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Rapid determination of designer benzodiazepines, benzodiazepines, and Z-hypnotics in whole blood using parallel artificial liquid membrane extraction and UHPLC-MS/MS

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Abstract

Benzodiazepines (BZD) and Z-hypnotics are frequently analyzed in forensic laboratories, and in 2012, the designer benzodiazepines (DBZD) emerged on the illegal drug scene. DBZD represent a particular challenge demanding new analytical methods. In this work, parallel artificial liquid membrane extraction (PALME) is used for sample preparation of DBZD, BZD, and Z-hypnotics in whole blood prior to UHPLC-MS/MS analysis. PALME of BZD, DBZD, and Z-hypnotics was performed from whole blood samples, and the analytes were extracted across a supported liquid membrane (SLM) and into an acceptor solution of dimethyl sulfoxide and 200 mM formic acid (75:25, v/v). The method was validated according to EMA guidelines. The method was linear throughout the calibration range ($R^2 > 0.99$). Intra- and inter-day accuracy and precision, as well as matrix effects, were within the guideline limit of $\pm 15\%$. LOD and LLOQ ranged from 0.10 to 5.0 ng mL^{-1} and 3.2 to 160 ng mL^{-1}, respectively. Extraction recoveries were reproducible and above 52%. The method was specific, and the analytes were stable in the PALME extracts for 4 and 10 days at 10 and -20 °C. No carry-over was observed within the calibration range. PALME and UHPLC-MS/MS for the determination of DBZD, BZD, and Z-hypnotics in whole blood are a green and low-cost alternative that provides high sample throughput (96-well format), extensive sample clean-up, good sensitivity, and high reproducibility. The presented method is also the first method incorporating analysis of DBZD, BZD, and Z-hypnotics in whole blood in one efficient analysis.

Keywords Designer benzodiazepines · Benzodiazepines · Z-hypnotics · Parallel artificial liquid membrane extraction · LC-MS/MS · Whole blood samples

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Introduction

Benzodiazepines (BZD) have been used clinically since the 1960s as anxiolytics, sedatives, hypnotics, anticonvulsants, and muscle relaxants [1]. The Z-hypnotics zopiclone and zolpidem are structurally different from BZD, but act via the same γ-aminobutyric acid type-A (GABA$_A$) receptor [2]. In addition to the therapeutic effects, BZD can cause synergistic effects when consumed together with other sedatives, antidepressants, neuroleptics, morphine-like substances, and especially alcohol [1]. This can lead to hospitalization and even death. BZD are frequently detected in cases of «Driving Under the Influence of Drugs» (DUID) [3], and in drug-facilitated crimes [4], where misuse of BZD often is implied. For these reasons, BZD are commonly analyzed in both clinical and forensic laboratories.

Designer benzodiazepines (DBZD) can be previously marketed BZD, metabolites of classic BZD, BZD marketed in only some countries, or structural analogues to therapeutically used BZD. Data about pharmacological and toxicological effects might not yet be available [5]. In 2012, the first DBZD not prescribed in any country, pyrazolam, was detected on the illegal drug scene [6]. For now, DBZD contribute only a fraction to the total number of designer drugs, but their prevalence has increased during the past 5 years [2, 7]. Analysis of BZD at a routine hospital laboratory, with positive immunoassay samples and negative confirmation for prescription BZD, revealed that 40% of the samples were positive for DBZD [8]. This confirms the importance of including DBZD in analytical methods for BZD. In Norway, DBZD are the most prevalent NPS found in DUID cases [9, 10].

Analytical methodologies for the determination of BZD in biological samples have been reviewed [1]. Analysis is primarily performed with LC-MS/MS, GC-MS/MS, or LC-UV, after sample preparation with liquid-liquid extraction (LLE), solid-phase extraction (SPE), or protein precipitation [1]. The use of supported liquid extraction (SLE) [11, 12] and miniaturized techniques such as solid-phase microextraction (SPME) [13], liquid-phase microextraction (LPME) [14], and dispersive liquid-liquid microextraction (DLLME) [15–17] is also worth mentioning.

In the review by Persona et al. [1], it is stated that development of new methods should be focused on reduction of the number of sample preparation steps, sample amount taken for analysis, consumption of time and reagents, and in general the costs of the whole analytical process, along with increase of specificity, accuracy, and sensitivity of the method. A sample preparation method fulfilling these criteria is parallel artificial liquid membrane extraction (PALME), a microextraction technique introduced in 2013 [18]. In PALME, analytes are extracted from an aqueous donor solution (sample), across an organic supported liquid membrane (SLM), and into an aqueous acceptor solution (extract). The extraction is facilitated by a pH gradient, and can be compared to LLE with back-extraction.

PALME meets the requirements of high throughput and high sensitivity. First, PALME is performed in a commercially available 96-well format. Second, extensive sample clean-up is achieved by preventing charged compounds and larger molecules like proteins and phospholipids from transferring across the SLM [19]. PALME is easy to operate, and semi-automation is possible by using a 96 channel pipette. The aqueous extracts are directly compatible with LC-MS/MS, and the consumption of organic solvents is kept to a minimum (< 5 μL per sample). Besides being a green sample preparation technique that provides extensive sample clean-up and a high sample throughput, PALME is a low-cost alternative to existing sample preparation techniques such as SPE, SLE, or phospholipid removal plates, which in our experience are 5 to 10 times more expensive.

One particular challenge in forensic analysis is the use of whole blood of varying quality as matrix. In SPE, a protein precipitation or dilution is usually necessary before extraction to avoid clogging the column, and especially autopsy blood can pose problems in both SPE and SLE [20–22]. In PALME, this issue is eliminated because the whole blood stays in the 96-well donor plate (Fig. 1, lower panel) and does not need to pass through a column.

The present work describes development and validation of a PALME procedure combined with UHPLC-MS/MS analysis for sensitive detection and quantification of DBZD, BZD, and Z-hypnotics in whole blood.

