Journal of Chromatography B, 1048 (2017) 77–84

Contents lists available at ScienceDirect

Journal of Chromatography B

journal homepage: www.elsevier.com/locate/chromb

Parallel artificial liquid membrane extraction of new psychoactive substances in plasma and whole blood

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ARTICLE INFO

Article history:
Received 15 December 2016
Received in revised form 8 February 2017
Accepted 10 February 2017
Available online 14 February 2017

Keywords:
Liquid-phase microextraction
Parallel artificial liquid membrane extraction
New psychoactive substances
Plasma samples
Whole blood samples

ABSTRACT

Parallel artificial liquid membrane extraction (PALME) was combined with ultra-high performance liquid chromatography-mass spectrometry (UHPLC–MS) and the potential for screening of new psychoactive substances (NPS) was investigated for the first time. PALME was performed in 96-well format comprising a donor plate, a supported liquid membrane (SLM), and an acceptor plate. Uncharged NPS were extracted from plasma or whole blood, across an organic SLM, and into an aqueous acceptor solution, facilitated by a pH gradient.

MDAI (5,6-methylenedioxy-2-aminoindane), methylone, PFA (para-fluoroamphetamine), mCPP (meta-chlorophenylpiperazine), pentedrone, methoxetamine, MDPV (methylenedioxypyrovalerone), ethylphenidate, 2C-E (2,5-dimethoxy-4-ethylphenethylamine), bromo-dragonfly, and AH-7921 (3,4-dichloro-*N*-{[1-(dimethylamino)cyclohexyl]methyl}benzamide) were selected as representative NPS. Optimization of operational parameters was necessary as the NPS were novel to PALME, and because PALME was performed from whole blood for the very first time. In the PALME method developed for plasma, NPS were extracted from a 250 μL alkalized donor solution consisting of 125 μL plasma sample, 115 μL 40 mM NaOH, and 10 μL internal standard. In the PALME method from whole blood, the 250 μL alkalized donor solution consisted of 100 μL whole blood, 50 μL deionized water, 75 μL 80 mM NaOH, and 25 μL internal standard. In both methods, extraction was accomplished across an SLM of 5 μL dodecyl acetate with 1% trioctylamine (w/w), and further into an acidic acceptor solution of 50 μL 20 mM formic acid. The extraction was promoted by agitation at 900 rpm and was carried out for 120 min. Method validation was performed and the following parameters were considered: linearity, limits of quantification (LOQ), intra- and inter-day precision, accuracy, extraction recoveries, carry-over, and matrix effects. The validation results were in accordance with FDA guidelines.

1. Introduction

New psychoactive substances (NPS) are occasionally referred to as “legal highs”. This term has received criticism as it may be incorrectly interpreted because of the word “legal” [1]. Still, the term is based on the fact that NPS resemble other controlled substances or “illegal highs” in molecular structure and pharmacological effects, but might not be subjected to regulatory control themselves [2]. The prevalence and the usage of NPS has increased dramatically the last 10–15 years, and it has become a severe social problem internationally as NPS represent a major health risk [3]. The increase is mainly due to on-line sales on the internet, where the products are frequently promoted as harmless everyday products, like bath salts [4]. This is unfortunate as NPS can be more toxic and potent than the controlled substances they resemble. In addition, the pharmacological effect may have a considerably slower onset. The latter may confuse the user and lead to a higher intake in order to achieve the desired effect more quickly, resulting in toxicity or worst case scenario: fatality [5]. The development and creativity behind the production and spreading of NPS is groundbreaking and has put the international system for drug control in a pinch [6]. All initiatives directed towards combatting the NPS phenomenon is therefore of great importance and the need for solid forensic analytical methods to achieve accurate identification of new substances is obvious. In the literature, liquid chromatography (LC-), gas chromatography

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http://dx.doi.org/10.1016/j.jchromb.2017.02.010
1570-0232/

(GC-), and capillary electrophoresis (CE-) coupled to tandem mass spectrometry (-MS/MS) are reported as suitable methods for analyzing NPS in whole blood, serum, urine [7–9], and saliva [10,11]. Limitations of the existing methods makes development of new techniques interesting and important, with special emphasis on providing high throughput, extensive clean-up prior to LC–MS/MS, and a simple work-flow [12].

Parallel artificial liquid membrane extraction (PALME) was introduced in 2013 as a new microextraction technique in the 96-well format [13]. The PALME principle is similar to the closely related technique hollow-fiber liquid-phase microextraction (HF-LPME), where a pH gradient is used to facilitate mass transfer of uncharged analytes across an organic supported liquid membrane (SLM). The commercially available equipment that was used for PALME in this paper is depicted in Fig. 1 and is comprised by a donor plate, an acceptor plate, and a top lid. The donor plate houses an aqueous donor solution (sample) and the acceptor plate houses an aqueous acceptor solution (PALME extract). In the bottom of the acceptor plate, a flat porous filter material of polyvinylidene fluoride (PVDF) is located, in which an organic solvent is immobilized to form the SLM. The pH gradient arises from pH adjustment of the donor and acceptor solution. When PALME is performed for basic analytes, the pH in the donor solution is adjusted sufficiently high to maintain the analytes non-charged. In this way the solubility of the basic compounds in the aqueous donor is reduced, thereby transferring them into the organic SLM. To further promote mass transfer across the SLM and into the aqueous acceptor solution, the pH in the acceptor is adjusted sufficiently low to ensure ionization of the basic compounds. This is performed to increase the solubility of the basic compounds in the aqueous acceptor, and to prevent the basic compounds from back-extracting to the organic SLM.

