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Determination of psychoactive drugs in serum using conductive vial electromembrane extraction combined with UHPLC-MS/MS

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ABSTRACT

Conductive vial electromembrane extraction (EME) with prototype equipment was applied for the first time to extract lipophilic basic drugs from serum. With this equipment, traditional platinum electrodes were replaced with sample and acceptor vials made from a conductive polymer, making the electrodes fully integrated and disposable. EME was combined with UHPLC-MS/MS, and a method to determine selected psychoactive drugs (alimemazine, amitriptyline, atomoxetine, clomipramine, doxepin, duloxetine, fluvoxamine, levomepromazine, nortriptyline and trimipramine) and metabolites (desmethyl clomipramine and desmethyl doxepin) in serum was developed, optimized, and validated. Extractions were carried out with 50 V for 15 min from serum samples (100 μL) diluted 1:3 with formic acid (0.1% v/v), using 2-nitrophenyl octyl ether as the supported liquid membrane (SLM), and formic acid (0.1% v/v, 300 μL) as acceptor phase.

Using conductive vial EME, the extraction of lipophilic drugs reached exhaustive or near-exhaustive conditions, with recoveries in the range 75–117%. The method demonstrated excellent accuracy and precision, with bias within ± 6%, and intra- and inter-day CVs ranging 0.9 – 6% and 2 – 6%, respectively. In addition, acceptor phases were completely free of glycerophosphocholines. EME-UHPLC-MS/MS was successfully applied in determination of psychoactive drugs in 30 patient samples, and the results were in agreement with the current hospital routine method at St. Olav University Hospital (Trondheim, Norway).

Obtaining comparable results to well-established routine methods is highly important for future implementation of EME into routine laboratories. These results thus serve as motivation for further advancing the EME technology. Until now, EME has been carried out with laboratory-build equipment, and the introduction of commercially available standardized equipment is expected to have a positive impact on future research activity.

1. Introduction

Miniaturization of the analytical procedure has been one of the major advancements in analytical chemistry the last decades. Within sample preparation, a number of microextraction techniques have been introduced as alternatives to traditional techniques like solid phase extraction (SPE) and liquid–liquid extraction (LLE). These include solid phase microextraction (SPME) [1], single drop microextraction (SDME) [2], dispersive liquid–liquid microextraction (DLLME) [3], hollow fiber liquid phase microextraction (HF-LPME) [4], and electromembrane extraction (EME) [5], among others. With reduced volumes of sample and organic solvents, microextraction provides sample clean-up and enrichment, and is scaled for modern chromatography. Due to the low consumption of organic solvents, microextraction is also highly relevant in a green chemistry perspective.

EME is a form of liquid phase microextraction (LPME) that introduces the concept of electrokinetic migration across a supported liquid membrane (SLM) to extract ionisable compounds from complex samples [5]. The samples in EME are aqueous, e.g. biological fluids and environmental waters [6,7]. In EME, the sample is separated from a

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clean acceptor phase by the SLM. The latter consists of an organic solvent immobilized in a polymeric membrane. Application of an electric field across the SLM facilitates electrokinetic migration of charged analytes from the sample, through the SLM and into the acceptor phase, which is compatible for further analysis with ultra-performance liquid chromatography-tandem mass spectrometry (UHPLC-MS/MS) or related instrumental techniques. Ionization of target analytes is ensured by adjusting the sample and acceptor pH. An external power supply delivers the electric field, and the direction and magnitude alters the selectivity of extraction. The acceptor phase is cathodic for extraction of basic drugs (cations), whereas polarity is reversed in extraction of acidic compounds. Operational parameters affecting the performance and selectivity are the physiochemical properties of the SLM, sample and acceptor pH, extraction voltage, and extraction time [8]. The extraction process is tuned for target analytes by optimizing these conditions.

EME was first introduced in 2006 using a hollow fiber based SLM [5]. From this, a number of technical configurations have evolved [7], including flat membranes [9], micro-chip systems [10] and multi-well EME [11,12]. Although several alternative electrode arrangements and materials have been reported [13–16], the electric field is most commonly coupled with stationary platinum wires [17]. So far, EME has been conducted with laboratory-build equipment. However, an EME prototype aimed for commercialization was recently developed [18]. Unlike previous EME configurations, the electric field is here coupled through a conductive polymeric material. By housing the sample and acceptor phase in vials made from the conductive material, electrodes are fully integrated. The sample and acceptor vial are connected by a leak tight interface holding a flat porous membrane that supports the SLM. The electrode vials are disposable, and thus cross contamination from electrodes is avoided.

The development of prototype EME equipment opens the door for potential future implementation in routine laboratories. Previous studies have reported successful EME of a variety of drugs from biological matrices [19–27], indicating great potential for clinical applications. However, coupling the electric field with conductive vials has not been done previously, and no data on optimization, validation, and reliability in conjunction with biological samples are available for this technical configuration. The aim of the present study was therefore to explore the prototype EME technology in a clinical setting. An EME procedure combined with UHPLC-MS/MS analysis for psychoactive drugs and metabolites from patient serum was developed. The work was carried out at St. Olav University Hospital in Trondheim, Norway, where the method was validated and compared to the current hospital method in routine operation. The experimental work was focused on the potential for exhaustive extraction, and to investigate if data obtained with prototype equipment for conductive vial EME met the regulatory requirements and the quality level expected for a routine laboratory.

