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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

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ABSTRACT

To date, thorough clean-up of complex biological samples remains an essential part of the analytical process. The solid phase extraction (SPE) technique is the well-known standard, however, its main weaknesses are the labor-intensive and time-consuming protocols. In this respect, dispersive liquid–liquid microextractions (DLLME) seem to offer less complex and more efficient extraction procedures. Furthermore, ionic liquids (ILs) – liquid salts – are emerging as new promising extraction solvents, thanks to their non-flammable nature, negligible vapor pressure and easily adaptable physiochemical properties. In this study, we investigated whether ILs can be used as an extraction solvent in a DLLME procedure for the extraction of a broad range of benzodiazepines and benzodiazepine-like hypnotics in whole blood samples. 1.0 mL whole blood was extracted using an optimized 30-min IL-based DLLME procedure, followed by LC-ESI(+)-MS/MS analysis in scheduled MRM scan mode. The optimized analytical method was successfully validated for 7-aminoflunitrazepam, alprazolam, bromazepam, clobazam, clonazepam, clotiazepam, diazepam, estazolam, ethyl loflazepate, etizolam, flurazepam, lormetazepam, midazolam, oxazepam, prazepam, temazepam, triazolam, zolpidem and zopiclone. The method showed good selectivity for endogenous interferences based on 12 sources of blank whole blood. No benzodiazepine interferences were observed, except for clorazepate and nordiazepam, which were excluded from the quantitative method. Matrix-matched calibration curves were constructed covering the whole therapeutic range, including low toxic plasma concentrations. Accuracy and precision results met the proposed acceptance criteria for the vast majority of compounds, except for brotizolam, chlordiazepoxide, cloxazolam, flunitrazepam, loprazolam, lorazepam and nitrazepam, which can only be determined in a semi-quantitative way. Recoveries were within the range of 24.7%–127.2% and matrix effects were within 20.0%–92.6%. Both parameters were tested using 5 sources of whole blood and coefficients of variance were below 20%. Overall, the applicability of ILs as promising solvents for the extraction of benzodiazepines in whole blood samples has been proven. Moreover, a fast and easy IL-based DLLME procedure was developed for the quantification of 19 benzodiazepines and benzodiazepine-like hypnotics.

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1. Introduction

In toxicology, the analysis of complex biological samples remains a challenging task. The introduction of a thorough sample clean-up step has become inevitable. Matrix components need to be eliminated in order to avoid possible interferences during quantification and to minimize the chance of polluting the analytical instrument. Moreover, a good sample preparation step can result in analyte enrichment and thus a final analytical method with improved sensitivity [1–3]. To date, the solid phase extraction (SPE) technique is one of the most frequently used sample preparation techniques in the biomedical field. Thanks to its high

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specificity and the ability of thoroughly eliminating matrix components, SPE has become a popular extraction technique. However, long extraction times, complex multi-step procedures and expensive columns make this technique less attractive. In this respect, the use of liquid–liquid extractions (LLE), is a more interesting alternative. Although, from a historical perspective, LLE is an older technique, it consists of faster and less complex protocols when compared to SPE. However, LLE procedures consume large volumes of hazardous volatile organic solvents (VOS) [1–5]. In this respect, two current trends in sample preparation seem to offer new perspectives.

The first trend is the use of microextractions. Microextractions are defined by the small volumes (μL range) of extraction solvents that are used and the high enrichment factors that go with it. A popular technique is the dispersive liquid–liquid microextraction (DLLME) technique. It is performed by adding a small volume of the immiscible extraction solvent to the sample. Both phases are mixed in order to obtain a fine dispersion and thus a high contact surface between the extraction solvent and the sample containing the analyte. The higher the contact surface, the better the transfer of analyte toward the extraction solvent. Eventually both phases are separated by centrifugation. The extraction solvent is collected and analyzed [1,6–9].

A second trend in sample preparation is the use of alternative extraction solvents other than the conventional VOS. Several disadvantages are associated with the use of VOS, such as their flammable nature and environmental pollution due to their volatility. Moreover, they are non-specific when it comes to extracting specific compounds from a complex matrix [4,6]. An interesting group of alternative extraction solvents are ionic liquids (ILs). ILs are liquid salts with melting points generally below 100 °C and consist entirely of ions. Typically, an organic cation is combined with an organic/inorganic anion. ILs are characterized by negligible vapor pressures, their non-flammable nature, high thermal stability and most interestingly, easily tunable physicochemical properties. This last feature makes it possible to easily introduce certain chemical groups into the chemical structure of the IL, resulting in task-specific ionic liquids (TSILs). This means that, as extraction solvents, TSILs can represent a step forward in the extraction of certain classes of drugs that were – up till now – very difficult to be extracted with the conventional solvents [4,7,10–14].

The combination of both previously discussed trends results in a state-of-the-art extraction technique; the IL-based DLLME technique, characterized by its efficient extraction protocols, using novel extraction solvents. So far, the use of IL-DLLME has been documented widely for the extraction of metal ions from aqueous and biological samples [4]. Not only metal ions, but also the extraction of drugs present in biological samples such as urine, blood, saliva and nails has been successfully demonstrated. When it comes to blood as a frequently analyzed sample matrix, it is often serum and plasma that are being studied [1,7,8]. Whole blood studies are difficult to find, despite its relevance in forensic settings, since plasma and serum are often not available due to cell lysis [2]. The largest groups of studied organic analytes in the context of IL-based DLLME are sulfonamides, alkaloids, pesticides, fungicides and NSAIDs [1,7,8]. To our knowledge, no benzodiazepines (BZDs) have been studied. However, they form an important class of drugs in toxicology. BZDs are often detected in real case samples, because of their widespread prescription due to broad therapeutic windows. Also in illegal circuits BZDs have been used for many years in combination with alcohol, opiates and other illegal drugs [15,16]. More recently, the rapidly growing problem of psychoactive (designer) BZDs has been the center of attention [17–19].

Two established techniques for benzodiazepine extraction from whole blood samples are SPE and LLE. SPE generally has high extraction yields around 100%, except for the more polar compounds as 7-aminoclonazepam, 7-aminoflunitrazepam and 7-aminonitrazepam, respectively 35%, 38% and 59% [20]. Moreover, SPE precision data show rather high variation, especially zolpidem and zopiclone have proven to be challenging analytes [20,21]. An additional drawback of the SPE technique are its time-consuming and complex procedures. Next to SPE, several LLE extraction procedures have been published concerning benzodiazepine determination in whole blood. Montenarh et al. have recently developed a simple LLE procedure that showed good validation results for 16 benzodiazepines [22]. Again, zolpidem and zopiclone showed deviating results. For zolpidem, matrix effects were obtained with poor repeatability and values greater than 130%, while the LOD values of zopiclone were too high (0.3 mg/mL) for the detection of therapeutic levels in whole blood. Another major concern was the still extensive 3-step protocol (2 × extraction + 1 × evaporation) associated with the use of rather high volumes of organic extraction solvent (2 mL). Apart from traditional SPE and LLE protocols, several microextraction techniques have been developed, however, they only focus on a small group of benzodiazepines and generally urine and plasma are assessed instead of whole blood [23].

Overall, the extraction of BZDs in whole blood samples remains an actual topic in toxicology, whereas it is difficult to extract a broad range of physicochemical differing compounds in one single step. Also, with the need for faster and more environmentally-friendly techniques, it is interesting to further investigate micro-extraction techniques in combination with novel extraction solvents. Therefore, the aim of this study was to develop a fast and simple IL-DLLME procedure for the extraction of a broad range of BZDs and BZD-like hypnotics from whole blood samples, while focusing on the proof-of-concept application of ILs as promising extraction solvents in toxicology.

