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Chiral Analysis of Methadone and its Main Metabolite, EDDP, in Postmortem Brain and Blood by Automated SPE and Liquid Chromatography–Mass Spectrometry

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We developed a method based on liquid chromatography coupled with tandem mass spectrometry to quantify individual enantiomers of methadone and its primary metabolite, R/S-2-ethyl-1,5-dimethyl-3,3-diphenylpyrrolinium (EDDP), in postmortem blood and brain tissue. Samples were prepared with a Tecan Evo robotic system. Precipitation was followed by solid-phase extraction, evaporation and reconstitution in the mobile phase. Enantiomers were fully separated with liquid chromatography on a chiral $\alpha(1)$ -acid glycoprotein column. A Quattro micro mass spectrometer was used for detection in the positive ion mode with an electrospray source. The lower limit of quantification in brain tissue was 0.005 mg/kg for methadone and 0.001 mg/kg for EDDP enantiomers; the maximum precision was 17% for both compounds; accuracy ranged from 94 to 101%. In blood, the limit of quantification was 0.001 mg/kg for all compounds, the total relative standard deviation was <15%, and the accuracy varied from 95 to 109%. Brain ($n = 11$) and blood ($n = 15$) samples were analyzed with intermediate precision that varied from 7.5 to 15% at 0.005 mg/kg and from 6.8 to 11.3% at 0.25 mg/kg for all compounds. Method development focused on producing a clean extract, particularly from brain samples. The method was tested on authentic brain and femoral blood samples.

Introduction

Methadone is the primary drug used in maintenance treatment of individuals with drug dependence in Denmark; in addition, it is the most common cause of death in poisoning cases among drug addicts (1, 2). Methadone has increasingly been used as a chronic pain medication, and fatal poisonings have also occurred in that context (3). In most countries, methadone is administered as a racemic mixture of R- and S-enantiomers, but the R-form is responsible for the major part of the drug effect (4–6).

Methadone provides analgesic and euphoric effects by stimulating the μ - and δ -opioid receptors in the central nervous system (4). It is excreted by the kidneys, and it is primarily metabolized to the inactive metabolite, R/S-2-ethyl-1,5-dimethyl-3,3-diphenylpyrrolinium (EDDP), which is observed in considerably lower concentrations than methadone in blood (7, 8). Recent studies have described methods for chiral separation of the two enantiomers (9–23); however, methadone is commonly measured with achiral methods in routine settings. A variety of methods have also been published for measuring methadone and EDDP in various types of media, including adipose matrices, brain tissue, breast milk and larva (24–26); however, to our knowledge, only one of those methods separated the enantiomers (24).

The active form of methadone can be identified and quantified in the target organ by analyzing brain samples. The protected position of the brain and its low metabolic activity may

delay postmortem putrefaction and decomposition processes. Thus, the brain matrix offers an advantage over other postmortem specimens (27, 28), because postmortem and perimortem methadone concentrations in brain may be less influenced by the time between death and analysis than methadone concentrations in other tissue types.

We previously developed a chiral method for quantifying methadone in postmortem blood samples based on liquid–liquid extraction (29). Attempts to apply this method to brain samples resulted in large variations in retention time (R_t) on the $\alpha(1)$ -acid glycoprotein (AGP) chiral column over a limited number of injections, and restitution of the column required a long flushing time. The aim of this work was to produce a reliable chiral method for quantifying both methadone and the metabolite, which may occur in low concentrations. We focused on the development of a solid-phase extraction (SPE) procedure that provided clean extracts to ensure stable chromatography.

Apparatus

Sample preparation, including SPE, was automatically performed on a Tecan Freedom Evo 150 robotic platform (Tecan, Männedorf, Switzerland) equipped with the following modules: a Tecan liquid handling arm with eight independent pipetting channels that used disposable filter tips; a Tecan robot manipulator arm for plate movements, a Tecan Te-Vacs vacuum station for SPE; a Tecan Te-Shake orbital plateshaker, a Mettler Toledo XP203S balance for weighing samples (Mettler Toledo, Switzerland), a Porvair Ultravap evaporator (Norfolk, UK), and a FluidX Xat-384 automated thermal sealer (Cheshire, UK). All modules were programmed and controlled with Tecan Evoware 2.3 SP3. The platform setup was similar to that presented by Andersen *et al.* (30), with a few modifications due to a difference in the size of the platform. The platform used during validation was a 150-cm model, and therefore, it did not include an on-system centrifuge; instead, a Centrifuge 5804 R (Eppendorf, Hamburg, Germany) was used. The SPE column was a Strata-X-C 33u polymeric strong cation 96-well plate with 30 mg/well (Phenomenex, Torrance, CA). Before analysis, brain samples were homogenized on a Gentlemacs Dissociator from Miltenyi Biotec (Bergisch Gladbach, Germany).

A chiral AGP guard column (10 \times 4.0 mm i.d., 5 μ m) and a chiral AGP analytical column (100 \times 4.0 mm i.d., 5 μ m) from Supelco (Bellefonte, PA) were used in series for separation on the liquid chromatography (LC) system. The LC system consisted of an 1100 series high-performance liquid chromatography (HPLC) system from Agilent Technologies (Waldbronn, Germany). It was equipped with a binary pump, an auto

sampler and a temperature-regulated column compartment. A Quattro micro (tandem quadrupole mass spectrometer, MS-MS) from Waters (Manchester, UK) was coupled to the HPLC system. Data were acquired in the positive ion mode with an electrospray ionization (ESI) source using MassLynx software 4.1. Quantification was based on ion chromatograms extracted with QuanLynx.