Material and methods

Chemicals and solvents

Methanol, acetonitrile, and formic acid (HCOOH) were all of LC-MS grade. Methanol, acetonitrile, and dimethyl sulfoxide (DMSO) were purchased from Merck. HCOOH, 2-undecanone, and dihexyl ether were purchased from Sigma-Aldrich. AnalaR® ammonium formate was purchased from BDH Laboratory Supplies, trioctylamine was from Cognis, and deionized water was purified with a Milli-Q water purification system from Millipore. Alprazolam, bromazepam, flunitrazepam, clonazepam, and nitrazepam were purchased from Sigma. Deschloroetizolam, diclazepam, flubromazepam, flubromazolam, clonazolam, meclonazepam, and the internal standards $^{13}C_6$-diazepam, $^{13}C_6$-clonazepam, $^{13}C_6$-N-desmethyldiazepam, and $^{13}C_6$-oxazepam were purchased from Chiron. Diazepam, phenazepam, oxazepam, zolpidem, zopiclone, and N-desmethyldiazepam were purchased from Lipomed. Etizolam was from LGC, and alprazolam-d5, flunitrazepam-d7, lorazepam-d4, nitrazepam-d5, zolpidem-d6, lorazepam, and midazolam were purchased from Cerillant. Zopiclone-d8 was purchased from Toronto Research Chemicals Inc.

Standard solutions

Stock solutions of each analyte were made in methanol or acetonitrile. Due to photosensitivity and possibility for degradation, zopiclone and zolpidem were dissolved in acetonitrile and protected from light. The stock solutions were stored at 4 °C.

Three working solutions were prepared with lower dosed BZD (8.0 μg mL^{-1}), higher dosed BZD (30 μg mL^{-1}, except oxazepam 200 μg mL^{-1}), and Z-hypnotics (zolpidem 50 μg mL^{-1}, zopiclone 10 μg mL^{-1}), see Electronic Supplementary Material (ESM), Table S1. The working solutions for lower and higher dosed BZD were prepared in methanol, and the working solution for Z-hypnotics was prepared
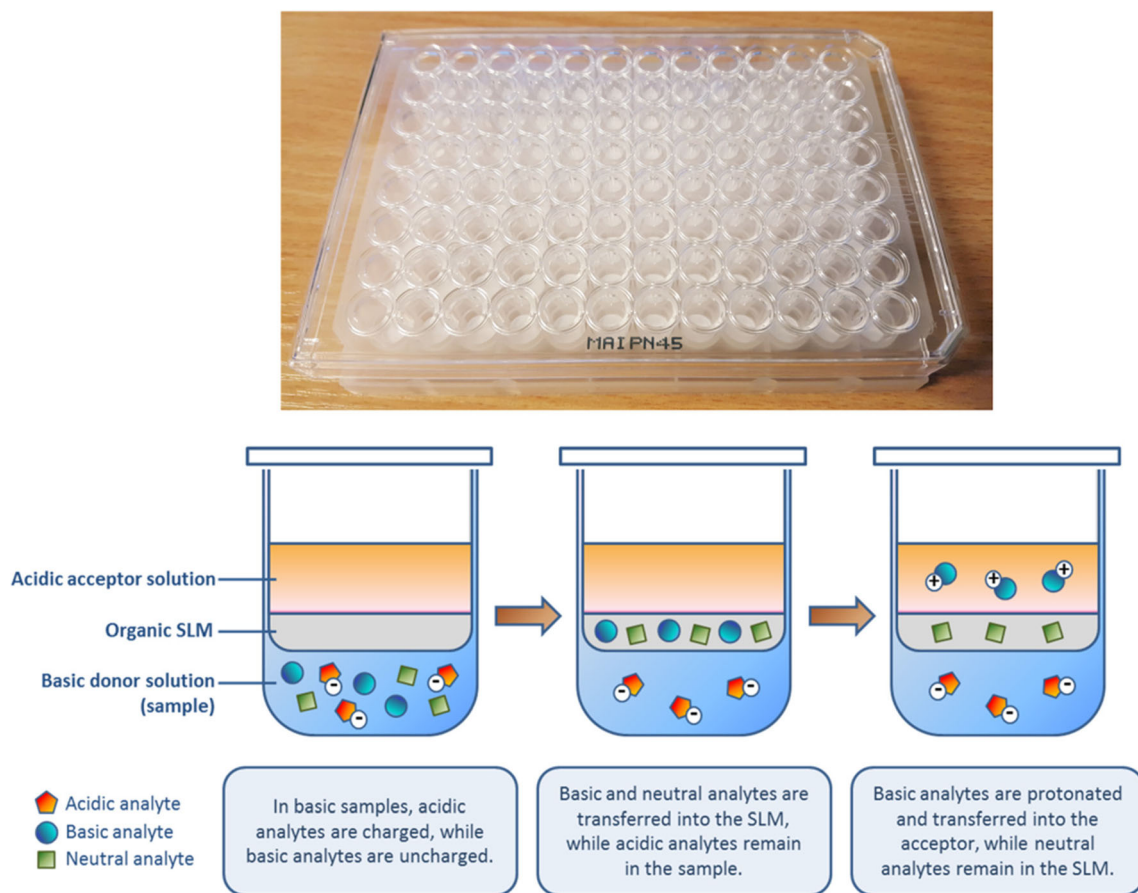
Fig. 1 96-well plates used for PALME (upper panel) and schematic illustration of the extraction process for PALME of basic analytes (lower panel)

in acetonitrile. The working solutions were further diluted with deionized water to prepare standard solutions with different concentrations that were used to spike drug-free whole blood (1:9, v/v). The standard solutions were stored at 4 °C. Standards containing the analytes in question used for routine analysis were used for the analysis of real samples.

Internal standards

The pure BZD internal standards were dissolved in methanol and the Z-hypnotic internal standards in acetonitrile, and a mixture of all internal standards was prepared in deionized water and stored at 4 °C. See ESM Table S2 for chemical characterization and concentrations. In accordance with literature demonstrating improved ability to compensate for ion suppression effects for ^{13}C-labeled internal standards [23] compared to deuterated, we used the ^{13}C-labeled internal standards for all compounds where this was available in our laboratory.

Whole blood samples

Human whole blood with sodium fluoride and heparin as additives was supplied by the Blood Bank of Oslo (Oslo University Hospital) and stored in brown glass bottles at −20 °C. The blood was thawed prior to preparation of calibration standards and quality controls (QC). In addition, forensic samples from DUID, drug-facilitated sexual assaults, and autopsy cases were analyzed as part of the routine casework at Oslo University Hospital and compared to routine methods. Samples from DUID or sexual assault cases were received in 5.0 mL BD Vacutainer® Blood Collection Tubes (BD Vacutainer Systems) containing 4.0 mg mL^{-1} sodium fluoride and sodium heparin (28 IU mL^{-1}). The forensic autopsy cases were received in 25 mL tubes (Sterilin) containing 200 mg potassium fluoride.