2. Materials and methods

2.1. Chemicals and reagents

Analytical reference standards of 7-aminoflunitrazepam.d7 (1 mg/mL), alprazolam (1 mg/mL), alprazolam.d5 (1 mg/mL), chlordiazepoxide.d5 (0.1 mg/mL), clobazam (1 mg/mL), clonazepam (1 mg/mL), clonazepam.d4 (1 mg/mL), diazepam (1 mg/mL), diazepam.d5 (1 mg/mL), flunitrazepam.d7 (0.1 mg/mL), flurazepam (1 mg/mL), lorazepam (1 mg/mL), lorazepam.d4 (1 mg/mL), lormetazepam (1 mg/mL), midazolam (1 mg/mL), midazolam.d4 (0.1 mg/mL), nitrazepam (1 mg/mL), nitrazepam.d5 (0.1 mg/mL), nordiazepam (1 mg/mL), nordiazepam.d5 (1 mg/mL), oxazepam (1 mg/mL), oxazepam.d5 (1 mg/mL), prazepam (1 mg/mL), prazepam.d5 (0.1 mg/mL), temazepam (1 mg/mL), temazepam.d5 (1 mg/mL), triazolam.d4 (0.1 mg/mL), zolpidem.d7 (0.1 mg/mL) and zopiclone (1 mg/mL) were purchased from Cerilliant (Round Rock, Texas, USA). 7-Aminoflunitrazepam (1 mg/mL), bromazepam (1 mg/mL), chlordiazepoxide (1 mg/mL), estazolam (1 mg/mL), estazolam.d5 (0.0999 mg/mL), etizolam (1 mg/mL), flunitrazepam (1 mg/mL), triazolam (1 mg/mL) and zolpidem tartrate (1 mg/mL) were purchased from LGC (Molsheim, France). Powder form reference standards of brotizolam (10 mg) and loprazolam mesilate (50 mg) were obtained from EDQM, Council of Europe (Strasbourg, France) and the British Pharmacopoeia Commission Laboratory (Teddington, UK), respectively. Ethyl loflazepate (100 mg) was purchased from Sanofi-Aventis (Diegem, Belgium). All powder form reference standards were diluted in methanol to obtain a final concentration of 1 mg/mL. Clorazepate, clotiazepam

and cloxazolam were extracted from their commercially available tablet forms, respectively, Tranxene®, Clozan® and Akton®, and diluted to a final concentration of 1 mg/mL in methanol. Methanolic standard stock solutions were prepared at different concentration levels by mixing reference standards of the 26 BZDs and 2 BZD-like hypnotics. A separate methanolic standard stock solution of all 16 deuterated analogues was prepared with a final concentration of 5 μg/mL. All standard solutions were stored at −20 °C. As ILs, 1-butyl-3-methylimidazolium hexafluorophosphate (BMIm PF_6) (99.5%), 1-hexyl-3-methylimidazolium hexafluorophosphate (HMIm PF_6) (99%) and 1-methyl-3-octylimidazolium hexafluorophosphate (OMIm PF_6) (99%) were purchased from IOLITEC Ionic Liquids Technologies GmbH (Heilbronn, Germany). All solvents and mobile phase additives were LC–MS grade quality. Methanol and acetonitrile were obtained from Biosolve (Valkenswaard, The Netherlands). Acetic acid, ammonium acetate, 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 were prepared as follows: pH 4.0: 10 mM of ammonium acetate, adjusted to pH 4.0 with acetic acid; pH 6.0: 10 mM of ammonium acetate, adjusted to pH 6.0 with acetic acid; 10 mM of ammonium bicarbonate, adjusted to pH 8.0 with ammonium hydroxide.

2.2. Biosamples

Blank donor whole blood was obtained from the blood transfusion centre (Gasthuisberg, Leuven, Belgium). To all blood samples, 1% sodium fluoride (Merck, Darmstadt, Germany) was added and samples were stored at −20 °C. In order to check for the absence of BZDs and BZD-like hypnotics before using the donor whole blood samples in experiments, they were analyzed using a validated SPE-LC–MS/MS method [21]. Positive donor whole blood samples were not included in the optimization and validation experiments. Experiments were performed using blank donor whole blood, spiked with a standard stock solution of 26 BZDs and 2 BZD-like hypnotics.

2.3. Method optimization

In order to optimize the IL-DLLME sample preparation procedure, parameters that could influence extraction efficiencies were evaluated. The assessed parameters were the type of IL added (BMIm PF_6, HMIm PF_6, OMIm PF_6), dilution and pH adjustment of the whole blood sample (no dilution, addition of 1.0 mL aqueous buffer pH 4.0, pH 6.0, pH 8.0), volume of IL added (20 μL, 40 μL, 60 μL, 80 μL, 100 μL) and collected (practically assessed), extraction method (rotary mixer, ultrasonic bath), extraction time (5 min, 10 min, 20 min, 40 min, 60 min) and dilution of the final extract (1:10, 1:20, 1:50 in methanol). All conditions for all parameters were tested in triplicate, process efficiencies (PE) were calculated and statistically evaluated using the Kruskal–Wallis test ($\alpha = 0.05$) in combination with a Dunn's multiple comparison test ($\alpha = 0.05$). PE values were calculated by dividing peak areas of pre-extraction spiked samples by peak areas of pure standards in methanol, both at a concentration of 100 ng/mL.

2.4. Final IL-DLLME sample preparation procedure

1.0 mL whole blood was transferred into a conical bottom glass tube and spiked with 20 μL standard stock solution of 16 deuterated BZD analogues (5 μg/mL). In a next step, the sample was diluted to 2.0 mL with aqueous buffer pH 8.0. Subsequently, 60 μL IL, BMIm PF_6, was added as an extraction solvent and the tube was rotated for 5 min, using a rotary mixer at 50 rpm. This mixing step results is a fine dispersion of the IL phase in the blood sample, enabling the efficient transfer of analyte toward the IL phase. In order to induce phase separation, the tube was centrifuged for 6 min at 3500 rpm. Two phases were formed: the upper blood phase and the lower IL phase. Finally, 10 μL of the separated IL phase was collected and diluted 1:10 in methanol in a vial. Vials were placed in the cooled autosampler and 10 μL was injected into the LC system. A simplified overview of the final IL-DLLME procedure is shown in Fig. 1.

2.5. Final LC–MS/MS analysis

A Shimadzu Prominence Ultra-Fast Liquid Chromatograph XR System (Shimadzu Benelux, Jette, Belgium) in combination with a Kinetex® Biphenyl LC Column (100 mm × 2.1 mm, 2.6 μm particle size) (Phenomenex, Utrecht, The Netherlands) were used for the separation of compounds in time. Aqueous buffer pH 8.0 and methanol were used as mobile phases A and B, respectively. A gradient elution program was set up, starting at 20% B, following a linear gradient reaching 90% B in 9 min. This condition was kept constant for 2 min, followed by a linear decrease, back to 20% B in 1 min. The starting conditions were kept constant for another 2 min in order to re-equilibrate the system. The total analytical run time was 14 min. Flow rate, column oven, autosampler cooler and sample injection volume were set at 0.5 mL/min, 45 °C, 10 °C and 10 μL, respectively. The first 5 min of the chromatographic run, the LC effluent was transported toward the waste, by means of a switching valve. This step was introduced in order to avoid high IL signals reaching the MS and therefore, the durability of the system was enhanced. For the remaining 9 min, the effluent was sent toward the Turbo V ion source, where it was ionized using an electrospray ionization (ESI) probe (Sciex, Halle, Belgium). Source parameters were set as follows: curtain gas: nitrogen, 25 psi; nebulizing gas: nitrogen, 55 psi; heater gas: nitrogen, 55 psi; ion source temperature: 550 °C; ion source voltage: +5500 V. A 3200 QTRAP mass spectrometer (Sciex, Halle, Belgium) was operated in scheduled multiple reaction monitoring (sMRM) scan mode to detect analyte ions. MRM transitions, retention times and other compound-dependent parameters are presented in Table 1. Fig. 2 shows a final chromatogram of a processed sample.

2.6. Data acquisition and processing

Data acquisition and processing was performed on a Dell Precision™ 390 Workstation equipped with Analyst software version 1.5.1. (Sciex, Halle, Belgium). All statistical analyses were performed using GraphPad Prism software version 6.0 (GraphPad Software, La Jolla, CA, USA).

2.7. Method validation

The validation of the analytical IL-DLLME-LC–MS/MS method was performed following internationally accepted validation guidelines for bioanalytical methods [24–26]. The following parameters were evaluated: selectivity, linearity, accuracy, precision, limit of quantification (LOQ), limit of detection (LOD), recovery, matrix effect and stability. All validation experiments were performed using blank donor whole blood, spiked with a standard stock solution of 26 BZDs and 2 BZD-like hypnotics at concentrations as shown in Table 2.

