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Fast and easy extraction of antidepressants from whole blood using ionic liquids as extraction solvent

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ABSTRACT

This study aims to prove that ionic liquids (ILs) can be used as extraction solvents in a liquid-liquid microextraction, coupled to LC-MS/MS, for the quantification of a large group of antidepressants in whole blood samples. The sample preparation procedure consisted of adding 1.0 mL aqueous buffer pH 3.0 and 60 μL of IL (1-butyl-3-methylimidazolium hexafluorophosphate) to 1.0 mL whole blood. Subsequently, a 5-min rotary mixing step was performed followed by centrifugation. The lower IL phase was collected, diluted 1:10 in methanol and 10 μL was injected into the LC-MS/MS. The following analytes were included in the full-quantitative method: agomelatine, amitriptyline, bupropion, clomipramine, dosulepin, doxepin, duloxetine, escitalopram, fluoxetine, imipramine, maprotiline, mianserin, mirtazapine, nortriptyline, paroxetine, reboxetine, trazodone and venlafaxine. Selectivity was checked for 10 different whole blood matrices. Additionally, possible interferences of deuterated standards or other antidepressants were evaluated. Overall, no interferences were found. For each analyte a matrix-matched calibration curve was constructed (7 levels, n = 6), covering therapeutic and low toxic concentrations. Accuracy and precision were evaluated over eight days, at three concentration levels (n = 2). Bias, repeatability and intermediate precision results met with the proposed validation criteria, except for fluvoxamine, which was therefore only included in the semi-quantitative method. LOQs were set at the lowest calibrator concentration and LOD values were - for most analytes - within a range of 1–2 ng/mL. Recoveries (RE) and matrix effects (ME) were evaluated for five types of donor whole blood, at two concentration levels. RE values were within a range of 53.11–132.98%. ME values were within a range of 61.92–123.24%. In conclusion, this study proves the applicability of ILs as extraction solvents for a large group of antidepressants in complex whole blood matrices.

1. Introduction

When complex biological samples need to be analyzed, sample preparation is the first thing that comes to mind. This prior cleaning step secures removal of interfering matrix components, which results in the reliable quantification of analytes. In addition, method sensitivity can be enhanced thanks to the implementation of an analyte concentration step [1–3]. Given the importance of sample preparation, it is understandable that the field is rapidly evolving, with the focus on more efficient and green alternatives [4–6].

In the 1990s, microextraction was introduced as a promising sample preparation technique [4]. Compared to conventional solid phase extraction (SPE) and liquid-liquid extraction (LLE) methods, microextractions have the advantage of being more environmentally friendly, simple and cost-effective. Also, high concentration factors are obtained thanks to the smaller solvent volumes that are consumed [4–7]. Typically, volumes smaller than 100 μL are used [7]. Over the last decade, different types of microextraction procedures were developed which can be divided in two major classes: solid phase-based and liquid phase-based microextractions. The spectrum of solid phase-based techniques ranges from a simple polymer coated fiber that is lowered into the sample, to a sorbent packed microsyringe or a sorbent coated stir bar [4]. Apart from their ability to efficiently remove matrix components, solid phase-based extraction techniques require expensive fibers and

Abbreviation: BM_3Amm Tf_2N, butyltrimethylammonium bis(trifluoromethylsulfonyl)imide; BMIm PF_6, 1-butyl-3-methylimidazolium hexafluorophosphate; BMPyrr Tf_2N, 1-butyl-1-methylpyrrolidinium bis(trifluoromethylsulfonyl)imide; HMIm PF_6, 1-hexyl-3-methylimidazolium hexafluorophosphate; IL, ionic liquid; IL-DLLME, IL-based dispersive liquid-liquid microextraction; OMIm PF_6, 1-methyl-3-octylimidazolium hexafluorophosphate

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thin layer coatings that are often only meant for one-time usage to avoid carryover [1,8]. In this perspective, liquid phase-based microextractions seem more appealing. They consist of simple, fast and low-cost procedures [7]. Currently, dispersive liquid-liquid microextractions (DLLME) are being widely applied, for extraction of water, soil, food and biological samples [6]. The technique was introduced in 2006 and is based on a ternary solvent system. The organic extraction solvent and disperser solvent are simultaneously injected into the aqueous sample, creating a fine dispersion and thus a high contact surface that favors the transfer of the analyte towards the extraction solvent. Recent developments have shown that the disperser solvent can be replaced by a physical mixing step to obtain a fine dispersion. To date, ultrasonic irradiation and vortex agitation are most commonly applied, resulting in greener procedures [1,4,6,7,9,10].

Next to improving extraction techniques, researchers have been looking for more favorable extraction solvents that can enhance extraction yields and have less impact on the environment, compared to conventional organic solvents [9]. Ionic liquids (ILs) have been proposed as a novel class of promising extraction solvents. They are molten salts, composed of organic and inorganic ions. The first IL reported was ethylammonium nitrate, however, this compound was found to be instable and therefore did not have any significant applications. In 1992, the first air- and moisture-stable ILs were available. Today, they are applied in electrochemistry, nanoparticle production, catalysis processes, analytical chemistry, etc. [11–18]. Moreover, researchers recently discovered their potential for application in pharmaceuticals as drug delivery systems or even IL-based drugs [18]. So, what makes these ILs so interesting? Their non-flammable nature, good thermal stability and negligible vapor pressures are part of the explanation. In addition, their easily tunable physicochemical properties provide researchers with infinite possibilities [11–18]. When we zoom in on analytical chemistry, more specifically, DLLME applications, several types of ILs have been tested as ideal extraction solvents. In conventional IL-DLLME, nonpolar ILs are used. Requirements are low viscosity, high density, water immiscibility and good interaction with the analyte [1,9,10,13,14,17]. Often imidazolium-, ammonium-, pyrrolidinium-, pyridinium- and phosphonium-based ILs are studied, as they are commercially available at relatively low costs [9,14].