PALME equipment and procedure

The equipment used for PALME comprised a 96-well donor plate of polypropylene with 0.50 mL wells from Agilent, and a 96-well acceptor plate from Millipore with polyvinylidene fluoride (PVDF) serving as support for the SLM. The pore size of the PVDF material was 0.45 μm and the internal diameter 6.0 mm.

The donor wells were filled with 250 μL donor solution (sample) consisting of a sample aliquot of 100 μL whole blood, 130 μL 50 mM phosphate buffer (pH 7.5), and

20 μL internal standard. The SLM was prepared by pipetting 4.0 μL of the mixture 2-undecanone and dihexyl ether (1:1, *w/w*) with 1% trioctylamine (*w/w*) onto the PVDF material, with the acceptor plate turned upside down. After about 5 s, the acceptor plate was turned back and the acceptor wells were filled with 150 μL acceptor solution containing DMSO mixed with 200 mM HCOOH (75:25, *v/v*). The donor and acceptor plates were clamped together, and a Platemax pierceable aluminum sealing film from Axygen was placed on top to prevent evaporation from the acceptor wells during extraction. The whole setup was placed on a Vibramax 100 platform shaker (Heidolph Instruments) which promoted the extraction by providing agitation of 900 rpm. PALME was carried out for 60 min. After PALME, the extracts were transferred to a Nunc 96-well Polypropylene MicroWell plate (Thermo Fisher Scientific) with 450 μL pointed wells. The extracts were diluted 1:1 (*v/v*) with deionized water before UHPLC-MS/MS analysis.

UHPLC-MS/MS

Ultra-high performance liquid chromatography (UHPLC) coupled to tandem mass spectrometry (-MS/MS) was performed with an Acquity UHPLC instrument and a Xevo TQS triple quadrupole from Waters. Chromatography was performed on a 100-mm Acquity UPLC® HSS T3 column, also from Waters. The analytes and the internal standards were separated with gradient elution mobile phases comprising ammonium formate buffer (pH 3.1) as mobile phase A and methanol as mobile phase B. The gradient started with 5% mobile phase B at 0.00 min, and continued with 60% B at 0.20 min, 80% B at 2.50 min, 98% B at 2.51 min, and 5% B at 3.10 min. The latter condition was maintained for 0.20 min giving a run time of 3.30 min and a total cycle time of 4.1 min. The column temperature was 65 °C, and the flow rate was 0.50 mL min^{-1}. The injection volume was 2.0 μL for each sample.

MS/MS acquisition was performed in the multiple reaction monitoring (MRM) mode with detection of positive ions generated by electrospray ionization. The ion spray voltage was 1.0 kV and the ion source temperature was 150 °C. The MRM transitions and the collision energies used for the model analytes and the internal standards are provided in Supplementary Tables 1 and 2, respectively. Data acquisition was accomplished with MassLynx 4.1 SCN 905 from Waters.

Method validation

A set of experiments were performed to determine optimal conditions for PALME of BZD, DBZD, and Z-hypnotics in whole blood. Composition of the sample, SLM, and acceptor solution was optimized before optimal extraction time was determined. The final PALME procedure combined with UHPLC-MS/MS analysis was validated according to the European Medicine Agency (EMA) Guideline on Bioanalytical Method Validation [24].

Linearity

Ten calibrators of BZD, DBZD, and Z-hypnotics were prepared in whole blood in a concentration range given in Table 1. Weighted standard curves (1/*x*) were constructed for each analyte by plotting the area ratio analyte/IS against the calibrator concentration, and corresponding R^2 values and the back-calculated concentrations for the calibration standards were evaluated.

Selectivity and carry-over

The selectivity of the method was evaluated in accordance with EMA guidelines by evaluating blank samples from six different sources of whole blood. In addition, PALME of whole blood spiked with 159 potentially interfering compounds (ESM Table S3) was performed in accordance with suggestions from the German society of forensic toxicologists [25]. Retention time and signal intensity were evaluated for the chromatographic peaks with similar *m/z* transitions as the BZD, DBZD, and Z-hypnotics.

Potential carry-over from possible overdose cases was investigated by injecting a high-concentrated standard (25 times higher than the highest calibration level) prior to the injection of three extracted blood samples with no analytes added (zero blood). The signal intensity from the zero blood was evaluated and compared to the signal intensity at LLOQ.

Accuracy and precision

Intra- and inter-day accuracy and precision were determined by extracting calibrators and QC samples. The QC concentrations were measured by interpolating the area ratio analyte/IS against the calibration curve. For intra-day accuracy and precision, ten replicates of the QC samples were extracted and analyzed. For inter-day accuracy and precision, three replicates of the QC samples were extracted and analyzed at eight consecutive days. Accuracy was expressed as the deviation between measured analyte concentration and theoretical value (% bias), with a deviation limit of ± 15% (± 20% at LLOQ). Precision was expressed as the coefficient of variation (CV) of the measured values, and was targeted not to exceed 15% (or 20% at LLOQ).