2.7.1. Selectivity

Determining selectivity is necessary to check if the optimized analytical method is able to differentiate between the analyte and

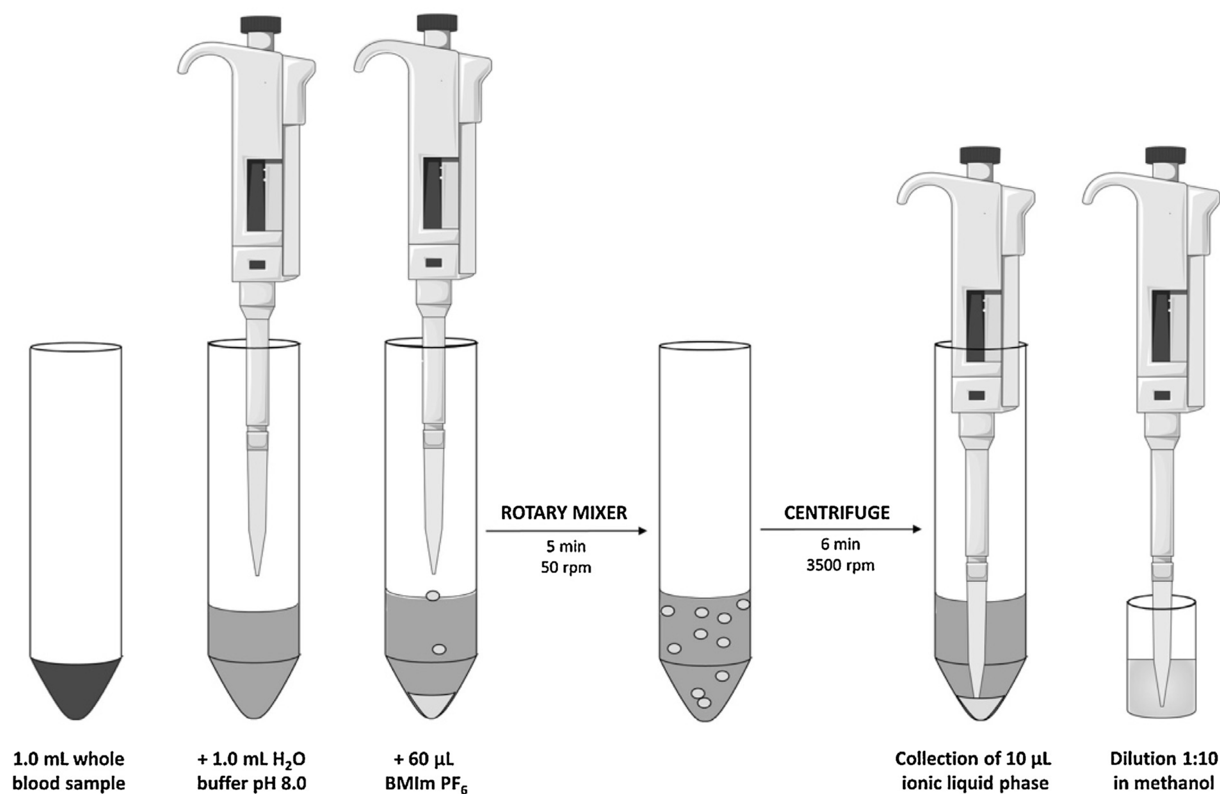

Fig. 1. Simplified overview of the final IL-DLLME sample preparation procedure.

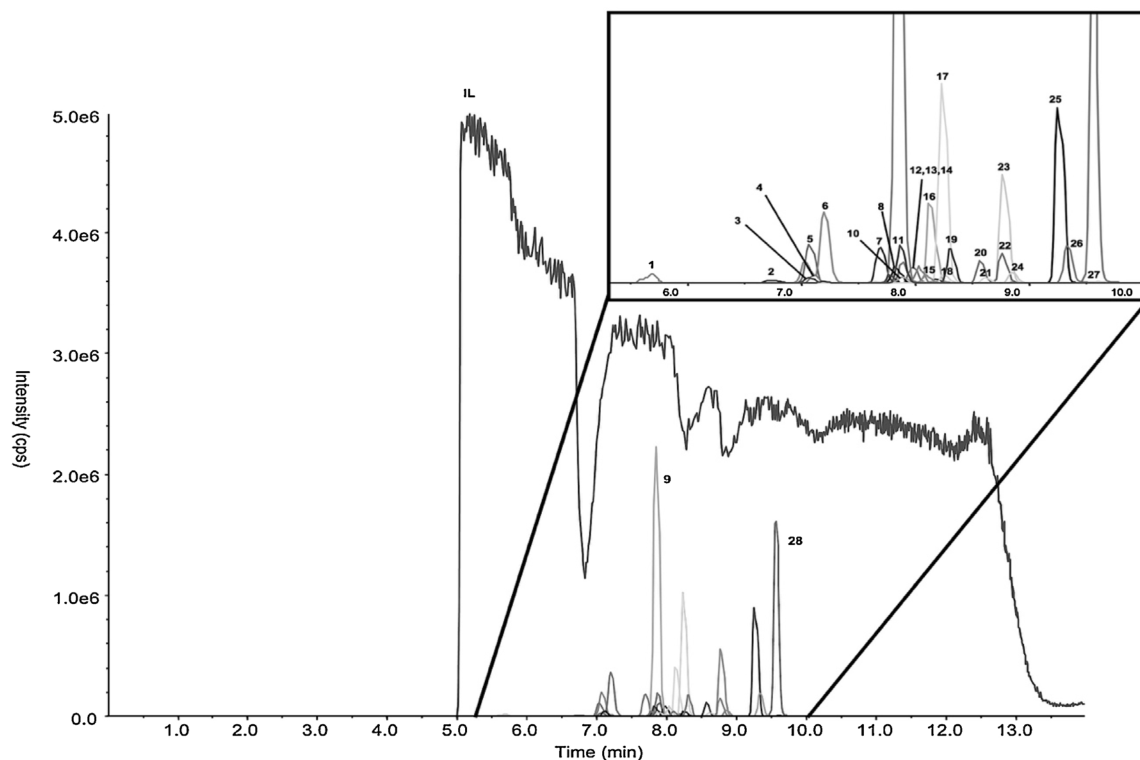

Fig. 2. Final chromatogram of a processed sample (whole blood spiked with 250 ng/mL of standard). During the first 5 min of the chromatographic run, the effluent was transported toward the waste. Signals of the IL, 26 BZDs and 2 BZD-like hypnotics are shown. (1) 7-Aminoflunitrazepam; (2) bromazepam; (3) lorazepam; (4) clonazepam; (5) nitrazepam; (6) oxazepam; (7) chlordiazepoxide; (8) flunitrazepam; (9) clobazam; (10) zopiclone; (11) clorazepate; (12) nordiazepam; (13) estazolam; (14) lormetazepam; (15) triazolam; (16) temazepam; (17) zolpidem; (18) alprazolam; (19) ethyl loflazepate; (20) cloxazolam; (21) brotizolam; (22) midazolam; (23) diazepam; (24) etizolam; (25) clotiazepam; (26) flurazepam; (27) loprazolam; (28) prazepam.

Table 1
Retention times, MRM transitions and compound-dependent MS settings for each analyte and deuterated internal standard.