Overall, IL-DLLME procedures have proven to be efficient, simple and cheap extraction protocols. Additionally, they have the advantage of using low volatile solvents that can be structurally fine-tuned to optimize extraction yields. In toxicology, however, IL-DLLME is hardly studied. The research domain of toxicology holds several challenges: multi-analyte approaches are needed that take into account complex matrix compositions. Moreover, developed methods need to have a sufficient sensitivity, as often, only trace amounts of the analytes are present [19]. To our knowledge, only two toxicologically relevant drug classes have been studied when it comes to IL-DLLME of biological samples: benzodiazepines [20] and methamphetamine [21,22], respectively, in whole blood and urine. Another important drug class, frequently detected in clinical and forensic screening procedures, are antidepressants. This large group consists of several structurally different compounds, generally divided in five subclasses based on their mechanism of action: monoamine oxidase (MAO) inhibitors, selective serotonin reuptake inhibitors (SSRI), selective norepinephrine reuptake inhibitors (SNRI), atypical antidepressants, and an important class - named after its chemical structure -, tricyclic antidepressants (TCA). Most antidepressants are relatively safe drugs, however, caution should be taken when combinations are prescribed. Especially, the increased risk of suicide at the start of therapy and the risk of relapse, can play a role in forensic toxicological cases [23]. To date, analysis of antidepressants in biological samples is usually performed using LC-MS/MS devices. As sample preparation techniques, several well-known procedures are applied: LLE [24], SPE [25] and protein precipitation (PP) [26]. All three have their own drawbacks; high solvent consumption, complex and tedious procedures, and high analyte losses, respectively.

Overall, sample preparation techniques are rapidly evolving, and the research field of toxicology should keep up pace. Liquid-liquid microextractions in combination with IL solvents, seem to offer promising perspectives. Consumption of low volumes of non-volatile solvents results in greener procedures, fast and easy protocols will save time and money, and most importantly, ILs are polyvalent solvents that can be modeled to better future extraction applications. The extensive study of this novel technique in clinical and forensic toxicology is of great importance. Therefore, with this research, we aim at developing and validating a fast and efficient IL-DLLME method, coupled to LC/MS-MS analysis, for the quantification of a wide range of antidepressants in whole blood.

2. Materials and methods

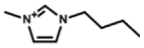
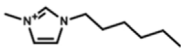
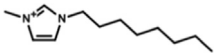
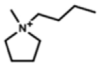
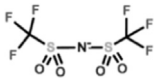
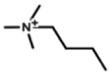
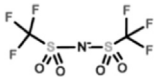
2.1. Chemicals and reagents

Methanolic reference standards of 14 antidepressants were purchased from LGC Standards (Wesel, Germany): amitriptyline hydrochloride (1 mg/mL), bupropion hydrochloride (1 mg/mL), clomipramine hydrochloride (1 mg/mL), doxepin hydrochloride (1 mg/mL), duloxetine hydrochloride (1 mg/mL), fluoxetine hydrochloride (1 mg/mL), imipramine hydrochloride (1 mg/mL), mianserin hydrochloride (1 mg/mL), mirtazapine (1 mg/mL), nortriptyline hydrochloride (1 mg/mL), reboxetine mesilate (1 mg/mL), paroxetine maleate (1 mg/mL), trazodone hydrochloride (1 mg/mL) and venlafaxine (1 mg/mL). Escitalopram oxalate and dosulepin hydrochloride were purchased from LGC Standards as powder standards and were dissolved in methanol to obtain a final concentration of 1 mg/mL. Three antidepressants were obtained as their commercially available tablet forms: Valdoxan® 25 mg (agomelatine), Floxyfral® 100 mg (fluvoxamine) and Ludiomil® 25 mg (maprotiline). Tablets were extracted with methanol and diluted to a final concentration of 1 mg/mL. A standard stock mix of all 19 antidepressants was prepared at 10 µg/mL in methanol. Three methanolic deuterated reference standards were purchased from LGC Standards: amitriptyline.d6 hydrochloride (100 µg/mL), doxepin.d3 hydrochloride (1 mg/mL) and nortriptyline.d3 hydrochloride (100 µg/mL). A standard stock mix of all three deuterated antidepressants was prepared at 10 µg/mL in methanol, and was used as internal standard. All reference standards and stocks were stored at − 20 °C. As extraction solvents, five ILs were obtained from IOLITEC Ionic Liquids Technologies GmbH (Heilbronn, Germany): 1-butyl-3-methylimidazolium hexafluorophosphate (BMIm PF_6, 99.5%), 1-hexyl-3-methylimidazolium hexafluorophosphate (HMIm PF_6, 99%), 1-methyl-3-octylimidazolium hexafluorophosphate (OMIm PF_6, 99%), 1-butyl-1-methylpyrrolidinium bis(trifluoromethylsulfonyl)imide (BMPyrr Tf_2N, 99%), butyltrimethylammonium bis(trifluoromethylsulfonyl)imide (BM_3Amm Tf_2N, 99%). Chemical structure and physicochemical properties of each IL can be found in Table 1. Methanol and acetonitrile LC-MS grade were purchased from Biosolve (Valkenswaard, The Netherlands). Ammonium formate, ammonium bicarbonate, formic acid and ammonium hydroxide LC-MS grade were purchased from Sigma-Aldrich (Bornem, Belgium). The aqueous mobile phases were prepared as follows: pH 3.0: 10 mM ammonium formate, adjusted with formic acid; pH 7.0: 10 mM ammonium bicarbonate, adjusted with formic acid; pH 11.0: 10 mM ammonium bicarbonate, adjusted with ammonium hydroxide. MilliQ water was obtained from a Milli-Q Water Purification System (Millipore, Brussels, Belgium).

2.2. Biosamples

Blank whole blood samples were obtained from Blood Transfusion Centre Leuven (Gasthuisberg, Leuven, Belgium). Approval for usage in this study was permitted by the Committee for Medical Ethics UZ Leuven. All samples were stored at − 20 °C and 1% sodium fluoride was added. Before use, blood samples were screened for the presence of

Table 1
Chemical structure and physicochemical properties of five tested ionic liquids: cation, anion, appearance, viscosity and density.

