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ABSTRACT

A specific and sensitive capillary liquid chromatography-ultraviolet detection (cap-LC-UV) method in combination with a micro-extraction by packed sorbent (MEPS) sample clean-up procedure has been developed and validated for the simultaneous analysis of chlorpromazine, olanzapine and their flavin-containing monooxygenase (FMO) mediated N-oxides in rat brain microdialysates. Chromatographic separation was obtained on an Acclaim Pepmap RP C18 column with an ID of 300 µm. An injection volume of 20 µL was used to inject the largely aqueous samples and was shown to have no influence on the obtained peak shape of the compounds of interest. Optimal conditions for MEPS extraction were obtained on a mixed-mode M1 (80% C8, 20% SCX) cartridge after diluting microdialysate samples with phosphate buffer pH 2.5 (1:3 v/v). The method was validated and lower limits of quantification (LLOQ) were determined at 0.5 nM for all compounds. Linearity was demonstrated between the LLOQ and 1 µM for all compounds (R^2 > 0.995). MEPS recoveries were between 92 and 98%, with intra- and interday variabilities below 15%. The applicability of the developed method was successfully demonstrated by analysing rat brain microdialysates. The capillary LC-UV method in combination with MEPS sample treatment provides a simple, sensitive method to quantify all compounds of interest in 45 min and can be applied for routine therapeutic monitoring and pharmacokinetic studies of olanzapine, chlorpromazine and their respective N-oxides.

Keywords: Olanzapine; Chlorpromazine; FMO; Capillary LC; microdialysis; MEPS

1. Introduction

Psychotic disorders, such as schizophrenia and bipolar disorder, are severe mental diseases that are estimated to affect as much as 3% of the world's population [1]. Schizophrenia is characterized by a combination of negative symptoms (social withdrawal, little emotion, lack of motivation), positive symptoms (disorganized thoughts and speech, delusions, hallucinations) and cognitive dysfunction. The disease is also associated with an increased suicide rate, emphasizing the need for optimized treatment in order to adequately manage psychotic episodes and behavioural symptoms of schizophrenic patients [2]. Psychotic disorders are treated with antipsychotic drugs, one of the fastest growing drug classes in pharmaceutical industry [3]. The first generation of antipsychotic drugs, known as the typical antipsychotics, were developed in the 1950s and include chlorpromazine, haloperidol and phenothiazine [4]. Due to their pharmacological action on D_2- and D_4-receptors, most first generation antipsychotics suffer from a number of severe side-effects, such as extrapyramidal symptoms and tardive dyskinesia. Due to the negative influence of these side-effects on patient adherence, several second generation antipsychotics were developed from the 1970s onward in an attempt to reduce these side-effects. These 'atypical' antipsychotics (risperidone, olanzapine, clozapine, quetiapine,…) exhibit a lower activity on D_2- and D_4-receptors and therefore have less extrapyramidal side-effects [5]. Although the precise mechanism of action of antipsychotics is still largely unknown, it has been suggested that the antipsychotic activity is predominantly mediated by transiently blocking dopaminergic (D_2) receptors in the brain. It is, however, possible that serotonin receptor (5HT2A) antagonism can also contribute to the therapeutic effect, especially in the case of atypical antipsychotics [6,7].

Chlorpromazine (CPZ) was the first antipsychotic drug to be developed in 1951. This phenothiazine (first generation) antipsychotic drug is still widely used for the treatment of schizophrenia, anxiety and tension [8]. After intake, CPZ is extensively metabolized and a large number of metabolites are formed, of which chlorpromazine N-oxide (CPZ-O), chlorpromazine sulfoxide, N-desmethyl-chlorpromazine and 7-hydroxy-chlorpromazine are most predominant [9]. Olanzapine (OLA) on the other hand is a second generation thienobenzodiazepine antipsychotic, which is currently one of the most widely prescribed antipsychotic drugs for the treatment of schizophrenia and bipolar disorder. It is very effective in reducing both the positive and negative symptoms of schizophrenia, with a low incidence of extrapyramidal symptoms, but is frequently associated with metabolic side effects and weight gain [10]. Olanzapine is extensively metabolized in the body, predominantly via cytochrome P450 (CYP) oxidative pathways and UGT1A4- (uridine diphosphate-glucuronosyltransferase 1A4) mediated glucuronidation, but the flavin-containing monooxygenase (FMO) system also plays an important role. The major metabolites are olanzapine 10-N-glucuronide, 4-N-desmethyl olanzapine

(CYP1A2 mediated) and olanzapine N-oxide (FMO mediated), none of which show pharmacological activity [11].

Flavin-containing monooxygenase (FMO) is a multigene family of microsomal enzymes located in the endoplasmic reticulum of different organs throughout the body [12,13]. They catalyse the NADPH-dependent oxidation of a number of xenobiotics containing a nucleophilic heteroatom (such as N, S or P). FMOs are characterized by a high *in vitro* catalytic efficiency and are mostly expressed in the kidneys (FMO-1), lungs (FMO-2, FMO-3), liver (FMO-3) and intestine (FMO-1), but are also present in the brain (FMO-2 being the most abundant) [14]. Contrary to CYP and UGT1A4 enzymes, FMO has not been studied extensively. Although FMO has been shown to catalyse the conversion of OLA to OLA-O *in vitro*, the role of FMO for the *in vivo* oxidation of OLA is less clear [12]. Expression of FMO in the human brain indicates a potential relevance for FMO in the central nervous system. It has been suggested that FMO can significantly be involved in the local metabolism and modulation of the pharmacological activity of psychoactive drugs [15]. Relatively little is known concerning substrate preferences for the different FMOs, although FMO-3 tends to prefer oxidation of smaller nucleophilic heteroatoms compared to FMO-1, which prefers drugs with bulkier side-chains [16].

Extensive interindividual variations of serum concentrations of OLA and its metabolites are observed in routine therapeutic drug monitoring (TDM) [17,18]. These large interindividual variations can be explained by influences such as gender, smoking habits, genetic variants and drug interactions. It has been shown that co-administration of certain CYP1A2 inducers or inhibitors can also result in considerable changes in systemic OLA exposure of the patient, with e.g. a 2-3 fold increase of OLA serum concentrations in fluvoxamine-treated patients and a 40-70% reduction in carbamazepine-treated patients [18,19]. A number of UGT1A4 polymorphisms have also been associated with reduced serum OLA concentrations [20]. Finally, certain FMO-1 polymorphisms were found to be associated with increased serum OLA concentrations, while a number of FMO-3 polymorphisms were shown to result in a high variability of OLA N-oxide concentrations [21]. These large variations of OLA and its metabolites make therapeutic drug monitoring (TDM) of OLA and its metabolites desirable in order to provide optimal dosage of OLA during antipsychotic therapy to minimize adverse effects and optimize patient adherence [19,22].