Limits of detection and quantification

The limits of detection (LODs) were determined by scalar dilutions of the lowest calibration standard (÷ 2, ÷ 5, ÷ 10, and ÷ 20) in whole blood. LOD was the lowest concentration providing a signal to noise ratio (S/N) equal to or greater than

Table 1 Validation data: calibration range, correlation coefficients, LLOQs, extraction recoveries, intra- and inter-day accuracy and precision, and matrix effects

Compound QC—ng mL⁻¹	Calibration range (ng mL⁻¹)	Linearity Correlation coefficient (R^2), (slope and intercept)	LLOQ (ng mL⁻¹)	Extraction recovery, $n = 6$ (%)	Intra-day, $n = 10$		Inter-day, $n = 24$		Matrix effects, (CV) (%)
					CV (%)	Bias (%)	CV (%)	Bias (%)	
Alprazolam	15–600	0.9926 ($y = 1.0531x + 5.6407$)	15						
QC 1–24					3	4	7	0	−13 (3)
QC 2–50				60	6	7	8	−2	−13 (3)
QC 3–200				55	5	10	7	3	−10 (3)
QC 4–462				67	8	2	6	3	−6 (4)
Bromazepam	15–600	0.9976 ($y = 0.0127x + 0.008$)	15						
QC 1–24					6	3	8	2	−1 (2)
QC 2–50				68	5	−3	6	−2	1 (3)
QC 3–200				68	7	4	8	2	2 (2)
QC 4–462				70	8	−3	6	1	0 (2)
Deschloroetizolam	2.0–80	0.9998 ($y = 0.0147x - 0.0115$)	2.0						
QC 1–3.2					6	14	10	5	−13 (2)
QC 2–6.7				74	5	8	8	−3	−6 (3)
QC 3–26.7				70	7	10	8	1	−7 (3)
QC 4–62				70	8	5	7	2	−6 (2)
Diazepam	15–600	0.9998 ($y = 0.025x - 0.0904$)	15						
QC 1–24					4	16	8	8	−6 (3)
QC 2–50				92	4	7	8	7	−4 (2)
QC 3–200				88	6	8	8	8	−5 (3)
QC 4–462				89	8	4	6	7	−5 (1)
Diclazepam	2.0–80	0.9993 ($y = 0.0145x - 0.0174$)	2.0						
QC 1–3.2					4	17	8	9	−6 (4)
QC 2–6.7				99	5	4	7	−4	−2 (3)
QC 3–26.7				95	6	6	8	−1	−2 (3)
QC 4–62				93	8	5	7	3	−4 (2)
Etizolam	2.0–80	0.9995 ($y = 0.0528x - 0.0479$)	2.0						
QC 1–3.2					3	18	10	6	−9 (3)
QC 2–6.7				85	5	7	8	−4	−6 (4)
QC 3–26.7				79	6	12	8	2	−4 (3)
QC 4–62				78	8	6	7	2	−4 (2)
Phenazepam	2.0–80	0.9998 ($y = 0.0038x - 0.0032$)	2.0						
QC 1–3.2					6	16	10	7	−11 (10)
QC 2–6.7				104	6	2	7	−4	−7 (4)
QC 3–26.7				102	7	14	9	3	3 (4)
QC 4–62				98	7	8	7	3	−2 (3)
Flubromazepam	2.0–80	0.9996 ($y = 0.002x - 0.0013$)	2.0						
QC 1–3.2					6	16	9	6	−3 (0)
QC 2–6.7				102	7	2	7	−4	2 (8)
QC 3–26.7				101	7	9	9	0	−3 (3)
QC 4–62				98	8	5	7	2	−2 (2)
Flubromazolam	2.0–80	0.9991 ($y = 0.0114x + 0.0029$)	2.0						
QC 1–3.2					4	18	11	4	−12 (6)
QC 2–6.7				66	7	12	8	1	−11 (3)
QC 3–26.7				65	7	19	9	5	−7 (4)
QC 4–62				68	9	7	8	1	−8 (2)
Flunitrazepam	2.0–80	0.9994 ($y = 0.4247x - 0.3597$)	2.0						
QC 1–3.2					6	13	8	1	−9 (3)
QC 2–6.7				101	3	7	11	−7	−3 (2)
QC 3–26.7				98	6	6	12	−6	−5 (3)
QC 4–62				101	9	4	11	1	−5 (2)
Clonazepam	2.0–80	0.9996	2.0						

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Table 1 (continued)

Compound QC—ng mL⁻¹	Calibration range (ng mL⁻¹)	Linearity Correlation coefficient (R^2), (slope and intercept)	LLOQ (ng mL⁻¹)	Extraction recovery, $n=6$ (%)	Intra-day, $n=10$ CV (%)	Bias (%)	Inter-day, $n=24$ CV (%)	Bias (%)	Matrix effects, (CV) (%)
QC 1–3.2		($y = 0.063x - 0.0232$)			6	9	7	1	−2 (5)
QC 2–6.7				100	5	8	6	0	−3 (4)
QC 3–26.7				101	5	8	6	2	−2 (3)
QC 4–62				104	8	4	6	2	−3 (1)
Clonazepam	2.0–80	0.9996	2.0						
QC 1–3.2		($y = 0.0068x - 0.0037$)			5	15	10	6	−3 (3)
QC 2–6.7				53	6	6	8	−1	−3 (3)
QC 3–26.7				52	6	12	10	2	−2 (2)
QC 4–62				53	7	2	8	2	−3 (1)
Lorazepam	15–600	0.9992	15						
QC 1–24		($y = -3\mathrm{E}\text{-}05x^2 + 0.0548x + 0.245$)			9	9	12	5	3 (5)
QC 2–50				88	7	10	11	12	3 (3)
QC 3–200				85	6	9	9	10	7 (1)
QC 4–462				86	11	−7	7	−1	3 (1)
Meclonazepam	2.0–80	0.9996	2.0						
QC 1–3.2		($y = 0.0031x + .0003$)			11	13	12	4	2 (0)
QC 2–6.7				100	7	15	10	1	−1 (6)
QC 3–26.7				103	7	17	9	4	−5 (5)
QC 4–62				102	6	10	8	3	−2 (3)
Midazolam	15–600	0.9996	15						
QC 1–24		($y = 0.0229x - 0.0839$)			3	15	8	7	−10 (4)
QC 2–50				100	5	6	8	−3	−9 (3)
QC 3–200				97	6	13	8	4	−6 (3)
QC 4–462				96	3	11	7	4	−6 (2)
N-desmethyldiazepam	15–600	1.0000	15						
QC 1–24		($y = 0.0417x - 0.0959$)			4	10	7	3	−5 (3)
QC 2–50				98	4	8	7	−1	−5 (3)
QC 3–200				96	6	8	7	2	−3 (2)
QC 4–462				95	7	6	5	2	−4 (1)
Nitrazepam	15–600	0.9992	15						
QC 1–24		($y = 0.1102x + 0.2403$)			5	3	7	1	−2 (2)
QC 2–50				99	6	6	6	2	−1 (2)
QC 3–200				97	5	8	4	4	−2 (3)
QC 4–462				96	9	4	5	3	−2 (1)
Oxazepam	100–4000	0.9999	100						
QC 1–160		($y = 0.0045x - 0.0219$)			4	6	7	1	7 (3)
QC 2–333				71	4	7	5	0	7 (2)
QC 3–1333				72	6	6	5	2	7 (2)
QC 4–3077				73	8	2	5	2	4 (1)
Zolpidem	25–1000	0.9999	25						
QC 1–40		($y = 0.0051x - 0.0202$)			3	13	10	5	−5 (2)
QC 2–83				75	5	1	8	−3	−3 (3)
QC 3–333				68	5	6	7	3	−2 (4)
QC 4–769				71	9	5	7	2	−1 (3)
Zopiclone	5.0–200	0.9986	5.0						
QC 1–8.0		($y = 0.0264x + 0.015$)			2	16	16	3	−1 (2)
QC 2–16.7				58	6	7	12	13	−3 (4)
QC 3–67				67	5	0	11	3	−4 (4)
QC 4–154				77	9	−8	9	−2	−5 (4)