	Cation	Anion	App.	Visc.(cP,25°C)	Dens.(g/mL, 24°C)
1-butyl-3-methylimidazolium hexafluorophosphate			liquid	267	1.37
1-hexyl-3-methylimidazolium hexafluorophosphate			liquid	465	1.30
1-octyl-3-methylimidazolium hexafluorophosphate			liquid	608	1.24
1-butyl-1-methylpyrrolidinium bis(trifluoromethylsulfonyl)imide			liquid	72	1.40
Butyltrimethylammonium bis(trifluoromethylsulfonyl)imide			liquid	100	1.40

App.: appearance; visc.: viscosity; dens.: density. Information retrieved from Technical Data Sheets (IOLITEC).

antidepressants, using a routine PP-LC-MS/MS method, developed in the lab of Pharmacology and Toxicology (KUL, Leuven, Belgium). Prior to optimization and validation experiments, whole blood was spiked at different concentration levels using the standard stock mix (10 µg/mL).

2.3. Sample preparation optimization

The DLLME sample preparation method for benzodiazepine determination in whole blood, published by De Boeck et al. [20], was used as a reference method to start the optimization process. Like benzodiazepines, antidepressants are also basic analytes. This predicts that a similar DLLME protocol will be needed to efficiently extract the large number of antidepressants from whole blood matrices. In short, the benzodiazepine protocol consists of extracting 1.0 mL whole blood using 60 µL of IL extraction solvent: BMIm PF_6. Prior to adding the extraction solvent, 1.0 mL of pH 8.0 aqueous buffer was used to deprotonate the basic analytes. A 5-min rotary mixing step was followed by phase separation through centrifugation. 10 µL of the lower IL phase was collected and diluted 1:10 in methanol.

The optimization process consisted of assessing five extraction parameters, chronologically: pH of whole blood sample, type of IL added, volume of IL added, extraction time and dilution of the IL extract prior to analysis. The first two parameters were tested simultaneously in a matrix model, as they are presumed to influence one another. All experiments were performed in triplicate, using blank whole blood, spiked at 100 ng/mL with standard stock mix (10 µg/mL). Conditions tested are shown in Table 2.

Process efficiency (PE) results and relative standard deviations (RSD) were calculated for the tested conditions of each parameter, and presented as boxplots (Fig. 2). Statistical analysis was performed on PE results to evaluate whether significant differences were found among the tested conditions, in order to select the most favorable condition to proceed with. A Kruskal-Wallis test ($\alpha = 0.05$), followed by a Dunn's multiple comparison test ($\alpha = 0.05$) was performed.

2.4. Final sample preparation procedure

In Supplementary material (Fig. S1), a schematic overview is given of the final optimized sample preparation procedure. In a first step, 1.0 mL of whole blood was transferred into a conical bottom glass tube. Internal standard was added by spiking 10 µL of deuterated stock mix (10 µg/mL). 1.0 mL of aqueous buffer pH 3.0 was added, followed by the addition of 60 µL BMIm PF_6. A rotary mixing step of 5 min was introduced to obtain a dispersion of IL in the aqueous blood phase. Thanks to this step, transfer of the analyte towards the IL phase is ensured. To separate both aqueous and IL phase, a 6-min centrifugation step was needed and subsequently, 10 µL IL was collected. Prior to LC-MS/MS analysis, the extract was diluted 1:10 in methanol.

Table 2
Optimization of DLLME procedure: extraction parameters and tested conditions for each parameter.

Extraction parameter	Tested conditions
pH whole blood	3.0, 7.0, 11.0
Type of IL added	BMIm PF_6, HMIm PF_6, OMIm PF_6, BMPyrr Tf_2N, BM_3Amm Tf_2N
Volume of IL added	40 µL, 60 µL, 80 µL, 100 µL
Extraction time	1 min, 5 min, 10 min, 15 min
Dilution factor of collected IL	1:5 in MeOH, 1:10 in MeOH, 1:20 in MeOH

N = 3. Aqueous buffer pH 3.0, 7.0 and 11.0 were prepared by dissolving respectively 10 mM ammonium formate, 10 mM ammonium acetate and 10 mM ammonium bicarbonate in water and adjusting to pH with an appropriate acid or base. BMIm PF_6: 1-butyl-3-methylimidazolium hexafluorophosphate; HMIm PF_6: 1-hexyl-3-methylimidazolium hexafluorophosphate; OMIm PF_6: 1-methyl-3-octylimidazolium hexafluorophosphate; BMPyrr Tf_2N: 1-butyl-1-methylpyrrolidinium bis(trifluoromethylsulfonyl)imide; BM_3Amm Tf_2N: butyltrimethylammonium bis(trifluoromethylsulfonyl)imide; MeOH: methanol.

2.5. Final LC-MS/MS analysis

The apparatus consisted of a Shimadzu Prominence Ultra-Fast Liquid Chromatograph XR System (Shimadzu Benelux, Jette, Belgium) in combination with a tandem mass spectrometer: 3200 QTRAP (Sciex, Halle, Belgium). The developed LC-MS/MS method was adapted from Montenarh D. et al. [24]. Compounds were separated on a Kinetex® Biphenyl LC column (150 mm × 2.1 mm, 2.6 µm particle size) (Phenomenex, Utrecht, The Netherlands) using a gradient elution of mobile phases A and B, respectively aqueous buffer pH 3.0 and acetonitrile. The following gradient was established: 0–11 min: 5–70% B; 11–12 min: 70% B; 12–13 min: 70–5% B; 13–15 min: 5% B. As high IL signals are reached in the beginning of the chromatographic run, a Valco valve was installed to spare the mass spectrometer from unwanted IL contamination. The first 4 min LC effluent was transported towards waste, as can be seen in the final chromatogram in Fig. 1. Other LC parameters were set as follows: flow rate: 0.4 mL/min; injection volume: 10 µL; column oven: 35 °C; autosampler cooler: 15 °C. The mass spectrometer was operated in scheduled multiple reaction monitoring (sMRM) mode and a Turbo V electrospray ionization source was used in positive mode. For each antidepressant and deuterated analogue, two MRM transitions were selected; quantifier and qualifier.