Chemicals

Racemic (*rac*) mixtures of *rac*-methadone (HCl salt, 99.99%), *rac*-EDDP (HClO₄ salt, 99.99%), and *rac*-EDDP-D₃ (ClO₄ salt, 99%) were purchased from Lipomed (Arlesheim, Switzerland); *rac*-methadone-D₃ (99%) was obtained from Cerilliant (Round Rock, TX). R-methadone (HCl salt, 99.9%) was acquired from Sanofi-Aventis (Frankfurt, Germany). Organic solvents were HPLC or liquid chromatography–mass spectrometry (LC–MS) grade from Fischer (Leicestershire, UK), and formic acid (~98%) was from Sigma-Aldrich (Steinheim, Germany). Analytic-grade hydrochloric acid (fuming 37%), ammonium solvent (25%), ammonium acetate, potassium dihydrogenphosphate, and sodium hydroxide were purchased from Merck (Darmstadt, Germany). All experiments included water that was purified with the Millipore Synergy UV water purification system (Millipore A/S, Copenhagen, Denmark).

Distinguishing between R- and S-enantiomers of EDDP was performed by injecting R-methadone extracts treated with the relevant enzyme to produce a detectable amount of R-EDDP (17). These enzymes included baculovirus-infected insect cell microsomes containing the cDNA-expressed P450 CYP isoenzymes (2B6, 2D6 and 3A4), UltraPool human liver microsomes (HLM) 150, pooled human liver S9, and NADPH regeneration system solutions A and B, all purchased from BD Biosciences (Woburn, MA).

Controls and standards

We prepared standard stock solutions of 1 mg/mL *rac*-methadone in methanol (MeOH), 1 mg/mL *rac*-EDDP in MeOH, 0.1 mg/mL methadone-D₃ in MeOH and 0.1 mg/mL EDDP-D₃ in MeOH. A mixed calibration standard solution of 5 mg/L R-methadone, S-methadone, R-EDDP and S-EDDP was made by diluting the stock solution with 50:50 v/v MeOH–water. The internal standard (IS) work solution was prepared in 50:50 v/v MeOH–water. This was used to dilute the stock solutions described previously to a 0.5 mg/L concentration of each methadone-D₃ and EDDP-D₃ enantiomer.

Control blood samples were prepared with blood obtained from the local blood bank. Control brain samples were prepared with a pooled brain homogenate of both grey and white matter from negative samples. Blood and brain quality controls (QC) were prepared by spiking a known mass of sample into low (0.005 mg/kg) and high (0.25 mg/kg) levels of each methadone and EDDP enantiomer. QC samples were stored at –80°C. Control samples were included in every run.

Method development

During the method development phase, the two primary priorities were to achieve a highly clean final extract (to enable stable

chromatography) and the highest possible recovery of EDDP (EDDP concentrations tend to be low in authentic samples).

First, we precipitated with MeOH, acetonitrile (ACN)–H₂O, and ACN, then we tested extracting with phosphate buffer (0.1M, pH 6), with or without SPE. We also tested the buffer extraction with a precipitation step after reconstitution with hexane. The precipitation quality was evaluated based on the clarity of the supernatant after centrifugation and the degree of ion suppression during injection of the final reconstituted extracts. The general extent of ion suppression was examined on a C18 column. This allowed the application of an organic phase gradient up to 100% (data not shown). We investigated ion suppression separately in the final analysis on the chiral column.

Recovery of methadone and EDDP was compared on different SPE columns (HLB, HXC and Strata-X-C). We tested different organic solvents to wash the SPE column, including MeOH, ACN, 50:50 v/v ACN–H₂O and 80:20 v/v toluene–ethylacetate. The quality of each wash was evaluated based on the loss of analytes and the peak signals measured at 184 > 184 *m/z* and 104 > 104 *m/z*, which are commonly used to measure phospholipids (31).

The release of analytes from the SPE column was examined with two different elution solvents, including 5%, 25% NH₃-solution in MeOH (MeOH–NH₃, suggested by the manufacturer) and a 4%, 25% NH₃-solution in ACN (ACN–NH₃). The SPE columns were loaded with analyte-fortified brain ACN supernatants and eluted in singles with volumes of 100, 200, 300, 400, 500, 600, 700 and 900 µL MeOH–NH₃ or ACN–NH₃, respectively; IS was added before evaporation. The analyte profiles at each volume of eluate were used as an expression of recovery.

For LC separation of the compounds, we used a gradient of 2-propanol in 15 mM ammonium acetate buffer with 0.01% formic acid (pH 5.3).

Validation

The LC–MS–MS method was validated according to Peters *et al.* (32) and in-house procedures. The validation parameters included selectivity, ion suppression, matrix effect, recovery, extract stability, linearity, limit of detection (LOD), lower limit of quantification (LLOQ), higher limit of quantification (HLOQ), accuracy, precision and carry-over.

Selectivity was examined by injecting 2 mg/L of standard solutions that contained acetyl-methadol, nicotine, caffeine, tetrahydrocannabinol (THC), THC-acid, 62 other common drugs of abuse, benzodiazepines and metabolites, basic drugs and metabolites, acidic or neutral drugs, and the methadone metabolites, nor-methadone and R/S-2-ethyl-5-methyl-3,3-diphenylpyrrolone (EDMP). The examined drugs are listed in Table 1.