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3. The lower limits of quantification (LLOQs) were determined from accuracy and precision data for the lowest calibration standard, with CV values within the guideline limits, in addition to an S/N equal to or greater than 10.

Extraction recovery and matrix effects

Endogenous compounds (e.g., phospholipids) present in blood samples may cause matrix effects in LC-MS, namely ion suppression or enhancement. Previous findings have concluded that PALME of plasma samples provides extracts free from phospholipids [19]. Still, potential matrix effects caused by other compounds should be investigated for new PALME applications.

The extraction efficiency of PALME and potential matrix effects were determined by analyzing three sets of samples spiked with analytes at three concentration levels; six replicates of zero blood from six different blood batches spiked with analytes before PALME (set 1), four replicates of the six blood batches spiked with analytes after PALME in a concentration equal to 100% recovery (set 2), and two replicates of neat standard solutions spiked with analytes equal to the concentrations in set 2, but without PALME and external influence from the blood matrix (set 3). In all cases, internal standards were added after PALME.

Extraction recoveries were calculated as the ratio between the peak areas obtained for the pre-spiked samples in set 1 and the peak areas obtained for the post-spiked samples in set 2. The matrix effects were quantified according to Matuszewski et al. [26] as the ratio between the peak areas obtained for the post-spiked samples in set 2 and the peak areas obtained for the neat standard solutions in set 3. The quantified matrix effects and their corresponding RSD values were targeted not to exceed ± 15%.

Stability

The stability of BZD, DBZD, and Z-hypnotics in the acceptor solution after PALME was evaluated by extracting calibrators and QC samples and storing them at 10 and − 20 °C after an initial UHPLC-MS/MS analysis. The solutions stored at 10 °C were reanalyzed after 4 days, and the solutions stored at − 20 °C were reanalyzed after 10 days. Percentage deviation from the initial concentrations was determined with an acceptance limit of ± 15%.

Comparison of PALME-UHPLC-MS/MS with routine methods at Oslo University Hospital

Blood samples from DUID, drug-facilitated sexual assault, and autopsy cases were used to demonstrate the applicability of the method for BZD, DBZD, and Z-hypnotics. Samples are received from police districts across the whole of Norway (DUID, DFSA) as part of police investigations into impairment due to medications or illegal drugs in crime suspects or crime victims and from forensic medical centers in Norway (forensic autopsy toxicology). For 50 samples screened positive with a UHPLC-MS/MS method [20] extended with DBZD, the results obtained by the presented method were analyzed in parallel with routine confirmation methods at Oslo University Hospital [11, 27, 28] as part of the routine casework. The samples represented 35 DUID cases, 15 autopsy cases, and one possible drug-facilitated sexual assault (DFSA). Several samples were positive for more than one component, which resulted in 97 concentration pairs for comparison. The results obtained were anonymized and a Bland-Altman analysis [29, 30] of the agreement between the routine methods and the PALME-UHPLC-MS/MS method was performed with Sigmaplot 13.0 (Systat Software Inc.).

Results and discussion

Optimization of PALME

Donor solution (sample)

PALME is based on passive diffusion of analytes from an aqueous sample and across an organic SLM. Therefore, the analytes should be uncharged in the sample to increase the affinity for the SLM. For this purpose, sodium hydroxide is frequently used as donor solution in PALME [18, 19, 31]. As seen in Supplementary Table 1, most BZD and DBZD possess relatively low pKa values. It was thus expected that the analytes would remain uncharged even at neutral conditions. Nevertheless, the viscosity of whole blood could possibly affect the extraction efficiency, and dilution with sodium hydroxide (pH 12), carbonate buffer (pH 9.5), and phosphate buffer (pH 7.5) was investigated.

The results were similar for the sample solutions prepared with carbonate buffer and phosphate buffer, and it was decided to use phosphate buffer (50 mM) at physiological pH conditions. Extraction from undiluted whole blood was also tested, but this was less efficient due to viscosity issues and reduced effect of agitation. Increased viscosity will counteract the effect of agitation during extraction, which primarily is performed to maintain convection in the sample. It was therefore decided that optimal sample composition included dilution of whole blood with phosphate buffer (pH 7.5), and PALME of BZD, DBZD, and Z-hypnotics was performed with neutral sample conditions.

Supported liquid membrane

Seven organic solvents with 1% trioctylamine (w/w) were tested as potential SLMs, either pure or in combination.

Trioctylamine served to avoid non-specific binding of BZD, DBZD, or Z-hypnotics to the PVDF support material in the SLM. The selected solvents were bis(2-ethylhexyl) phosphite, dihexyl ether, dodecyl acetate, hexadecane, isopentylbenzene, undecanol, and 2-undecanone. The solvents were selected based on previous experience in our laboratory.

The main purpose of the SLM solvent is to provide sufficient affinity for the analytes to facilitate their transfer from the sample and into the SLM, and then release them into the aqueous acceptor; otherwise, the analytes could be trapped inside the SLM. Therefore, selecting a proper solvent for the SLM is an important step of the optimization.