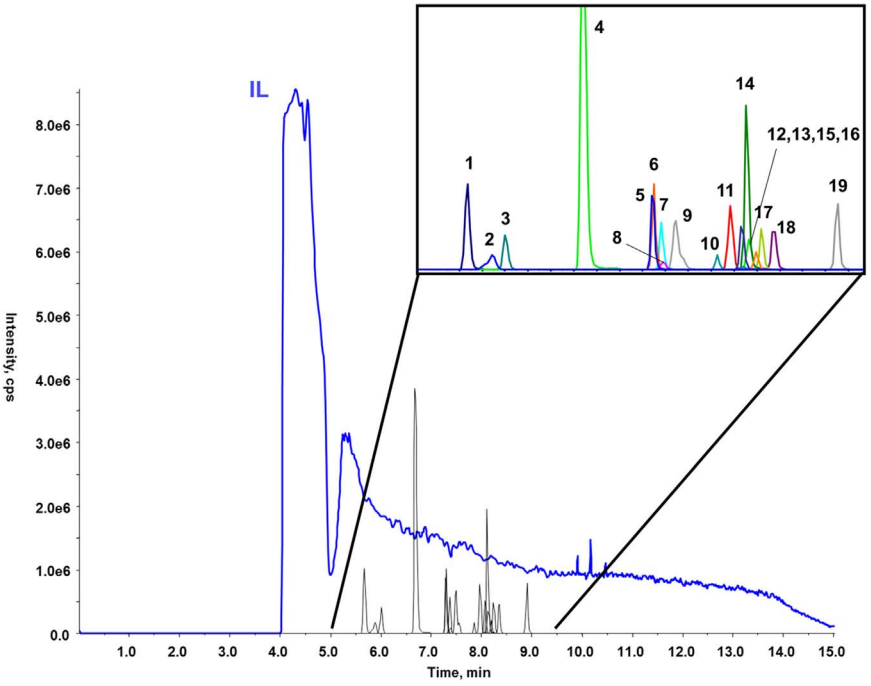


Fig. 1. Chromatogram of a processed sample. Whole blood spiked at 100 ng/mL for 19 antidepressants, except for trazodone: 1000 ng/mL; IL: 1-butyl-3-methylimidazolium hexafluorophosphate; (1) mirtazapine; (2) bupropion; (3) venlafaxine; (4) trazodone; (5) escitalopram; (6) reboxetine; (7) mianserin; (8) fluvoxamine; (9) doxepin; (10) paroxetine; (11) agomelatine; (12) dosulepin; (13) fluoxetine; (14) imipramine; (15) nortriptyline; (16) duloxetine; (17) maprotiline; (18) amitriptyline; (19) clomipramine. First 4 min of the LC effluent was sent towards waste to avoid MS contamination.

Table 3
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 mass *MRM 2* (Da)	CE (V)
Agomelatine	7.9	56	7.0	16.0	224.2	185.0	21	141.0	63
Amitriptyline	8.3	46	8.5	28.0	278.2	105.2	31	233.2	21
Amitriptyline.d6	8.3	36	5.0	14.0	284.3	233.1	23	117.1	33
Bupropion	5.8	26	26.0	24.0	240.1	131.1	35	184.1	17
Clomipramine	8.9	21	11.0	16.0	315.1	86.3	23	58.1	71
Dosulepin	8.0	46	4.0	18.0	296.2	223.1	31	218.1	31
Doxepin	7.4	36	11.0	16.0	280.1	107.3	29	235.0	25
Doxepin.d3	7.4	46	7.0	16.0	283.2	165.2	75	107.1	33
Duloxetine	8.2	16	3.5	18.0	298.1	154.2	11	123.0	59
Escitalopram	7.2	46	10.5	18.0	325.2	109.1	29	234.2	39
Fluoxetine	8.0	26	10.5	22.0	310.1	148.1	15	91.2	89
Fluvoxamine	7.3	31	6.5	36.0	319.2	71.0	31	200.2	33
Imipramine	8.1	26	11.0	16.0	281.2	86.0	23	58.0	57
Maprotiline	8.2	31	11.5	22.0	278.2	250.1	25	191.1	53
Mianserin	7.3	31	12.0	16.0	265.2	58.1	43	208.2	27
Mirtazapine	5.6	41	9.5	20.0	266.1	195.1	27	194.2	59
Nortriptyline	8.1	36	10.0	24.0	264.2	233.0	21	117.0	27
Nortriptyline.d3	8.1	36	5.5	18.0	267.4	233.0	19	117.0	27
Paroxetine	7.8	46	7.0	26.0	330.1	69.9	39	192.2	35
Reboxetine	7.2	16	8.0	12.0	314.2	176.2	19	91.0	37
Trazodone	6.6	31	12.0	18.0	372.1	148.1	45	176.1	37
Venlafaxine	5.9	36	8.0	18.0	278.2	58.1	37	260.2	17

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 60 s. Underlined transitions were used for quantification. Transitions are described by Montenarh et al. [23], those not described were optimized manually by direct infusion.

Transitions were set as described by Montenarh et al. [24], those not described were optimized manually by direct infusion. All MRM transitions, retention times (RT) and other compound-dependent settings can be found in Table 3. Source-dependent parameters were set as follows: curtain gas: nitrogen, 10 psi; nebulizing gas: nitrogen, 50 psi; heater gas: nitrogen, 50 psi; ion source temperature: 600 °C; ion source voltage: 5500 V.

2.6. Data acquisition and processing

A Dell Precision™ 390 Workstation with Analyst 1.5.1. software (Sciex, Halle, Belgium) was used for data acquisition and processing. GraphPad Prism 6.0 software was used for statistical analysis (GraphPad Software, La Jolla, CA, USA).