Ion suppression was examined by constantly injecting analytes through a T-piece at 0.1 ng/min, and simultaneously injecting various extracts from negative cases or solutions with co-eluting drugs. Extracts from femoral blood (*n* = 5), white brain matter (*n* = 1), grey brain matter (*n* = 1), a mixture of white and grey brain matter (*n* = 6) and a blank mobile phase were injected together with a standard that contained the four compounds of interest. Matrix effects were examined by first

Table I

Drugs and Metabolites Examined for Interference During the Selectivity Experiment, Listed Alphabetically

6-Monoacetylmorphine	Methadone, nor-
3,4-Methylenedioxyamphetamine	Methamphetamine
3,4-Methylenedioxyamphetamine	Methotrimeprazine
Alprazolam	Methylenedioxyethylamphetamine
Amitriptyline	Methylphenidate
amphetamine	Metoclopramide
Benzoylcegonine	Metoprolol
Bromazepam	Mianserine
Buprenorphine	Mirtazapine
Buprenorphine, nor-	Morphine
Caffeine	Nicotine
Chlordiazepoxide	Nitrazepam
Chlorprothixen	Nitrazepam, 7-amino
Chlorprothixen, dem-	Norfluoxetine
Citalopam, dem-	Nortriptyline
Citalopram	Orphenadrine
Clonazepam	Orphenadrine, dem-
Clonazepam, 7-amino	Oxazepam
Clozapine	Oxycodone
Cocaine	Paroxetine
Codeine	Promethazine
Diazepam	Propranolol
EDDP	Quetiapine
EMDP	Sertraline
Evo- α -acetylmethadol	THC
Flunitrazepam	THC-acid
Flunitrazepam, 7-amino	Tramadol
Fluoxetine	Tramadol- <i>o</i> -desmethyl
Fluoxetine, nor-	Triazolam
Ketamine	Venlafaxine
Ketobemidone	Zaleplon
Lamotrigine	Zolpidem
Lidocaine	Zopiclone
Lorazepam	Zuclopenthixol
Methadone	

evaluating the peak area of extracts that had been fortified with analyte (0.04 mg/L) after extraction and then comparing with the peak areas of injected pure standards. Seven brain extracts and eight blood extracts were examined. Extraction recoveries were determined by comparing peak areas of samples fortified before and after the precipitation and SPE steps.

The linearity of the four compounds in brain tissue was evaluated based on seven levels of fortification (ranging from 0.0005–2.00 mg/kg) in brain homogenates, each level performed in triplicate, nine levels of fortification (ranging from 0.0001–2.00 mg/kg) in blood performed in triplicate, and blanks.

Accuracy, precision, LLOQ and HLOQ were assessed on the basis of quadruple determinations of seven fortified blood samples (ranging from 0.0005–2.0 mg/kg) and six fortified brain samples (ranging from 0.001–2.0 mg/kg) performed on three different days. The controls were fortified as a pool, portioned out, and stored at -80°C until analysis.

Final analytical procedure

Sample preparation

Samples were postmortem femoral blood and frontal lobe brain tissue obtained from an autopsy. Femoral blood samples were stabilized with 10 mg sodium fluoride.

For brain analysis, 2–4 g of grey matter was obtained from a frontal lobe sample that had been stored at -20°C between

Table II

Gradient Used on the LC–MS–MS System*

Time (min)	A (%)
0.0	5
7.5	5
8.5	12
13.0	12
13.1 [†]	20
19.0	20
19.1	5
26.0	5

*Note: Solvent A, 2-propanol; solvent B, 15 mM ammonium acetate with 0.01% formic acid (pH = 5.3).

[†]This gradient step has a rinsing function necessary during brain sample injection.

autopsy and sampling. The grey matter was homogenized undiluted at room temperature and stored at -80°C in aliquots of 0.5–1 g until analysis. Calibration samples based on triple measurements of a blank matrix and 0.5 mg/kg of a matrix fortified with each enantiomer was included in each run. With a positive displacement pipette, brain samples were transferred manually to a scale and weights were recorded. For blood samples, 200 μL of each sample was weighed during automated transfer to a 2-mL, 96-well plate placed on the balance; the weights were recorded automatically. A volume of 20 μL IS solution was added to each well, followed by precipitation of the samples with 900 μL ACN added slowly over 3 min during orbital shaking of the plate. The plate was centrifuged for 5 min at 3,600 rpm, while the SPE columns were preconditioned and equilibrated with 900 μL MeOH and 900 μL water. After centrifugation, 900 μL supernatant was transferred to the preconditioned SPE columns together with 100 μL 0.1M phosphate buffer (adjusted to pH 6 with NaOH). Columns were consecutively washed with 900 μL 0.1M HCl solution, $2 \times 900 \mu\text{L}$ MeOH, and 100 μL freshly prepared ACN containing 4% v/v 25% NH_3 solution. Next, 20 μL 2% formic acid in water was added to the collection wells, followed by elution with 400 μL freshly prepared ACN containing 4% v/v 25% NH_3 solution. The eluate was evaporated to dryness with 35°C N_2 and reconstituted with 3 min of orbital shaking in 100 μL mobile phase (MP, 20% 2-propanol in 15 mM ammonium acetate, 0.01% formic acid, pH 5.3).