First, the organic solvents were tested separately with 1% trioctylamine (*w/w*). The results showed potential for 2-undecanone, dihexyl ether, isopentylbenzene, and bis(2-ethylhexyl) phosphite, but none of these were effective for all the analytes. In a next set of experiments, combinations of 2-undecanone with dihexyl ether and isopentylbenzene, and dihexyl ether with isopentylbenzene and bis(2-ethylhexyl) phosphite were prepared by mixing the solvents in a 1:1 weight by weight ratio before 1% trioctylamine was added. The results were generally improved with the solvent combinations, and the most effective solvent combination was 2-undecanone and dihexyl ether (1:1, *w/w*) with 1% trioctylamine (*w/w*).

Acceptor solution

The PALME process involves two critical steps: (1) mass transfer of analytes from the aqueous sample and into the organic SLM (sample/SLM interface), and (2) mass transfer of analytes from the SLM and into the aqueous acceptor solution (SLM/acceptor interface). As seen from Supplementary Table 1, the analytes of interest possess log P values > 2.5, except zopiclone. Because of this, analyte transfer from the sample and into the SLM was expected to be feasible. However, many BZD and DBZD possess low pKa values. The second step was therefore expected to be more critical, as ionization at the SLM/acceptor interface is a prerequisite to facilitate analyte transfer from the SLM and into the acceptor solution. The pH in the acceptor solution should therefore be sufficiently low. For this purpose, different concentrations of HCOOH were tested (10–1000 mM). The aim was to shift the equilibrium towards protonated state in the acceptor solution. The process efficiency increased with increasing HCOOH concentration for most analytes, with a few exceptions (zolpidem and alprazolam). Final acid concentration was set to 200 mM HCOOH (data not shown).

Further on, the possibility of modifying the acceptor solution with organic solvents was investigated. The aim was to increase analyte solubility by making the acceptor more organic (without dissolving the SLM). DMSO, acetonitrile, and methanol were separately added to 200 mM HCOOH in the volume ratios 25:75 and 50:50 and compared to acceptor solutions of pure 200 mM HCOOH. The SLM was not stable with acceptor solutions modified with acetonitrile and methanol, and the extracts were not analyzed. DMSO on the other hand was found to be highly beneficial for the process efficiency, and additional volume ratios of DMSO added to 200 mM HCOOH were therefore tested (60:40, 70:30, 75:25, and 100:0). Pure DMSO was not successful as the acceptor volume substantially decreased during extraction, which indicated dissolution and leakage of the SLM. From the results, it was concluded that 200 mM HCOOH with 75% DMSO was the optimal composition of the acceptor solution.

Dilution of acceptor solutions containing DMSO was necessary prior to analysis because poor chromatography was observed when the amount of DMSO in the extracts exceeded 60% (data not shown). This issue was completely eliminated by diluting the extracts 1:1 (*v/v*) with deionized water.

Extraction time

PALME was performed with optimized conditions for 5, 15, 30, 60, 120, and 180 min. Time curves with signal intensity plotted against extraction time are shown in Fig. 2. The extraction time clearly affected the recovery of BZD, DBZD, and Z-hypnotics from whole blood. Although some analytes benefited from longer extraction time than 60 min, most analytes reached equilibrium within this time, and 60 min was considered optimal extraction time with respect to both throughput and recovery.

Method validation

Linearity

Linear calibration curves with corresponding $R^2 > 0.99$ were obtained for all the analytes throughout the concentration range, except for lorazepam where $R^2 > 0.99$ was obtained with a quadratic calibration curve. The slope and intercept for the calibration curves are given in Table 1 together with corresponding R^2 values. Evaluation of the back-calculated concentrations of the calibration standards showed that the concentration deviations were within ± 15% of the nominal value (± 20% for LLOQ). In addition, more than 50% of the calibration standard replicates tested per concentration level fulfilled these criteria. This is in accordance with the EMA guideline.

Selectivity and carry-over

No interfering peaks were found in extracted blank samples. Standards containing potential interfering compounds were analyzed with no co-elution or interference with the measurement of BZD, Z-hypnotics, or the internal standards when

Fig. 2 Time curves constructed
with signal intensity plotted
against extraction time (5, 15, 30,
45, 60, 120, and 180 min). RSD
values below 15% ($n = 4$)
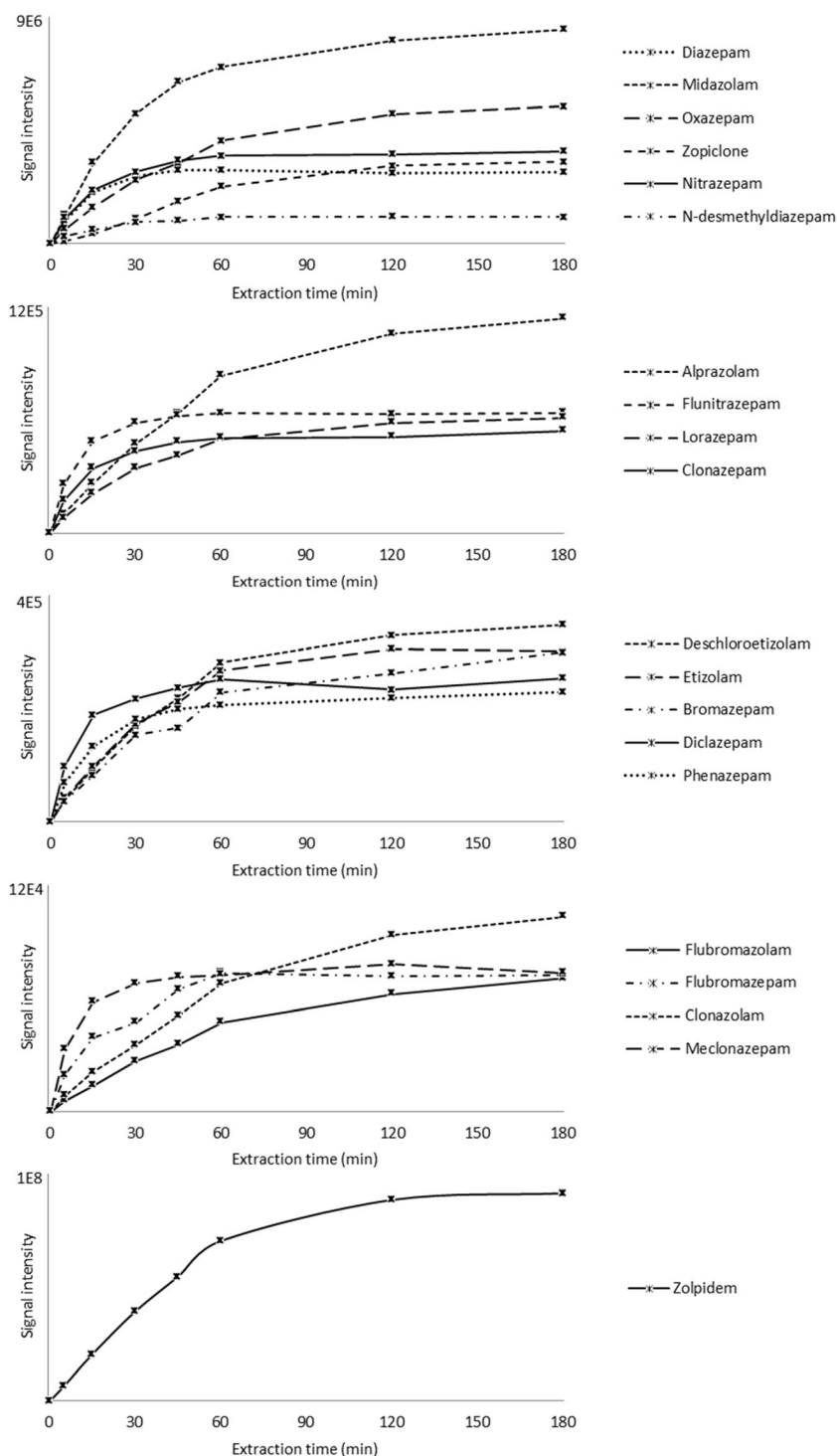
monitoring the MRM transitions selected. Thus, the specific-
ity of the method was considered satisfactory. No problems
with carry-over were found within the calibration range. The
possibility of very high-concentrated samples (e.g., autopsy
overdose cases) was evaluated with a concentration 25 times
higher than the highest calibration level. Approximately half
of the compounds had carry-over exceeding 20% of the
LLOQ sample. Thus, potential carry-over should be