Microdialysis is a powerful *in vivo* sampling technique to measure unbound (free) drug concentrations over time in extracellular fluid from virtually any tissue in the body, including the brain. The technique uses a microdialysis probe containing a semi-permeable membrane which is inserted into the selected tissue followed by continuous perfusion with a suitable perfusate. Small molecules will be transported over the semipermeable membrane through passive diffusion, while

larger bio-macromolecules are excluded. As a consequence, microdialysis samples generally only require minimal sample clean-up prior to analysis compared to e.g. plasma or urine samples. Microdialysis sampling has been applied for *in vivo* sampling of analytes in blood, brain and other tissues [23–25]. It is minimally invasive, offers the possibility of real-time sampling and since no fluid loss occurs, long term sampling is possible without interfering with the pharmacokinetic behaviour of the drug [26]. Because the concentration of a drug can be determined in a specific tissue of interest and since only the active free fraction of the drug is sampled, microdialysis is very suitable for establishing pharmacokinetic and pharmacodynamic profiles of drugs. The efficacy of antipsychotics is determined by the capability of penetrating the blood brain barrier and the capability of binding to the action targets. Therefore, *in vivo* microdialysis sampling in the brain in combination with total plasma concentration can provide relevant information concerning the distribution of unbound, active drug within the tissue of interest [27]. Microdialysis has been successfully applied for the collection of intracephalic neurotransmitter samples in specific brain areas [25,28], but also for the determination of drug and metabolite levels in animal studies [27,29,30]. A number of studies have been conducted where the effect of antipsychotics, including OLA and CPZ, on extracellular acetylcholine, dopamine and histamine levels in different brain regions was studied using *in vivo* microdialysis [31–33]. *In vivo* microdialysis was also employed to study the effect of a number of antipsychotics (including CPZ and OLA) on the neurotensine efflux in dopamine terminal regions [34]. Microdialysis samples generally only yield small sample volumes (µL-range) with low concentrations of analytes (diluting effect of the dialysis procedure). As a consequence, a sensitive analytical method is required to quantify all analytes of interest and their metabolites [35].

Since only limited sample volumes are available, the use of microextraction techniques is appealing for sample clean-up. Microextraction by packed sorbent (MEPS) is a sample preparation technique based on the miniaturization of conventional solid-phase extraction (SPE). 1-4 mg of packing sorbent is integrated in a gas-tight syringe which can be reused more than 100 times for urine or plasma samples [36]. MEPS entails a number of advantages such as ease of use, reduction of solvent volumes, low cost compared to conventional SPE procedures and suitability for small sample volumes [36,37]. MEPS can be performed both off- and on-line and has been used extensively in bioanalysis for a variety of sample matrices, e.g. for the extraction of antidepressants, antibiotics and antipsychotics [38–40].

Several analytical methods for the determination of OLA, CPZ or both in biological matrices have been published. A number of methods based on gas chromatography for the determination of either OLA or CPZ in rat plasma have been described [41,42]. Several LC methods have been published for the determination of OLA or CPZ in either plasma or brain tissue using different detection systems:

electrochemical detection for CPZ in brain tissue [43] and OLA in human plasma [44,45]; coulometric detection for OLA in rat brain [46]; UV detection for CPZ in plasma [47–49] and OLA in serum and in plasma [47,50–53]. Nevertheless, most analytical methods for the determination of antipsychotics in biological samples utilize LC-MS [5,54–58]. There are several reports of LC–MS methods for the simultaneous determination of OLA and CPZ in plasma [54–57], serum [58] and blood [5], but none in brain microdialysates. Zheng et al. developed a method for the quantification of OLA in rat brain microdialysates using LC-MS [59]. The simultaneous quantification of five antipsychotics, including CPZ and OLA, in rat plasma using LC-UV was performed by Zhang et al. but to the best of our knowledge, no method has been described for the simultaneous separation, detection and quantification of CPZ, OLA and their respective N-oxides [47]. UV detection is simpler, more robust, cheaper and more widely available than mass spectrometry. In this article, it will be demonstrated that capillary LC-UV can be an interesting technique for the analysis of samples with limited sample volumes when mass spectrometry equipment is not available.

A high proportion of psychiatric patients are on more than one antipsychotic drug which emphasizes the need for analytical methods that can detect multiple antipsychotics. Both OLA and CPZ are substrates of FMO resulting in the N-oxidation of the drugs. The structures of OLA, CPZ and their respective N-oxides olanzapine N-oxide (OLA-O) and chlorpromazine N-oxide (CPZ-O) are shown in Fig. 1. In contrast to e.g. CYP enzymes, drastic inhibition of FMO has not been reported and inhibition of FMO is most likely to be competitive. Possible *in vivo* inhibition of FMO is suggested to be influenced by a combination of dietary factors and polymorphic variants of the FMOs. Furthermore, it is suggested in literature that CPZ does not only function as an FMO-substrate, but also as an inhibitor of FMO, especially in higher concentrations [60,61]. As a consequence, it is interesting to investigate whether co-administration of CPZ and OLA can have an influence on the N-oxidation of OLA and the concentration of OLA in the brain.

The aim of the present study is to develop and validate a simple, specific and sensitive capillary LC-UV method for the simultaneous separation and quantification of CPZ, OLA and their FMO-mediated N-oxides in rat brain microdialysates. Sensitivity limits that can be obtained by UV detection using miniaturized column diameters and optimized injection volumes are investigated. In order to eliminate all UV interferences, an additional sample clean-up by MEPS extraction is developed. After validation, the applicability of the developed method is demonstrated by quantifying OLA, OLA-O, CPZ and CPZ-O levels in rat brain microdialysate samples. OLA and CPZ are also co-administered to rats in order to investigate the potential inhibitory effect of CPZ on FMO-mediated OLA-O formation.

2. Material and methods

2.1. Chemicals and reagents

Analytical standards of olanzapine (OLA) and chlorpromazine (CPZ) were obtained from Sigma-Aldrich (Steinheim, Germany); chlorpromazine N-oxide (CPZ-O) was purchased from Hangzhou Sage Chemical Co. Ltd (Hangzhou, China); olanzapine N-oxide (OLA-O) was acquired from TLC Pharma Chem Inc (Vaughan, Canada). Dimethyl sulfoxide (DMSO) and methanol (MeOH) were from Acros Organics (Geel, Belgium); Acetonitrile (ACN), ammonium acetate, triethylamine (TEA), sodium chloride and hydrochloric acid (37% w/w) were purchased from Fisher Scientific (Loughborough, UK); Ammonia solution 25% (w/w) and potassium chloride were obtained from Chem Lab NV (Zedelgem, Belgium); Calcium chloride and magnesium chloride were purchased from Sigma Aldrich (Belgium); Glacial acetic acid was acquired from VWR chemicals (Fontenay-sous-Bois, France) and dihydrogen potassium phosphate from Merck KGaA (Darmstadt, Germany). Ultrapure water was obtained using a Milli-Q apparatus from Millipore (Milford, MA, USA). A 691 pH meter (Metrohm, Antwerpen, Belgium) was used for pH adjustments. All reagents were of analytical grade or better.

2.2. Preparation of solutions

The dialysis perfusate was a Ringer's solution consisting of 2.5 mM KCl, 1.18 mM $MgCl_2$, 1.26 mM $CaCl_2$ and 125 mM NaCl [62]. Standard stock solutions of OLA, OLA-O, CPZ and CPZ-O with a concentration of 100 µM were prepared by dissolving the pure substance in DMSO. These solutions were kept at -20°C. Different stock solutions of OLA, OLA-O, CPZ and CPZ-O were prepared daily in Ringer's solution at concentrations of 100 nM and 5 µM. Working standard solutions were obtained by spiking Ringer's solution with these primary stock solutions to obtain OLA, OLA-O, CPZ and CPZ-O concentrations between 0.5 nM and 1 µM. The samples for MEPS recovery determination were prepared in an analogous manner to obtain concentrations of 2 nM, 100 nM and 1 µM for OLA, OLA-O, CPZ and CPZ-O (corresponding to final concentrations of 0.5 nM, 25 nM and 250 nM after 4 times dilution of the MEPS eluate). For stability testing, standard solutions of 20 nM and 100 nM were prepared in the same manner. All calibration standards were stored at -80°C prior to analysis. All evaluation of data and calculations were performed in MS Excel (Microsoft Corporation, Seattle, WA, USA).