LC–MS–MS analysis

A gradient of 2-propanol and freshly prepared 15 mM ammonium acetate buffer with 0.01% formic acid (pH 5.3) was applied to the column for complete separation of the methadone and EDDP enantiomers (Table II). The gradient started with 5% 2-propanol for 7.5 min, followed by an increase to 12% over 1 min, and then it was held at 12% for 4.5 min before implementing a rapid increase to 20% over 0.1 min; then, the 20% 2-propanol was held until reaching a total run time of 19 min; next, the gradient was decreased over 0.1 min to 5%; after 26 min, the system was fully stabilized and ready for a new injection. The flow was set to 0.400 mL/min, and 10 μL of reconstituted eluate was injected into the LC–MS–MS system. The column temperature was 25°C . MS conditions were optimized by running a large variety of cone and collision energies during the injection of a system control. We selected the

Table III

Chromatography and MS-MS Conditions of the Method*

	R _t (min)	Cone (V)	Collision 1 (V)	Transition 1 MRM	Collision 2 (V)	Transition 2 MRM	Ion Ratio MRM2/MRM1
R-Methadone	12.32	26	14	310.1 > 265.1	26	310.1 > 105.1	2.98
R-Methadone-D3	12.25	25	15	313.15 > 268.1			
S-Methadone	14.48	26	14	310.1 > 265.1	26	310.1 > 105.1	2.98
S-Methadone-D3	14.58	25	15	313.15 > 268.1			
R-EDDP	8.63	45	30	278.1 > 234.1	24	278.1 > 249.15	2.08
R-EDDP-D3	8.58	43	30	281.1 > 234.1			
S-EDDP	10.63	45	30	278.1 > 234.1	24	278.1 > 249.15	2.08
S-EDDP-D3	10.55	43	30	281.1 > 234.1			

*Note: Positive ion mode was used.

parameters that provided the highest stable signal (Table III). The source and desolvation temperatures were 120 and 400°C, respectively. The calibration curves comprised the peak analyte area/peak IS area versus concentration.

The AGP column was washed with 25% 2-propanol at 30°C for 30 min at the end of each series. The LC-MS-MS system without the AGP column was washed with pure 2-propanol after every 500 injections; this resulted in more stable chromatography over time.

Results

Method optimization

The ACN precipitation resulted in the highest clarity of supernatant, and ion suppression was very low when combined with SPE. Therefore, we selected this procedure.

All of the examined SPE columns demonstrated good methadone recovery. The Strata-X-C column was chosen for further development, because it provided the best EDDP recovery (data not shown). Both the 80:20 v/v toluene-ethylacetate and the 50:50 v/v ACN-H₂O washing solutions reduced the analyte recovery and had little effect on the presence of phospholipids. Both ACN and MeOH efficiently removed phospholipids, and washing with 1,800 µL was more effective than washing with 900 µL. Washing with ACN reduced the EDDP recovery, and therefore MeOH was selected as the organic wash for the SPE column. An additional wash with 0.1M HCl did not reduce recovery.

When MeOH-NH₃ was used for elution, both methadone and EDDP were observed in the first 100 µL, and they were fully eluted after 300 and 700 µL, respectively (Figure 1). When ACN-NH₃ was applied, neither compound was observed in the first 100 µL, and both were fully eluted after 300 µL. During the MeOH-NH₃ elution, a large increase in phospholipids was observed over the first 500 µL; in contrast, with the ACN-NH₃ elution, only a weak increase was observed after the first 100 µL. We chose to use the ACN-NH₃ elution, and we decided not to collect the first 100 µL (which made this a small “wash step”); instead, we only collected the next 400 µL.

The necessity of adding formic acid to the eluate before evaporation was evaluated based on the final recovery of methadone and EDDP. Without formic acid, EDDP was unstable, and the compound was almost completely lost when the eluate was left overnight before evaporation. Adding 20 µL of 2% formic acid to the collection tray before elution resulted in stable recovery of the compounds (data not shown).

For LC, a slow gradient increase from 5 to 12% of 2-propanol was necessary to ensure stable ionization. This slow increase resulted in a slightly longer run time, but provided a more stable signal for R-methadone, which eluted during the gradient step. A 20% 2-propanol step was included in the gradient to clean the column before the next injection. The highest recommended organic percentage (25%) was applied to the column at the end of each run at 30°C for 30 min; this ensured good retention time stability and long column life. Retention time varied slightly between runs, depending on the exact pH of the ammonium acetate buffer; however, within each run, variations in retention time did not exceed ±0.1 min among 70 injections, which included 48 brain extracts.

Validation

The selectivity study did not reveal any interfering peaks in the *m/z* tracks monitored for enantiomers of methadone and EDDP. No ion suppression was observed for the mobile phase or the extracts. A representative example is shown in Figure 2.

The matrix effects of the samples were nearly identical for the IS and analytes. The respective matrix effects for R-methadone and S-methadone were 5.8 ± 0.5% and 4.3 ± 0.8% in brain, and 8.4 ± 9.3% and 6.6 ± 10.3% in blood. For R-EDDP and S-EDDP, the respective matrix effects were 10.7 ± 0.8% and 11.4 ± 0.6% in brain, and -8.8 ± 10.0% and -9.6 ± 11.2% in blood.

The methadone extraction recoveries were 67 and 78% in brain and blood, respectively. The EDDP extraction recovery was, on average, 62% for both brain and blood. Due to the lack of complete ACN supernatant transfer and the matrix effects, in routine analyses, the total signal recoveries were, on average, 55% in brain and 53% in blood for EDDP, and 64% in brain and 62% in blood for methadone.

The linearity results are given in Table IV. Non-linearity was observed for methadone above 1 mg/kg, but EDDP did not exhibit non-linearity over the examined range. However, an intercept setoff was observed for the two EDDP enantiomers, due to low EDDP levels in the IS. Thus, a two-point calibration line was used (0 and 0.5 mg/kg). Five of the calibrators examined were as a minimum between the final LLOQ and HLOQ.