2. Methods and materials

2.1. Chemicals

The reference substances for each analyte were purchased from two separate sources for calibration and quality control. Doxepin, desmethyl doxepin (DM-doxepin), nortriptyline, and trimipramine were purchased from Sigma Aldrich (St. Louis, MO, USA). Atomoxetine, duloxetine, fluvoxamine, and nortriptyline were obtained from Chiron (Trondheim, Norway). Alimemazine, amitriptyline, doxepin, DM-doxepin, fluvoxamine, and levomepromazine were from Toronto Research Chemicals (TRC, Toronto, Canada). Atomoxetine and duloxetine were obtained from Lilly Research laboratories (Indianapolis, IN, USA). Alimemazine and trimipramine were from Rhone-Poulenc Rorer (Strasbourg, France). Clomipramine and desmethyl clomipramine (DM-clomipramine) were obtained from Novartis (Basel, Switzerland). Amitriptyline, DM-clomipramine, and levomepromazine were purchased from Lundbeck (Copenhagen, Denmark), Lipomed (Arlesheim, Switzerland), and United States Pharmacopeia (Rockville, MD, USA), respectively.

Deuterated internal standards amitriptyline-d3, atomoxetine-d3, clomipramine-d6, doxepine-d3, duloxetine-d7, fluvoxamine-d3, nortriptyline-d3, and trimipramine-d3 were from TRC. Alimemazine-d6, DM-clomipramine-d3, DM-doxepin-d3, and levomepromazine-d3 were from Sigma Aldrich (Schnelldorf, Germany), Lipomed, Cerilliant (Round Rock, TX, USA), and SynFine Research (Richmond Hill, Canada), respectively.

2-nitrophenyl octyl ether, ammonium hydroxide solution (28–30%, ACS reagent), and ammonium formate (≥99.995%, trace metal basis) were purchased from Sigma Aldrich. Formic acid (99.0%, Optima™ LC-MS Grade) was obtained from Fischer Scientific (Leicestershire, UK). Methanol (LC-MS grade) was purchased from Merck (Darmstadt, Germany). Type 1 water was obtained from a Milli-Q purification system from Millipore (Molsheim, France). External quality control samples (n = 8) were obtained from LGC Standards Proficiency Testing (Bury, UK). Human blank serum was obtained from healthy blood donors (St. Olav University Hospital, Trondheim, Norway).

2.2. Preparation of solutions

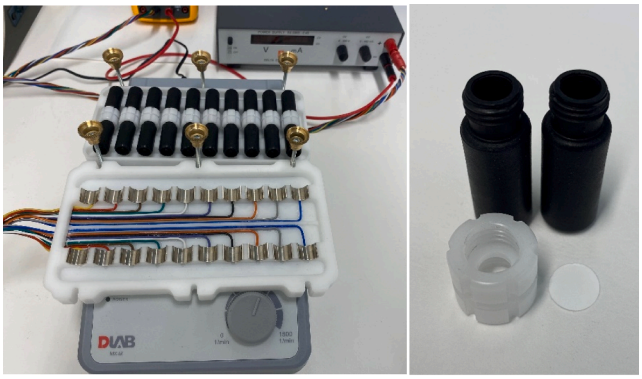
Two stock solutions (1–5 mM) in methanol were prepared for each analyte; one for calibration and the other for quality control (QC). Stock solutions were combined and diluted in methanol to obtain two sets of working solutions for each calibration and QC concentration level. The stock and working solutions were stored at 4 °C. Calibrator and QC samples in serum were prepared by spiking analyte free serum with working solutions (1:100 v/v) at each concentration level. These were stored at −20 °C until use. The internal standard solution containing all deuterated standards (100 – 200 ng/mL) was prepared in 20% methanol in water (v/v) and stored at 4 °C. Calibrator, quality control and internal standard concentrations of each analyte are provided in Supplementary Table S1 and S2.

Formic acid solution (0.1%, v/v) was prepared by diluting formic acid (99.0%) in type 1 water. A 50 mM ammonium formate solution was prepared by dissolving ammonium formate salt in type 1 water. Mobile phase A, 5 mM ammonium formate, was prepared by diluting the 50 mM solution, and adjusted to pH 10.1 ± 0.1 with ammonium hydroxide solution (28–30%).

2.3. EME set-up and procedure

Electromembrane extraction was performed with a prototype device developed by Extraction Technologies Norway AS (ETN, Ski, Norway). The device is depicted in Fig. 1a along with a schematic illustration in Fig. 1b. Sample and acceptor vials made from a conductive polymer were used as electrodes. The vials were supplied by the company ETN, and no further information on the conductive material is available. A maximum of ten EME units operated in parallel, and each EME unit consisted of one sample and one acceptor vial, connected by a leak-tight interface that held a flat polypropylene membrane (Fig. 1a). The sample and acceptor phase were contained in the sample and acceptor vial, respectively. Each vial had a total volume of 600 µL, and could be filled with working volumes in the range from 200 to 350 µL. The polypropylene membrane (ACCUREL®, 254 mm wide, 110 or 168 µm thick, MEMBRANA, Wuppertal, Germany) was saturated with the organic solvent of choice to create the SLM. EME units were placed horizontally in the device with horizontal agitation to ensure contact between the liquid phases and the membrane. A video demonstrating the assembly of EME units is available as Supplementary Material. The electric field was delivered by a power supply that connected to the vial surfaces via the device lid (Fig. 1a). To extract basic drugs, the acceptor was made cathodic and the sample anodic. Protonated basic drugs were extracted across the SLM and into the acceptor phase by electrokinetic migration (Fig. 1b).

A series of optimization experiments were carried out during method



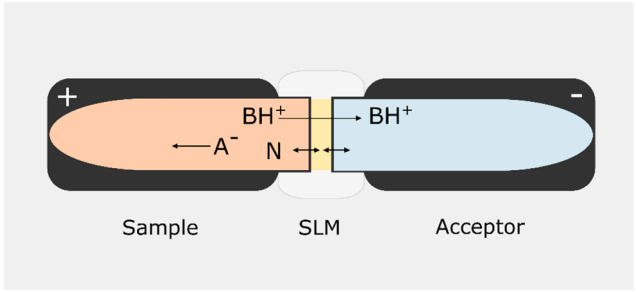


Fig. 1. a) EME set up with conductive vial electrodes (left). Each EME unit consists of a sample and acceptor vial made from a conductive polymer, separated by a leak-tight interface holding a flat polypropylene membrane (right). b) Schematic illustration of EME unit coupled for extraction of protonated basic drugs.

development using serum spiked to the middle QC concentration (QC2). These experiments were performed with extraction voltage 100 V for 15 min if not stated otherwise. The final, optimized EME procedure was as follows. Aliquots of serum (100 µL, spiked or analyte free), internal standard solution (25 µL) and 0.1% formic acid (v/v, 175 µL) were added to the sample vial. The acceptor phase, 0.1% formic acid (v/v, 300 µL), was added to the acceptor vial. The SLM was prepared by pipetting 9 µL NPOE on the flat polypropylene membrane (168 µm) contained in the leak-tight interface. EME units were assembled and placed in the EME device. Extraction was carried out with 50 V for 15 min. The agitation rate was 875 rpm. After extraction, the acceptor phases were analysed with UHPLC-MS/MS.