2.3. Instrumentation and chromatographic conditions

Chromatographic analysis was performed on an UltiMate 3000 series RSLC nano system, equipped with a binary capillary pump, degasser, autosampler, thermostatted column compartment and a variable wavelength UV detector (Dionex, Amsterdam, the Netherlands). Data acquisition and peak processing were performed using Chromeleon version 6.8 chromatography data software (Dionex).

An Acclaim Pepmap RSLC C18 capillary column (150 mm x 300 µm i.d., 2 µm, 100 Å) equipped with a C18 Pepmap100 µ-precolumn (5 mm x 300 µm i.d., 5 µm, 100 Å), both from Thermo Fisher Scientific (Erembodegem, Belgium) was used for analysis. The column was thermostatted at 40°C and samples were kept at 5°C in the autosampler. A volume of 20 µL of MEPS extract was injected into the capillary LC system. Mobile phases consisted of 10 mM ammonium acetate with 0.05% triethylamine, adjusted to pH 3.00 ± 0.01 using acetic acid (A), and acetonitrile (B). The separation was performed in gradient mode using the following time program: 10% B (min 0); 10% B (min 3); 16% B (min 12); 31% B (min 12.1); 31% B (min 23); 35% B (min 38); 35% B (min 40); 90% B (min 40.1); 90% B (min 45). A flow rate of 4 $\mu L.min^{-1}$ was applied and UV detection was performed at 254 nm.

2.4. MEPS procedure

The sample pretreatment procedure was performed using MEPS on a BIN (Barrel Insert and Needle Assembly) containing a cartridge with 4 mg of solid phase M1 (80% C8; 20% SCX) material, inserted into a 250 µL gas-tight syringe from SGE Analytical Science (Melbourne, VIC, Australia).

The final sample preparation method was obtained by testing different sample diluents and varying the ratios of sample dilution, eluent composition and volumes. The final MEPS conditions are as follows: the MEPS sorbent was first activated with 100 µL of eluent (5% ammonia in 80% methanol (v/v)) and 100 µL of methanol to ensure reproducible retention of the analytes [37]. The sorbent was then conditioned using a 1:3 mixture (v/v) of Ringer's solution and 0.833 M phosphate buffer pH 2.5 (Ph. Eur.) (4 x 100 µL). 50 µL microdialysate sample was mixed with 150 µL phosphate buffer pH 2.5, vortexed for 60 s and centrifuged for 60 s at 14,000 x *g*. Then, the mixture was drawn through the syringe and ejected in the same vial three times at an approximate flow rate of 5 $\mu L.s^{-1}$. The sorbent was washed once with 100 µL 5% acetic acid (v/v) and once with 100 µL of a mixture of methanol and water (10:90, v/v) to remove matrix-related interferences. Then, the analytes were eluted using 50 µL of a solution containing 5% ammonia in 80% methanol (v/v). The resulting extract was diluted with 150 µL of mobile phase (A) (10 mM NH_4Ac, 0.05% triethylamine, pH 3.00) in order to reduce the fraction of methanol prior to injection. 20 µL of the resulting solution was injected onto the capillary LC system. All MEPS steps, including activation, conditioning, loading, elution and washing were carried out manually. Between every extraction, the sorbent was washed with 4 x 100 µL water followed by 4 x 100 µL methanol. This step was included to decrease memory effects (carry-over) and also resulted in simultaneous conditioning of the sorbent for the next extraction.

2.5. Microdialysis procedure

Microdialysis sampling was performed at the Department of Analytical Chemistry of Anadolu University (Eskisehir, Turkey). All microdialysis apparatus were obtained from CMA (Solna, Sweden).

Microdialysis was performed on adult male Wistar rats weighing 250-350 g (obtained from the Laboratory Animal Centre at Osmangazi University (Eskisehir, Turkey)). The rats were housed in a temperature-controlled room under a 12 h light-dark cycle with free access to food prior to microdialysis sampling. All animal experiments were performed in accordance with the principles of animal care and use approved by the ethical committee of Anadolu University (Approval File No. 2012-33). Anaesthesia of rats was achieved by intra-peritoneal (i.p.) injection with urethane (1.5 $g.kg^{-1}$) throughout the complete experimental period [63]. A total of six rats were used in the experiments. Either 200 $mg.kg^{-1}$ CPZ (2 rats), 50 $mg.kg^{-1}$ OLA (2 rats) or a combination of 200 $mg.kg^{-1}$ CPZ and 25 $mg.kg^{-1}$ OLA (2 rats) was given by i.p. injection. Following anaesthesia, the rats were mounted on a stereotaxic frame (David Kopf Instruments, Tujunga, CA, USA). Two areas of the brain were tested for placement of the microdialysis probe: the "bulbus olfactorius" and the "prefrontal cortex". The probe coordinates for both brain areas relative to the bregma were: prefrontal cortex (Anterior Posterior (AP) +0.2, lateral (L) -2.5, ventral (V) -2.5) and bulbus olfactorius (AP +5.7, L -1.5, V -2.5), according to the Paxinos and Watson atlas [64]. The bulbus olfactorius was selected for further experiments due to its high density of D_2-receptors. It has been proposed that OLA and CPZ exert their antipsychotic effect by antagonizing this receptor [65–67]. Furthermore, a higher concentration of OLA and CPZ was found in the bulbus olfactorius compared to the cortex during preliminary tests. Initial microdialysis experiments were performed with a CMA/12 (PC) microdialysis probe, with a cut-off of 20 kDA and a membrane length of 2 mm. However, the resulting concentrations of OLA-O and CPZ-O in the obtained microdialysis samples were too low to be adequately quantified (below LLOQ) and therefore not suitable for pharmacokinetic analysis. It was therefore decided to use a microdialysis probe (CMA/12 (PAES)) with a cut-off of 100 kDA and a membrane length of 3 mm, which provided better probe recovery results for OLA-O and CPZ-O. The latter microdialysis probe was therefore applied for all microdialysis samples in the present study (including blank microdialysates). A thermostatically controlled heating pad (CMA150) maintained body temperature between 37.1 and 37.3 °C during the entire experiment. The probe was conditioned by perfusing with Ringer's solution for 1 h at a flow rate of 1.0 $\mu L.min^{-1}$ prior to sample collection. Microdialysis samples were collected at the same perfusion rate at 4°C and a temporal resolution of 1 h. Rat brain microdialysis samples were stored at -80 °C prior to MEPS treatment and capillary-LC-UV analysis.

2.6. Method validation

The developed method was validated for specificity, linearity, lower limit of quantification (LLOQ), accuracy, precision and stability according to ICH and bioanalytical guidelines [68,69].

2.6.1. MEPS recovery, precision and accuracy

50 µL of Ringer's solution was spiked with 10 µL standard stock solution to obtain three different concentrations (high-intermediate-low) of OLA, OLA-O, CPZ and CPZ-O (2 nM, 100 nM and 1 µM). 140 µL of phosphate buffer pH 2.5 was added, the sample was vortexed for 60 s and centrifuged for 60 s at 14,000 x *g*. The resulting samples were subjected to the MEPS procedure as described above and injected into the Cap-LC system. The percentage MEPS recovery was calculated for each analyte by taking the ratio of the resulting peak areas after MEPS and the peak areas from standard solutions with the same concentration prepared in Ringer's solution. Bias percentage values of the spiked MEPS samples were calculated using the following formula: Bias % = [(concentration found – concentration spiked) / concentration spiked] x 100.