Up to date, PALME of non-polar basic drugs [13], polar basic drugs [14], and non-polar acidic drugs [15] from human plasma has been reported, but the number of analytes included in these papers has been very limited. In other words, only a few PALME applications have been published up to date. However, the numerous advantages associated with PALME call for expansion into new applications to further investigate its potential. The 96-well format provides high throughput as it enables extraction of 96 samples simultaneously, efficient sample clean-up is achieved as the supported liquid membrane discriminates larger molecules like proteins and charged compounds, the work-flow is simple, and the low consumption of organic solvents represents a green chemistry approach to sample preparation [13,16].

In this work, PALME was investigated for the first time for the potential screening of new psychoactive substances (NPS) in plasma. This work also investigated PALME directly from whole blood for the first time. The work is a preliminary study, with focus on general screening for NPS substances which differs substantially in terms of molecular properties. Focus was directed towards optimization of operational parameters and validation. Additionally, the porous PVDF filter of the commercially available acceptor plates, used to support the SLM, was not optimal as it caused non-specific binding of analytes, which consequently counteracted the extraction performance, as previously observed [13]. Therefore, the challenges associated with the PVDF material was prioritized early in the optimization process.

2. Experimental

2.1. Chemicals

Methanol and sodium hydroxide were purchased from Merck Millipore (Darmstadt, Germany). Formic acid, dodecyl acetate, hexadecane, isopentylbenzene, 2-nonanone, dihexyl ether, 2-nitrophenyl octyl ether, and trioctylamine were from Sigma-Aldrich (St. Louis, MO, USA). Ammonium formate was from VWR (Radnor, PA, USA), and deionized water was purified with a MilliQ water purification system from Millipore (Molsheim, France).

MDAI was kindly donated from the National Criminal Investigation Service (NCIS/KRIPOS, Oslo, Norway). Methylone, pentedrone, and 2C-E were from Lipomed (Arlesheim, the Netherlands). PFA, 6-APB, and bromo-dragonfly, as well as the internal standards $^{13}C_6$-MDA (3,4-methyl-dioxyamphetamine), $^{13}C_6$-PMA (4-methoxyamphetamine), $^{13}C_6$-MDEA (3,4-methylenedioxy-*N*-ethylamphetamine), and $^{13}C_6$-PMMA (para-methoxymetamphetamine) were from Chiron AS (Trondheim, Norway). mCPP was from Sigma-Aldrich (Steinheim, Germany), methoxetamine was from LGC (Teddington, Great Britain), and MDPV was from National Measurement Institute (Montvale, New Jersey, USA). Ethylphenidate and AH-7921 were from Cayman Chemical Company (Ann Arbor, Michigan, USA).

2.2. Standard solutions

Stock solutions of each analyte dissolved in methanol was supplied by the Norwegian Institute of Public Health (NIPH, Oslo, Norway), and stored at −20 °C. The stock solutions were used to prepare working solutions containing all the analytes: working solution A was used in the experiments with PALME from plasma, and working solution B was used in the experiments with PALME from whole blood.

Working solution A comprised 10 μg mL^{-1} of each analyte in deionized water. The solution was stored at 5 °C, and used to spike drug-free plasma. Standard solutions were prepared by diluting working solution A with 20 mM formic acid.

For practical reasons, working solution B was prepared with different concentrations of the analytes: MDAI (9 μg mL^{-1}), methylone (10 μg mL^{-1}), PFA (8 μg mL^{-1}), mCPP (10 μg mL^{-1}), 6-APB (9 μg mL^{-1}), pentedrone (5 μg mL^{-1}), methoxetamine (6 μg mL^{-1}), MDPV (14 μg mL^{-1}), ethylphenidate (6 μg mL^{-1}), 2C-E (10 μg mL^{-1}), bromo-dragonfly (15 μg mL^{-1}), and AH-7921 (8 μg mL^{-1}). Working solution B was stored at −20 °C, and was further diluted with deionized water to prepare standard solutions that were used to spike drug-free whole blood.

2.3. Internal standards

$^{13}C_6$-MDA served as internal standard for all the analytes in the PALME experiments from plasma. In the PALME experiments from whole blood, the analytes were allocated one of the following internal standards: $^{13}C_6$-MDA (pentedrone), $^{13}C_6$-PMA (MDAI, methylone, PFA, mCPP, 6-APB, methoxetamine, and ethylphenidate), $^{13}C_6$-2C-B (2C-E), $^{13}C_6$-MDEA (MDPV and bromo-dragonfly), and $^{13}C_6$-PMMA (AH-7921). The internal standards were dissolved in deionized water and stored at 4 °C.