During initial optimization experiments, process efficiency (PE%) was the main output parameter. Process efficiency was calculated for each analyte by comparing analyte peak areas in the extracted acceptor phase to peak area in a spiked neat acceptor phase (corresponding to 100% analyte transfer). The following equation was used:

$$PE\% = \frac{Area_{acceptor}}{Area_{ref}} \times 100\% \quad (1)$$

$Area_{acceptor}$ and $area_{ref}$ are the analyte peak area in the extracted acceptor phase and in the spiked neat acceptor phase (0.1% formic acid v/v), respectively.

2.4. Sample preparation - routine method at St. Olav University hospital

The target drugs are routinely analysed at the Department of Clinical Pharmacology at St. Olav University Hospital in Trondheim, Norway. The analytes are included in one common method consisting of two sub-methods. Alimemazine, amitriptyline, atomoxetine, clomipramine, DM-clomipramine, doxepin, DM-doxepin, fluvoxamine, levomepromazine, nortriptyline, and trimipramine are included in extraction method 1, whereas duloxetine is analysed with extraction method 2. The two methods are divided into two aliquots with additional sample preparation and UHPLC-MS/MS analysis after a first common sample preparation step. The routine method is accredited according to the Norwegian Accreditation standard for medical laboratories (ISO15189:2012), and was regarded as a reference method for comparison.

Automated sample preparation was performed using a pipetting robot (Hamilton Microlab Star, Hamilton Company, Bonaduz, Switzerland). Serum samples (100 µL) and internal standard solution (25 µL) were transferred to a 96-well phospholipid removal plate (Ostro Protein Precipitation & Phospholipid Removal Plate, 25 mg, Waters). Ice-cold acetonitrile containing 1% formic acid (v/v, 375 µL) was added to the wells and mixed with the sample for protein precipitation. Samples were filtrated into a 96-well collection plate (2 mL, Waters) using a positive pressure unit (Positive Pressure 96-processor, Waters). For extraction method 1, aliquots of filtrate (30 µL) were transferred to a 1 mL 96-well collection plate (Waters), diluted with 120 µL 30% methanol in water. The remaining filtrates (extraction method 2) were evaporated to dryness under ambient air at 40 °C (for 40 min, UltraVap, Portvair Science, Norfolk, UK) and reconstituted in 30% methanol in water (100 µL).

2.5. UHPLC-MS/MS

Ultra high performance liquid chromatography-tandem mass spectrometry (UHPLC-MS/MS) was used for separation and detection of target analytes. For both EME and the routine method, the selected psychoactive drugs and metabolites were analysed using two UHPLC-MS/MS methods. Alimemazine, amitriptyline, atomoxetine, clomipramine, DM-clomipramine, doxepin, DM-doxepin, fluvoxamine, levomepromazine, nortriptyline, and trimipramine were analysed in method 1, whereas duloxetine was analysed with method 2 due to a lower instrument response for this analyte.

Chromatographic separation was achieved using an Acquity UPLC® BEH C18 (2.1 × 50 mm, 1.7 µm particles) analytical column and an Acquity UPLC® BEH C18 (2.1 × 5 mm, 1.7 µm particles) pre-column on an Acquity UPLC® I-Class instrument, all from Waters (Milford, MA, USA). The mobile phase was 5 mM ammonium formate with pH 10.1 (A) and methanol (B). Gradient elution was carried out with a flow rate of 0.6 mL/min and column temperature 50 °C in both method 1 and 2. Method 1 gradient started with 60% B and continued to 90% B at 0.9 min, 98% B at 1.9 min, and 60% B at 3 min. Method 2 gradient was 55% B at 0 min, 70% B at 1 min, 98% B at 2 min, and 55% B at 3 min. In method 1, the injection volume for the EME method was 0.4 µL and for the routine method 2 µL. In method 2, the injection volume was 5 µL for both EME and the routine method.

Detection was achieved using Xevo TQ-S tandem mass spectrometer (Waters, Manchester, UK) equipped with Z-spray electrospray interface. Positive ionization (ESI+) was performed in the multiple reaction monitoring (MRM) mode. The capillary voltage was set to 1 kV and the ion source temperature was 120 °C in method 1 and method 2. The cone gas flow was 150 L/h. The desolvation gas (nitrogen) was delivered with a flow rate of 1000 L/h and was heated to 600 °C in method 1 and to 450 °C in method 2. Two MRM transitions were used for all analytes except DM-clomipramine. MRM transitions, cone voltage, collision energies and retention times for target analytes and internal standards are presented in Supplementary Table S3. The EME and routine method operated in the same quantification range. For the EME method, five calibration standards were used, applying linear calibration curves with 1/x weighing. In the routine method, four calibration standards were used, and quadratic calibration curves with 1/x weighing were applied.

2.6. Method validation

After a series of experiments optimizing the SLM, sample pH, extraction time and voltage, the final EME-UHPLC-MS/MS method was

validated in accordance with guidelines given by the U.S. Department of Health and Human Services, Food and Drug Administration [28]. In some cases, more confining, internally established acceptance criteria were used. Two UHPLC-MS/MS instruments were used interchangeably during validation.