To determine the repeatability (*intraday precision*), assays were performed in triplicate within the same day. Assays were also repeated three times over three days to obtain intermediate precisions (*interday precision*). Both *interday* and *intraday* precision are expressed as percentage relative standard deviation values (RSD %).

2.6.2. Linearity, lower limit of quantification

Calibration standards (standard solutions of 0.5-1-2.5-10-20-50-250 nM) were prepared by spiking Ringer's solution with stock solutions of analytes. The analyte peak areas obtained were plotted against the corresponding concentrations of the analytes in the range of 0.5 nM – 250 nM. Calibration curves were constructed with the least-square method using weighted linear regression analysis (w=1/x). The values for the limit of quantification (LLOQ) were defined as the lowest concentration of analyte for which a signal-to-noise ratio (S/N) ≥ 10 was obtained and which had acceptable accuracy and precision. Following regulatory guidelines for bioanalytical method validation of exogenous analytes, acceptance limits of 15 % were proposed for the current study [70].

2.6.3. Specificity and selectivity

The specificity and selectivity of the method was evaluated by comparing chromatograms from blank rat microdialysis samples, rat microdialysis samples spiked with OLA, OLA-O, CPZ and CPZ-O and real microdialysis samples obtained from rats that were injected (i.p.) with OLA and CPZ. Four blank rat microdialysis samples were pooled and different aliquots were taken for specificity determination.

2.6.4. Stability

Short- and long-term stability of the analytes in microdialysates was evaluated by spiking standard solutions into Ringer's solution at two concentrations (20 and 100 nM for all analytes). For short-term stability, spiked samples were kept in the autosampler for 24 h (5°C). Long-term stability was determined after storage of spiked samples for 20 days in a freezer at -80 °C. Freeze and thaw stability was also assessed for spiked samples stored at -80 °C for 24 h, by thawing and refreezing the

samples 3 times prior to analysis. Stability results were evaluated by comparing chromatographic peak areas of OLA, OLA-O, CPZ and CPZ-O with those of standard solutions with the same concentration from freshly prepared stock samples (n=3).

3. Results and discussion

3.1. Optimization of chromatographic conditions

An Acclaim Pepmap RSLC C18 capillary column (150 mm x 300 µm i.d., 2 µm, 100 Å) was selected for analysis because all analytes were sufficiently retained on this column and good peak shapes in combination with an adequate resolution for the critical pair (CPZ/CPZ-O) were obtained. Method development focused on obtaining a rugged chromatographic separation with sufficient resolution between the critical pair, CPZ and CPZ-O. Initial optimization experiments were performed using a generic gradient wherein the percentage of organic modifier was varied from 5% to 95% in 30 min, at a flow rate of 4 µL/min and using an injection volume of 2 µL. The effect of several parameters, including choice and concentration of additive, mobile phase pH and type and concentration of organic modifier were investigated to optimize the proposed capillary-LC-UV method. ACN, MeOH and a mixture of ACN:MeOH 50:50 (v/v) were tested as organic modifiers, but only ACN yielded sufficient resolution between CPZ and CPZ-O. Ammonium formate and ammonium acetate (at different concentrations) were investigated as mobile phase additives at different pH values. The use of ammonium acetate yielded a higher sensitivity and reproducibility compared to ammonium formate. Different concentrations of 5, 10 and 20 mM were tested and 10 mM ammonium acetate was chosen as optimum concentration in terms of efficiency and total run time. A low mobile phase pH (pH 3.00) was chosen since higher pH values gave rise to increased peak tailing and reduced signal for some of the analytes. However, at this low pH value, all analytes were positively charged. Addition of a silanol masking agent was therefore required to prevent excessive peak tailing. Triethylamine (TEA) in a concentration of 0.05% (v/v) gave the best results in terms of peak shapes. Different flow rates (4.0 µL.min^{-1} and 8.0 µL.min^{-1}) were tested and the lower flow rate was chosen since it led to an improved resolution between CPZ and CPZ-O. This was at the cost of a longer run time. However, this was not considered to be a problem. The final method hence entailed a gradient using mobile phases consisting of 10 mM ammonium acetate at pH 3.00 with the addition of 0.05 % TEA (A) and ACN (B) at a flow rate of 4.0 µL.min^{-1}. A stepwise gradient was subsequently applied in order to maximize resolution between the antipsychotics (OLA/CPZ) and their respective N-oxides. The following multistep gradient program was used in the final separation method: 10% B (min 0); 10% B (min 3); 16% B (min 12); 31% B (min 12.1); 31% B (min 23); 35% B (min 38); 35% B (min 40); 90% B (min 40.1); 90% B (min 45). Using these chromatographic conditions, baseline separation for

all compounds was obtained with sufficient resolution for the critical pair CPZ and CPZ-O (R_s > 4) (Fig. 2).

To increase the sensitivity of the method, the effect of different injection volumes (5 and 20 µL, using 5 µL and 20 µL sample loops in full loop mode to ensure robust sample injections) and different concentrations of organic modifier in the sample solution on the peak shape of OLA and OLA-O was investigated (see Tables S-1 and S-2 in the Supplementary Information). Since these compounds are the least retained, their peak shape will be affected most by different injection conditions. As can be deduced from Tables S-1 and S-2, the use of a larger injection volume increased both retention times (due to the larger flow path caused by the larger loop volume) and peak widths of the compounds. Table S-1 and Figs. 3 (A-C), however, clearly demonstrate that an injection volume of 20 µL could be applied without resulting in excessive band broadening and loss of separation, as long as the percentage of MeOH in the sample solution did not exceed 20%. For higher percentages of MeOH in the sample solvent, distorted peak shapes and smaller peak areas were obtained for OLA and OLA-O.

For ACN (Table S-2 and Figs. 3 (B-D)), distorted peaks were observed at much lower concentrations (12.5% ACN for an injection volume of 5 µL and 10% ACN for an injection volume of 20 µL, respectively). These observations can be explained by the fact that the analytes can be adequately trapped at the head of the column when the elution strength of the sample solvent is weaker than the elution strength of the mobile phase at the moment the analytes elute from the column (14% ACN for OLA and 16% ACN for OLA-O). Using the transfer rule of Schoenmakers et al., it can be calculated that these mobile phase compositions correspond with 25% MeOH for the elution of OLA and 28% MeOH for the elution of OLA-O, respectively [71]. This explains why higher percentages of MeOH in the sample solvent can be applied, before peak shapes become distorted.

3.2. MEPS optimization

When microdialysate rat samples were directly injected onto the capillary LC, a number of interferences were present in the chromatogram that prohibited quantification of all analytes. In order to eliminate these interferences, an off-line microextraction by packed sorbent procedure (MEPS) was developed. The MEPS technique exhibits a number of advantages over other potential sample clean-up methods (SPE, LLE). The use of MEPS significantly decreases the time required for sample pretreatment (i.e. 15 min vs 45 min for SPE) and also reduces the amount of solvents used. MEPS is very suitable for the analysis of small sample volumes (which is always the case for microdialysis samples), with good recovery and high repeatability. Furthermore, the small quantity of sorbent in the MEPS BIN (4 mg vs 30-100 mg in the case of SPE) will enable easy and effective

washing between samples which reduces the possibility of carry-over. An additional benefit of this sample pretreatment step is that it will increase column longevity.