	RT (min)	DP (V)	EP (V)	CEP (V)	Q1 mass (Da)	Q3 mass *MRM 1* (Da)	CE (V)	Q3 mas *MRM 2* (Da)	CE (V)
7-Aminoflunitrazepam	5.6	58	10.0	30	284.1	135.0	39	226.2	37
7-Aminoflunitrazepam.d7	5.6	58	10 .0	18	291.2	138.3	37	230.2	41
Alprazolam	8.2	66	7.5	30	309.2	205.2	53	281.2	30
Alprazolam.d5	8.2	66	7.5	19	314.2	210.2	53	286.2	30
Bromazepam	6.8	53	7.5	32	316.0	182.2	43	209.2	33
Brotizolam	8.6	56	11.0	43	393.1	314.1	32	210.1	57
Chlordiazepoxide	7.7	53	5.5	30	300.1	227.2	31	283.8	19
Chlordiazepoxide.d5	7.7	53	5.5	19	305.1	232.1	31	288.2	19
Clobazam	7.9	50	8.0	30	301.2	259.2	30	224.2	45
Clonazepam	7.1	61	7.5	31	316.1	270.0	34	214.0	54
Clonazepam.d4	7.1	61	7.5	19	320.1	274.0	34	218.0	54
Clorazepate	7.9	65	10.0	18	271.0	140.1	50	165.0	50
Clotiazepam	9.2	45	9.0	35	319.1	154.1	41	291.0	30
Cloxazolam	8.6	68	9.0	35	349.1	305.0	35	140.0	50
Diazepam	8.8	63	8.5	30	285.2	193.2	44	154.1	37
Diazepam.d5	8.8	63	8.5	18	290.2	198.2	44	154.0	37
Estazolam	8.0	65	10.0	18	295.0	267.1	50	241.0	50
Estazolam.d5	8.0	65	10.0	18	300.1	272.2	50	/	/
Ethyl loflazepate	8.3	55	5.0	20	363.1	261.2	47	289.2	25
Etizolam	8.8	65	10.0	20	343.1	314.2	35	259.1	45
Flunitrazepam	7.8	40	7.5	31	314.2	268.1	25	239.1	40
Flunitrazepam.d7	7.8	40	7.5	19	321.2	275.1	25	246.1	40
Flurazepam	9.3	43	7.5	43	388.2	315.0	31	317.0	27
Loprazolam	9.5	68	9.5	43	465.2	252.1	57	408.0	33
Lorazepam	7.0	50	5.0	30	321.1	275.0	25	229.2	35
Lorazepam.d4	7.0	50	5.0	19	325.1	279.1	27	233.2	37
Lormetazepam	8.0	50	10.0	32	335.1/337.1	289.1	30	291.1	30
Midazolam	8.7	71	7.0	35	326.2	291.1	36	249.1	47
Midazolam.d4	8.7	71	7.0	19	330.2	295.1	36	253.1	47
Nitrazepam	7.1	65	9.0	29	282.2	236.1	34	180.2	51
Nitrazepam.d5	7.1	65	9.0	18	287.2	241.1	34	185.2	51
Nordiazepam	7.9	58	4.5	18	273.0	142.0	37	208.2	33
Nordiazepam.d5	7.9	58	4.5	18	278.0	142.0	39	167.2	37
Oxazepam	7.2	45	7.5	31	287.2	241.0	30	269.0	25
Oxazepam.d5	7.2	45	7.5	18	292.2	246.0	30	274.0	25
Prazepam	9.5	60	10.0	37	325.2	271.0	32	140.1	48
Prazepam.d5	9.5	60	10.0	19	330.2	276.0	32	213.0	48
Temazepam	8.2	46	6.5	30	301.2/303.2	255.0	29	257.0	32
Temazepam.d5	8.2	46	6.5	19	306.2/308.2	260.0	29	262.0	32
Triazolam	8.1	76	7.5	33	343.1	239.1	59	308.1	38
Triazolam.d4	8.1	76	7.5	20	347.1	243.1	59	312.1	38
Zolpidem	8.2	56	5.5	32	308.2	235.2	47	236.2	38
Zolpidem.d7	8.2	56	5.5	19	315.2	242.2	47	243.2	38
Zopiclone	7.8	33	4.0	56	389.2	245.1	25	217.1	43

RT: retention time; DP: declustering potential; EP: entrance potential; CEP: collision cell entry potential. The collision cell exit potential (CXP) was set at 4.0 V. Since we used a scheduled MRM mode, all MRM transitions were measured during a compound-specific detection window of 100 s. The target scan time was set at 0.9 s. Underlined transitions were used for quantification. The majority of MRM transitions were published by Verplaetse et al. [21], MS settings for the remaining compounds were determined by direct infusion.

other possible structurally related compounds present in the matrix, as for instance deuterated analogues. Eight different types of ante-mortem blank whole blood samples and four different types of post-mortem blank whole blood samples were extracted using the IL-DLLME protocol, analyzed and checked for interferences. Two zero samples were prepared by spiking 2 different types of ante-mortem blank whole blood samples with 20 μL standard stock solution of 16 deuterated analogues (5 μg/mL). Zero samples were extracted, analyzed and checked for interferences. In order to check for mutual interferences of benzodiazepines, methanolic standard solutions were made for all 28 compounds at high concentration levels (highest calibrator concentration). These stock solutions were checked for possible interferences with other analytes included in the analytical method.

2.7.2. Linearity

For reliable quantification, the relation between the analyte concentration and the analyte response signal is demonstrated. The choice of an appropriate calibration model was made based on selecting the models with the lowest back-calculated values (within 25% of nominal concentration) and comparing these models based on precision and accuracy data. The final model was selected based on the best – within specification – precision and accuracy results. The following models were investigated: linear least squares un-weighted and weighted ($1/x$, $1/x^2$) regression models and quadratic least squares un-weighted and weighted ($1/x$, $1/x^2$) regression models. Calibrators were matrix-based ($n = 6$ different types of donor blood) and 7 concentration levels were used to construct calibration curves. Furthermore, for each analyte, an appropriate internal standard (ISTD) was selected. For 15 analytes, deuterated analogues were available. For all remaining analytes, the three most appropriate ISTDs were selected based on a similarity in behavior during ionization and chromatographic separation. Matrix effect, recovery, competition of co-eluting compounds and retention times were taken into account when choosing the internal standards. A comparison was

Table 2
Nominal concentrations of quality controls used for validation.

	Level 1	Level 2	Level 3	Level 4	Level 5	Level 6	Level 7	Linearity
		LOW		MED			HIGH	Accuracy and precision
		LOW	LOW	LOW	HIGH	HIGH	HIGH	Recovery and matrix effect
		LOW				HIGH		Stability
7-Aminoflunitrazepam	2.0	5.0	10.0	25.0	50.0	100.0	250.0	
Alprazolam	2.0	5.0	10.0	25.0	50.0	100.0	250.0	
Bromazepam	10.0	20.0	50.0	100.0	250.0	500.0	1000.0	
Brotizolam	2.0	5.0	10.0	25.0	50.0	100.0	250.0	
Chlordiazepoxide	50.0	100.0	250.0	500.0	1000.0	1500.0	2000.0	
Clobazam	50.0	100.0	250.0	500.0	1000.0	1500.0	2000.0	
Clonazepam	2.0	5.0	10.0	25.0	50.0	100.0	250.0	
Clorazepate	10.0	20.0	50.0	100.0	250.0	500.0	1000.0	
Clotiazepam	50.0	100.0	250.0	500.0	1000.0	1500.0	2000.0	
Cloxazolam	10.0	20.0	50.0	100.0	250.0	500.0	1000.0	
Diazepam	50.0	100.0	250.0	500.0	1000.0	1500.0	2000.0	
Estazolam	10.0	20.0	50.0	100.0	250.0	500.0	1000.0	
Ethyl loflazepate	10.0	20.0	50.0	100.0	250.0	500.0	1000.0	
Etizolam	2.0	5.0	10.0	25.0	50.0	100.0	250.0	
Flunitrazepam	2.0	5.0	10.0	25.0	50.0	100.0	250.0	
Flurazepam	2.0	5.0	10.0	25.0	50.0	100.0	250.0	
Loprazolam	2.0	5.0	10.0	25.0	50.0	100.0	250.0	
Lorazepam	2.0	5.0	10.0	25.0	50.0	100.0	250.0	
Lormetazepam	2.0	5.0	10.0	25.0	50.0	100.0	250.0	
Midazolam	10.0	20.0	50.0	100.0	250.0	500.0	1000.0	
Nitrazepam	10.0	20.0	50.0	100.0	250.0	500.0	1000.0	
Nordiazepam	10.0	20.0	50.0	100.0	250.0	500.0	1000.0	
Oxazepam	50.0	100.0	250.0	500.0	1000.0	1500.0	2000.0	
Prazepam	50.0	100.0	250.0	500.0	1000.0	1500.0	2000.0	
Temazepam	10.0	20.0	50.0	100.0	250.0	500.0	1000.0	
Triazolam	2.0	5.0	10.0	25.0	50.0	100.0	250.0	
Zolpidem	10.0	20.0	50.0	100.0	250.0	500.0	1000.0	
Zopiclone	2.0	5.0	10.0	25.0	50.0	100.0	250.0	

All concentrations are given in ng/mL.Underlined concentrations indicate the concentration levels that were used for recovery and matrix effect tests.

made between calibration curves constructed using the selected ISTDs, and finally, the ISTD attaining the best back-calculated values was selected.