2.6.1. Limit of detection and quantification, calibration curves

Limit of detection (LOD) was estimated from scalar dilutions (1:5, 1:10, 1:20, 1:30 and 1:50) of analytes in serum, based on a signal to noise ratio (S/N) above three for both the qualification and quantification ion. The lower limit of quantification (LLOQ) was the lowest calibration concentration for each analyte. Blank serum was spiked to the LLOQ concentration and analysed on ten days to evaluate precision and accuracy at the LLOQ-level. Requirements at the LLOQ-level were coefficient of variation (CV) $\leq$ 20%, bias $\pm$ 20%, and S/N $\geq$ 10.

Linear calibration based on peak-area ratios of analyte relative to the internal standard were applied with 1/x weighing, excluding the origin in UHPLC-MS/MS method 1, and including the origin in method 2. Linearity was evaluated from five-point calibration curves with three parallels at each concentration level. The calibration curve was evaluated based on coefficient of correlation (R), residuals and back-calculated concentrations for each analyte.

2.6.2. Precision and bias

Intra- and inter-day precision were evaluated using QC samples at three concentration levels. Intra-day precision was determined from six replicates per concentration level, whereas inter-day precision was determined by analysing one sample at each concentration level for ten days. Requirements were CV $\leq$ 10% and $\leq$ 15% for intra- and inter-day precision, respectively.

Accuracy was studied with internal QC samples, and external control samples obtained from LGC Standards were included. Acceptable bias was $\pm$ 15% ($\pm$20% at LLOQ). Inter-day accuracy at three concentration levels was calculated using precision data (n = 10). For external quality control samples (n = 8), the bias acceptance criterion was $|Z| \leq 2.1$. All analytes except alimemazine and levomepromazine were included in external controls.

2.6.3. Extraction recovery and matrix effects

Extraction recovery was assessed at the low and high QC concentration with six replicates per concentration. Recovery was determined by comparing analyte signal relative to the internal standard in samples spiked with analyte pre- and post-extraction. Internal standard was added post-extraction in both cases. The following equation was used

$$\text{Recovery} = \frac{Response_{\text{pre-spiked}}}{Response_{\text{post-spiked}}} \times 100\% \quad (2)$$

where *response* relates to the analyte area relative to internal standard area.

Matrix effects (ME) were assessed at the low and high QC concentration level with six samples (from different individuals) per concentration. The analyte peak area in post-extraction spiked acceptor phases were compared with the peak area in neat acceptor phases spiked to the same fortification level using the following equation

$$\text{ME} = \frac{Area_{\text{post-spiked}}}{Area_{\text{ref}}} \times 100\% \quad (3)$$

Internal standard corrected matrix effects were calculated by replacing the analyte area with the response relative to IS in equation (3).

2.6.4. Carry-over and auto sampler stability

Instrument carry-over was investigated by inspecting chromatograms of blank serum extract injected after the highest calibration standard. Carry-over was assessed by comparing the signal in the blank sample to the lowest calibrator.

To study analyte stability on the auto sampler, extracted QC samples (n = 4 at each level) and calibrators were left on the auto sampler (10 °C) and reinjected together with fresh calibrators after one, two and six days. Analyte concentration was determined both from the original (reinjected) and from freshly prepared calibrators, and compared with day zero.

2.7. Application: Comparison with routine method at St. Olav University hospital

To evaluate the applicability of the method, 30 authentic serum samples obtained from patients under treatment of psychoactive drugs were analysed. The samples have been to our laboratory for routine therapeutic monitoring. Assay results were compared with the routine method at St. Olav University Hospital. Samples were stored for four weeks at 4 °C prior to being freeze stored (-20 °C) for one to five weeks before re-analysis with EME and the routine method. The anonymized serum samples were prepared and analysed with both EME and the routine method on the same day, using the same UHPLC-MS/MS instrument. Analysis was carried out over two days employing different instruments each day. According to the Regional Committee of Research Ethics, no formal approval is needed for a brief presentation of routine results as part of a methodological article.

To evaluate agreement between EME and the routine method, Bland-Altman analysis of agreement [29] was performed with MedCalc Statistical Software version 17.5 (MedCalc Software bvba, Ostend, Belgium).

2.8. Detection of phospholipids

Presence of phospholipids in sample extracts obtained with EME and the hospital routine method was investigated by detecting the MRM transition m/z 184 > 184, applying in-source collision induced fragmentation (75 V collision voltage, 2 eV collision energy). UHPLC-MS/MS conditions were otherwise the same as UHPLC-MS/MS method 1 described in Section 2.5, with an injection volume of 2 µL. The supernatant of a serum sample subjected to protein precipitation (PPT) served as a reference to the serum phospholipid content. PPT was performed by adding serum (100 µL), internal standard solution (25 µL) and acetonitrile containing 1% formic acid (v/v, 375 µL) to an Eppendorf tube before vortex mixing (15 sec) and centrifuging (3 min, 11 000 rpm).

3. Results and discussion

3.1. Working principle

Electromembrane extraction was performed with sample and acceptor vials of conductive polymer, which served as electrodes. The EME device is depicted along with a schematic illustration in Fig. 1a and b, respectively. The sample vial housed the serum samples (100 µL) spiked with basic drugs. Internal standard and formic acid (0.1%, v/v) were added to the sample to a total volume of 300 µL. Formic acid was added to increase recovery as described below. The SLM consisted of 9 µL NPOE held by capillary forces in the pores of a flat polypropylene membrane. The acceptor phase was 300 µL 0.1% formic acid housed in the acceptor vial. The electric field was coupled through the conductive vials making the sample anodic and the acceptor phase cathodic. The system was agitated, and protonated basic drugs were extracted across the SLM and into the acceptor phase by electrokinetic migration. After extraction, acceptor phases were collected and analysed with UHPLC-MS/MS.