2.7. Method validation

The optimized IL-DLLME-LC-MS/MS method was validated according to internationally accepted validation guidelines for bioanalytical methods [27–29]. Blank whole blood was spiked at different concentration levels, as described in Table 4. Selectivity, linearity, accuracy, precision, sensitivity, matrix effect, recovery and stability were evaluated.

Selectivity was assessed by extracting 10 sources of blank whole

Table 4
Nominal concentrations of spiked whole blood samples, used for validation.

	Level 1	Level 2	Level 3	Level 4	Level 5	Level 6	Level 7
Agomelatine	10.0	25.0	50.0	100.0	300.0	600.0	1000.0
Amitriptyline	10.0	25.0	50.0	100.0	300.0	600.0	1000.0
Bupropion	10.0	25.0	50.0	100.0	300.0	600.0	1000.0
Clomipramine	10.0	25.0	50.0	100.0	300.0	600.0	1000.0
Dosulepin	10.0	25.0	50.0	100.0	300.0	600.0	1000.0
Doxepine	10.0	25.0	50.0	100.0	300.0	600.0	1000.0
Duloxetine	10.0	25.0	50.0	100.0	300.0	600.0	1000.0
Escitalopram	10.0	25.0	50.0	100.0	300.0	600.0	1000.0
Fluoxetine	10.0	25.0	50.0	100.0	300.0	600.0	1000.0
Fluvoxamine	10.0	25.0	50.0	100.0	300.0	600.0	1000.0
Imipramine	10.0	25.0	50.0	100.0	300.0	600.0	1000.0
Maprotiline	10.0	25.0	50.0	100.0	300.0	600.0	1000.0
Mianserin	10.0	25.0	50.0	100.0	300.0	600.0	1000.0
Mirtazapine	10.0	25.0	50.0	100.0	300.0	600.0	1000.0
Nortriptyline	10.0	25.0	50.0	100.0	300.0	600.0	1000.0
Paroxetine	10.0	25.0	50.0	100.0	300.0	600.0	1000.0
Reboxetine	10.0	25.0	50.0	100.0	300.0	600.0	1000.0
Trazodone	250.0	500.0	800.0	1000.0	1300.0	1600.0	2000.0
Venlafaxine	10.0	25.0	50.0	100.0	300.0	600.0	1000.0

All concentrations are given in ng/mL. Accuracy and Precision were tested at LOW (Level 1), MED (Level 4) and HIGH (Level 6) concentrations. Recovery and Matrix effects were tested at LOW (Level 2) and HIGH (Level 6) concentrations. Stability was tested at LOW (Level 2) and HIGH (Level 6) concentrations.

blood and two zero-samples (blank whole blood spiked with internal standard). Extracts were checked for matrix and internal standard interferences, respectively. To test whether analytes showed mutual interferences, methanolic standard stocks were analyzed of all 19 antidepressants individually, at high therapeutic concentrations. These were checked for mutual interferences at the measured transitions. In a next step, linearity was evaluated. Matrix-matched calibration curves were constructed for each analyte, based on 7 calibration levels (n = 6 different sources of whole blood). Selection of a proper deuterated standard was based on obtaining repeatable results and thus low RSD values. To select the ideal calibration model, six models were tested and compared based on back-calculated values. The model with back-calculated values closest to the nominal concentration was selected, as this generated the optimal precision and accuracy results. Models that were tested: linear least squares unweighted and weighted ($1/x$, $1/x^2$) and quadratic least squares unweighted and weighted ($1/x$, $1/x^2$) models.

Accuracy and precision were assessed in duplicate at 3 concentration levels (LOW, MED, HIGH), on each of eight days. Results were analyzed using one-way ANOVA, with 'days' as grouping variable. Mean square between days (MS_B) and mean square within days (MS_W) values were used to calculate bias, repeatability (Rep) and intermediate precision (Int prec). Bias should be within ± 15% (± 20% near LOQ) of the nominal concentration and precision should be lower than 15% (20% near LOQ).

Sensitivity was assessed by determining limit of quantification (LOQ) and limit of detection (LOD). The lowest concentration level that was included in the calibration curve (Table 4 - *Level 1*) was set as LOQ for each analyte. Each LOQ was evaluated in precision and accuracy tests. LODs were determined by constructing a linear unweighted calibration curve near the LOQ. It was constructed using the second lowest calibrator, LOQ and zero samples.

Recovery (RE) and matrix effect (ME) were determined at two concentration levels (LOW, HIGH) for five sources of whole blood. Three sets of samples were prepared: (A) pure standards in methanol, (B) post-extraction spiked samples and (C) pre-extraction spiked samples. To meet with proposed guidelines, ME values should be within 85–115% (80–120% near LOQ).

As a final validation step, processed sample stability was evaluated under three conditions: storage in a cooled autosampler, on-bench storage at room temperature (RT) and freeze/thaw stability. All three parameters were tested at two concentration levels (LOW, HIGH) in duplicate. To determine autosampler stability, processed samples were placed in the cooled autosampler and injected each hour, for a 7-h time span. Linear regression analysis was performed on the hourly measured concentrations, to detect instability. A slope that significantly differs from zero ($p \leq 0.05$) indicates instability. Bench-top stability was determined by placing processed samples for 3 h on the bench at RT. Instability was detected by comparing the initial concentration to the concentration after 3 h. Freeze/thaw stability was determined by analyzing processed samples initially and after 3 freeze/thaw cycles of 23.5 h at – 20 °C and 0.5 h at RT. The initial concentration was compared to the concentration after the freeze/thaw cycles. For the last two stability parameters, concentration ratios were calculated (stability sample/control sample × 100). Whole blood stability of antidepressants has already been thoroughly tested and reported by Montenarh et al. [24] and Roemmelt et al. [26], therefore it was not further investigated in this study.