2.7.3. Accuracy and precision

Accuracy tests were performed to provide an indication whether the measured concentration equals the theoretical sample concentration. In addition, both repeatability and intermediate precision were assessed, representing variability within a day and the total of within and between day variance, respectively. Daily spiked QC samples were tested at LOW, MED and HIGH concentration levels. All 3 levels were evaluated on each of eight days (n = 2 types of different donor blood). Accuracy was calculated as bias and repeatability and intermediate precision were calculated as a relative standard deviation, RSD_{rep} and $RSD_{int\ pr}$, respectively. Calculations were performed using the following formulas [24]:

$$Bias(\%) = \frac{X - \mu}{\mu}.100$$

$$RSD_{rep}(\%) = \frac{\sqrt{MS_W}}{X}.100$$

$$RSD_{int\ pr}(\%) = \frac{\sqrt{\frac{MS_B+(n-1).MS_W}{n}}}{X}.100$$

X represents the mean calculated concentration and μ represents the nominal concentration. A one-way ANOVA was performed on the calculated concentrations, using 'days' as the grouping variable, in order to obtain mean square within day (MS_W) and mean square between day (MS_B) values (n = number of observations each day).

2.7.4. Limit of quantification (LOQ) and limit of detection (LOD)

The LOQ was assessed by analyzing the lowest calibrator used for linearity testing (n = 6 different types of whole blood). The signal-to-noise ratio (S/N) was evaluated and should be greater or equal to 10. The LOD was evaluated based on a specific linear unweighted calibration curve that was constructed near the LOQ, using the second lowest calibrator, LOQ and zero samples as calibrators. The following formula was used for calculating the LOD [24]:

$$LOD = \frac{3 * SD_{intercept}}{slope}$$

$SD_{intercept}$ represents the standard deviation of the y-intercept.

2.7.5. Recovery and matrix effect

Extraction yields are evaluated as recovery (RE) and possible ion suppression or ion enhancement due to matrix components are evaluated as matrix effect (ME). Two concentration levels (LOW, HIGH) of a methanolic standard (A), post-extraction spiked sample (B) and a pre-extraction spiked sample (C) are tested (n = 5 types of different donor blood). Absolute analyte peak areas are used to calculate ME and RE using the following formulas [24]:

$$ME = \frac{B}{A}.100$$

$$RE = \frac{C}{B}.100$$

2.7.6. Stability

Processed sample stability, bench top stability and freeze/thaw stability were evaluated. Processed sample stability was tested by analyzing a processed sample at LOW and HIGH concentration, after 0 h, 1 h, 2 h, 3 h, 4 h, 6 h and 8 h of storage in the 10 °C cooled autosampler. Regression analysis was performed over the 8 h time span. A significantly negative slope ($p < 0.05$) indicates a significant decrease of the analyte concentration and thus instability. Bench-top stability was tested by analyzing processed samples at LOW and HIGH concentrations (n = 3) after 3 h of storage on the bench-top at room temperature. The concentration after 3 h was compared with the 0 h control measurement (ratio 3 h/0 h). Freeze/thaw stability was evaluated by analyzing processed samples at LOW and HIGH concentrations (n = 3) after 3 freeze/thaw cycles of 23 h at −20 °C and 0.5 h at room temperature. The concentration after 3 cycles was compared with the control measurement (ratio freeze–thaw/control).

3. Results and discussion

3.1. Method optimization

3.1.1. Type of IL

When selecting the appropriate IL, several requirements were listed in order to proceed with the ideal extraction solvent for BZD and BZD-like hypnotics, present in a whole blood matrix. The IL needed to have a higher density than water, so after centrifugation, it would form the lower phase in the conical tube, which is easier for IL collection. Furthermore, the ILs needed to have a workable viscosity, be commercially available and immiscible with water. Of course, good affinity for the BZD structure is required. Three imidazolium based ILs were tested, only differing in their alkyl chain length: BMIm PF_6, HMIm PF_6 and OMIm PF_6. BMIm PF_6 had significantly ($p \leq 0.05$) higher PE values compared to the other tested ILs. Moreover, BMIm PF_6 also had a lower viscosity, which made it easier to perform the extraction procedure. Overall, BMIm PF_6 was chosen as the ideal extraction solvent. PE (%) results are shown in Fig. 3A.

3.1.2. Dilution and pH adjustment of whole blood sample

In order to evaluate the influence of pH on BZD extraction yields, 1.0 mL H_2O buffer was added and compared to the condition

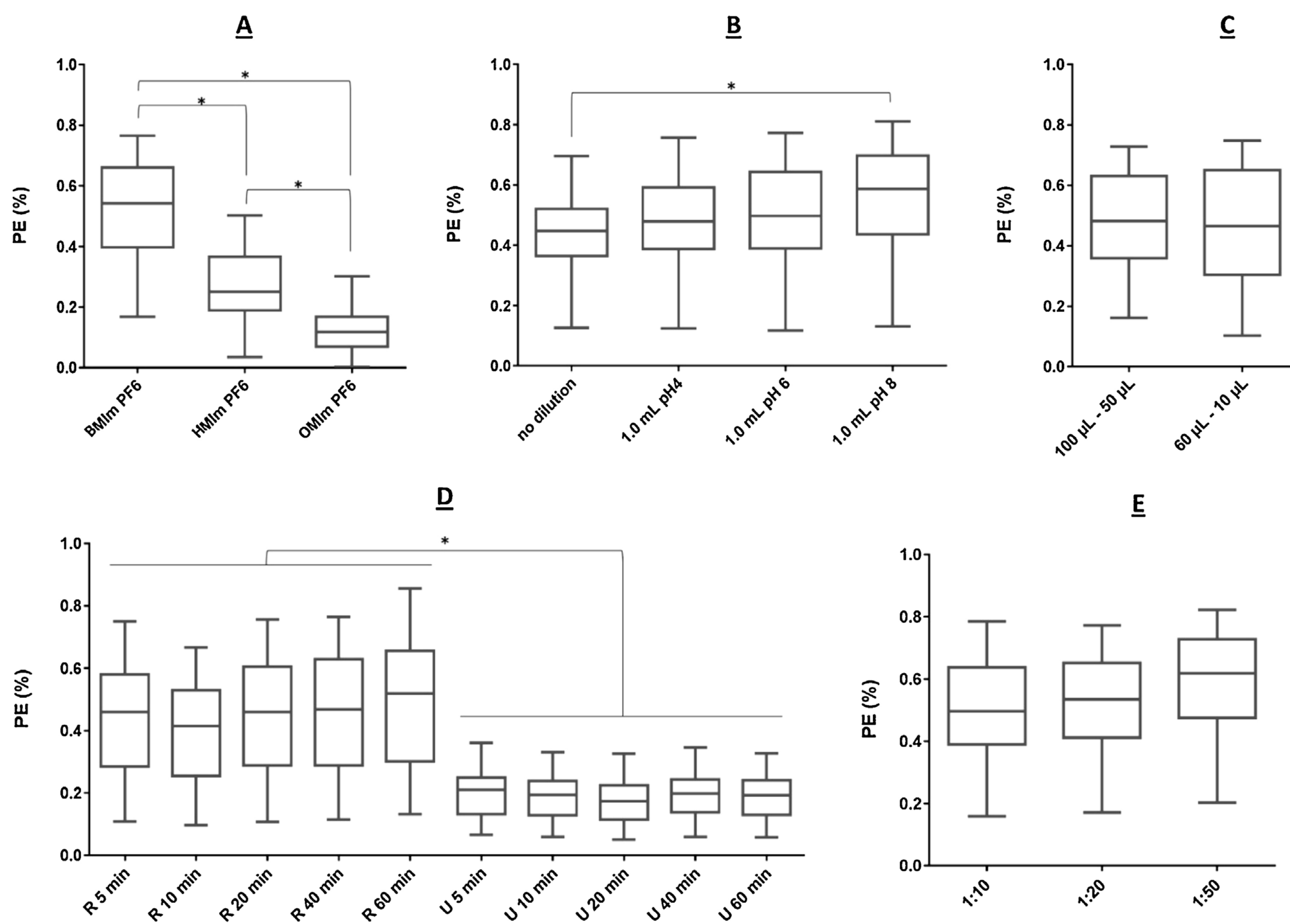

Fig. 3. Process efficiency (PE (%)) results of the IL-DLLME optimization experiments, presented as boxplots. (A) Type of IL (BMIm PF_6: 1-butyl-3-methylimidazolium hexafluorophosphate; HMIm PF_6: 1-hexyl-3-methylimidazolium hexafluorophosphate; OMIm PF_6: 1-methyl-3-octyl-imidazolium hexafluorophosphate); (B) dilution and pH adjustment of whole blood sample; (C) volume of IL added and collected; (D) extraction method and time (R: rotation; U: ultrasonic bath); (E) dilution of the extract in methanol. Each boxplot represents the total of the 28 tested compounds. Whiskers represent the minimal and maximal PE values that were obtained. * indicates a statistical significant difference ($p \leq 0.05$).