3.2. Optimization

An EME method for extraction of ten psychoactive drugs and two metabolites from serum was developed and optimized. The latter

included thickness and volume of the SLM, sample pH, applied voltage, and extraction time. Optimization experiments were performed with spiked serum, and output parameters were process efficiency/recovery and repeatability. Process efficiencies/recoveries exceeding 85% were considered as exhaustive extraction, and the acceptance criterion for repeatability was set to CV less than 15%.

NPOE is regarded as the first choice SLM solvent for nonpolar basic drugs (log P > 2), and was therefore selected as the SLM solvent with no further optimization [8,17]. The SLM was optimized based on support membrane thickness and NPOE volume. Flat polypropylene support membranes, 110 and 168 μm thick, were tested and process efficiencies were almost unaffected by membrane thickness. However, 168 μm was selected due to a higher degree of robustness. With this membrane, the current was stable throughout 15 min of extraction, while the current sometimes increased at the end with the thinner support membrane. To optimize the SLM volume, NPOE volumes in the range 6–10 μL were tested and the process efficiencies were unaffected by the NPOE volume within this range. An NPOE volume of 9 μL was selected to completely fill the pore volume of the support membrane.

The target analytes are basic drugs with pKa values ranging from 8.9 to 10.5 (chemicalize.com), and are to a large extent protonated at physiological pH. Therefore, initial extractions were performed with the serum samples diluted 1:3 (v/v) with type 1 water. For the majority of analytes, exhaustive extraction was not obtained in this case. By diluting the sample with formic acid (0.1%, v/v), the sample pH was reduced by two units, and the process efficiencies increased drastically and exhaustive extraction (PE > 85%) was achieved. This was most probably due to suppression of drug-protein binding upon acidification, and/or an ion pair effect of formate ions in the sample.

Voltage was optimized in the range from 0 to 150 V based on 15-minute extractions. Recoveries as function of voltage are presented in Fig. 2. Without the electric field (0 V), a few analytes were partially extracted by passive diffusion, but recoveries were less than 11%. Recoveries increased upon the application of electric field, as this facilitated electrokinetic migration of positively charged analytes into the acceptor phase. Recoveries increased with voltage up to 25–50 V. Above this level, extraction was no longer limited by voltage, and there was no further increase in recovery. Therefore, 50 V was selected as the final extraction voltage.

Finally, extraction time was optimized in the range 5 to 30 min based on 50 V extractions. Analyte recoveries as a function of time are displayed in Fig. 3. Recoveries increased rapidly during the first five minutes, and after 15 min, the majority of analytes were extracted exhaustively (recovery > 85%). Although the system was not yet at equilibrium, 15 min was selected as the final extraction time. Extractions longer than this increased time consumption and sacrificed precision.

Shifts in pH and bubble formation are unwanted side effects that can arise from the current in EME. Excessive current can cause electrolysis in the sample and acceptor, which produces gas bubbles, and generates H^+ and OH^- at the anode and cathode, respectively [30]. Significant changes in acceptor pH can cause back-extraction to the SLM following analyte deprotonation [31]. As a preventative measure, extraction currents were kept below 50 μA, as recommended previously [17]. Specifically, the measured current, after an initial current peak (corresponding to the condenser effect [32]), was between 0.6 and 4 μA, depending on analyte concentration (Figure S1). The currents produced with conductive vial EME were thus comparable to what has been reported with traditional EME configurations using NPOE in the SLM [20,32]. From Figs. 2 and 3, no sign of analyte back-extraction was observed. Verification of pH before and after extraction supported that pH conditions were stable.

So far, EME has been performed with equipment produced in-house by individual laboratories. By contrast, the current data were obtained with industrially made prototype equipment, specifically developed for EME application. The EME device was straightforward to operate, and experimental data were highly repeatable. Previous studies using flat porous membranes filled with NPOE to extract similar analytes from plasma reported voltages in the range from 100 to 300 V [9,11,12]. With the current system, it appears that exhaustive extraction could be obtained with lower voltage, possibly due to the different coupling of the electric field. The conductive vial technology also offer the advantage of disposable electrodes. Thus, cross-contamination from re-used platinum electrodes is avoided.

The presented results are the first published using EME with conductive vials to extract pharmaceuticals from biological samples. This prototype technology was recently tested by extracting model analytes spiked in hydrochloric acid [18]. In accordance with this pilot study, the response to operational parameters in the present work closely paralleled traditional EME devices. With conductive polymer electrodes, serving as both electrodes and vials, the recovery as a function of voltage (Fig. 2) was similar to what was previously reported using traditional platinum electrodes [33]. In addition, the time-recovery relationship (Fig. 3) followed the same trend as with traditional EME devices [19,24,34]. This indicates that although the conductive polymer electrode configuration is novel, previous knowledge on EME is applicable also with conductive vial EME.

3.3. Validation

The EME-UHPLC-MS/MS method for determination of selected psychoactive drugs in serum was validated with respect to linearity,

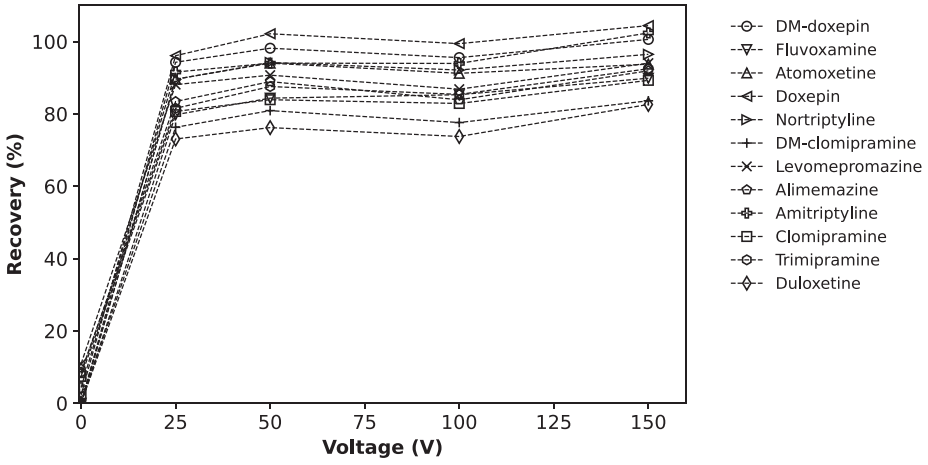


Fig. 2. Recovery (%) as a function of applied voltage (n = 3). All CVs were below 9%. Extraction time was 15 min.