3. Results and discussion

3.1. Sample preparation optimization

3.1.1. pH whole blood + type of IL added

To achieve high extraction yields, the ideal extraction solvent (IL) needs to be selected. As the class of ILs consists of an infinite amount of possible anion – cation combinations, some requirements need to be defined to select suitable candidates for the extraction application. First, the IL needs to be hydrophobic, a workable viscosity is necessary to allow accurately pipetting and the IL should have a higher density than water so it forms the lower phase, which is easier to collect. In total, five ILs were selected from different classes (Table 1). BMIm PF_6, HMIm PF_6 and OMIm PF_6 consist of the commonly used imidazolium cation and hydrophobic hexafluorophosphate cation. These were selected as they are evaluated in several extraction applications and give insight into the contribution of the alkyl chain length on extraction yields. As pH 11.0 was included as a test condition, two specific base stable ILs were selected: BMPyrr Tf_2N and BM_3Amm Tf_2N. The choice of this high pH testing condition is attributed to the high pKa (± 9) of antidepressants. Process efficiency results for each pH value are presented as boxplots in Fig. 2A, B, C. Statistical analysis showed that BMIm PF_6 gives significantly ($p \leq 0.05$) higher PE values compared to the other tested extraction solvents. This can be partly explained by the different retention of the tested ILs on the LC column, and thus possible co-elution of the IL peak with the analyte peaks. Fig. 3 shows the

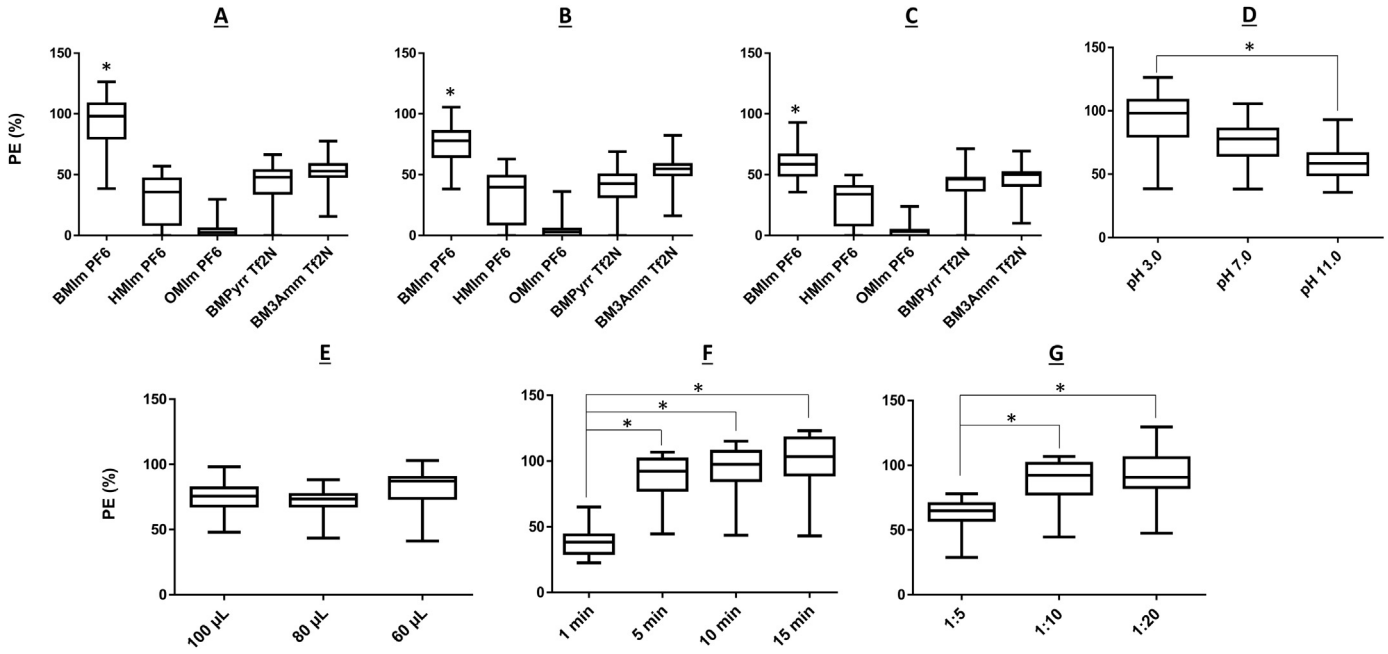


Fig. 2. Method optimization: Boxplots of process efficiency results for each evaluated extraction parameter. (A) type of IL added at pH 3.0; (B) type of IL added at pH 7.0; (C) type of IL added at pH 11.0; (D) BMIm PF_6 (1-butyl-3-methylimidazolium hexafluorophosphate) at pH 3.0, 7.0 and 11.0; (E) volume of IL added; (F) extraction time; (G) dilution factor of collected IL. * indicates a significant ($p \leq 0.05$) difference between conditions. Remark: (A), (B) and (C) do not show statistical comparison between groups.

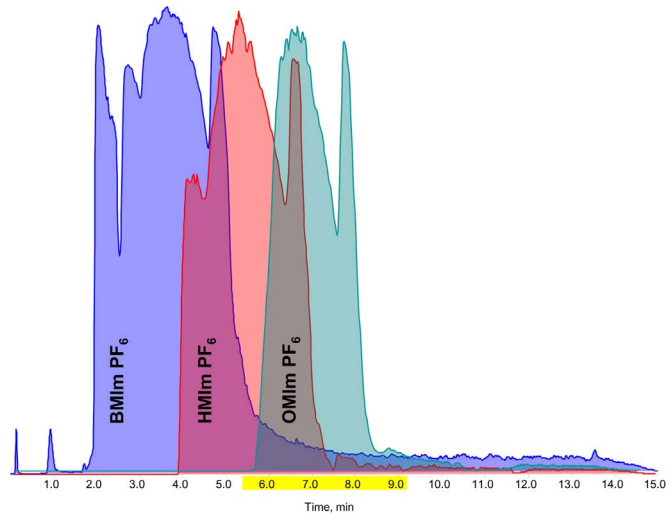


Fig. 3. Chromatographic retention profile of imidazolium-based ILs. BMIm PF_6: 1-butyl-3-methylimidazolium hexafluorophosphate; HMIm PF_6: 1-hexyl-3-methylimidazolium hexafluorophosphate; OMIm PF_6: 1-methyl-3-octylimidazolium hexafluorophosphate. In yellow, the retention range of 19 tested antidepressants in given. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

chromatographic elution of three imidazolium-based ILs. As can be seen, the longer the alkyl chain, the more retention, the more interference with antidepressant elution (5–10 min) and ionization. It was found that BMIm PF_6 interferes the least, thanks to short retention times. Another explanation for the better BMIm PF_6 results could be that polar antidepressant substituents rather interact with more polar ILs.