2.4. Biological matrices

Human whole blood with sodium fluoride and heparin as additives was supplied by the Blood Bank of Oslo, Oslo University Hospital, Norway. Plasma was stored in plastic tubes at −25 °C, and whole blood was stored in brown glass bottles at −20 °C. The biological matrices were thawed prior to preparation of the biological samples.

2.5. PALME procedure

PALME was performed using a 96-well donor plate of polypropylene with 0.5 mL wells from Agilent (Santa Clara, CA, USA), and a 96-well acceptor plate from Millipore (Billerica, MA, USA) with

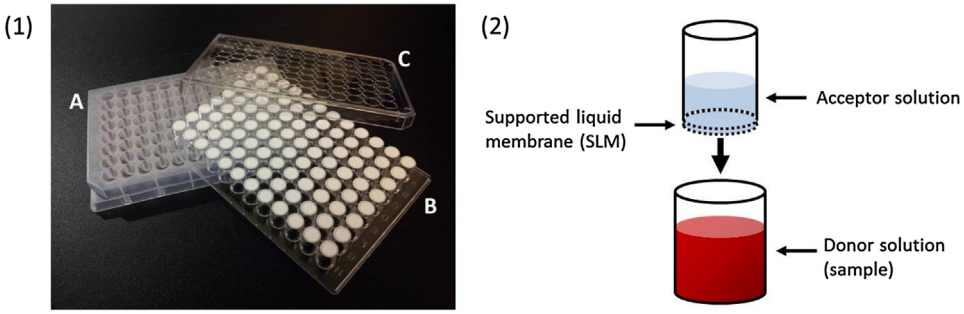

Fig. 1. Picture of commercially available equipment used for PALME (1), depicting the donor plate (A), the acceptor plate (turned upside down) with PVDF material (B), and the top lid (C). Schematic illustration of a PALME extraction unit (2).

polyvinylidene fluoride (PVDF) as support for the liquid membrane (SLM). The pore size of the PVDF material was 0.45 μm and the internal diameter 6.0 mm. A lid was used to prevent evaporation of the acceptor solutions during PALME.

When performing PALME from plasma, sample aliquots of 125 μL were pipetted into the donor wells in the donor plate. This was either blank plasma or plasma spiked with the analytes. To alkalize the samples, 115 μL of 40 mM NaOH was added to the sample. Finally, 10 μL of internal standard was added, making a final sample volume of 250 μL.

When performing PALME from whole blood, sample aliquots of 150 μL were pipetted into the donor wells in the donor plate. This was either 100 μL whole blood spiked with 50 μL standard containing the analytes, or 100 μL whole blood and 50 μL deionized water (blank blood). To alkalize the samples, 75 μL of 80 mM NaOH was added. Finally, 25 μL of internal standard were added, making a final sample volume of 250 μL.

To prepare the SLM, 5 μL dodecyl acetate with 1% trioctylamine (w/w) was pipetted onto the PVDF material in the acceptor plate. Finally, 50 μL of acceptor solution comprising 20 mM formic acid was added to the wells in the acceptor plate. The donor and acceptor plate was clamped together, and the whole set-up was placed on a platform shaker (Vibramax 100, Heidolph Instruments, Schwabach, Germany), providing agitation of 900 rpm to promote the extraction. PALME was carried out for 120 min.

2.6. UHPLC–MS/MS analysis of plasma samples

UHPLC–MS/MS analysis of PALME extracts obtained from plasma samples was performed with a chromatographic system consisting of a Dionex UltiMate 3000 RS Pump, Column Compartment, and Autosampler, and a Thermo Scientific LTQ XL Linear Ion Trap Mass Spectrometer from Thermo Scientific (San Jose, CA, USA). Chromatographic separation was obtained with an Acquity UPLC® HSS T3 column from Waters (Wexford, Ireland) with a length of 100 mm, an inner diameter of 2.1 mm, a particle size of 1.8 μm, and a pore size of 100 Å. The column temperature was 40 °C. Data acquisition and processing was performed using Xcalibur version 2.2 SP148 software from Thermo Scientific.

Mobile phase A and B comprised 20 mM formic acid and methanol in the ratios 95:5 (v/v) and 5:95 (v/v), respectively. Throughout the analysis, the mobile phases were pumped with an overall flow rate of 0.3 mL min^{-1} following a pre-set gradient: 15% B at 0.00 min, 50% B at 15 min, 100% B at 15.1 min, 100% B at 16.9 min, 15% B at 17 min, and 15% B at 20 min. Total run time was 20 min, and the injection volume was 2.0 μL. Ionization was performed with electrospray ionization (ESI) in the positive ionization mode (ESI+) with a voltage of 5 kV. Analysis was performed using selected reaction monitoring (SRM). The transitions and collision energies are shown in Table 1.