Since the compounds of interest exhibited both ionic and hydrophobic features, a M1 MEPS cartridge, consisting of 80% C8 and 20% SCX material, was selected for sample clean-up. Prior to MEPS extraction, microdialysis samples were diluted with an aqueous solution in order to adjust the pH of the sample to a more acidic pH. This was necessary to ensure all analytes were ionized and could be adequately retained on the M1-cartridge through (cat)ionic interactions. Different sample diluents were tested for pH adjustment: 5% acetic acid, 5% formic acid, 0.1 M KH_2PO_4 (pH 4.1) and phosphate buffer (pH 2.5). Dilution with 150 µL phosphate buffer (pH 2.5) yielded the best recoveries for all analytes and was therefore selected for further experiments. For analyte elution, the pH of the eluent was increased to pH=11.5 with ammonia to neutralize all compounds. MeOH was additionally added to the eluent to decrease hydrophobic interactions between the analytes and the sorbent. Different eluent solutions were evaluated by varying the concentrations of ammonia and MeOH. Optimal recoveries were obtained with 5% NH_4^+ in 80% MeOH.

Since a relatively high amount of organic modifier was required to elute the MEPS cartridge, direct injection of the MEPS eluate onto the Cap-LC was not possible. It was therefore evaluated whether the sample could be evaporated and reconstituted in mobile phase A. This, however, resulted in high sample variation and low recoveries due to compound degradation during the evaporation and reconstitution step. It was therefore decided to simply dilute the MEPS extract four times with mobile phase (A) in order to decrease the amount of organic modifier. Previous experiments already demonstrated that the percentage of MeOH in the sample solvent could amount up to 20% without affecting the separation of the compounds of interest. Elution with 50 µL of eluent followed by dilution with 150 µL mobile phase (A) resulted in good recoveries for all analytes (> 90 %, see also section 3.4.3) with an acceptable repeatability and this procedure was therefore chosen for the final MEPS protocol. The final MEPS protocol was able to remove all potential interfering compounds, as is demonstrated by the absence of any interfering peaks in the chromatographic trace shown in Fig. 4a. With one MEPS-cartridge, it was possible to perform over 100 extractions without any loss in performance. By rinsing with 4 x 100 µL H_2O and 4 x 100 µL MeOH, no carryover was observed, even at the highest concentration.

3.3. Method validation

3.3.1. Specificity and selectivity

The specificity of a method is the ability to measure and discriminate the analyte from potential interfering compounds, either exogenous or endogenous, in the sample matrix. Chromatograms of blank rat microdialysate, blank rat microdialysate spiked with 20 nM OLA, OLA-O, CPZ and CPZ-O and rat microdialysate after i.p. injection of OLA and CPZ are shown in Fig. 4. The specificity of the method is demonstrated by the good resolution obtained between the peaks of OLA and CPZ and their respective N-oxides, and by the absence of interfering peaks from endogenous compounds at the retention times of OLA, OLA-O, CPZ and CPZ-O.

3.3.2. Linearity and LLOQ

Calibration curves were set up for OLA, OLA-O, CPZ and CPZ-O and good linearity (determination coefficients $R^2 > 0.9950$) was found for all parent compounds and metabolites in the concentration range of 0.5 nM – 250 nM. Linearity parameters for the calibration curves in Ringer's solution are shown in Table 1. The lower limit of quantification (LLOQ) was set to 0.5 nM for all compounds since it was the lowest concentration resulting in a S/N ≥ 10 with an acceptable precision (< 15% for each compound). When microdialysis samples were eluted from the MEPS cartridge, a dilution step (1:4) was required to reduce the amount of MeOH in the extract prior to LC injection. As a result, the lowest concentration that could be detected in actual rat samples was 2 nM instead of 0.5 nM. These concentrations were found to be considerably lower than those obtained in previously reported OLA and CPZ assays using HPLC-UV (ranging between 5 nM and 330 nM) [47,48,50,52,53].

3.3.3. MEPS recovery, accuracy and precision

In order to verify the accuracy of the chromatographic separation method and sample preparation step (MEPS), a recovery study was carried out by spiking Ringer's solution with a low (LLOQ), intermediate and high concentration of analytes. At all concentration levels, satisfactory results were found with recovery values for the different analytes ranging from 90 to 97% (2 nM), 88 to 101% (100 nM) and 91 to 99% (1000 nM) (Table 2). Average recoveries were all between 92% (CPZ-O) and 98% (OLA-O) with acceptable RSD values, demonstrating the excellent extraction capabilities of the used MEPS-protocol.

The overall precision in Ringer's solution, defined by the % RSD, ranged from 2.45% to 8.57% for OLA, from 1.92% to 9.86% for OLA-O, from 4.93% to 13.12% for CPZ and from 4.42% to 13.25% for CPZ-O. Analytical accuracy, expressed as % bias, varied from -11.65 to 9.97% (Table 3). Since all obtained values were beneath the specified limits of 15%, we concluded that the combination of MEPS sample preparation with capillary LC-UV analysis resulted in a good analytical method with satisfactory

accuracy and precision. The MEPS sorbent could be used for the whole lifespan of the study since no clogging of the sorbent was observed and extraction yields of the analytes remained constant (number of samples: ± 120). This is contrary to serum or plasma samples, where it is necessary to discard and replace the MEPS cartridge after 50-100 extractions.

3.3.4. Stability

The stability of OLA, OLA-O, CPZ and CPZ-O in microdialysates was evaluated under different storage conditions (short term stability, long-term stability and freeze-thaw stability) at two concentrations (20 nM and 100 nM) by comparing spiked Ringer's solution after a specific storage condition with freshly prepared standards. The results of the stability study can be found in Table 3. The results demonstrate minimal loss of analytes, therefore OLA, OLA-O, CPZ and CPZ-O can be considered stable in microdialysate samples using the applied storage conditions.

3.4. Method application

To demonstrate the applicability of the developed Cap-LC-UV method, a number of actual rat microdialysate samples were analysed. Samples were collected at different time points after i.p. administration of either OLA (50 $mg.kg^{-1}$), CPZ (200 $mg.kg^{-1}$) or co-administration of OLA and CPZ (25 $mg.kg^{-1}$ and 200 $mg.kg^{-1}$). The concentration of OLA in the co-administration experiment was lower (25 $mg.kg^{-1}$) compared to the single injection of OLA (50 $mg.kg^{-1}$) to avoid overdosing the rats.

The concentration-time profiles for unbound OLA and OLA-O, and CPZ and CPZ-O in brain extracellular fluid (bulbus olfactorius) are shown in Fig. 5a and Fig. 5b, respectively. The developed method is able to detect and quantify both OLA, CPZ and their respective FMO-mediated N-oxides in rat brain microdialysates. It was observed that the concentration of OLA in the rat brain increased until 180 min, and declined thereafter. The concentration of OLA-O on the other hand increased during the entire measurement period. The concentration of CPZ in rat brain increased until 120 min, and subsequently started to decline. The concentration of CPZ-O increased at first and remained more or less constant from 120 min to 240 min. No clear correlation was found between a decline of the parent compound and a similar increase in the resulting N-oxide. Since FMO is also located in other organs in the body (lungs, intestine, liver), it is possible that an important part of the formation of N-oxides does not take place in the brain. This could explain the lack of correlation between the concentrations of the parent compound and metabolite in rat brain. In a follow-up study, longer time periods of micro sampling will be investigated.

