as promising solvents, research on their applicability for the extraction of complex matrices in the field of forensic toxicology is still scarce. Especially research on postmortem matrices is important, as high variability in composition may have a significant influence on extraction yields, matrix effects and thus quantification (16).

Recently, De Boeck et al. validated an IL-DLLME procedure, coupled to liquid chromatography—tandem mass spectrometry (LC-MS/MS) for the quantification of benzodiazepines (BZDs) and BZD-like hypnotics in blood (17). A toxicological relevant class of drugs was studied, as BZDs and BZD-like hypnotics are still frequently used and abused (18). From a forensic perspective, it is useful to evaluate whether the validated IL-DLLME method can be applied for analysis of postmortem blood samples.

The aim of this study was to investigate the applicability of a validated IL-DLLME-LC-MS/MS method (17) for the identification and quantification of BZDs in 11 relevant postmortem blood samples. Method comparison was performed, using SPE-LC-MS/MS (19) as a reference method.

Materials and Methods

Chemicals and Reagents

Analytical reference standards of deuterated BZDs were purchased as methanolic solutions from Cerilliant (Round Rock, Texas, U.S.A.): 7-aminoflunitrazepam.d7 (1 mg/mL), alprazolam.d5 (1 mg/mL), chlordiazepoxide.d5 (0.1 mg/mL), clonazepam.d4 (1 mg/mL), diazepam.d5 (1 mg/mL),flunitrazepam.d7 (0.1 mg/mL), lorazepam.d4 (1 mg/mL), midazolam.d4 (0.1 mg/mL), nitrazepam.d5 (0.1 mg/mL), nordiazepam.d5 (1 mg/mL), oxazepam.d5 (1 mg/mL), prazepam.d5 (0.1 mg/mL), temazepam.d5 (1 mg/mL), triazolam.d4 (0.1 mg/ mL), and zolpidem.d7 (0.1 mg/mL). Estazolam.d5 (0.0999 mg/ mL) was obtained from LGC (Molsheim, France). A methanolic standard stock solution, containing all 16 deuterated analogs, was prepared at a final concentration of 5 µg/mL. This stock solution was used as internal standard (ISTD) in IL-DLLME procedures. N-methylclonazepam was obtained from Roche (Brussels, Belgium) and diluted to 600 ng/mL in methanol. This solution was used as ISTD in SPE procedures. All standard solutions were stored at -20°C. The IL, 1-butyl-3-methylimidazolium hexafluorophosphate (BMIm PF₆) (99.5%) was purchased from IOLITEC Ionic Liquids Technologies GmbH (Heilbronn, Germany). Solvents and mobile phase additives were LC-MS grade quality. Methanol was obtained from Biosolve (Valkenswaard, The Netherlands). Ammonium hydroxide and ammonium bicarbonate were purchased from Sigma-Aldrich (Bornem, Belgium). Water was purified using a Milli-Q water purification system (Millipore, Brussels, Belgium). Aqueous buffers pH 8.0 and pH 9.0 were prepared by adjusting a 10 mM ammonium bicarbonate solution in Milli-Q water to, respectively, pH 8.0 and pH 9.0 with ammonium hydroxide.

Biosamples

Real case postmortem blood samples were collected from the femoral vein during autopsies by Forensic Medicine (University Hospital of Leuven, Belgium) and were analyzed by Toxicology and Pharmacology (KU Leuven, Belgium) as part of an ongoing judicial inquiry in 2016–2017. Based on prior analysis, positive BZD cases were selected. In total, 11 positive postmortem blood samples were evaluated in this study, as is shown in Table S1. Use of postmortem blood samples in this study was approved by the Committee for Medical Ethics UZ Leuven. Postmortem blood samples were stored at -20° C.

Medidrug[®] benzodiazepine Quality Control (QC) serum samples were purchased from MEDICHEM (Steinenbronn, Germany) at three concentration levels: L1, L2, and L3. The lyophilized controls were dissolved in MilliQ water at the day of analysis, as described in the user guidelines.