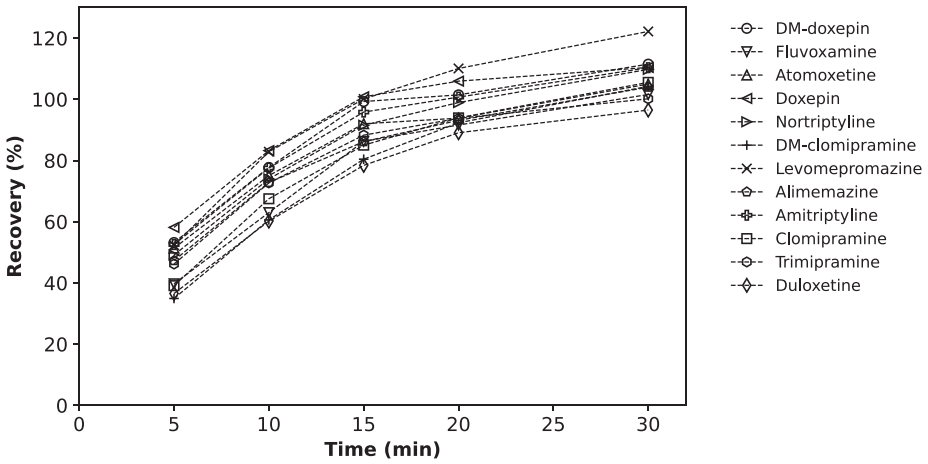


Fig. 3. Recovery (%) as a function of time (n = 4). CVs were ≤ 15%, ≤ 5% and ≤ 10% after 5, 10–20 and 30 min, respectively. Extraction voltage was 50 V.

limit of detection, precision, accuracy, recoveries, matrix effects, carry-over, and stability. A summary of validation parameters is presented in Table 1. The applicability of the method was evaluated by analysing 30 positive serum samples obtained from patients. Assay results were compared to parallel data obtained with the routine method at St. Olav University Hospital.

3.3.1. Limits of detection and quantification, linearity

Limit of detection (LOD) and lower limit of quantification (LLOQ) for each analyte are presented in Table 1. Estimated LODs ranged from 0.2 to 2 nM. The lowest calibration concentration represented LLOQ and ranged from 5 to 80 nM. Acceptable precision and accuracy were achieved at the LLOQ level with inter-day CV ≤ 10% and bias ± 9% (n = 10).

Calibration curves were linear, with R ≥ 0.9995, in the calibration range for each analyte (Table 1). Weighed residuals (1/x) were randomly distributed around zero, and back-calculated concentrations were within ± 7%.

3.3.2. Precision and accuracy

Precision and accuracy results are presented in Table 1. The method showed excellent precision with intra- and inter-day CVs ranging 0.9–6% and 2–6%, respectively. Acceptable accuracy was achieved, with inter-day bias within ± 6% for all analytes. External controls had Z-scores within ± 1, with the exception of one of two control samples for atomoxetine (Z = 2.9). The absolute difference between measured and theoretical value was 16%. The sample had previously been run with satisfactory results with the hospital routine method (Z = 1.4). Reanalysis with the routine method however paralleled the EME results (3% deviation), indicating a possible issue with the sample itself.

3.3.3. Recoveries and matrix effects

Recoveries are presented in Table 1. Analyte recoveries ranged from 75 to 117%, with a median recovery of 88% across analytes. Exhaustive extraction (recovery ≥ 85%) was achieved for DM-doxepin, fluvoxamine, atomoxetine, nortriptyline, doxepin, levomepromazine, amitriptyline, and trimipramine. Although not regarded exhaustively extracted, DM-clomipramine, and duloxetine had recoveries in the range of 75–84%. Alimemazine and clomipramine were extracted exhaustively at the low concentration (87–96%) but not at the high concentration (76–81%). Nevertheless, analyte recoveries showed high repeatability (CV ≤ 6%) for all analytes.

Calculated matrix effects (ME) ranged from 99 to 129% (CV ≤ 12%), and were more pronounced at the low concentrations (Table 1). At the high concentration, matrix effects were negligible. With internal standard correction, the matrix effects were in the range 94–104% (CV ≤ 6%), showing the capability of the internal standard to compensate matrix effects.

3.3.4. Instrument carry-over and auto-sampler stability

False positive results caused by carry-over were unlikely. The highest signal caused by carry-over was 9% of the lowest calibrator and was the case for DM-clomipramine. Extracted QC-samples and calibrators were stable at 10 °C (auto-sampler) for at least six days.

3.3.5. Application: Comparison with reference method at St. Olav University hospital

The EME-UHPLC-MS/MS method was successful in measuring selected psychoactive drugs and metabolites in 30 positive patient samples. The patient samples and external control samples (n = 8) were included in a method comparison of EME and the hospital routine method at the Department of Clinical Pharmacology at St. Olav University Hospital. The hospital routine method is accredited according to the Norwegian Accreditation standard for medical laboratories (ISO15189:2012), and was regarded as a reference method in the comparison. It has been used in therapeutic drug monitoring of the target analytes for the last seven years. The method, consisting of two sub-methods, is regularly tested through external control programs (LGC Standards Proficiency Testing), in which Z-scores within ± 2.1 are achieved. All analytes except levomepromazine and alimemazine are included in these proficiency tests.