Since it is suggested in literature that CPZ can potentially act as a competitive inhibitor for FMO N-oxidation, OLA and CPZ were also co-administered to rats. The concentration-time profiles for all

analytes after co-administration of OLA and CPZ can be found in Fig. 5c (OLA and OLA-O) and 5d (CPZ and CPZ-O). In these rats, a similar concentration-time profile was found for both OLA and OLA-O compared to the rats treated with OLA only. No significant differences in concentration of OLA and OLA-O were found at all investigated time points. Although no clear indication of an *in vivo* inhibitory effect of CPZ on OLA-O formation was observed, this observation should be confirmed by further research using a higher number of rats over a longer time period. It would also be interesting to compare the effect of FMO-mediated N-oxidation for varying concentrations of antipsychotics.

4. Conclusion

It is demonstrated that capillary LC-UV is an interesting technique for the analysis of samples with limited sample volumes when mass spectrometry equipment is not available. By decreasing the i.d. of the column, while maintaining a similar injection volume as on a conventional column, a clear increase in sensitivity can be obtained. This is the first study on the simultaneous determination of OLA, OLA-O, CPZ and CPZ-O in rat brain microdialysates. A capillary LC-UV method in combination with MEPS sample pretreatment was developed and subsequently validated. The MEPS sample pretreatment was relatively cheap, fast and provided good selectivity and reproducibility. The LLOQ of the method (0.5 nM) was sufficiently low to allow the analysis of low concentrated rat brain microdialysates, as demonstrated by the analysis of a number of actual rat brain microdialysates. This assay can be applied for the analysis of OLA, OLA-O, CPZ and CPZ-O in rat brain microdialysate samples collected during clinical and pharmacokinetic studies.

Conflict of Interest Statement

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Figure 1: Structures of chlorpromazine (CPZ) and olanzapine (OLA) and their conversion into chlorpromazine N-oxide (CPZ-O) and olanzapine N-oxide (OLA-O) by flavin-containing monooxygenase (FMO).

Figure 2: UV chromatogram for the separation of a mixture of 20 nM OLA, OLA-O, CPZ and CPZ-O in Ringer's solution. Final chromatographic conditions of the method: UV detection: 254 nm; flow rate 4 µL/min; injection volume 20 µL; temperature 40°C. Mobile phases: (A) 10 mM ammonium acetate with 0.05% triethylamine, pH 3.00 (A); (B) ACN. Gradient program: 10% B (min 0); 10% B (min 3); 16% B (min 12); 31% B (min 12.1); 31% B (min 23); 35% B (min 38); 35% B (min 40); 90% B (min 40.1); 90% B (min 45).

Figure 3: Comparison of the effect of different injection volumes (5 and 20 µL) and different percentages organic modifier (ACN or MeOH) on the peak shape of OLA and OLA-O. For the different injection volumes, different concentrations were used to obtain the same mass loading: 5 µL (80 nM) and 20 µL (20 nM). All samples are dissolved in Ringer's solution with the specified amount of organic modifier. A signal offset of 11-14% was applied to overlay the different chromatograms to increase the clarity of the figure. (A) Comparison of different percentages MeOH for an injection volume of 5 µL. (B) Comparison of different percentages ACN for an injection volume of 5 µL. (C) Comparison of different percentages MeOH for an injection volume of 20 µL. (D) Comparison of different percentages ACN for an injection volume of 20 µL.

Figure 4: Chromatograms obtained after MEPS sample treatment of (a) blank rat microdialysate collected before i.p. drug administration; (b) blank rat microdialysate spiked with OLA, OLA-O, CPZ and CPZ-O (20 nM); (c) microdialysis sample after i.p. administration of OLA (25 $mg.kg^{-1}$) and CPZ (200 $mg.kg^{-1}$) collected at 180 min, resulting in following concentrations: 46.4 nM (OLA); 10.0 nM (OLA-O); 16.5 nM (CPZ); 13.5 nM (CPZ-O). Different time segments in the chromatograms are separated using vertical dashed lines.

Figure 5: Concentration-time profiles for different rat brain (bulbus olfactorius) microdialysate samples. (A) concentration-time profiles of OLA and OLA-O for two rats after i.p. administration of OLA (50 $mg.kg^{-1}$); (B) concentration-time profiles of CPZ and CPZ-O for two rats after i.p. administration of CPZ (200 $mg.kg^{-1}$); (C) concentration-time profiles of OLA and OLA-O for two rats after i.p. co-administration of OLA (25 $mg.kg^{-1}$) and CPZ (200 $mg.kg^{-1}$); (D) concentration-time profiles

of CPZ and CPZ-O for two rats after i.p. co-administration of OLA (25 mg.kg^{-1}) and CPZ (200 mg.kg^{-1}). Concentrations for rat 1 are shown in grey, concentrations for rat 2 in black (all figures).

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Table 1: Linearity parameters for the calibration curves of OLA, OLA-O, CPZ and CPZ-O in Ringer's solution.

Compound	Linearity range (nM)	Equation coefficients (y = ax + b)[A]		R^{2} [B]	LLOQ[C]
		a	b		
OLA	0.5 – 250	0.0461 ± 0.000052	-0.0021 ± 0.00050	0.9954	0.5
OLA-O	0.5 – 250	0.0294 ± 0.000050	-0.0060 ± 0.00048	0.9950	0.5
CPZ	0.5 – 250	0.0261 ± 0.000017	-0.0023 ± 0.00017	0.9985	0.5
CPZ-O	0.5 – 250	0.0317 ± 0.000010	0.0098 ± 0.000097	0.9996	0.5

[A] y = analyte peak area; x= analyte concentration (nM); a and b ± standard deviation

[B] R^2 = determination coefficient

[C] Lower limit of quantification (nM)

Table 2: MEPS recovery, precision and accuracy of OLA, OLA-O, CPZ and CPZ-O in Ringer's solution.

		Trueness		Precision		
Spiked conc. (nM)	Final conc.[A] (nM)	Calculated conc. (nM)	Relative bias (%)	Repeatability (% RSD)	Intermediate precision (% RSD)	MEPS Recovery (%)[B]
OLA						
2 nM	0.5 nM	1.79	-10.52	2.45	8.57	89
100 nM	25 nM	105.29	5.29	4.08	3.53	101
1000 nM	250 nM	1061.83	6.18	4.27	5.85	97
Mean						**96**
OLA-O						
2 nM	0.5 nM	2.18	9.02	3.55	9.86	96
100 nM	25 nM	109.97	9.97	1.92	9.25	100
1000 nM	250 nM	1092.69	9.27	4.53	5.56	99
Mean						**98**
CPZ						
2 nM	0.5 nM	1.77	-11.65	12.86	2.67	97
100 nM	25 nM	89.47	-10.53	11.30	5.85	92
1000 nM	250 nM	1082.97	8.30	4.93	13.12	91
Mean						**93**
CPZ-O						
2 nM	0.5 nM	1.80	-10.21	8.40	8.44	96

100 nM	25 nM	91.71	-8.29	13.25	4.06	88
1000 nM	250 nM	990.63	-0.94	4.42	5.95	92
Mean						**92**

RSD %: relative standard deviation in %.

conc. = concentration

[A] Final concentration: obtained after 4 x dilution of the MEPS eluate, before injection on the column
[B] Recovery MEPS (%): ratio of peak areas after MEPS and peak areas from standard solutions with the same concentration prepared in Ringer's solution.