Table 1
MS parameters for LC–MS/MS analysis of PALME extracts from plasma samples.

Compound	SRM transitions		Collision energy (%)
	Precursor	Fragment	
MDAI	178.1	161	33
Methylone	208.1	190	34
PFA	154.2	137	18
mCPP	197.2	119	27
6-APB	176.1	159	39
Pentedrone	192.2	174	32
Methoxetamine	248.1	203	49
MDPV	276.3	205	50
Ethylphenidate	248.2	84.1	35
2C-E	210.2	178.1	45
Bromo-dragonfly	294.2	277	31
AH-7921	329.2	190.1	31
^{13}C6-MDA	186.1	169.1	35

2.7. UHPLC–MS/MS analysis of whole blood samples

UHPLC–MS/MS analysis of PALME extracts obtained from whole blood samples was performed using an integrated system from Waters (Milford, MA, USA) with an Acquity™ Ultra Performance LC. Chromatographic separation was performed at 65 °C on an Acquity UPLC® HSS T3 column with a length of 100 mm, an inner diameter of 2.1 mm, a particle size of 1.8 μm, and a pore size of 100 Å. Data acquisition was performed using MassLynx 4.1 software (Waters Corp., Milford, MA, USA), and data were processed with the TargetLynx quantification program (Waters Corp.).

Mobile phase A and B consisted of 10 mM ammonium formate (pH 3.1) and methanol, respectively. The flow rate was 0.5 mL min^{-1}, and the analysis was performed with the following gradient: 2.5% B at 0.00 min, 35% B at 6.75 min, 65% B at 8.00 min, 100% B at 8.01 min, 100% B at 9.00 min, and 2.5% B at 9.01 min. Total run time was 11 min, and the injection volume was 3.0 μL. MS detection was performed on a Waters Quattro Premier XE triple-quadrupole MS, (Waters Corp., Milford, MA, USA). Ionization was performed with electrospray ionization in the positive mode with a voltage of 1 kV. Analysis was performed using selected reaction monitoring. The SRM transitions and collision energies are presented in Table 2.

2.8. Method validation of PALME from plasma

The following parameters were taken into consideration: linearity, limits of quantification (LOQ), intra- and inter-day precision, accuracy, extraction recoveries, carry-over, and matrix effects.

The linearity of the method was studied for each analyte on eight concentration levels (n = 4) ranging from 5 to 1000 ng mL^{-1}. Weighted quadratic calibration curves (1/x) were constructed by the computer software Xcalibur. The LOQ for each analyte

Table 2
MS parameters for LC–MS/MS analysis of PALME extracts from whole blood samples.

Compound	SRM transitions			
	Precursor	Fragment 1	Fragment 2	Collision energy (%)
MDAI	178.1	161	131	18
Methylone	208.1	190.1	160	20
PFA	154.2	137.2	109	10
mCPP	197.2	154.1	119.1	30
6-APB	176.1	159.1	131	14
Pentedrone	192.2	174.1	132	18
Methoxetamine	248.1	203.1	121	15
MDPV	276.3	175.1	126.1	28
Ethylphenidate	248.2	84.1	56	28
2C-E	210.2	193.1	178.1	20
Bromo-dragonfly	294.2	198.1	141.1	30
AH-7921	329.2	284.2	190.1	28

was determined from scalar dilutions of the analytes in plasma (0.5–1000 ng mL^{-1}). The LOQ was the concentration value giving an S/N = 10. Intra-day precision and accuracy was studied at three concentration levels and with ten replicates, whereas inter-day precision was determined over three consecutive days at three concentration levels and with four replicates.

The extraction recoveries were determined on two concentration levels. Eight replicates of blank plasma were spiked with analytes before PALME and compared to four replicates of post extraction spiked samples. The extracts from the plasma samples that were spiked after PALME represented 100% recovery and were used to determine the extraction recoveries from the plasma samples that were spiked before PALME.

Potential carry-over in the UHPLC–MS/MS was investigated by injecting a ten times more concentrated sample than the highest standard of 1000 ng mL^{-1}, followed by the injection of three blank plasma samples. After UHPLC–MS/MS, the chromatograms obtained from the blank plasma samples were investigated for potential chromatographic peaks, serving as proofs of carry-over.

Finally, to evaluate any potential ion suppression or enhancement (matrix effects) due to the plasma matrix, acceptor solutions from extracted blank plasma (spiked with analytes after PALME, n = 2) were compared with untreated acceptor solutions (spiked with analytes without performing PALME, n = 2). This was performed for two concentration levels, and the matrix effects were quantified as described in section 2.10. (Calculations).

2.9. Method validation of PALME from whole blood

Method validation from whole blood was carried out similarly to the validation from plasma. Details are given in Table 6.

2.10. Calculations

Recovery (R%) was calculated according to the following equation for each analyte:

$$R\% = \frac{n_a final}{n_d initial} \times 100\% = \frac{V_a}{V_d} \times \frac{C_a final}{C_d initial} \times 100\%$$

where n_d is the initial number of analyte moles present in the donor solution (sample), and n_a is the final number of analyte moles collected in the acceptor solution. V_d and V_a represents the donor and acceptor volume, respectively, whereas C_d initial and C_a final represents the initial and final analyte concentration in the donor and acceptor solution.