Accuracy and precision data are presented in Table V. Precision is presented as the total relative standard deviation (%RSD) over all 12 measurements; accuracy is based on the theoretically calculated fortified values. The chosen criteria included the maximum %RSD, set to 20% at LLOQ and 15% at

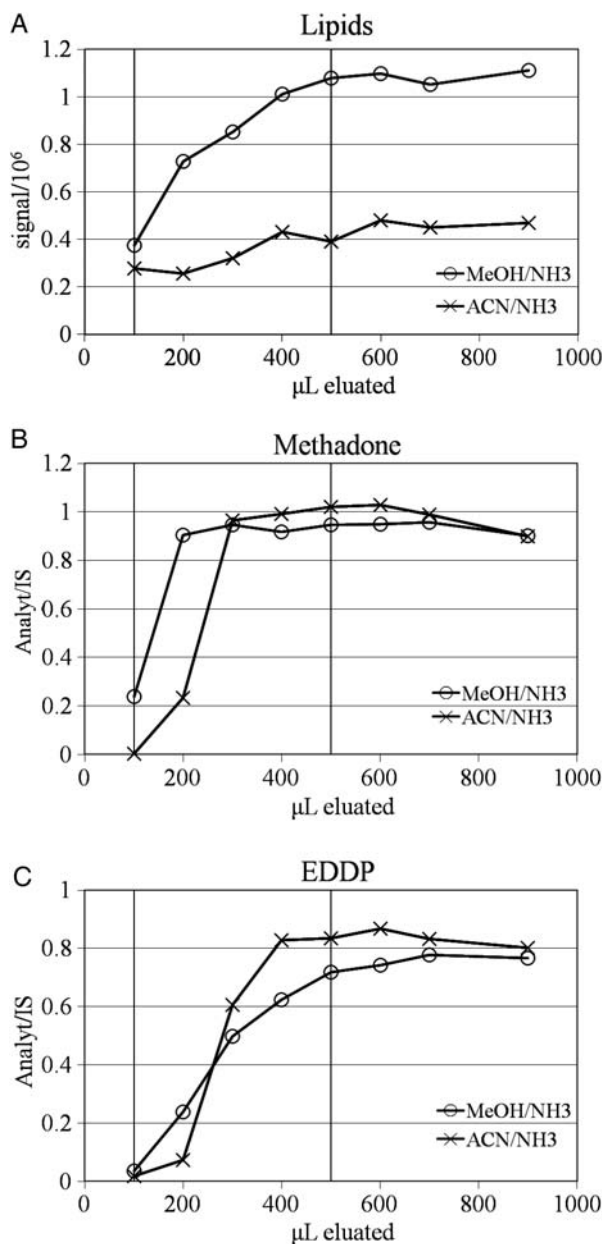


Figure 1. Response of EDDP or methadone in collected volumes during elution with 5% 25% NH₃ solution in methanol (MeOH–NH₃) (A); 4% 25% NH₃ solution in ACN (ACN–NH₃) (B); total signal for all peaks in 104 > 104 *m/z* chromatogram (C). Volumes collected of the ACN–NH₃ eluate in the final method are indicated with the two black lines.

levels above LLOQ, and maximum accuracy, set to 80–120% at LLOQ and 85–115% at levels above LLOQ. For brain tissue, a %RSD up to 17% over LLOQ was acceptable at some levels due to the complexity of the matrix. In brain tissue, the LLOQ was 0.005 mg/kg for the methadone enantiomers and 0.001 mg/kg for the EDDP enantiomers. In blood, the LLOQ was 0.001 mg/L for all analytes. Based on the non-linearity observed previously for methadone enantiomers (linear up to 1 mg/kg, but low accuracy at 2 mg/kg), the HLOQ was considered to be 1 mg/kg for both methadone enantiomers. Samples with higher concentrations were diluted and reanalyzed. The HLOQ for EDDP enantiomers

was 2 mg/kg in both matrices. The LOD was 0.0005 mg/kg in blood and brain for all analytes (based on three times the standard deviation at LOQ). Intermediate precision was observed for blood and brain samples tested over 15 and 11 days, respectively. Table VI presents precision measurements for the two QC levels, 0.005 and 0.25 mg/kg (6.8–15%). The ion ratio deviated less than ± 15% at 0.0005 mg/kg for all compounds over the 12 precision and accuracy measurements.

Stability of fortified blood samples was examined at high and low levels. Over three thaw/freeze cycles, both methadone and EDDP concentrations fell by 20–25%. In addition, the metabolite, EDMP, was detected in the sample. Brain tissue extracts from the first day of the precision and accuracy experiments were reanalyzed after seven days at 20°C, 10 days at –20°C, or 163 days at –20°C. We found no relevant differences compared to a fresh system control. Carry-over was examined by injecting 2 mg/L standards, followed by blank mobile phase. We observed 0.01–0.03% carry-over for all four compounds.

Application

The described method was applied to authentic brain and femoral blood samples. The sum of methadone enantiomer concentrations was compared with achiral measurements from 68 femoral blood samples that had been completed earlier using an accredited in-house method (33). A correlation of $R^2 = 0.93$ was observed (Figure 3). When the results from diluted samples (dilution factors between 2 and 10) were compared to those from undiluted samples, a correlation of $R^2 = 0.996$ was observed for the blood samples, and a correlation of $R^2 = 0.993$ for the brain samples.

In 11 authentic cases, we compared concentrations of white and grey brain matter, and the ratios of compound concentration in grey matter to that in white matter were 1.4 ± 0.5 for R-EDDP, 1.6 ± 0.6 for S-EDDP, and 0.7 ± 0.2 for both methadone enantiomers. White matter had to be diluted before extraction, because otherwise, its consistency resulted in poor precipitation. Therefore, grey matter alone was used in further studies. Table VIII shows brain and femoral blood measurements from six authentic cases.

The same AGP column was used during method development, validation and analysis of brain samples. The column showed no sign of wear through approximately 1,000 brain extract injections. However, the front column was changed several times during this period. Chromatography was stable among different columns bought from the same manufacturer.