monitored in routine analysis, especially in cases where over-
doses are implied.

Accuracy and precision

Excellent reproducibility was demonstrated for the determina-
tion of BZD, DBZD, and Z-hypnotics in whole blood using
PALME and UHPLC-MS/MS. Intra- and inter-day precision
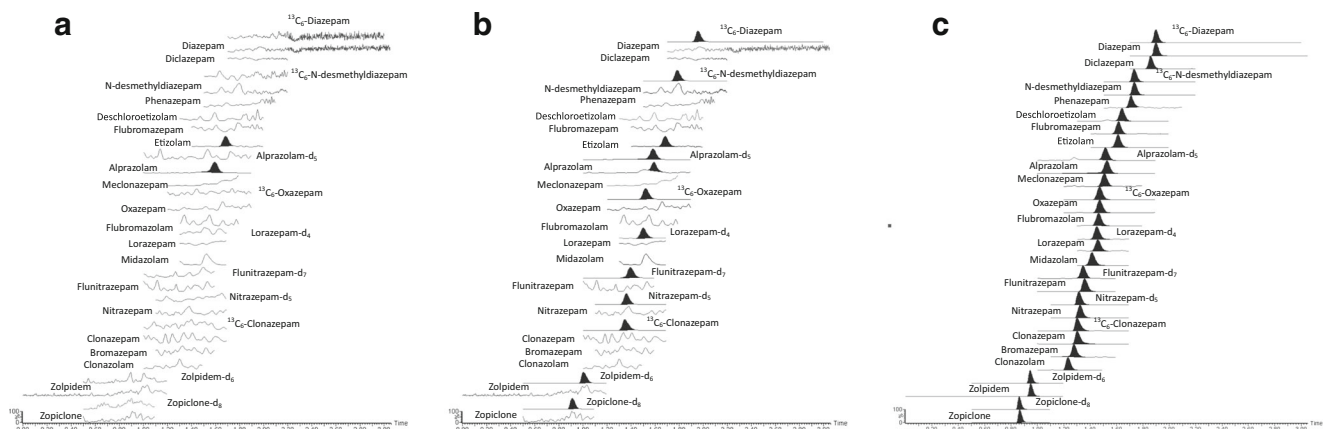
Fig. 3 Chromatograms of (**a**) blank whole blood, (**b**) whole blood spiked with internal standards, and (**c**) whole blood spiked with calibrator 1 and internal standards

was within 15% with CVs ranging from 2–11% and 4–12%, respectively. Intra- and inter-day accuracy was within ± 15% for almost all the analytes. The exceptions were intra-day flubromazolam (19% bias) and meclonazepam (17% bias) in QC 3, but these deviations were most likely due to random errors during analysis.

Limits of detection and quantification

Even with diluted PALME extracts, LODs ranging from 0.10 to 5.0 ng mL^{-1} were obtained. The LLOQs were equal to the lowest calibration standard, with values ranging from 2.0 to

100 ng mL^{-1} (Table 1). For the higher dosed BZD, and in particular oxazepam, a lower LLOQ and LOD would however be expected for our method if lower concentrations had been evaluated. A chromatogram of calibrator 1 is shown in Fig. 3, together with blank sample and blank sample with internal standard.