IL-DLLME-LC-MS/MS Method

The used IL-DLLME-LC-MS/MS method was previously described by De Boeck et al. (17) In summary, 1 mL blood was extracted using 60 µL of IL (BMIm PF₆) by means of a 5 min rotary mixing step. The collected IL extract was diluted in MeOH and injected into the LC-MS/MS instrument. It should be noted that for complex blood samples, it was necessary to first remove the upper blood layer prior to IL collection, to avoid matrix contamination. Separation of compounds was obtained on a Kinetex[®] Biphenyl column (100 mm \times 2.1 mm, 2.6 μ m) (Phenomenex, Utrecht, The Netherlands). A gradient elution was performed with mobile phase solvents (A) aqueous buffer pH 8.0 and (B) methanol: 0-9 min: 20-90% B; 9-11 min: 90% B; 11-12 min: 90-20% B; 12-14 min: 20% B. The triple quadrupole MS was operated using positive electrospray ionization, in scheduled multiple reaction monitoring (sMRM) mode. Medidrug[®] benzodiazepine QC serum samples (L1, L2, and L3) were analyzed at the beginning of the analytical run and checked for their appliance with acceptance criteria, stated in the certificate of analysis. More details on sample preparation, LC-MS/MS settings, data acquisition/processing, and construction of calibration curves were described by De Boeck et al. (17).

SPE-LC-MS/MS Method

The used SPE-LC-MS/MS method was previously described by Verplaetse et al. (19) In summary, 0.5 mL blood was extracted using mixed-mode SPE cartridges: Bond Elut Plexa PCX, 60 mg, 3 mL (Varian, Sint-Katelijne-Waver, Belgium). Cartridges were eluted in two steps: 3×1 mL acetone–chloroform (1:1) and 3×1 mL 2% ammoniated ethyl acetate. Next, the evaporated and reconstituted extract was injected into the LC-MS/MS instrument. Separation was obtained on an Acquity C18 column (50 × 2.1 mm, 1.7 µm) (Waters, Zellik, Belgium). A gradient elution was performed with mobile phase solvents (A) aqueous buffer pH 9.0 and (B) methanol: 0–10 min: 25– 90% B; 10–11 min: 90% B; 11–11.5 min: 90–25% B; 11.5– 13 min: 25% B. More details on sample preparation, LC-MS/ MS settings, data acquisition/processing, and construction of calibration curves were described by Verplaetse et al. (19).

Method Comparison

Eleven BZD postmortem cases were selected (Table S1). Postmortem blood was analyzed using IL-DLLME-LC-MS/MS (17) and SPE-LC-MS/MS (19) on the same day. Each sample was analyzed a single time, as only small sample volumes were available for this study. Both methods were compared for the identification and quantification of BZDs and BZD-like hypnotics. Qualitative comparison was performed based on the