Table 3
Chemical characterization of the model analytes.

Compound	Formula	pKa	log P	Molecular weight
MDAI	$C_{10}H_{11}NO_2$	10.0	1.1	177.2
Methylone	$C_{11}H_{13}NO_3$	8.0	1.2	207.2
PFA	$C_9H_{12}FN$	10.0	2.0	153.2
mCPP	$C_{10}H_{13}ClN_2$	8.9	2.2	196.7
6-APB	$C_{11}H_{13}NO$	10.0	2.0	175.2
Pentedrone	$C_{12}H_{17}NO$	8.2	2.6	191.3
Methoxetamine	$C_{15}H_{21}NO_2$	8.1	2.9	247.3
MDPV	$C_{16}H_{21}NO_3$	7.3	3.0	275.3
Ethylphenidate	$C_{15}H_{21}NO_2$	9.1	2.6	247.3
2C-E	$C_{12}H_{19}NO_2$	9.7	2.2	209.3
Bromo-dragonfly	$C_{13}H_{12}BrNO_2$	9.8	2.9	294.1
AH-7921	$C_{16}H_{22}Cl_2N_2O$	9.5	4.0	329.3

Matrix effects (ME) were calculated according to Matuszewski et al. [17] as follows:

$$ME\% = \frac{B}{A} \times 100\%$$

where B is the peak area obtained from spiking an extract acquired after extraction from blank whole blood or plasma., and A is the peak area obtained from a neat standard solution.

3. Results and discussion

Parallel artificial liquid membrane extraction (PALME) was for the first time performed for new psychoactive substances (NPS) in plasma and whole blood. Optimization of operational parameters was performed before the final methods were validated. Twelve different NPS were selected as model analytes, and were all hydrophobic bases, covering a broad log P range from 1.1 to 4.0 (see Table 3 for exact log P values, molecular formulas, pKa values, and molecular weights).

PALME was performed with commercially available equipment. This was definitely an advantage, but the PVDF filter in the bottom of the acceptor plates was not optimal, based on previous observations [13]. Non-specific binding of analytes to the PVDF material sacrificed the linearity as the extraction recoveries decreased with decreasing analyte concentration. Polypropylene is an alternative filter material used in HF-LPME [18], and in the first PALME paper the PVDF material was replaced with polypropylene. This improved the extraction performance, but 96-well plates with polypropylene filters with appropriate dimensions are not yet commercially available. Optimization of PALME performance with PVDF filters was therefore investigated in detail.

3.1. Optimization of operational parameters (PALME from plasma)

Different operational parameters were studied and optimized. First, different organic solvents were tested as potential liquid membranes. Six pure solvents were selected based on earlier experiences with HF-LPME and PALME [13,19]. In all cases, 5 μL solvent was pipetted onto the PVDF material and was immobilized by capillary forces, without further optimization of the SLM volume. The solvents were non-volatile and immiscible with water to prevent partial evaporation during PALME, and to avoid leakage of the SLM to the donor/acceptor solution. Extraction recoveries (n = 4) after 30 min of PALME are presented in Table 4. Hexadecane was inefficient, probably because it was too hydrophobic. Isopentylbenzene, dodecyl acetate, and 2-nonanone provided the highest recoveries. Isopentylbenzene was not tested further for boiling point reasons (195 °C), and 2-nonanone was not selected due to water solubility (1.1 g/L) [20].Thus, dodecyl acetate (water solubility 0.02 g/L,

Table 4
Recovery data for PALME from plasma with different organic solvents serving as SLM.

	% Recovery (% RSD), n = 4					
Compound	Dihexyl ether	2-nonanone	Dodecyl acetate	Isopentyl- benzene	2-nitrophenyl octyl ether	Hexadecane
MDAI	16 (5)	57 (3)	20 (8)	51 (13)	10 (14)	1 (26)
Methylone	51 (7)	87 (3)	64 (2)	89 (2)	39 (4)	8 (11)
PFA	73 (2)	83 (3)	69 (4)	93 (5)	45 (10)	35 (10)
mCPP	87 (4)	90 (4)	90 (4)	102 (2)	76 (6)	35 (6)
6-APB	75 (5)	73 (3)	79 (5)	97 (4)	57 (4)	26 (10)
Pentedrone	101 (3)	100 (3)	110 (2)	110 (4)	101 (3)	94 (2)
Methoxetamine	104 (7)	104 (5)	110 (2)	112 (2)	103 (3)	83 (6)
MDPV	88 (11)	57 (5)	97 (6)	92 (14)	99 (6)	65 (12)
Ethylphenidate	97 (2)	89 (7)	102 (4)	103 (5)	94 (6)	66 (7)
2-CE	17 (8)	44 (7)	28 (4)	50 (8)	23 (9)	1 (20)
Bromo-dragonfly	53 (7)	17 (10)	67 (7)	74 (12)	67 (9)	23 (12)
AH-7921	65 (14)	28 (5)	79 (8)	72 (11)	85 (3)	30 (16)
Mean values	60 (6)	68 (6)	68 (6)	75 (12)	58 (10)	33 (14)