Table 3
Overview of the practical assessment of the volume of IL added and collected.

Volume of IL added	20 μL	40 μL	50 μL	60 μL	80 μL	100 μL
Volume of IL collected[a]	xx	xx	x	10 μL	30 μL	50 μL

[a] Largest volume of IL possible to be collected in a repeatable and accurate manner. xx: no phase separation was obtained and therefore it was not possible to collect the IL; x: it was not possible to collect a certain IL volume in a repeatable and accurate manner.

of no dilution. Buffer pH 4.0, pH 6.0 and pH 8.0 were tested. As expected, the highest PE values were obtained by adding a more basic buffer (pH 8.0), since deprotonation of BZDs is promoted under basic conditions, resulting in uncharged molecules that are more easily extracted toward the organic IL phase. These findings were statistically confirmed, since 1.0 mL pH 8.0 gave significantly ($p \leq 0.05$) higher PE values compared to the condition of no dilution. Moreover, diluting the whole blood sample by adding 1.0 mL buffer also seemed to improve the ease with which the lower IL phase could be collected, thanks to the reduced viscosity of the whole blood sample. Overall, a 1.0 mL dilution of the whole blood sample with H_2O pH 8.0 was chosen as the ideal extraction condition. PE (%) results are shown in Fig. 3B.

3.1.3. Volume of IL added and collected

The volume of IL added and collected was practically assessed. Volumes of 20 μL, 40 μL, 60 μL, 80 μL and 100 μL IL were added and after centrifugation it was evaluated whether it was still possible to repeatedly and accurately collect a defined volume of the lower IL phase. Ideally, the smallest volume of IL possible should be added, since this results in the highest enrichment factor of the analyte. The ideal volume of IL added was found to be in-between 40 μL and 60 μL, so an additional volume of 50 μL was tested. As can be seen in Table 3, the addition of 60 μL IL, was the smallest volume possible that still allowed the collection of 10 μL IL in a repeatable and accurate manner, and therefore, it was chosen as the optimal condition. This reduction in volume of extraction solvent that was added results in a higher enrichment factor (1.67 times higher), compared to the initial conditions of 100 μL IL addition and 50 μL IL collection. In order to check whether the small extraction solvent volume influences PE values, both the initial and optimized conditions were compared. No significant effect was seen ($p > 0.05$); PE (%) results are shown in Fig. 3C.

3.1.4. Extraction method and time

Two commonly used techniques were evaluated as possible extraction methods: ultrasonic bath mixing and rotary mixing. A time range from 5 min to 60 min was tested, no longer extraction times were evaluated since the aim of this study is to develop a fast extraction procedure. The experiments revealed that using a rotary mixer gave significantly ($p \leq 0.05$) better extraction results compared to the use of an ultrasonic bath. These findings were confirmed when both extraction methods were visually evaluated: rotary mixing showed a much better mixing between the blood sample and the IL extraction solvent. When evaluating extraction times, no significant ($p > 0.05$) differences were seen, indicating that the extraction equilibrium is rapidly attained. Overall, 5 min of rotary mixing was chosen as the ideal extraction condition. PE (%) results are shown in Fig. 3D.

3.1.5. Dilution of the extract

After collection of the IL extract, it was evaluated whether diluting the extract would reduce matrix effects and therefore yield higher PE extraction values. Additionally, a higher dilution factor means less ionic liquid that is injected into the mass spectrometer (MS) and therefore less contamination. However, when comparing all tested dilution factors (1:10, 1:20, 1:50 in methanol) no significant ($p > 0.05$) difference in PE values was detected, which means no significant improvements are detected for higher dilution factors. PE (%) results are shown in Fig. 3E. Eventually, the lowest dilution factor – 1:10 – was chosen as the optimal condition, because of the higher analyte signals that were obtained. In order to prevent high IL signals from reaching the MS and thus prevent contamination, a diverter valve was introduced as described in Section 2.5.

3.2. Method validation

3.2.1. Selectivity

No interfering peaks were observed for both blank samples and zero samples. As MRM 1 of both oxazepam and nitrazepam. d5 consisted of the same precursor ion mass (Q1 mass) and product ion mass (Q3 mass), MRM 2 was used to quantify both compounds in order to avoid possible errors, since retention times only differed ±0.1 min. No benzodiazepine interferences were found, except for nordiazepam and clorazepate, which can be explained by the co-elution of chromatographic peaks and closely related ion masses used for identification. Moreover, clorazepate is known to be rapidly decarboxylated into its primary metabolite: nordiazepam, which can be an explanation for the fact that in the developed method clorazepate and nordiazepam show similar transitions [27,28]. Based on these interference observations, both nordiazepam and clorazepate were excluded from the quantitative method.

3.2.2. Linearity

Final calibration ranges, calibration models, ISTDs and coefficients of determination (R^2) are shown in Table 4. Calibration ranges include therapeutic and (low) toxic plasma concentrations: 2 ng/mL–250 ng/mL: 7-aminoflunitrazepam, alprazolam, brotizolam, clonazepam, etizolam, flunitrazepam, flurazepam, loprazolam, lorazepam, lormetazepam, triazolam and zopiclone; 10–1000 ng/mL: bromazepam, cloxazolam, clorazepate, estazolam, ethyl loflazepate, midazolam, nitrazepam, nordiazepam, temazepam, zolpidem; 50–2000 ng/mL: chlordiazepoxide, clobazam, clotiazepam, diazepam, oxazepam, prazepam [29]. Overall, heteroscedastic datasets were observed, which can be explained by the fact that wide concentration ranges were tested. Heteroscedasticity indicates the need of using an appropriate weighing factor. Overall, The lowest back-calculated values, best residual plots and best precision/accuracy data were obtained with the weighted $1/x^2$ linear and quadratic models. The choice of these models is confirmed in literature as the most commonly used for LC–MS/MS applications [25,30]. For all analytes back-calculated values were obtained within 75%–125%, expect for brotizolam, clobazam, cloxazolam, flunitrazepam, lorazepam, nitrazepam and zopiclone. For these compounds it was difficult to construct proper calibration curves, as can be seen from their slightly deviating R^2 values, indicating concentration-dependent behavior.

3.2.3. Accuracy and precision

Accuracy and precision data are shown in Table 4. All accuracy data (bias %) were within the acceptable range of 15% and 20% near the LOQ, except for brotizolam, chlordiazepoxide, cloxazolam, flunitrazepam, loprazolam and zolpidem. The same acceptance criteria were used to evaluate the obtained precision data (RSD_{rep} and $RSD_{int\ pr}$). Results for all analytes complied with specifications, except for brotizolam, chlordiazepoxide, cloxazolam, loprazolam, lorazepam, nitrazepam and zopiclone. Overall, the largest deviating accuracy and precision results were observed for brotizolam, chlordiazepoxide, cloxazolam, loprazolam and nitrazepam. This can be partially explained by the calibration curves that showed

Table 4
Final calibration ranges, calibration models, internal standards (ISTD), coefficients of determination (R^2), limits of quantification (LOQ), limits of detection (LOD), accuracy results presented as bias and precision results presented as relative standard deviation values of repeatability (RSD_{rep}) and intermediate precision $_{int\ prec}RSD_{int\ pr}$).