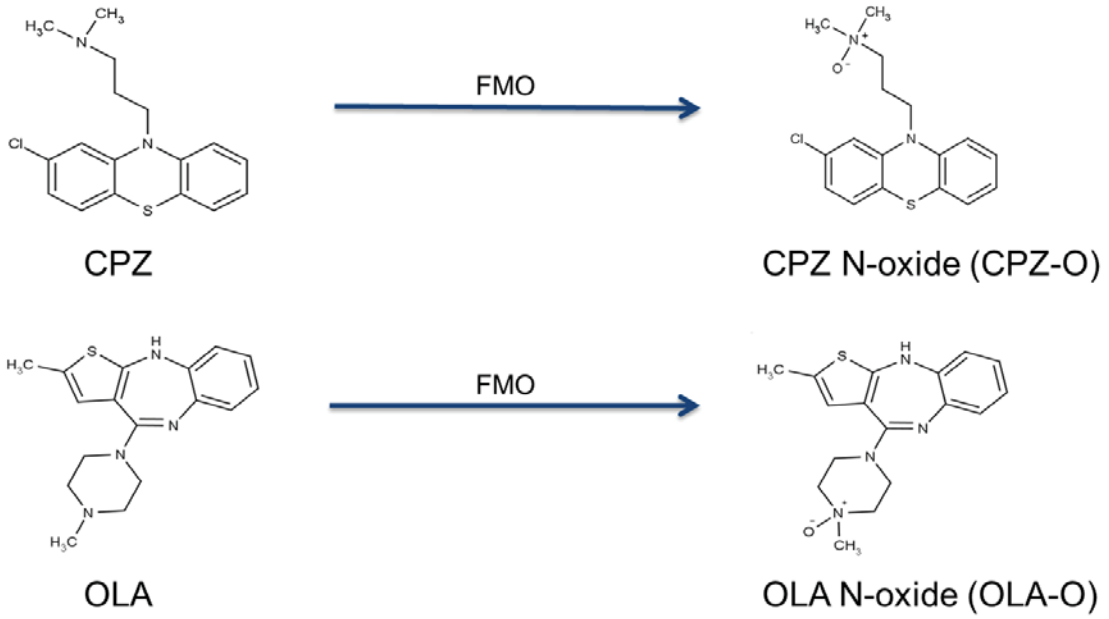
Table 3: Short-term, long-term and freeze/thaw stability of OLA, OLA-O, CPZ and CPZ-O in Ringer's solution (n=3).

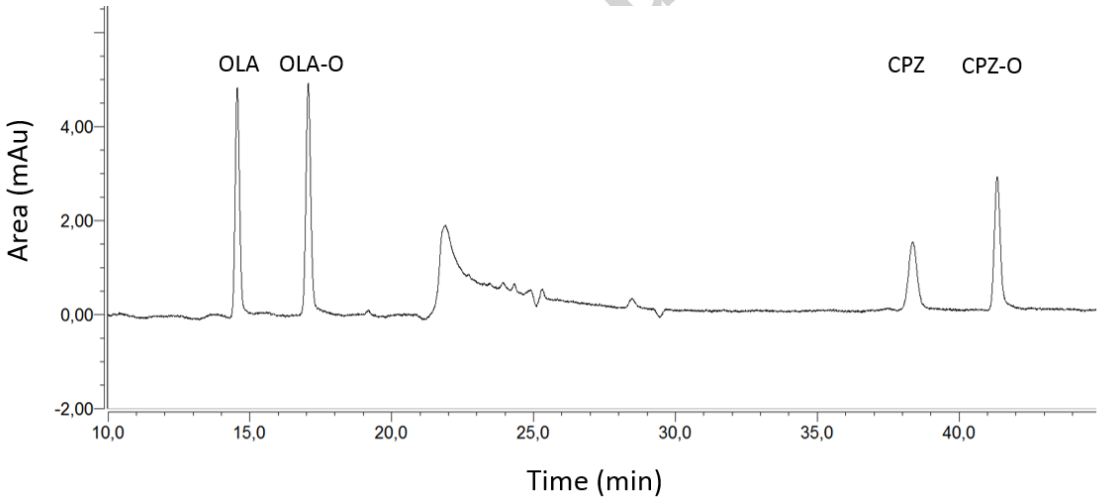
	Short-term stability (24 h, 5°C)		Long-term stability (20 days, - 80 °C)		Freeze-thaw stability	
	% remaining	RSD %	% remaining	RSD %	% remaining	RSD %
OLA						
20 nM	97.36	2.18	99.91	4.50	99.29	4.50
100 nM	96.78	1.08	99.96	0.41	98.23	2.13
OLA-O						
20 nM	98.93	0.91	99.27	1.94	98.90	1.94
100 nM	99.98	1.10	99.19	0.61	98.93	0.11
CPZ						
20 nM	99.32	1.19	99.64	7.18	93.73	12.88
100 nM	94.51	7.34	98.58	1.18	97.12	1.54
CPZ-O						
20 nM	99.51	1.33	100.77	1.47	99.54	1.47
100 nM	98.09	1.13	97.50	1.08	99.94	1.31

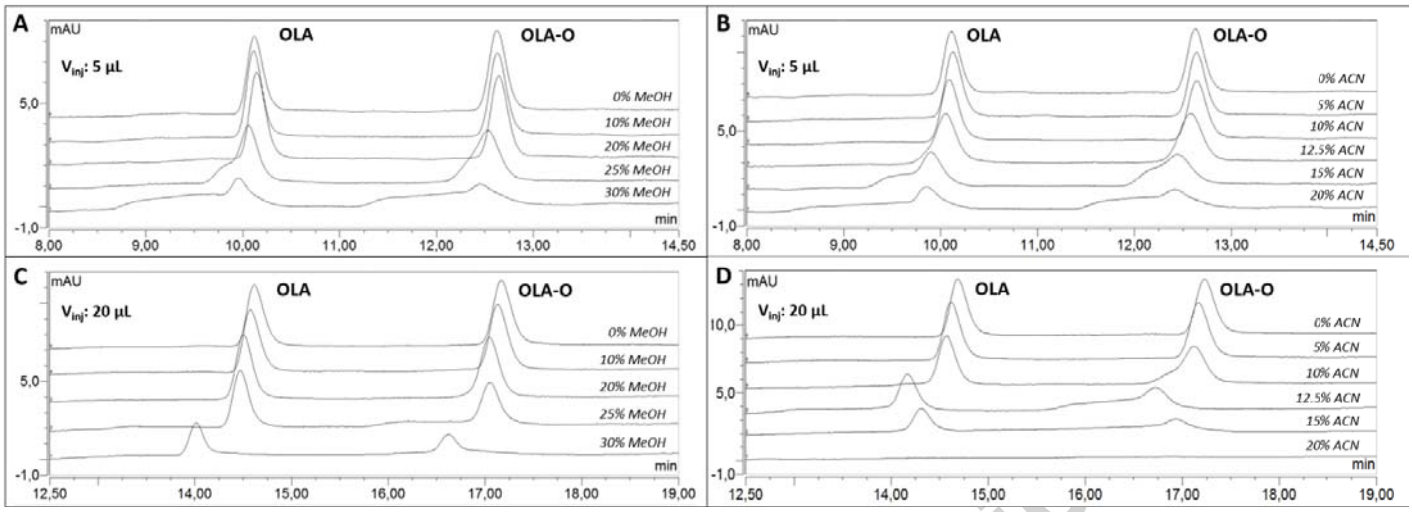
RSD %: relative standard deviation in %. % remaining = (peak area analyte after storage condition/peak area of freshly prepared solution of analyte) x 100.