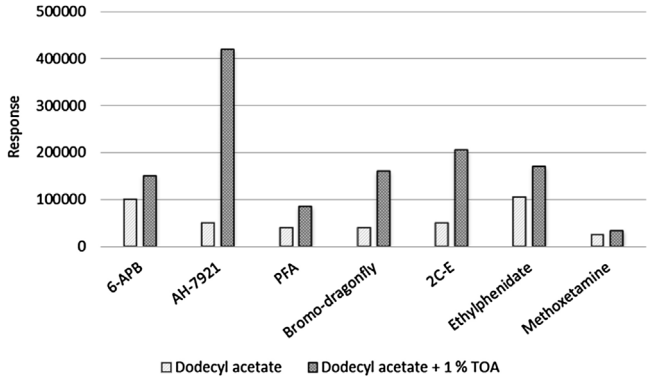

Fig. 2. The effect of trioctylamine (TOA) added to the supported liquid membrane. Extraction performance from PALME experiments from plasma with (A) SLM = dodecyl acetate, and (B) SLM = dodecylacetate + 1% TOA.

boiling point 265 °C) was selected for the remaining experiments [20].

As seen from Table 4, acceptable extraction recoveries were obtained for all the analytes. Still, in some initial experiments, significant non-specific binding to the PVDF material was observed. Particularly, recoveries were to some extent improved by increasing the total load of NPS in the samples. To circumvent this, trioctylamine (TOA) was added to the organic solvent serving as SLM (dodecyl acetate) to mask the PVDF binding sites. To evaluate the effect of TOA in the liquid membrane, PALME was performed both with and without the addition of 1% TOA (w/w). The 1% concentration of TOA was selected without further optimization, based on earlier experiences with related substances (unpublished work). The percentage addition of TOA to the organic solvent was not further optimized in this work. The results are shown in Fig. 2 for a representative selection of eight analytes (n = 4). The figure shows that the effect of TOA was analyte dependent. For example, the effect of TOA was appreciable for the compound AH-7921, whereas for methoxetamine the effect was less significant. Still, addition of 1% TOA to the SLM was considered beneficial for most analytes, and the overall extraction performance was improved substantially. Dodecyl acetate with 1% TOA was therefore chosen as optimal SLM for the continued work.

In a final optimization experiment, the extraction time was addressed. PALME was performed from plasma, and the extraction recoveries were measured after 5 min, 20 min, 45 min, 60 min, 90 min, 120 min, and 180 min (n = 4). Fig. 3 shows the extraction recoveries for pentedrone, methylone, and 2C-E. These analytes were selected to illustrate three kinetic patterns: some analytes reached equilibrium within 45 min (pentedrone, MDPV, AH-7921, mCPP, methoxetamine, and ethylphenidate), some analytes continued to increase even after 45 min of PALME (methylone, bromo-dragonfly, PFA, and 6-APB), and finally, some analytes were extracted with very slow kinetics (2C-E and MDAI) with no equilibrium established, even after three hours of PALME. The analytes with rapid kinetics were mostly with log P > 2.5, but kinetics was not only related to log P. Other chemical descriptors were also considered, such as polar surface area, number of hydrogen binding donor and acceptor sites, and number of rotational bonds, but the current data set was insufficient to develop a clear relation between extraction kinetics and molecular properties. The latter compounds were relatively polar substances (low log P). The final extraction time was set to 120 min as a compromise between throughput and recovery.

3.2. Results from the method validation of PALME from plasma

Successful PALME from plasma was achieved from a 250 μL alkalized donor solution comprising the plasma sample (125 μL), the internal standard (10 μL), and 40 mM NaOH (115 μL). The analytes were extracted across an SLM of 5 μL dodecyl acetate with 1% TOA (w/w), and finally into an acidic acceptor solution of 20 mM formic acid [13]. PALME was performed for 120 min, and the extraction was promoted with agitation at 900 rpm [13]. The final PALME method was validated for the twelve NPS presented in Table 3, and with ^{13}C MDA as internal standard. The validation was performed according to FDA guidelines, and the results are summarized in Table 5.

The calibration curves showed that the method was linear in the range from 5 to 1000 ng mL^{-1}, and quadratic correlation coefficients >0.990 were obtained for all the analytes, except for MDAI (r^2 = 0,988). Table 5 shows that the LOQs were 0.78 ng mL^{-1} or lower for all the analytes, and this confirmed that quantification was feasible down to the 5 ng mL^{-1} point of the calibration curve. Intra- and inter-day precision was also satisfactory, and within the range 4–13% RSD and 5–20% RSD, respectively. The accuracy was within ±20%.