The methods were compared using Bland-Altman analysis [29,35]. The Bland-Altman diagram is presented in Fig. 4. The method difference (y-axis) is given relative to the mean. Some samples contained more than one analyte and the total number of concentration pairs included in the comparison was 55. The mean bias ± SD was −1.1 ± 6.1%. Four samples were outside the 95% limit of agreement [-13.0, 10.8]. However, all samples were within ± 20% difference. These results showed that the EME-UHPLC-MS/MS method for determining serum concentrations of selected psychoactive drugs was in agreement with the hospital routine method.

3.3.6. Comparison with earlier EME publications

Tricyclic antidepressants are used commonly as model analytes in

Table 1
Calibration range, coefficient of correlation (R), limit of detection (LOD), lower limit of quantification (LLOQ), intra- and inter-day precision, accuracy, recoveries and matrix effects of psychoactive drugs in serum.

Analyte, concentration (nM)	*Range (nM)*	*Linearity (R)*	*LOD (nM)*	*LLOQ (nM)*	*Intra-day precision[a] (CV)*	*Inter-day precision[b] (CV)*	*Accuracy[c] (bias%)*	*External control bias (%), (z-value)*	*Recovery[d] (CV)*	*ME[e] (CV)*	*IS corrected ME (CV)*
DM-doxepin	10–2000	1.0000	0.33	10				4 (0.3)			
20					2	2	5		95 (3)	101 (5)	97 (3)
500					1.2	5	5				
1000					1.1	4	1.9		87 (3)	101 (3)	100 (4)
Fluvoxamine	25–2000	0.9999	1.25	25				−5 (-0.2)			
50					3	2	−3		92 (3)	112 (6)	97 (3)
500					1.8	3	−3				
1000					2	3	−1.7		84 (3)	102 (1.6)	99 (3)
Atomoxetine	40–4000	0.9995	0.8	40				9 (0.9)			
80					3	3	−1.3	16 (3)	96 (3)	101 (5)	99 (3)
1000					1.7	4	−1.8				
2000					2	5	−1.4		85 (3)	101	99 (3)
Nortriptyline	20–2000	0.9999	1	20				3 (0.2)			
40					3	4	−0.3		91 (3)	109 (5)	101 (1.2)
500					2	5	0.5				
1000					1.1	5	−3		84 (4)	102 (1.8)	100 (4)
Doxepin	10–2000	0.9999	2	10				3 (0.2)			
20					1.4	4	4		92 (4)	99 (4)	98 (5)
500					1.0	4	0.6				
1000					1.5	4	−2		90 (3)	99 (3)	99 (4)
DM-clomipramine	10–2000	0.9999	0.2	10				3 (0.2)			
20					2	4	−1.3		84 (2)	125 (4)	100 (3)
500					1.5	5	−2				
1000					2	5	−4		75 (4)	105 (2)	100 (6)
Levomepromazine	5–1000	0.9997	0.5	5				–			
10					6	4	−6		117 (4)	123 (12)	98 (5)
250					1.0	4	−5				
500					1.1	5	−3		88 (6)	101 (5)	99 (4)
Alimemazine	10–1000	0.9999	2	10				–			
20					4	5	−3		95 (4)	109 (9)	102 (6)
250					2	5	0.3				
500					2	6	1.4		76 (6)	101 (4)	94 (5)
Amitriptyline	10–2000	0.9999	0.5	10				3 (0.3)			
20					4	5	0.4		95 (5)	108 (6)	97 (3)
500					2	3	−0.4				
1000					1.3	4	−4		90 (1.4)	105 (3)	104 (4)
Clomipramine	10–2000	0.9999	1	10				13 (1.0)			
20					0.9	5	4		87 (5)	129 (5)	94 (4)
500					1.7	4	3				
1000					1.6	5	−0.2		81 (5)	103 (3)	98 (3)
Trimipramine	10–2000	0.9997	1	10				4 (0.3)			
20					2	4	−3		90 (5)	113 (4)	101 (4)
500					1.9	4	−1.6				
1000					1.1	5	−1.5		88 (6)	102 (3)	99 (4)
Duloxetine	10–500	0.9997	0.2	10				7 (0.7)			
20					3	3	−5		83 (1.8)	108 (5)	100 (1.9)
125					1.6	4	−3				
250					1.5	4	−1.0		78 (3)	100 (2)	99 (3)

[a] Intra-day precision was determined at three concentration levels with n = 6.
[b] Inter-day precision was determined at three concentration levels with n = 10.
[c] Accuracy was determined from inter-day precision data (n = 10).
[d] Recovery was determined at two concentration levels with n = 6.
[e] Matrix effect (ME) was determined at two concentration levels with n = 6.

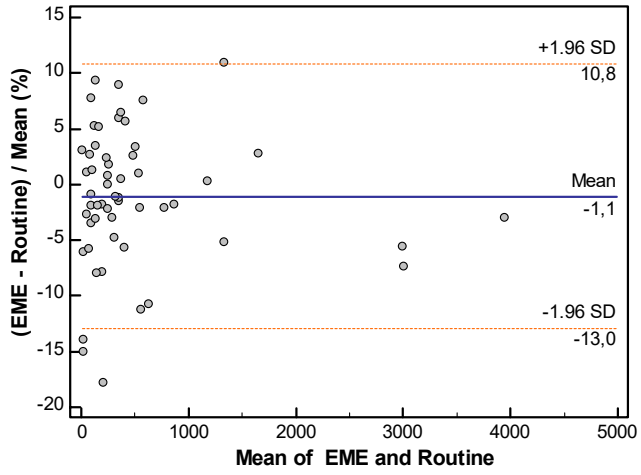


Fig. 4. Bland-Altman diagram comparing EME and the hospital routine assay results for patient samples and external controls.