	Benzodiazepine	IL-DLLME Conc.	IL-DLLME Clinical	SPE Conc.	SPE Clinical
	(-like hypnotic)	(ng/mL)	Interpretation	(ng/mL)	Interpretation
Case 1	Lorazepam	5	<ther< td=""><td>8</td><td><ther< td=""></ther<></td></ther<>	8	<ther< td=""></ther<>
	Lormetazepam	35	>Ther	40	>Ther
	Nordiazepam	756	Ther	429	Ther
	Oxazepam	67	<ther< td=""><td>105</td><td><ther< td=""></ther<></td></ther<>	105	<ther< td=""></ther<>
	Prazepam	<50 (LOO)	<ther< td=""><td>18</td><td><ther< td=""></ther<></td></ther<>	18	<ther< td=""></ther<>
Case 2	Alprazolam	18	Ther	15	Ther
	Diazepam	<50 (LOO)	<ther< td=""><td>20</td><td><ther< td=""></ther<></td></ther<>	20	<ther< td=""></ther<>
	Lorazepam	4	<ther< td=""><td><2 (LOO)</td><td><ther< td=""></ther<></td></ther<>	<2 (LOO)	<ther< td=""></ther<>
	Lormetazepam	<0.19 (LOD)	<ther< td=""><td><2 (LOO)</td><td><ther< td=""></ther<></td></ther<>	<2 (LOO)	<ther< td=""></ther<>
	Nordiazepam	2166	Tox	1318	>Ther
	Oxazepam	214	Ther	220	Ther
	Temazenam	<10 (LOO)	<ther< td=""><td>3</td><td><ther< td=""></ther<></td></ther<>	3	<ther< td=""></ther<>
Case 3 Case 4	Ethyl loflazenate	<10 (LOQ)	?	8	?
	Flurazenam	117	- >Ther	369	Tox
	Lorazenam	<2 (LOO)	<ther< td=""><td><2 (LOO)</td><td><ther< td=""></ther<></td></ther<>	<2 (LOO)	<ther< td=""></ther<>
	Lormetazenam	20	Ther	26	Ther
	Tetrazenam	NIM	v	6	Ther
	Diazenam	362	Ther	287	Ther
	Lorozonom	302	Ther	287	Ther
	Lorazepain	41 5	Ther	6	Ther
	Nordiozonom	200	Ther	227	There
	Overenem	200 <50 (LOO)	Ther	337	Ther
	Tamaganam	<50 (LOQ)	<1 her	24	<1 ner
C 5	Diagona	43 <50 (LOO)	Ther	12	Ther
Case 5	Diazepam	<50 (LOQ)	<ther< td=""><td>13</td><td><1 ner</td></ther<>	13	<1 ner
	Nordiazepam	<10 (LOQ)	<1 ner	9	<1 ner
a (Temazepam	<10 (LOQ)	<1 her	<2 (LOQ)	<1 ner
Case 6	Zolpidem	14	<1her	NIQM	X
Case 7	Flurazepam	<2 (LOQ)	<ther< td=""><td>2</td><td><ther< td=""></ther<></td></ther<>	2	<ther< td=""></ther<>
	Zopiclone	3	<1 her	NIQM	X
Case 8	Diazepam	347	Ther	261	Ther
	Lorazepam	282	>Ther	425	Tox
	Nitrazepam	<10 (LOQ)	<ther< td=""><td>2</td><td><ther< td=""></ther<></td></ther<>	2	<ther< td=""></ther<>
	Nordiazepam	334	Ther	411	Ther
	Oxazepam	<50 (LOQ)	<ther< td=""><td>60</td><td><ther< td=""></ther<></td></ther<>	60	<ther< td=""></ther<>
	Temazepam	54	Ther	50	Ther
Case 9	Bromazepam	<10 (LOQ)	<ther< td=""><td>2</td><td><ther< td=""></ther<></td></ther<>	2	<ther< td=""></ther<>
	Flurazepam	3	<ther< td=""><td>7</td><td><ther< td=""></ther<></td></ther<>	7	<ther< td=""></ther<>
	Lorazepam	29	Ther	47	Ther
Case 10	Alprazolam	3	<ther< td=""><td>2</td><td><ther< td=""></ther<></td></ther<>	2	<ther< td=""></ther<>
	Clonazepam	<2 (LOQ)	<ther< td=""><td>< 2 (LOQ)</td><td><ther< td=""></ther<></td></ther<>	< 2 (LOQ)	<ther< td=""></ther<>
	Diazepam	<50 (LOQ)	<ther< td=""><td>< 2 (LOQ)</td><td><ther< td=""></ther<></td></ther<>	< 2 (LOQ)	<ther< td=""></ther<>
	Lorazepam	77	Ther	106	Ther
	Nordiazepam	<10 (LOQ)	<ther< td=""><td>< 2 (LOQ)</td><td><ther< td=""></ther<></td></ther<>	< 2 (LOQ)	<ther< td=""></ther<>
	Oxazepam	<50 (LOQ)	<ther< td=""><td>21</td><td><ther< td=""></ther<></td></ther<>	21	<ther< td=""></ther<>
	Zolpidem	203	>Ther	NIOM	Х
	Tetrazepam	NIM	X	8	<ther< td=""></ther<>
Case 11	Alprazolam	<2 (LOQ)	<ther< td=""><td>< 2 (LOO)</td><td><ther< td=""></ther<></td></ther<>	< 2 (LOO)	<ther< td=""></ther<>
	Lorazepam	<2 (LOO)	<ther< td=""><td>2</td><td><ther< td=""></ther<></td></ther<>	2	<ther< td=""></ther<>
	Lormetazenam	8	Ther	7	Ther
	Nordiazenam	1051	>Ther	353	Ther
	Oxazenam	<50 (LOO)	<ther< td=""><td>17</td><td><ther< td=""></ther<></td></ther<>	17	<ther< td=""></ther<>
	Onucopuin		- 1 1101	± /	11101