Discussion

Several methods have been published for chiral determination of methadone alone or in combination with EDDP and EDMP in various neat sample types, including serum, plasma and urine (9–13,17,18,20–23). Most methods applied liquid–liquid extraction (9,11,12,14,15,18,20–23,29), and more recent methods included LC–MS (10, 11, 16, 18, 20) or LC–MS–MS detection (12–14,29). By quantifying the R-methadone and S-methadone enantiomers separately, it is possible to perform a toxicological interpretation of the active R-enantiomer alone. Only very few publications contain data regarding the separate

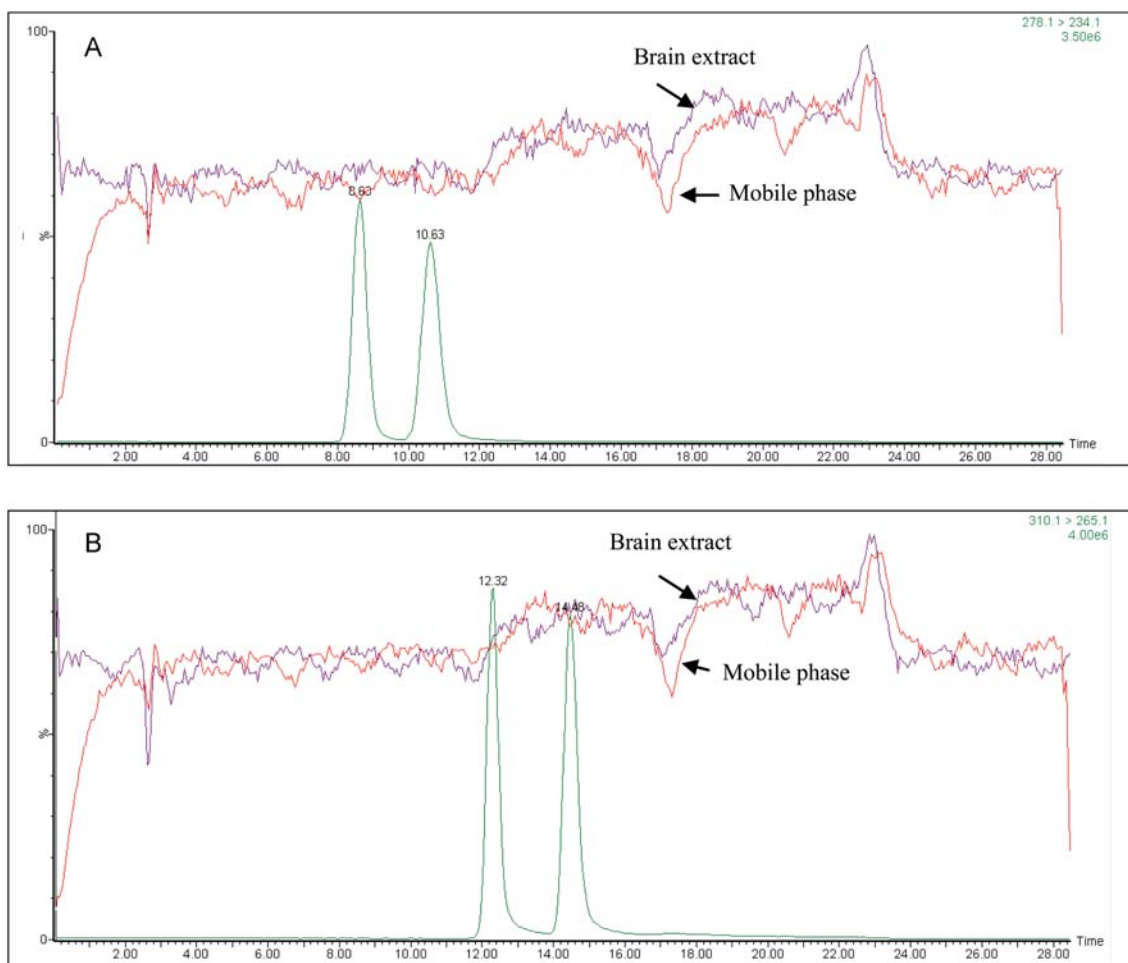


Figure 2. Ion suppression examination of the quantification tracks for EDDP and methadone: mobile phase and brain extract injected during 1 µg/min EDDP (A); methadone (B) compared to 0.1 mg/L injection.

Table IV

Linearity Properties for Methadone and EDDP in Blood and Brain*

	Methadone		EDDP	
	Blood	Brain	Blood	Brain
Range LLOQ–HLOQ (mg/kg)	0.001–1	0.005–1	0.001–2	0.005–2
R2	0.997	0.985	0.997	0.980
Slope (R/S) [†]	42.4/42.1	57.0/56.5	43.9/44.0	47.7/47.2
Intercept [†]	—	—	0.012/0.012	0.012/0.012

*Note: No variation was observed between the R- and S-enantiomers.

[†]Slope and intercept given is the average found over five analytical runs.

enantiomers in more complex matrices, like whole blood and tissue (21, 24, 29). In contrast, several achiral methods have been published (25,26,33–36). Measurement in matrices other than blood provides supplementary information that may be valuable for interpreting drug levels (27, 28).