To select the appropriate pH, all BMIm PF_6 results were grouped in Fig. 2D. Adjusting the blood sample to pH 3.0 results in significantly ($p \leq 0.05$) higher PE values, and thus 3.0 was chosen as optimal pH. This indicates that protonated antidepressants show good interaction with the IL. Note that PE values above 100% are obtained, which can be attributed to matrix enhancement.

3.1.2. Volume of IL added

The volume of IL added determines the analyte enrichment factor: the lower the volume added, the higher the concentration of the final extract. However, lowering the added IL volume is limited by the collection step of IL at the end of the procedure. Moreover, the IL volume added should still allow to accurately collect 10 µL of IL. From the tested volumes, it was seen that 60 µL was the smallest volume that still allowed 10 µL collection in an accurate and repeatable manner ($n = 3$ different whole blood matrices). Furthermore, it was evaluated whether the addition of such a small volume of IL comprises PE values. Fig. 2E shows PE results of 60 µL and larger volumes added. No significant differences ($p > 0.05$) were detected. Eventually, 60 µL was chosen as the optimal condition to proceed with.

3.1.3. Extraction time

A dispersion was obtained by physical agitation of the blood/IL mixture using a rotary mixer. Results for the tested extraction times are shown in Fig. 2F. Statistical analysis showed that after 1 min of rotation, significantly ($p \leq 0.05$) lower PE values were obtained. No significant ($p > 0.05$) differences were found between 5 min, 10 min and 15 min of rotation, so the shortest extraction time (5 min) was selected to proceed with. In literature, it has also been described that in LLE, shorter extraction times result in cleaner extracts, thanks to slow transfer of interfering matrix [30].

3.1.4. Dilution factor of collected IL

After collection of the IL, a dilution step is necessary. This is needed to lower the IL's viscosity and allow injection into the LC-MS device in a repeatable manner. Furthermore, ion suppression due to the IL signal can be expected as this is was mentioned by De Boeck et al. [20] and it was also detected during the IL selection procedure. By diluting the IL extract, ion suppression will be less pronounced. On the other hand, the less the IL extract is diluted, the higher the analyte peaks will be, resulting in enhanced sensitivity. A good balance between IL interference and sensitivity should be obtained. Fig. 2G shows PE results for the three tested dilution factors. Results show that the dilution did significantly ($p \leq 0.05$) affect PE values at a dilution factor of 1:5. This confirms the IL suppression theory. No significant ($p > 0.05$)

differences were detected between a 1:10 and 1:20 dilution. Finally, 1:10 in methanol was selected as the optimal dilution factor, as better sensitivity was obtained.

3.2. Method validation

3.2.1. Selectivity

When analyzing blank whole blood processed sampled, no matrix interferences were found. Tested zero-samples did not show any deuterated internal standard interferences. Also, no mutual antidepressant interferes were detected. Overall, it was concluded that the IL-DLLME-LC-MS/MS method has shown to be selective and thus the combination of quantifier and qualifier transitions have found to be selective.

3.2.2. Linearity

Calibration ranges, calibration models, selected deuterated internal standards and coefficients of determination (R^2) are shown in Table S1. Calibration ranges start at the lowest therapeutic plasma concentrations, ranging to low toxic plasma concentrations [31]. For 18 antidepressants calibration curves ranged from 10 ng/mL to 1000 ng/mL, except for trazodone, which ranged from 250 to 2000 ng/mL. Unweighted linear models did not seem to fit the obtained linearity data, as calibration ranges are quite broad. This was seen from the obtained heteroscedastic distributions. As is reported in literature [27,32], wide bioanalytical calibration ranges often need weighted and/or quadratic models to optimally describe the relation between concentration and area under the curve (AUC). In this study, both $1/x$ and $1/x^2$ weighted quadratic models were applied. For the majority of selected models, back-calculated values were within ± 25% of the nominal concentrations. Escitalopram, fluvoxamine and reboxetine showed values within ± 30% of the nominal concentrations. Furthermore, for the construction of calibration curves, each analyte was coupled to an appropriate internal standard. For each calibration level, ratios (AUC analyte / AUC deuterated analogue) showed RSD% values below 15%. Additionally, R^2 values were determined. All coefficients were ≥ 0.99, except for fluoxetine and fluvoxamine. In literature, R^2 is defined as a less suitable parameter to evaluate a calibration model [27]. However, it gives an indication of which compounds show less established calibration curves, as for instance fluvoxamine.

3.2.3. Accuracy and precision

Accuracy and precision results are shown in Table S1. Results show that for 18 antidepressants, bias met with the proposed criteria (± 15% of the nominal concentration (± 20% near LOQ)). Precision results needed to comply with the acceptance criteria of 15% and 20% near LOQ. For repeatability, results complied, with the exception of fluvoxamine (18.06%, HIGH). Intermediate precision showed deviating results for agomelatine (15.53%, HIGH) and fluvoxamine (20.34%, MED; 21.80%, HIGH). Overall it can be concluded that fluvoxamine cannot be included in the full-quantitative method, due to large accuracy and precision deviations. Fluvoxamine already showed poor quality calibration curves, which is most likely the explanation. As fluvoxamine was extracted from its commercially available tablet form (Floxyfral® 100 mg), co-formulants can interfere with LC-MS analysis or can alter the homogeneity of the extracted standard, which explains possible validation hurdles. Agomelatine was also extracted from its commercially available tablet form (Valdoxan® 25 mg), which may explain deviations. However, agomelatine was still included in the full quantitative method, as only a small intermediate precision deviation was seen at HIGH concentrations (600 ng/mL) and agomelatine's therapeutic range is far below this concentration (up to 300 ng/mL). Nonetheless, caution should be taken with highly concentrated samples. Also, a more thorough extraction procedure for tablet form pharmaceuticals is needed in the future.