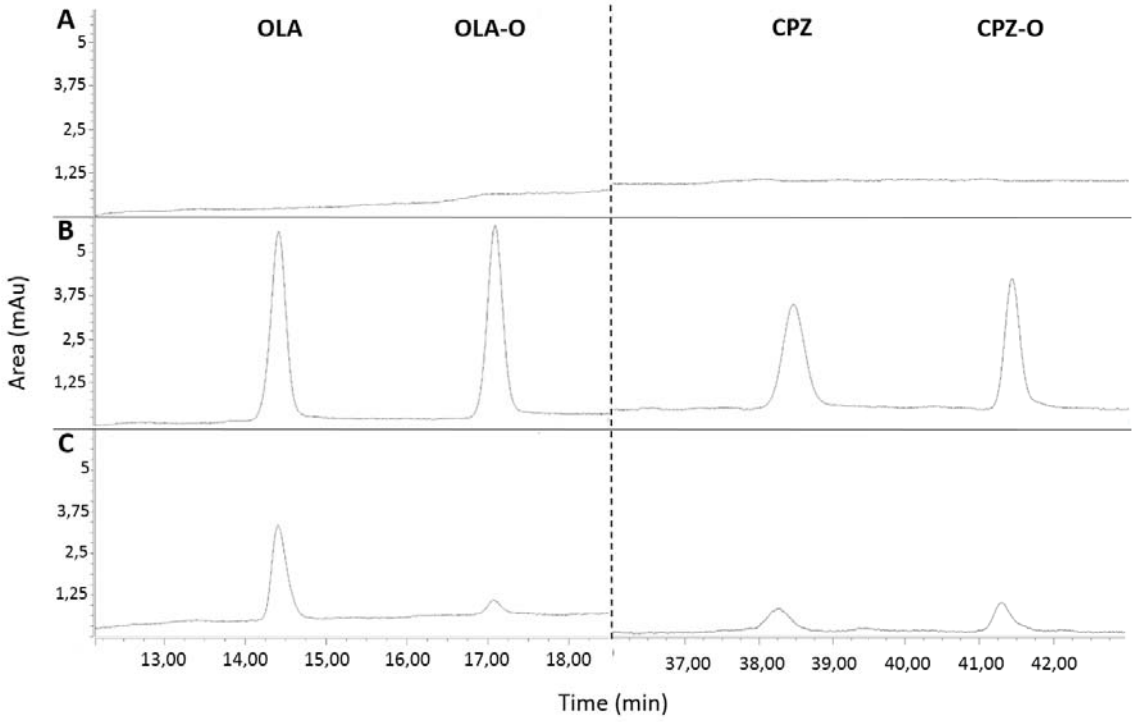
Highlights

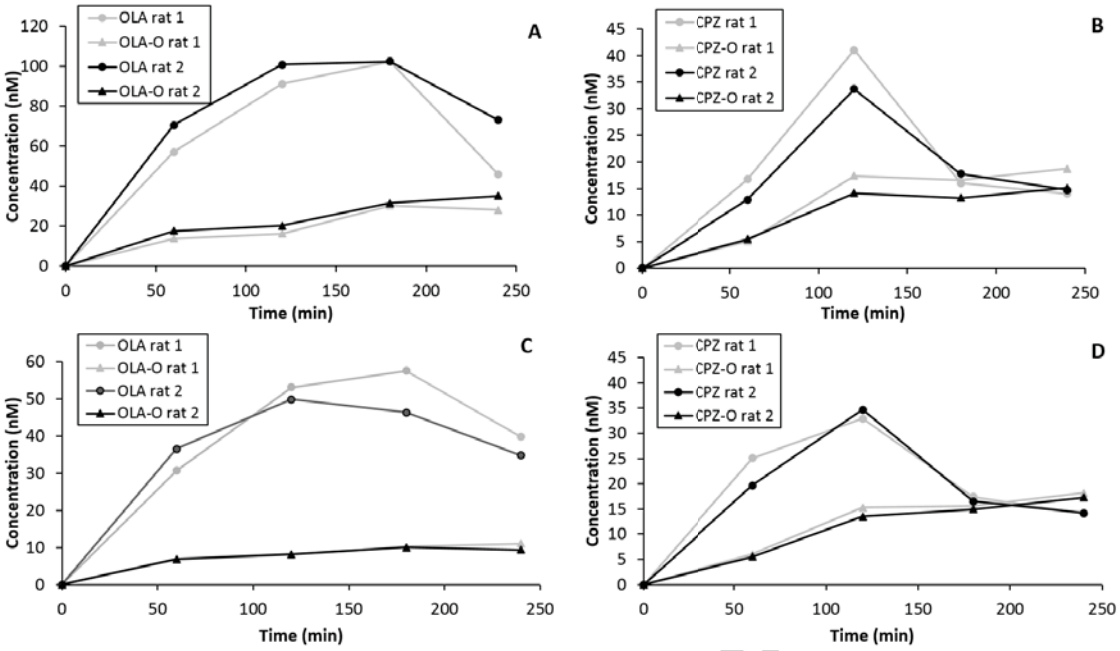
- A sensitive and quantitative method for OLA, CPZ and their N-oxides is developed
- The method is based on capillary UHPLC-UV with MEPS sample preparation
- LLOQ's of 0.5 nM are obtained for all compounds of interest
- Validation of the method is performed according to bioanalytical guidelines
- Applicability of the method is demonstrated on rat brain microdialysates


FMO
CPZ
CPZ N-oxide (CPZ-O)
FMO
OLA
OLA N-oxide (OLA-O)

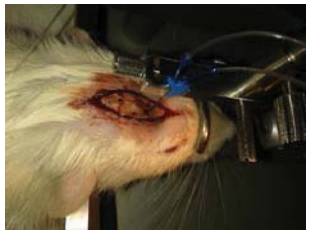

OLA
OLA-O
CPZ
CPZ-O
Area (mAu)
4,00
2,00
0,00
-2,00
10,0
15,0
20,0
25,0
30,0
35,0
40,0
Time (min)


A
mAU
OLA
OLA-O
V_{inj}: 5 µL
0% MeOH
10% MeOH
20% MeOH
25% MeOH
30% MeOH
min
B
mAU
OLA
OLA-O
V_{inj}: 5 µL
0% ACN
5% ACN
10% ACN
12.5% ACN
15% ACN
20% ACN
min
C
mAU
OLA
OLA-O
V_{inj}: 20 µL
0% MeOH
10% MeOH
20% MeOH
25% MeOH
30% MeOH
min
D
mAU
OLA
OLA-O
V_{inj}: 20 µL
0% ACN
5% ACN
10% ACN
12.5% ACN
15% ACN
20% ACN
min


A
OLA
OLA-O
CPZ
CPZ-O
B
C
Area (mAu)
Time (min)



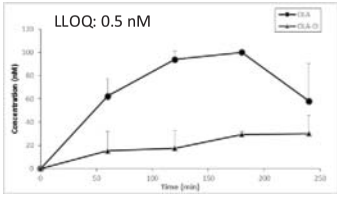
A sensitive capillary LC-UV method for the simultaneous determination of olanzapine, chlorpromazine and their FMO-mediated N-oxidation products in brain microdialysates



Rat microdialysis sampling

MEPS sample pretreatment

Capillary LC-UV



Pharmacokinetic profile