Extraction recoveries were determined at two concentration levels to investigate whether the recovery measurements were affected by analyte concentration. The recovery data in Table 5 shows that the NPS with log P > 2 obtained a higher recovery compared to the NPS with log P < 2. Still, all the analytes were extracted with high precision, proving that the NPS were successfully extracted from plasma.

It was observed no chromatographic peaks in the LC–MS/MS chromatograms from the blank plasma samples that were injected after the sample with high concentration of analytes, and therefore it was concluded that carry-over will not be likely to affect the ana-

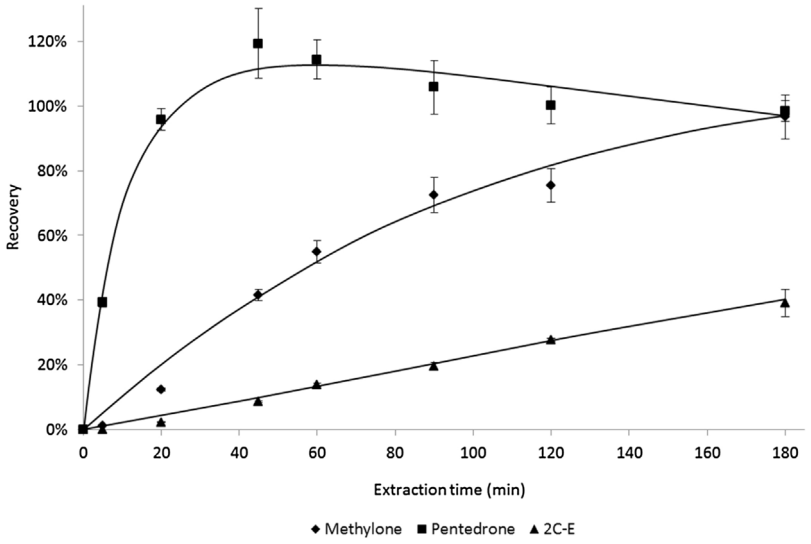

Fig. 3. Extraction recoveries from plasma versus extraction time (5–180 min) with error bars representing ± 1 standard error (SE).

Table 5
Validation data for the PALME method from plasma.

Compound	Concentration (ng mL^{-1})	Intra-day precision[a], RSD (%)	Inter-day precision[b], RSD (%)	Accuracy[c] (%)	Recovery[d] (%)	LLOQ (ng mL^{-1})	Calibration range (ng mL^{-1})	Linearity (r^2)
MDAI	7.5	11	9	20	26	0.7	5–1000	0.988
	75	8	13	11				
	750	13	14	6	25			
Methylone	7.5	9	12	−17	80	0.1	5–1000	0.992
	75	7	11	−3				
	750	8	13	−4	95			
PFA	7.5	7	9	−15	94	0.5	5–1000	0.996
	75	4	10	−4				
	750	9	14	−7	91			
mCPP	7.5	5	5	−12	94	0.8	5–1000	0.994
	75	6	10	5				
	750	6	20	−2	104			
6-APB	7.5	4	7	−10	86	0.5	5–1000	0.994
	75	5	10	5				
	750	8	11	−1	96			
Pentedrone	7.5	6	6	−15	109	0.4	5–1000	0.992
	75	5	11	6				
	750	9	11	−2	117			
Methoxetamine	7.5	8	5	−6	98	0.3	5–1000	0.993
	75	6	11	−7				
	750	9	10	−4	101			
MDPV	7.5	7	8	−10	100	0.1	5–1000	0.995
	75	7	11	−6				
	750	11	12	−8	99			
Ethylphenidate	7.5	5	7	−13	105	0.3	5–1000	0.999
	75	6	12	3				
	750	8	16	−1	109			
2C-E	7.5	5	9	−6	44	0.4	5–1000	0.992
	75	5	10	0				
	750	6	10	−4	52			
Bromo-dragonfly	7.5	5	5	−11	95	0.5	5–1000	0.997
	75	4	10	5				
	750	6	10	−2	107			
AH-7921	7.5	7	11	−6	101	0.2	5–1000	0.997
	75	8	13	−2				
	750	9	11	2	105			

[a] Intra-day precision was measured at three concentration levels (n = 10).
[b] Inter-day precision was measured at three concentration levels at three consecutive days (n = 4).
[c] Accuracy was measured from intra-day precision at three concentration levels (n = 10).
[d] Recovery was measured at two concentration levels (n = 8).

Table 6
Validation data for the PALME method from whole blood.