Extraction recovery and matrix effects

Extraction recovery for each analyte was determined at three concentration levels to ensure that it was unaltered at different analyte concentrations. This was confirmed for all analytes,
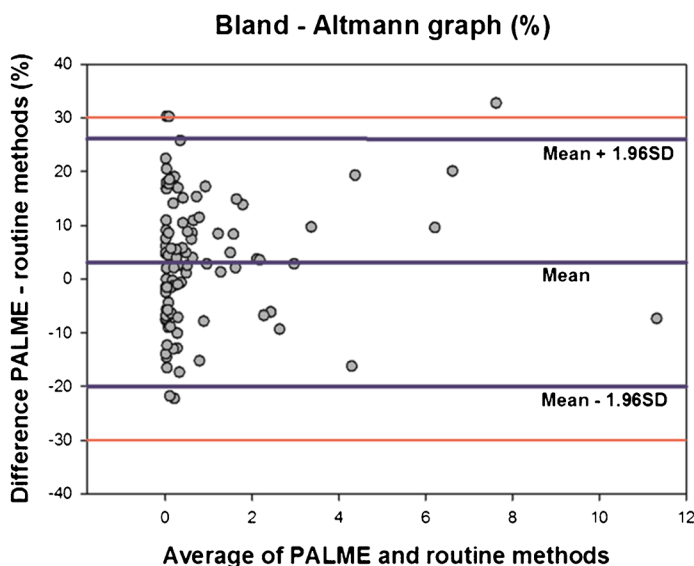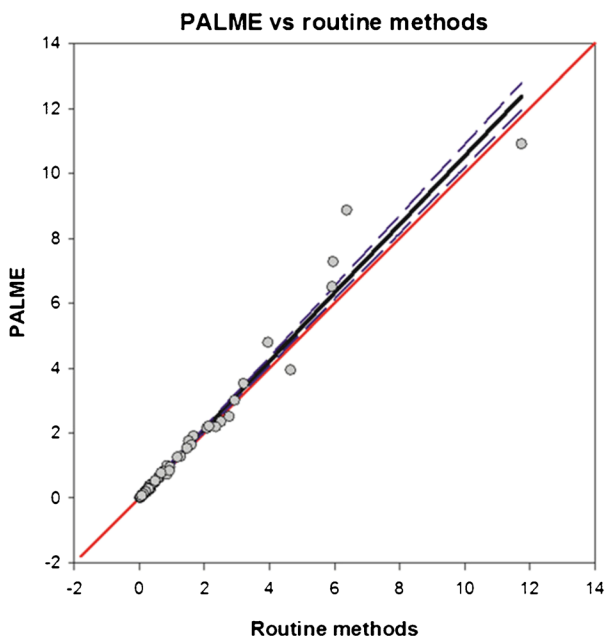
Fig. 4 Bland-Altman comparison between the concentrations obtained with the presented PALME-UHPLC-MS/MS method (*y*-axis) and the previously used methods (*x*-axis) (left panel), and Bland-Altman graph

showing the difference, expressed as percentages, between the presented method and the previously used method (*y*-axis), and average concentration (*x*-axis)

except for zopiclone, where the recovery increased from 58 to 77% from the lowest to the highest concentration level. Diclazepam, phenazepam, flubromazepam, flunitrazepam, clonazepam, meclonazepam, midazolam, *N*-desmethyldiazepam, and nitrazepam were exhaustively extracted with recoveries > 90%, whereas deschloroetizolam, diazepam, etizolam, lorazepam, oxazepam, and zolpidem were extracted with recoveries > 70%. Alprazolam, bromazepam, flubromazolam, and clonazolam were extracted with recoveries ranging from 52 to 68%. However, although not all analytes were exhaustively extracted, the recoveries were reproducible and allowed accurate determination of BZD, DBZD, and Z-hypnotics in whole blood. Additionally, the quantified matrix effects and their corresponding RSD values were all within ± 15%. The absence of interfering compounds from the blood matrix demonstrated the extensive clean-up obtained with PALME.

Stability

BZD, DBZD, and Z-hypnotics were found to be stable in the PALME acceptor solution for 4 and 10 days at 10 and − 20 °C, respectively. The deviations from the initial measured concentrations were within ± 15%.

Comparison of PALME-UHPLC-MS/MS with routine methods at Oslo University Hospital

Positive samples were found for all BZD and Z-hypnotics in the method, and for three DBZD (diclazepam, clonazolam, and etizolam), in total 14 of the 20 components in the method. A correspondence within ± 20% was found for 92% of the 97 concentration pairs.

The routine methods have showed satisfactory results in external proficiency tests during the last 3 years with *z*-scores ≤ ± 2 for all the BZD and Z-hypnotics, except fenazepam (not included by the organizers). For the DBZD, only etizolam and diclazepam have been included in proficiency tests so far, with *z*-scores ≤ ± 1 for etizolam and 2.06 for diclazepam. A Bland-Altman plot for method comparison [29, 30] is shown in Fig. 4, and the difference is shown as percentage of the mean of the PALME and routine method results. No particular practical problems with the extraction were found for the autopsy samples. Only three samples deviated more than 30% from the routine methods (marked with red lines in Fig. 4, right panel), a deviation which for our routine work would result in a re-analysis. Of these samples, two were zopiclone in autopsy blood where inherent compound instability could have contributed to the discrepancy.

Incurred sample reanalysis was not performed. This has however previously been performed with good results for the routine methods used for comparison. The good correspondence between the new method and the routine methods would suggest that the new method did not suffer from problems with correct quantification due to differences in protein binding, back-conversion of metabolites, etc. for real samples, compared to QC samples.

Method benefits

The presented method uses a limited amount of sample material (0.1 mL) compared to several other methods for analysis of BZD and Z-hypnotics in whole blood (0.5 to 1.0 mL) [11, 16, 17, 32] or 0.2 mL [33]. The new method does also benefit from semi-automation by use of a 96-channel pipette and a very short cycle time (4.1 min). Other comparable methods have longer cycle times (5.5 to 19 min) and more manual approaches [11, 16, 17, 32, 33]. The achieved LLOQ values are comparable to previously published methods for BZD using SPE [32], LLE [33] or DLLME [15–17], which report LLOQ values in the range 2.0 to 50 ng mL^{-1}, whereas higher LLOQ values were found compared to an SLE method [11] which report very low LLOQ values (0.20–17 ng mL^{-1}).

In a green chemistry perspective, the use of only 4.0 μL of organic solvent per sample represents an important and large step forward. In comparison, the method presented by De Boeck et al. uses 60 μL organic solvent per sample [16], while SPE, LLE, or SLE methods can use up to several milliliters. Compared to other extraction methods in the 96-well format, PALME does additionally represent a low-cost alternative.

Conclusion

The presented PALME-UHPLC-MS/MS method for the determination of DBZD, BZD, and Z-hypnotics in whole blood was found to be a green and low-cost alternative that provides high sample throughput, extensive sample clean-up, and good sensitivity and reproducibility. Good performance was found for real samples, and new DBZD can easily be incorporated as they enter the market. The method is to our knowledge the first method incorporating analysis of DBZD, BZD, and Z-hypnotics in whole blood in one efficient analysis.

Compliance with ethical standards

Conflict of interest The authors declare that they have no competing interests.

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