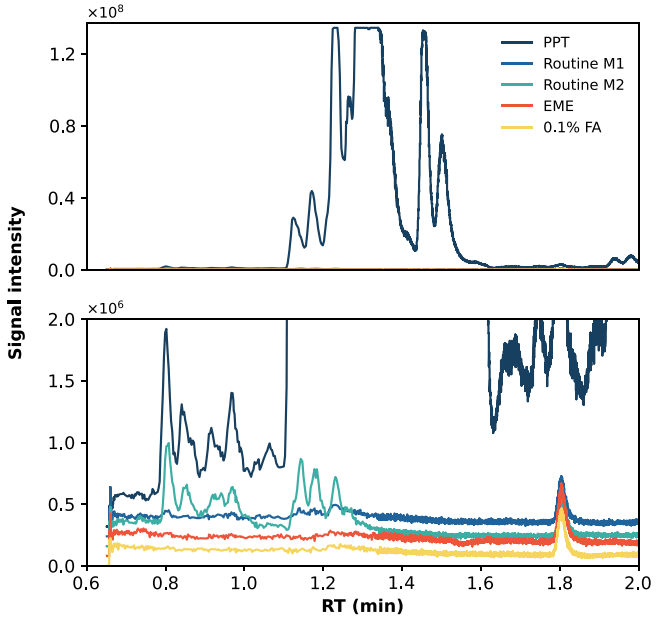


Fig. 5. UHPLC-MS/MS chromatograms (m/z 184 > 184) detecting phospholipids in extracts obtained with PPT, routine extraction method 1 (M1), routine extraction method 2 (M2), and EME. A neat acceptor phase (0.1% FA) was included as a blank. Upper panel represent full scale chromatograms while the lower panel displays a section of lower signal intensity to enable comparison.

EME of nonpolar basic drugs. When extracted from biological samples, recoveries are lower and precision is poorer compared to EME from acidified water samples. However, exhaustive and near-exhaustive extraction from plasma have been achieved previously [9,27]. Alimemazine, atomoxetine and levomepromazine have not been extracted with EME. Precision reported in the current paper was clearly improved compared to earlier publications. With amitriptyline as example, intra-day precision ranged between 1.3 and 4 % CV in the current work, while similar data reported with different laboratory built devices ranged between 5 and 10 % CV [9,11,36].

3.4. Phospholipid clean-up

The EME and hospital routine method were compared with respect to phospholipid removal. When introduced to an LC-MS system, phospholipids in serum, plasma, and blood, may reduce column lifetime, and pollute the mass spectrometer source causing significant matrix effects [37]. Efforts are therefore made to remove these compounds before biological samples are introduced to the instrument. Phospholipid removal was evaluated based on the remaining phospholipids in sample extracts obtained with EME and the hospital routine method. This was done using UHPLC-MS/MS to detect glycerophosphocholines (GPCho) in sample extracts, which are the most common phospholipids in plasma [37,38]. Specifically, in-source collision induced fragmentation and detection of the MRM transition m/z 184 > 184, was used, which corresponds to the common fragment ion of GPCho, lysophosphatidylcholines, and sphingomyelin [38,39]. The resulting chromatograms of EME acceptor phases and routine extracts were studied qualitatively. A sample subjected to protein precipitation (PPT) was included as a reference for serum phospholipids, and 0.1% v/v formic acid (same as neat acceptor phase) was included as a blank. Representative phospholipid chromatograms are shown in Fig. 5. Four EME acceptor phases from different serum samples were investigated, and the chromatograms were comparable.

Phospholipid removal was achieved with both EME and the routine method. Routine extraction method 1 and 2 employ a commercially available 96-well filtration plate specifically aimed to remove phospholipids. In EME, phospholipids are discriminated by their zwitterionic nature (at physiological pH) and/or low solubility in the SLM [40]. Chromatograms obtained with EME were comparable to that of 0.1% v/v formic acid, indicating very effective clean-up. With EME, glycerophosphocholines were completely retained in the sample during the extraction.

3.5. Future work

This study showed that by using industrially produced EME equipment, reliable data in accordance with recommended guidelines are obtainable. Serum concentrations of target psychoactive drugs and metabolites were successfully determined with great precision and accuracy using EME-UHPLC-MS/MS. In addition, acceptor phases were completely free of glycerophosphocholines. By using equipment of industrial standard, the EME performance matched the routine method. The introduction of standardized equipment is important for several reasons. Firstly, it is crucial for the implementation into any routine setting. In addition, allowing research to be conducted on a standard format is highly beneficial for further activity and interest in EME. Regarding the current potential for routine implementation, the presented conductive vial prototype device was straightforward and easy to use, but further advancements in sample throughput and automation is necessary to qualify for routine operation.

4. Conclusion

In this paper, prototype conductive vial electromembrane extraction (EME) was explored in the context of routine clinical analysis. EME was performed by coupling the electric field with sample and acceptor vials produced from conductive polymer, which made the electrodes fully integrated and disposable. An EME-UHPLC-MS/MS method for extracting psychoactive drugs, mainly antidepressants, from serum was successfully developed, optimized and validated. The obtained results demonstrated that these lipophilic drugs could be exhaustively extracted with excellent precision using the conductive vial configuration. Although the electrode configuration was fundamentally different from metal wires, the response to operational parameters closely paralleled data obtained with conventional EME [19,24,33,34]. Thus, the fundamental theory and experimental operation of EME reported in literature, apparently is also valid for conductive vial EME.

Data complied with validation requirements, and the EME-UHPLC-MS/MS method was in agreement with the routine method that employed commercially available sample preparation. EME is generally

regarded as compatible for automation in 96-well format [7], which is promising for future implementations. Further development into multi-well systems is also the aim for the conductive vial technology. The present results motivate further development of new and improved methods using EME. Future research should aim to utilize the unique features of EME for conditions that are otherwise challenging with conventional sample preparation.

Declaration of Competing Interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Coauthor Roger Trones is working in the company Extraction Technologies Norway, and this company is developing EME technology towards commercialization. All other authors declare that they have no known competing financial interests.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jchromb.2021.122926.

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