	Calibration range (ng/mL)	Calibration model	ISTD	R^2	LOQ (ng/mL)	LOD (ng/mL)	LOW			MED			HIGH		
							Bias (%)	RSD_{rep} (%)	$RSD_{int\ pr}$ (%)	Bias (%)	RSD_{rep} (%)	$RSD_{int\ pr}$ (%)	Bias (%)	RSD_{rep}(%)	$RSD_{int\ pr}$ (%)
7-Aminoflunitrazepam	2–250	1/x quadratic	7-Aminoflunitrazepam.d7	≥0.99	2	0.09	1.34	4.16	3.95	−0.35	3.50	6.58	7.80	2.30	4.55
Alprazolam	2–250	$1/x^2$ linear	Alprazolam.d5	≥0.99	2	0.28	0.23	7.85	6.76	8.01	4.88	4.72	7.07	5.04	7.04
Bromazepam	10–1000	$1/x^2$ linear	Triazolam.d4	≥0.99	10	0.67	1.70	8.36	18.00	3.91	9.60	15.46	7.71	7.50	13.03
Brotizolam	2–250	$1/x^2$ quadratic	Alprazolam.d5	0.98[a]	2	1.41	42.47[a]	9.47	33.97[a]	32.80[a]	5.13	25.95[a]	30.65[a]	4.35	21.07[a]
Chlordiazepoxide	50–2000	$1/x^2$ quadratic	Chlordiazepoxide.d5	≥0.99	50	4.74	−33.18[a]	6.14	18.20	−26.39[a]	7.09	16.38[a]	−14.79	5.76	8.98
Clobazam	50–2000	$1/x^2$ linear	Chlordiazepoxide.d5	0.95[a]	50	3.49	2.67	7.78	19.79	11.78	4.52	14.61	−4.36	0.72	13.22
Clonazepam	2–250	$1/x^2$ linear	Clonazepam.d4	≥0.99	2	0.25	−12.33	8.24	11.63	−8.00	5.55	11.71	3.76	2.81	11.00
Clorazepate	10–1000	$1/x^2$ linear	Nordiazepam.d5	≥0.99	10	0.69	0.51	2.75	5.14	−1.86	3.94	6.78	3.16	5.37	5.52
Clotiazepam	50–2000	1/x quadratic	Midazolam.d4	≥0.99	50	0.54	−2.98	4.81	13.62	1.44	5.16	10.26	3.96	2.09	12.11
Cloxazolam	10–1000	1/x quadratic	Midazolam.d4	0.97[a]	10	3.63	65.29[a]	10.27	28.74[a]	62.07[a]	10.40	21.49[a]	24.41[a]	4.68	14.70
Diazepam	50–2000	$1/x^2$ quadratic	Diazepam.d5	≥0.99	50	2.16	−5.11	5.31	8.09	−4.16	3.85	9.12	4.72	2.82	10.32
Estazolam	10–1000	$1/x^2$ linear	Estazolam.d5	≥0.99	10	0.40	3.44	4.04	5.74	1.68	2.92	6.40	7.03	2.05	5.03
Ethyl loflazepate	10–1000	$1/x^2$ linear	Estazolam.d5	0.98[a]	10	0.94	−0.50	6.27	10.18	5.84	6.52	7.54	0.05	3.55	15.60
Etizolam	2–250	$1/x^2$ quadratic	Midazolam.d4	≥0.99	2	0.26	−13.24	6.96	8.36	−12.85	3.91	13.56	−9.46	2.80	13.34
Flunitrazepam	2–250	$1/x^2$ linear	Flunitrazepam.d7	0.98[a]	2	0.18	15.39	8.13	10.37	20.73[a]	7.42	7.96	3.58	4.45	10.34
Flurazepam	2–250	1/x linear	Midazolam.d4	≥0.99	2	0.03	−11.32	3.64	6.73	−13.49	3.95	10.55	−11.34	1.72	12.57
Loprazolam	2–250	$1/x^2$ linear	Prazepam.d5	≥0.99	2	0.003	−14.09	6.59	10.34	−14.78	5.97	16.41[a]	−21.76[a]	8.43	19.87[a]
Lorazepam	2–250	$1/x^2$ linear	Lorazepam.d4	0.97[a]	2	0.52	−7.48	16.34	21.80[a]	15.64	8.75	11.23	2.77	2.26	7.05
Lormetazepam	2–250	1/x linear	Temazepam.d5	≥0.99	2	0.19	−7.53	6.94	9.67	−8.39	3.81	6.03	3.20	1.95	5.82
Midazolam	10–1000	1/x quadratic	Midazoldam.d4	≥0.99	10	0.50	−0.09	3.88	6.91	−0.95	5.14	9.46	1.72	1.95	6.07
Nitrazepam	10–1000	$1/x^2$ linear	Nitrazepam.d5	0.94[a]	10	0.61	−8.72	6.78	24.10[a]	12.29	6.08	19.40[a]	14.28	2.99	16.55[a]
Nordiazepam	10–1000	$1/x^2$ linear	Nordiazepam.d5	≥0.99	10	0.69	4.05	4.38	6.48	0.65	3.56	7.62	2.72	3.61	5.27
Oxazepam	50–2000	$1/x^2$ linear	Oxazepam.d5	≥0.99	50	1.78	−10.34	4.13	16.06	−13.85	6.46	15.97	−10.91	2.51	12.64
Prazepam	50–2000	$1/x^2$ linear	Prazepam.d5	0.97[a]	50	1.83	2.32	4.63	11.09	6.62	3.71	7.40	−12.08	2.02	3.54
Temazepam	10–1000	1/x quadratic	Temazepam.d5	≥0.99	10	0.46	−0.14	3.19	6.54	−1.12	3.74	7.05	4.62	2.65	5.66
Triazolam	2–250	1/x quadratic	Triazolam.d4	≥0.99	2	0.28	−4.94	5.76	7.45	1.35	4.13	6.76	12.99	7.49	7.55
Zolpidem	10–1000	quadratic	Zolpidem.7	≥0.99	10	0.43	−8.83	5.52	5.79	0.28	5.09	6.82	16.25[a]	1.42	4.21
Zopiclone	2–250	$1/x^2$ linear	Nordiazepam.d5	0.97[a]	2	0.33	0.01	6.97	9.24	0.82	4.15	12.84	−11.17	6.55	42.64[a]

[a] Marks all values that deviate from the proposed acceptance criteria.

Table 5
Matrix effects (ME) and associated coefficient of variance values (CV_{ME}), recoveries (RE) and associated coefficient of variance values (CV_{RE}), processed sample stability, bench-top stability and freeze/thaw stability results.