Precipitation and SPE

The removal of lipids from brain extracts was the primary focus of the present method development. This necessitated thorough examinations of the precipitation, SPE, washing and

Table V

Precision and Accuracy ($n = 12$) Results from the Method Validation*

Level mg/kg	R-methadone				S-methadone			
	%RSD		Accuracy %		%RSD		Accuracy %	
	Blood	Brain	Blood	Brain	Blood	Brain	Blood	Brain
~0.0005	(16)		(111)		(20)		(114)	
~0.001	10.0	(19)	101	(79)	11.7	(22)	104	(72)
~0.005	8.8	13.6	101	98	9.0	14.7	103	97
~0.05	11.5	10.4	103	96	12.4	11.7	107	95
~0.25	12.1	14.8	102	100	12.8	16.4	104	99
~1	11.9	15.8	97	95	12.0	17.2	98	94
~2	(11.0)	(14.9)	(87)	(93)	(11.7)	(16.3)	(88)	(91)

Level mg/kg	R-EDDP				S-EDDP			
	%RSD		Accuracy %		%RSD		Accuracy %	
	Blood	Brain	Blood	Brain	Blood	Brain	Blood	Brain
~0.0005	(24)		(123)		(24.7)		(125)	
~0.001	12.6	20	106	84	11.2	19	109	83
~0.005	11.0	7.8	99	99	10.5	8.7	100	99
~0.05	13.5	5.0	101	96	11.8	5.9	102	96
~0.25	12.7	9.0	100	99	12.4	9.6	101	99
~1	14.3	10.1	99	97	13.5	10.8	100	97
~2	12.6	9.5	95	101	11.9	10.5	95	101

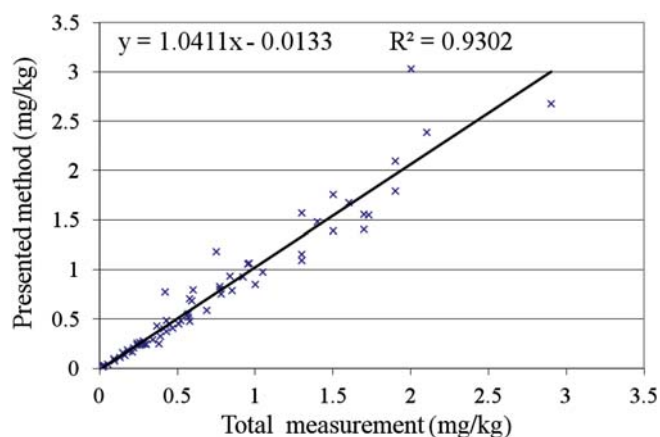
*Note: Levels where values are given in parentheses are not included in the quantitative range.

Table VI

Intermediate Precision for Low QC and High QC Measured in Brain (N = 11) and Blood (N = 15)

Level	R-methadone				S-methadone			
	%RSD		Accuracy %		%RSD		Accuracy %	
mg/kg	Blood	Brain	Blood	Brain	Blood	Brain	Blood	Brain
~0.005	11.7	15	98	96	9.9	15	96	98
~0.25	10.9	6.8	96	97	11.3	6.8	95	98

Level	R-EDDP				S-EDDP			
	%RSD		Accuracy %		%RSD		Accuracy %	
mg/kg	Blood	Brain	Blood	Brain	Blood	Brain	Blood	Brain
~0.0005	7.5	8.5	96	103	7.8	8.3	96	103
~0.25	9.6	9.2	94	108	9.8	9.0	94	108

**Figure 3.** Comparison of total methadone concentration measured with the presented method and in routine analysis ($n = 68$).

elution steps. Published works involving complex matrices emphasized the necessity of good sample cleanup. Choo *et al.* observed that precipitation of breast milk before SPE treatment helped remove proteins and lipids and resulted in a more stable SPE flow (25). When precipitation is conducted, a loss in recovery is expected, because analytes are captured in the protein precipitates (21, 37). Here, this loss was an accepted tradeoff for a cleaner final extract. The addition of IS to the samples before precipitation corrected for analyte loss during this step. A comparison of quality among precipitation methods was conducted on an achiral C18 column instead of the chiral AGP column. The more robust C18 column allowed a 100% organic phase that allowed chromatographic visualization of all potential contaminants and impurities in the extracts. This would not be possible on the AGP column, because a maximum 25% organic phase is recommended, thus potential contaminants trapped on the column would not be detected.

SPE has the advantages of a reduced sample volume requirement and the potential for automation. Bones *et al.* (38) thoroughly compared the extraction of compounds (including methadone and EDDP) from water samples with Strata-X, Strata-X-C and Strata-X-C-W columns. They achieved the best recovery of methadone and EDDP with a Strata-X-C column at pH 6 by eluting with 5% 25% NH_3 in MeOH. The SPE recoveries for methadone (76%) and EDDP (63%) were comparable

with those observed in the present study, despite our use of ACN-NH_3 as the elution medium. The Strata-X-C column has been used by others to extract methadone with EDDP and EDMP from breast milk (25) and by the manufacturer to extract these analytes from plasma. Those studies also noted the feasibility of using a high percentage of organic solvent in the wash step to ensure a high degree of cleanup without analyte loss. SPE chromatography revealed that ACN-NH_3 was a better elution solvent than the MeOH-NH_3 suggested by the manufacturer and used by other groups (17, 25, 38). Eluting with ACN-NH_3 resulted in a cleaner final extract; the eluted phospholipids could be minimized significantly by discarding the first 100 μL of the eluate, and collecting only slightly more volume than the amount needed for maximum recovery of the two analytes.

Chiral-LC-MS-MS

Other methods for the chiral separation of methadone and EDDP on an AGP column have generally used ACN (10, 12, 19, 22, 29) or 2-propanol (14, 16, 17, 21) as the organic phase in isocratic LC. Of the two, 2-propanol is cheaper and less harmful, and a 2-propanol gradient can achieve full separation of the enantiomers on the LC system after a relatively short time compared with other chiral methods (15, 16, 22, 23, 29). The final 10 min of the gradient functions as a rinsing step, and this might be excluded for examining neater samples (e.g., urine or plasma), which would result in an even shorter LC run time. Overall, chromatography provided good selectivity, with no detectable interfering compounds.