TABLE 1-IL-DLLME and SPE analysis results of 11 forensic postmortem whole blood cases.

IL-DLLME-LC-MS/MS method: De Boeck et al. (17); SPE-LC-MS/MS method: Verplaetse et al. (19); <LOQ: detected, but not quantified, as lower than limit of quantification; <LOD: not detected, as lower than limit of detection; NIM: compound not included in method; NIQM: compound not included in quantitative method; x: no clinical interpretation was possible, since no concentrations were determined. Clinical interpretations were determined according to Regenthal et al. (24); ?: no information was found in literature regarding plasma concentrations and clinical interpretation.

identification of BZDs. Quantitative comparison was performed using Bland–Altman analysis. The inherent imprecision of both methods (CV_{Method}) was used to calculate acceptance limits on the observed differences (20):

for both methods was set at 15% and 20% (near LOQ). The following acceptance criteria were obtained for Bland–Altman analysis (20):

LOW concentrations: 0 \pm 0.416 \cdot mean

MED/HIGH concentrations: 0 \pm 0.554 \cdot mean

The Bland–Altman analysis was graphically presented as a difference plot; % difference as a function of average concentration. % Difference was calculated as (100*(A - B)/average), where A and B are, respectively, concentrations determined using IL-DLLME-LC-MS/MS (17) and SPE-LC-MS/MS (19).

License

Both SPE and IL-DLLME methods complied with bioanalytical validation guidelines (21,22). Therefore, inherent imprecision



FIG. 1—Bland–Altman analysis of benzodiazepine measurements in 11 postmortem blood samples. % Difference was calculated as (100*(A – B)/average), where A and B are, respectively, concentrations determined using IL-DLLME-LC-MS/MS (17) and SPE-LC-MS/MS (19). Horizontal dotted lines indicate acceptance limits for LOW and MED/HIGH concentration samples. Deviating observations are indicated in red. In between brackets, case numbers are indicated. FLU: flurazepam LOR: lorazepam; NORD: nordiazepam; TEM: temazepam. [Color figure can be viewed at wileyonlinelibrary.com]

Results and Discussion

Of 11 analyzed postmortem samples, 51 BZDs and BZD-like hypnotics were observed using the SPE-LC-MS/MS method. IL-DLLME-LC-MS/MS was able to detect 48 of 51 BZDs. Moreover, lormetazepam concentration (case 2) was below the limit of detection and was not detected using IL-DLLME-LC-MS/MS. However, it should be noted that the observed concentration was subtherapeutic and of limited forensic relevance. Furthermore, tetrazepam was not detected (case 3 and 10), as it was not included in the IL-DLLME method, due to its suspension from the European market in 2013 (23). Overall, qualitatively, both methods gave comparable results. Table 1 shows all 51 BZD observations: alprazolam (n = 3), bromazepam (n = 1)clonazepam (n = 1), diazepam (n = 5), ethyl loflazepate (n = 1), flurazepam (n = 3), lorazepam (n = 8), lormetazepam (n = 5), nordiazepam (n = 7), nitrazepam (n = 1), oxazepam (n = 6), prazepam (n = 1), temazepam (n = 4), tetrazepam (n = 2), zolpidem (n = 2), and zopiclone (n = 1).