3.2.4. Sensitivity

LOQ and LOD data are shown in Table S1. Level 1 calibrators were selected as LOQ concentrations, which was 10 ng/mL for 18 antidepressants. Trazodone had a LOQ of 250 ng/mL. All LOQs were below the lowest therapeutic concentrations [31], except for agomelatine, which has 7 ng/mL as a lower limit of the therapeutic plasma range. LOD values ranged from 0.78 to 2.14 ng/mL and 35.15 ng/mL for trazodone.

3.2.5. Recovery and matrix effect

RE and ME results are shown in Table S2. RE values were within a range of 53.11–131.95% at LOW concentrations and 61.66–132.98% at high concentrations, with most compounds ranging within 80–115%. The lowest recoveries were obtained for the first eluting and thus most polar compounds, such as bupropion and venlafaxine. The less polar the compound, the better the interaction with BMIm PF_6. Furthermore, some antidepressants showed RE values above 100%. This could be attributed to the aqueous buffer pH 3.0 that was added before extraction and is known to protonate and thus charge the analyte. This charge could enhance ionization and therefore give higher AUC values for pre-extraction spiked samples. It should be noted that the obtained recoveries are comparable to those obtained with more complex and tedious SPE techniques [25]. Furthermore, a fast and simple PP procedure for determination of 39 antidepressants in whole blood showed RE values of 50–70%, indicating high analyte losses when compared to the newly developed IL-DLLME method [26]. Montenarh et al. [24] validated a conventional LLE procedure for the quantification of 33 antidepressants in whole blood. Here, RE values of mutually tested analytes are in a comparable range of ± 60 – 120%, except for amitriptyline (± 200%). Table S2 also shows that ME values are within a range of 61.92–123.24% at low concentrations and 77.12–97.64% at high concentrations. For most analytes, ion suppression was seen (80–90%), however, at low concentrations, fluvoxamine showed matrix enhancement. Interesting is that early eluting compounds are more prone to ion suppression, due to high IL signals. Possible hypotheses for the mechanism of ion suppression were already published [33]. Most likely, the non-volatile ILs change viscosity of the LC effluent droplets, which will prevent droplets from reaching the critical limit of transforming into gas phase. CV values for both ME and RE were found to be below 20% (LOW) and below 15% (HIGH). This implies that ME and RE are reproducible and can be considered during quantification. Note that fluvoxamine showed $CV_{RE,\ LOW}$ of 21.84%, which is an additional reason to exclude the compound from the quantitative method.

3.2.6. Stability

Autosampler stability, bench-top stability and freeze/thaw stability results are shown in Table S2. Processed sample stability results indicate that all compounds are stable for 7 h in the cooled autosampler, except for fluvoxamine and mianserin, respectively, 4 h and 6 h. Processed samples were also evaluated under bench-top conditions, all analytes were within 85–115%, with CV values lower than 15%, at both concentration levels. Freeze/thaw conditions gave comparable results, all antidepressants showed results within a range of 85–115%, with CV values lower than 15%, at both concentration levels. Escitalopram and reboxetine showed results near 120%, this may indicate that deuterated standards are more prone to freeze/thaw instability compared to the analyte itself. Caution should be taken, as escitalopram results were obtained with low repeatability (CV: 22.4%).

4. Conclusion

In this study, an IL-DLLME procedure was optimized and validated in combination with LC-MS/MS analysis for the quantification of 18 antidepressants in whole blood. The following analytes were included in the full-quantitative method: agomelatine, amitriptyline, bupropion, clomipramine, dosulepin, doxepin, duloxetine, escitalopram, fluoxetine,

imipramine, maprotiline, mianserin, mirtazapine, nortriptyline, paroxetine, reboxetine, trazodone and venlafaxine. The method was found to be selective, accurate and precise, with desired sensitivity. However, fluvoxamine was only included in the semi-quantitative method as accuracy and precision data did not meet with the proposed criteria. In general, good REs were obtained for the IL-based liquid-liquid microextraction technique, when compared to antidepressant research with other sample preparation techniques as LLE, SPE and PP [24–26]. However, IL-DLLME matrix suppression is rather pronounced (ME ± 80%). This can be attributed to the IL that sticks to the column and forms a broad peak across the chromatogram. Even though five ILs were tested, for none of these solvents it was possible to obtain chromatographic separation from the antidepressant peaks. This is one of the biggest disadvantaged of using ILs in LC-MS applications. Moreover, ILs have good solvation power [5], so it can be assumed that some proteins will be extracted from the whole blood matrix and can be a possible cause of the observed ion suppression. However, in this study, ME showed good repeatable results (CV < 15%), so ME can be accurately taken into account when constructing whole blood matrix-matched calibration curves. Interesting to note is that the same IL was found to be the ideal extraction solvent, compared to previous benzodiazepine research by De Boeck et al. [20]. This finding shows that BMIm PF_6 has a broad extraction range and therefore can be applied in possible screening applications. Overall, to our knowledge, this is the first paper that describes the use of ILs in a liquid-liquid microextraction for the quantification of antidepressants in complex biological samples. The optimized sample preparation method consisted of a minimal consumption of extraction solvent (60 μL) and an optimized processing time of less than 30 min. Compared to conventional SPE procedures, time and costs are clearly saved. Moreover, the use of ILs was evaluated for the extraction of antidepressants, which underlines the potential of IL usage in toxicology. In conclusion, IL-based bioanalytical research is still in its infancy, as with more ILs to come, current hurdles can be overcome, extraction yields can be optimized and more biodegradable and green solvents can be used.

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Conflicts of interest

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.talanta.2017.12.044.

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