Validation

The presented method is suitable for routine analyses of methadone and EDDP in brain and blood samples. Its quantitative range extends well below the therapeutic methadone level (0.05 mg/kg racemic) and into the highly toxic range. Because this method requires low sample volumes, it is possible to prepare samples in a compact 96-well setup. This results in a short sample preparation time, due to the automation of sample precipitation, extraction, evaporation and reconstitution. The selectivity of the method was thoroughly examined with a wide range of common drugs, and no interference was

Table VIIA

Measured Methadone and EDDP Enantiomers in Postmortem Brain and Blood from Six Deceased Drug Addicts*

Case History			Brain (mg/kg)				Blood (mg/kg)			
Case	Age	Sex	R-Mtd	S-Mtd	R-EDDP	S-EDDP	R-Mtd	S-Mtd	R-EDDP	S-EDDP
1	41	M	0.070	0.058	(0.0004)	(0.0005)	0.051	0.046	0.003	0.005
2	45	M	0.165	0.145	0.001	0.002	0.092	0.078	0.014	0.024
3	59	M	0.523	0.331	0.008	0.013	0.148	0.104	0.026	0.037
4	56	M	1.617	0.717	0.030	0.046	0.652	0.273	0.105	0.149
5	57	F	2.515	1.427	0.023	0.032	1.026	0.523	0.160	0.182
6	42	M	4.559	2.435	0.050	0.047	1.173	0.587	0.137	0.124

*Note: The samples are ordered from lowest to highest blood R-methadone concentration. Concentrations given in parenthesis are below LOQ. Mtd: methadone.

Table VIIB

Calculated R/S Ratios Based on Values Given in Table VIIA, with More Decimals than Given in Table VIIA Used For Calculation.

Case	R/S			
	Methadone		EDDP	
	Brain	Blood	Brain	Blood
1	1.20	1.12	0.71	0.67
2	1.14	1.18	0.67	0.59
3	1.58	1.43	0.62	0.70
4	2.25	2.38	0.65	0.70
5	1.76	1.96	0.71	0.88
6	1.87	2.00	1.06	1.10
Median	1.67	1.70	0.69	0.70

observed for methadone or EDDP. The accuracy and %RSD for the blood analysis were 95–109% and <15%, respectively, at all levels. In addition, a %RSD slightly greater than 15% was accepted in brain samples at two levels of methadone (16% for 0.25 mg/kg and 17% for 1 mg/kg). The 0.25 mg/kg level was examined over 11 days with a %RSD <10% for all analytes, indicating that the initial %RSD was slightly high and thus, it was valid to accept the two >15% values.

Because, in some cases, EDDP is present at very low concentrations, the low LLOQ (0.001 mg/kg in blood and brain samples) of the method represents an advantage for pharmacokinetic studies and other considerations, like distinguishing between acute or chronic intoxication. In addition, diluting the sample with buffer provided reliable results; thus, the HLOQ can easily be extended.

Evaluating the effect of thawing and freezing is more relevant for blood than for brain samples. Of the two matrices, only blood is currently used for routine analyses, and therefore, only blood is affected by freeze/thawing. In previous studies, no degradation of plasma samples was observed over three and five thaw/freeze cycles (10, 14). The degradation observed in the present study may be explained by the complexity of the whole blood samples. The loss of analytes observed over the thaw/freeze cycles highlights the importance of avoiding repeated thawing of samples.

The matrix effect showed very low variability for brain and high variability for blood. This probably depends on the optimization of extraction conditions. The use of an isotopic IS corrects for the matrix effect; we found very good agreement between methadone concentrations determined with this method and those determined with our in-house accredited method (Figure 3).

Wang *et al.* presented methadone enantiomer measurements in brain tissue with achiral quantification of EDDP (24). Their method was developed for plasma and applied to different mouse tissues to estimate the effects of P-glycoprotein on methadone distribution (24). In comparison, our method allowed chromatographic separation of both methadone and EDDP enantiomers in less than half the run time, and the LLOQ was 10 times lower than their LOD for both methadone and EDDP (0.001 versus 0.01 mg/kg) with only one-fifth of the amount of sample. Our method was developed for routine analyses of both brain and whole blood samples. To our knowledge, this is the first published method to include a chiral determination of methadone and EDDP in human brain tissue.

Application

The data presented from the six examined cases (Table VIIA) showed large inter-individual variations in the brain/blood ratios. The mean methadone brain/blood ratio (2.6 for both R and S) and the large inter-individual variation were on the same order as those reported by others (39–42). Only one publication has been found that has an R/S-methadone ratio in whole blood of 1.46 (29), which is very close to the ratio observed in the present study (median 1.70, Table VIIB). The R/S-methadone ratio that we observed in brain tissue (median 1.67) was very close to the value observed for blood, and it was consistent with the R/S-methadone ratio of 1.5 determined by Wang *et al.* in mouse brain (24). The R/S-EDDP ratio was previously characterized as <1 in plasma (13) and 0.9 in whole blood (29). Those values were consistent with our observed ratio of 0.70. We observed a large inter-individual variation in the methadone/EDDP ratio in blood among the 6 cases. Similar variation has been noted by others (7, 8). These very limited preliminary results indicated that the method provides reliable results.

Conclusion

A highly selective, sensitive, chiral method for determining methadone and EDDP levels has been developed for routine analysis of blood and brain tissue. Results observed in a preliminary examination of authentic postmortem blood samples were consistent with values published previously in the literature. To our knowledge, this is the first published method for the quantification of methadone and EDDP enantiomers in human brain tissue.

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