Twenty-four of 51 BZD observations were quantified, as they were within calibration ranges of both methods. Figure 1 shows the associated difference plot. The following outliers were detected: flurazepam (case 3 and 9), lorazepam (case 4 and 9), nordiazepam (case 1,2 and 11), and temazepam (case 4). Lorazepam and nordiazepam were only included in the semiguantitative method due to deviations during IL-DLLME validation experiments (17), which is a possible explanation for the observed differences compared to SPE-LC-MS/MS. However, flurazepam and temazepam also showed deviating values, despite acceptable validation data for both methods. Deviating results can be explained by the absence of postmortem samples in IL-DLLME-LC-MS/MS matrix effect validation experiments. In this case, it is difficult to predict how postmortem matrix composition will affect quantification, especially when high variability in sample composition is expected, owing to chemical and biological degradation processes (16). Another explanation for the deviating flurazepam and temazepam results could be the choice of the ISTD. Selecting an appropriate ISTD should ideally compensate for matrix effects associated with different types of analyzed samples. However, in the SPE-LC-MS/MS method, only one nondeuterated ISTD was chosen to correct for a large group of compounds. This may indicate that complex samples (such as case 3 and 4) can have a significant impact on BZD quantification. Especially in case of flurazepam, the choice of ISTD can be a potential issue, as flurazepam and ISTD have a 2.5 min retention difference. The IL-DLLME-LC-MS/MS method uses 16 deuterated analogs, which should be more effective in compensating matrix effects.

Once more, these findings proof that caution is needed when analyzing postmortem samples. Additionally, it seems valuable to include complex postmortem samples during linearity and matrix effect validation experiments. Furthermore, it should be noted that the SPE method was unable to quantify zolpidem and zopiclone concentrations as they were excluded from the quantitative method. This was attributed to the inability of the ISTD to compensate for high SPE sample preparation variability (19).

Conclusion

A validated IL-DLLME-LC-MS/MS method (17) for the quantification of BZDs and BZD-like hypnotics was evaluated for its applicability in forensic toxicology, moreover, for the analysis of 11 relevant postmortem blood samples. The IL-DLLME-LC-MS/MS method was cross-compared to a validated SPE-LC-MS/MS method (19) via Bland-Altman analysis. In total, 51 BZDs and BZD-like hypnotics were detected. Both methods gave comparable qualitative results. Quantitative results also showed a high level of agreement for both methods, except for flurazepam (case 3 and 9), lorazepam (case 4 and 9), nordiazepam (case 1, 2 and 11), and temazepam (case 4). Lorazepam and nordiazepam were not included in the full-quantitative IL-DLLME method. Based on flurazepam and temazepam deviations, it can be concluded that care should be taken when complex biological matrices are analyzed. Several biomolecules such as lipids, proteins, and sugars can alter matrix effects and thus influence quantification. Especially, the assessment of postmortem samples can be very challenging, as several degradation mechanisms are involved. The inclusion of postmortem samples during validation should be advised for forensic analytical methods. Furthermore, appropriate deuterated ISTDs should be selected to compensate for possible matrix effects. Overall, it can be concluded that the published IL-DLLME method has shown promise for its application in forensic toxicology. It should be noted that this is the first paper that focusses on postmortem blood samples, using the IL-based liquid-liquid microextraction technique.