Compound	Concentration (ng mL^{-1})	Intra-day precision[a], RSD (%)	Inter-day precision[b], RSD (%)	Accuracy[c] (%)	Recovery[d] (%)	LLOQ (ng mL^{-1})	Calibration range (ng mL^{-1})	Linearity (r^2)
MDAI	89	6	11	−3	11	44	44–266	0.997
Methylone	104	13	12	−8	31	52	52–311	0.990
PFA	77	8	7	−3	40	38	38–230	0.992
mCPP	98	9	8	−6	66	98	98–295	0.993
6-APB	88	5	6	−8	38	44	44–263	0.993
Pentedrone	48	8	9	−2	83	25	25–143	0.992
Methoxetamine	62	8	9	−6	86	62	62–185	0.990
MDPV	138	7	11	8	54	138	138–413	0.997
Ethylphenidate	62	10	7	1	63	62	62–185	0.989
2C-E	105	4	9	−9	3	105	105–314	0.996
Bromo-dragonfly	147	19	19	9	38	147	147–441	0.985
AH-7921	82	18	13	−3	32	82	82–247	0.970

[a] Intra-day precision was measured at one concentration level (n = 10).
[b] Inter-day precision was measured at one concentration level at seven consecutive days (n = 2).
[c] Accuracy was measured from inter-day precision at one concentration level (n = 2).
[d] Recovery was measured at one concentration level (n = 10).

lytical results with expectable concentrations in biological samples. The quantified matrix effects were satisfactory and within ±15%.

3.3. Experiments with PALME from whole blood

This was the first time PALME was performed from whole blood. For simplicity, we preferred to use the same experimental conditions as for the plasma samples, but due to compositional differences, the pH condition in the whole blood samples was particularly addressed. A rough inspection of the sample pH, using pH paper, revealed that the initial sample composition failed to alkalize the blood samples sufficiently. This was due to the buffer capacity of whole blood, providing a stable pH of 7.4. At this pH, some of the analytes with the highest pKa values were protonated and consequently prevented from entering the organic SLM. To ensure that all the basic analytes were uncharged in the donor solution, the samples were prepared as described in the experimental section, but with different concentrations of NaOH. The aim was to achieve a final sample pH of 12 and investigate how the elevated sample pH affected the extraction performance, using extraction recovery as success parameter. NaOH concentrations of 10 mM, 20 mM, 30 mM, 40 mM, 60 mM, 80 mM, 100 mM was tested (n = 4, data not shown). The highest recoveries were obtained with 75 μL 80 mM NaOH added to the sample, resulting in a final concentration of 24 mM NaOH in a final donor solution of 250 μL.

To investigate whether foaming and potential carry-over occurred between the donor wells in the donor plate during PALME, an entire 96-well plate was filled with blood samples. Every other donor well was filled with blank samples (blank whole blood) and blood samples spiked with analytes, respectively. No traces of analytes were detected in the blank samples after PALME, and this supported the absence of carry-over and cross contamination between individual donor wells.

3.4. Results from the method validation of PALME from whole blood

PALME of the twelve NPS (Table 3) from whole blood was performed from a 250 μL alkalized donor solution comprising the blood sample (100 μL), deionized water (50 μL), internal standard (25 μL), and 80 mM NaOH (75 μL). The analytes were extracted across an SLM of 5 μL dodecyl acetate with 1% TOA (w/w), and finally into an acidic acceptor solution of 20 mM formic acid [13]. PALME was performed for 120 min, and the extraction was promoted by agitation at 900 rpm [13]. The final PALME method was validated, and the results are summarized in Table 6.

The method was linear in the range from the LOQ of each analyte to the highest calibration level. Correlation coefficients >0.990 were obtained for all the analytes, except for ethylphenidate (0.988), bromo-dragonfly (0.985), and AH-7921 (0.970). Table 6 shows the LOQs for each analyte, and they were all considered satisfactory. Intra-day precision was within ±20% for MDAI, mCPP, 6-APB, pentedrone, methoxetamine, MDPV, 2C-E, and bromo-dragonfly, whereas inter-day precision was within ±20% for all the analytes. Accuracies were within ±9% and were in compliance with the requirements for bioanalytical method validation (<15%).

No chromatographic peaks were observed in the LC–MS/MS chromatograms from the blank blood in the carry-over experiment, or in the LC–MS/MS chromatograms from the specificity experiment. Therefore, it was concluded that the method was specific and provided no carry-over. The quantified matrix effects were satisfactory and within ±15%.

4. Conclusion

In this work, the potential of parallel artificial liquid membrane extraction (PALME) of new psychoactive substances (NPS) was demonstrated with commercially available equipment for the first time. Twelve compounds representing a selection of NPS were successfully extracted from plasma and whole blood. Based on initial optimization experiments and previous experiences, two separate PALME methods were developed for plasma and whole blood. The final methods were validated, and the results were in accordance to FDA guidelines.

This is the first PALME application developed for forensic analysis. Extensive sample clean-up, possibility for high throughput, and a simple work-flow are advantages associated with PALME. The aqueous extracts from PALME are directly compatible with LC–MS, and no evaporation and reconstitution is required. PALME can be performed with commercially available plates, and only 3 μL organic solvent is used per sample. The total consumption for processing 96 samples is therefore less than 0.3 mL, and this represents a very interesting approach to green sample preparation. Based on these general advantages, and the applications developed in this work, PALME is expected to be a valuable tool for forensic laboratories in the near future.

Acknowledgement

The Research Council of Norway is acknowledged for financial support through Grant no. 231917.

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