	LOW							HIGH						
	ME (%)	CV_{ME} (%)	RE (%)	CV_{RE} (%)	Processed sample stability (h)	Bench-top stability (%)	Freeze/ thaw stability (%)	ME (%)	CV_{ME} (%)	RE (%)	CV_{RE} (%)	Processed sample stability (h)	Bench-top stability (%)	Freeze/thaw stability (%)
7-Aminoflunitrazepam	20.8	1.5	83.2	11.1	6	103.3	100.8	22.6	1.8	79.4	9.2	8	104.7	111.0
Alprazolam	37.6	6.1	68.2	15.5	8	83.9	82.9	58.0	3.5	72.3	9.5	8	104.8	100.1
Bromazepam	20.0	5.2	24.7	15.7	8	112.6	116.4	22.4	1.3	26.0	14.4	8	100.6	101.7
Brotizolam	42.1	7.8	75.9	9.5	8	104.4	97.6	55.6	3.8	81.3	7.7	6	119.6	103.5
Chlordiazepoxide	41.7	2.9	43.1	28.9[a]	8	105.6	93.1	60.0	3.1	42.8	14.4	8	96.2	89.8
Clobazam	58.1	2.2	123.1	9.6	8	95.3	64.1[a]	76.3	1.0	127.2	4.5	<4[a]	94.5	70.4
Clonazepam	54.1	6.6	97.0	14.2	8	113.2	105.9	59.5	3.2	100.0	11.4	8	95.7	95.9
Clorazepate	46.8	3.6	54.6	16.7	8	88.7	93.4	65.6	1.7	60.8	11.3	8	98.5	95.3
Clotiazepam	57.5	3.8	80.0	13.9	8	92.7	127.8[a]	86.1	2.3	82.0	8.3	8	92.4	116.0[a]
Cloxazolam	43.3	2.0	66.4	19.6	8	87.6	104.2	58.2	1.5	66.4	11.5	8	90.8	101.7
Diazepam	50.9	2.4	95.0	10.3	8	95.9	89.9	75.7	1.6	96.0	8.2	8	99.0	95.5
Estazolam	37.2	3.0	85.3	6.7	8	100.1	108.4	45.6	2.1	84.0	7.4	8	99.8	98.7
Ethyl loflazepate	40.6	4.2	83.9	18.1	6	95.4	99.3	53.8	5.7	86.2	10.6	8	94.8	104.9
Etizolam	42.5	10.0	62.1	13.7	8	85.1	84.8	46.2	5.5	64.8	9.7	4[a]	92.4	75.8[a]
Flunitrazepam	61.6	0.9	117.7	11.9	8	93.9	86.1	72.1	4.0	124.0	5.3	8	75.7[a]	75.6[a]
Flurazepam	60.7	5.0	108.3	14.2	8	92.5	107.2	75.4	1.4	106.6	7.6	4[a]	91.4	97.9
Loprazolam	76.3	3.1	98.3	14.6	6	95.5	88.6	82.0	1.9	93.4	9.2	8	100.8	84.8
Lorazepam	44.6	5.8	50.2	17.0	8	81.1	103.7	45.4	2.5	51.6	12.2	8	93.4	97.7
Lormetazepam	32.6	6.4	114.0	11.3	8	98.8	91.9	45.7	3.6	108.3	4.5	4[a]	93.2	87.9
Midazolam	46.9	2.5	68.6	17.1	8	97.3	91.9	65.1	0.9	69.2	16.1[a]	4[a]	105.3	103.2
Nitrazepam	45.2	4.1	80.5	10.1	8	94.9	90.0	54.2	1.3	85.4	7.8	6	96.8	96.0
Nordiazepam	53.2	9.8	54.2	16.9	8	97.0	94.2	67.3	3.5	60.2	10.3	8	97.8	97.0
Oxazepam	50.5	1.8	35.9	17.5	8	119.4	115.5	60.8	1.5	39.9	14.0	4[a]	114.5	116.3
Prazepam	66.9	1.3	93.7	17.1	8	90.2	84.0	92.6	2.0	94.3	9.7	<4[a]	101.6	99.4
Temazepam	36.9	3.2	91.9	10.5	8	98.2	94.0	55.4	1.5	91.7	7.5	4[a]	103.8	99.3
Triazolam	27.2	5.1	90.2	12.4	8	112.0	111.3	35.5	3.6	83.6	8.3	8	93.2	94.2
Zolpidem	48.8	4.1	98.5	12.8	8	99.2	93.1	71.0	1.8	98.0	8.7	4[a]	99.5	99.4
Zopiclone	50.1	6.2	75.8	17.5	6	81.6	84.4	66.8	5.4	58.8	12.3	8	105.9	97.2

[a] Marks all values that deviate from the proposed acceptance criteria.

deviating R^2 for most of these components. Furthermore, the incomplete dissolution of the cloxazolam (Akton®) tablet and low solubility of cloxazolam as such could also be a possible explanation for the unacceptable high bias results over the whole tested concentration range (±60%). Moreover, brotizolam and loprazolam were purchased as powder standards, resulting in possible incomplete dissolved standard solutions. Overall, more accurate and precise results can be obtained by improving the tablet extraction procedure and lowering the concentration of the standard stock solutions, to improve dissolution. Furthermore, zolpidem and zopiclone were included in the quantitative method, as they only show deviating results at the HIGH concentration level, therefore a prior sample dilution is recommended for high concentration samples.

3.2.4. LOQ and LOD

LOQ and LOD values are shown in Table 4. The lowest calibrator used for linearity tests was proven to be the LOQ. All LOQ values were lower than the lowest therapeutic plasma concentrations, except for brotizolam with low therapeutic plasma concentrations of 1 ng/mL and LOQ of 2 ng/mL.

3.2.5. Recovery and matrix effect

Recoveries and matrix effects are shown in Table 5. Recoveries for all analytes were within a range of 24.7%–127.2%. The lowest recoveries were detected for the more polar compounds, such as bromazepam, lorazepam, oxazepam and chlordiazepoxide. The opposite is true for the nonpolar compounds that show recovery values close to 100%, including loprazolam, prazepam and flurazepam. Matrix effects were pronounced, ranging from 20.0% to 92.6%. These low values indicate ion suppression, especially for the compounds eluting in the beginning of the chromatogram. This can be partially explained by the presence of high IL signals at these short elution times, affecting the more polar compounds that were already listed above. Deuterated ISTDs were introduced to correct for these inconveniences (Table 4). Furthermore, coefficients of variance (CV) for matrix effects were all within the prescribed ranges of 15% and 20% near LOQ, which means ion suppression is repeatable at both tested concentrations (n = 5) and can therefore be taken into account for quantification when using matrix-matched calibration curves. Recovery tests also showed acceptable CV% values, except for chlordiazepoxide ($CV_{RE,\ LOW}$ = 28.9%) and midazolam ($CV_{RE,\ HIGH}$ = 16.1%).

3.2.6. Stability

Processed sample stability, bench-top stability and freeze/thaw stability results are shown in Table 5. For most analytes, processed sample stability results showed no significant decrease of concentration over a time of 6 h–8 h. Remarkably low stability (<4 h) was detected for clobazam and prazepam. Bench-top stability results were within a range of 80%–120%, with small deviations detected for flunitrazepam at high concentrations. Freeze/thaw stability results were also within a range of 80%–120%, with the exception of clobazam, clotiazepam, etizolam, and flunitrazepam. Overall, the proposed acceptance criteria (90%–110%) were not fulfilled for half of the tested BZD compounds, indicating instability of the processed samples. Several studies have already been performed regarding BZD stability in biofluids, indicating bad stability of N-oxides and nitro group containing compounds. [31,32] Instability of the processed samples could be explained by thermal instability, which is documented in Ref. [33], for N-4 oxides, 7-nitro compounds, alpha-hydroxy ketones and *N*-methyl-alpha-hydroxy ketones. Another explanation for the processed sample instability could be the presence of the IL,

which should be further investigated. Overall, it is recommended to immediately analyze the processed samples.

4. Conclusions

In this study, a new IL-DLLME procedure coupled to LC–MS/MS analysis was developed for the quantification of BZDs and BZD-like hypnotics in whole blood samples. The method was fully validated for the following compounds: 7-aminoflunitrazepam, alprazolam, bromazepam, clobazam, clonazepam, clotiazepam, diazepam, estazolam, ethyl loflazepate, etizolam, flurazepam, lormetazepam, midazolam, oxazepam, prazepam, temazepam, triazolam, zolpidem and zopiclone. These findings demonstrate the applicability of ILs as promising extraction solvents in toxicology. When using the commercially available BMIm PF_6 as an extraction solvent, high recoveries were obtained ranging from 80% to 100% for the vast majority of compounds, which are comparable to the results obtained with established SPE and LLE techniques [20–22]. Notable is the high recovery (±80%) that was obtained for the more polar compound 7-aminoflunitrazepam, which has shown to be a struggle in established SPE methods (20%–60%) [20,21]. Moreover, the use of ILs as extraction solvents reduces the environmental pollution thanks to their non-volatile nature. Another advantage of the developed IL-DLLME method is that it only requires 30 min to process a whole blood sample, while SPE procedures generally take about 3 h. Compared to other microextraction procedures, to our knowledge, this is the first to extract such a broad range of benzodiazepines from whole blood samples and the first to use an IL as the extraction solvent. Attention needs to be drawn to the observed ion suppression (20%–93%), probably due to the high IL signals that co-elute with the analyte. As matrix effects have shown to be repeatable (CV% < 15%) and the method has shown to be sufficiently sensitive, precise and accurate we can conclude that they do not affect quantification of the included analytes. Overall, this paper demonstrates the applicability of ILs for the extraction of compounds with high variability in physicochemical properties, from whole blood. Moreover, a fast and cost-effective IL-DLLME-LC–MS/MS method was developed and validated for the quantification of 19 BZDs and BZD-like hypnotics.

Author contributions

Experimental design: MDB, WD, JT, EC.
Conduct experiments: MDB, SM, EC.
Data analysis: MDB, SM, EC.
Manuscript writing: MDB.
Manuscript revision: SM, WD, JT, EC.

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