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References

- 1. Filippou O, Bitas D, Samanidou V. Green approaches in sample preparation of bioanalytical samples prior to chromatographic analysis. J Chromatogr B Anal Technol Biomed Life Sci 2017;1043:44-62.
- 2. Bylda C, Thiele R, Kobold U, Volmer DA. Recent advances in sample preparation techniques to overcome difficulties encountered during quantitative analysis of small molecules from biofluids using LC-MS/MS. Anal R Soc Chem 2014;139(10):2265-76.
- 3. Hayes RN. Bioanalytical methods for sample cleanup. BioPharm Int 2012;25(12):52-4.
- 4. Saraji M, Boroujeni MK. Recent developments in dispersive liquid-liquid microextraction. Anal Bioanal Chem 2014;406(8):2027-66.
- 5. Spietelun A, Marcinkowski Ł, de la Guardia M, Namieśnik J. Green aspects, developments and perspectives of liquid phase microextraction techniques. Talanta 2014;119:34-45.
- 6. Escudero LB, Castro Grijalba A, Martinis EM, Wuilloud RG. Bioanalytical separation and preconcentration using ionic liquids. Anal Bioanal Chem 2013;405(24):7597-613.
- 7. Rezaee M, Assadi Y, Hosseinia MRM, Aghaee E, Ahmadi F, Berijani S, et al. Determination of organic compounds in water using dispersive liquid-liquid microextraction. J Chromatogr A 2006;1116(1-2):1-
- 8. Trujillo-Rodríguez MJ, Rocío-Bautista P, Pino V, Afonso AM. Ionic liquids in dispersive liquid-liquid microextraction. Trends Anal Chem 2013;51:87-106.
- 9. Zainal-Abidin MH, Hayyan M, Hayyan A, Jayakumar NS. New horizons in the extraction of bioactive compounds using deep eutectic solvents: a review. Anal Chim Acta 2017;979:1-23.
- 10. Pena-Pereira F, Namieśnik J. Ionic liquids and deep eutectic mixtures: sustainable solvents for extraction processes. Chemsuschem 2014;7 (7):1784-800.
- 11. An J, Trujillo-Rodríguez MJ, Pino V, Anderson JL. Non-conventional solvents in liquid phase microextraction and aqueous biphasic systems. J Chromatogr A 2017;1500:1-23.
- 12. Torimoto T, Tsuda T, Okazaki KI, Kuwabata S. New frontiers in materials science opened by ionic liquids. Adv Mater 2010;22(11):1196-221.

- 13. Ho TD, Zhang C, Hantao LW, Anderson JL. Ionic liquids in analytical chemistry: fundamentals, advances, and perspectives. Anal Chem [Internet] 2014;86(1):262-85.
- 14. Fei ZF, Dyson PJ. The making of iLiquids the chemist's equivalent of the iPhone. Chem Commun 2013;49(26):2594-6.
- 15. Davis JH. Task-specific ionic liquids. Chem Lett 2004;33(9):1072-7.
- Verplaetse R, Tytgat J. Liquid chromatography tandem mass spectrometry in 16 forensic toxicology: what about matrix effects? TIAFT Bull 2011;41(1):8-16.
- 17. De Boeck M, Missotten S, Dehaen W, Tytgat J, Cuypers E. Development and validation of a fast ionic liquid-based dispersive liquid-liquid microextraction procedure combined with LC-MS/MS analysis for the quantification of benzodiazepines and benzodiazepine-like hypnotics in whole blood. Forensic Sci Int 2017;274:44-54.
- Levine B, editor. Principles of forensic toxicology. Washington, DC: AACC Press, 2013.
- 19. Verplaetse R, Cuypers E, Tytgat J. The evaluation of the applicability of a high pH mobile phase in ultrahigh performance liquid chromatography tandem mass spectrometry analysis of benzodiazepines and benzodiazepinelike hypnotics in urine and blood. J Chromatogr A 2012;1249:147-54.
- 20. Jensen AL, Kjelgaard-Hansen M. Method comparison in the clinical laboratory. Vet Clin Pathol 2006;35(3):276-86.
- 21. Polettini A, editor. Applications of LC-MS in toxicology. London, U.K.: Pharmaceutical Press, 2006.
- 22. Peters FT, Drummer OH, Musshoff F. Validation of new methods. Forensic Sci Int 2007;165(2-3):216-24.
- 23. European Medicines Agency; http://www.ema.europa.eu/ema/index.jsp? curl=pages/medicines/human/referrals/Tetraz epam_containing_medicinal_ products/human_referral_prac_000015.jsp&mid=WC0b0 1ac05805c516f (accessed January 16, 2018).
- 24. Regenthal R, Krueger M, Koeppel C, Preiss R. Drug levels: therapeutic and toxic serum/plasma concentrations of common drugs. J Clin Monit Comput 1999;15(7-8):529-44.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Table S1. Postmortem forensic cases: positive for benzodiazepines and benzodiazepine-like hypnotics.