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Enantiomeric methadone quantitation on real post-mortem dried matrix spots samples: Comparison of liquid chromatography and supercritical fluid chromatography coupled to mass spectrometry

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ABSTRACT

This study describes two bioanalytical methods for the quantitation of the two methadone enantiomers in dried matrix spots using high performance liquid chromatography coupled to tandem mass spectrometry (HPLC-MS/MS) and high performance supercritical chromatography tandem mass spectrometry (HPSFC-MS/MS). Dried matrix spots were obtained by spotting 10 µL of each sample fluid on a Whatman paper. Methadone and its main metabolite, EDDP, were extracted with 100 µL methanol and subsequently injected into the LC-MS/MS and SFC-MS/MS systems. Enantiomeric separation was achieved with AGP-column for the LC conditions and with Chiralpak IH-3 in SFC. The two methods were fully validated and 93 post-mortem samples were analysed with both analytical methods. Results from validation parameters and results obtained for all post-mortem samples were compared with a significant spearman correlation of $r_s = 0.9978$ for R-methadone and $r_s = 0.9981$ for S-methadone. The LC method provided better results in terms of uncertainty, retention factor and resolution, whereas SFC provides better sensitivity, with lower LOD. Median R-/S-methadone ratio in peripheral blood was found equal to 1.60 (N = 32), varying from 0.79 to 4.23. The reported values were in good agreement with previously published results.

Based on the results obtained here, SFC-MS/MS can be considered a reliable alternative to the widely used LC-MS/MS for the quantitation of methadone enantiomers in bioanalysis and should be evaluated for other bioanalytical methods. Both methods can be easily and quickly used in toxicological routine analysis for the methadone quantitation in human fluids matrices, even if considering that the polysaccharide coated column IH-3 used in SFC does not allow the enantiomeric EDDP separation.

1. Introduction

Methadone is a µ-opioid receptor agonist similar to morphine and is administered for chronic pain and opioid related dependence [1,2]. R-methadone has a higher µ and δ opioid receptor activation and a greater analgesic activity compared to the S-methadone [2]. Methadone is also shown to increase QT dispersion as well as QT interval [3,4] mainly through the S-methadone enantiomer, because of its 3.5-fold more potent hERG channel blockage compared to the R-methadone [5]. In this context, even if the use of R-methadone enantiomer is suggested [6], methadone is still clinically administered in the racemic form. Due to the high interindividual variability in R-/S-methadone stereoselective

Abbreviations: ACN, acetonitrile; AGP, alpha 1-acid glycoprotein; CB, cardiac blood; DBS, dried blood spot; DMS, dried matrix spot; EDDP, 2-Ethylidene-1,5-dimethyl-3,3-diphenylpyrrolidine; ESI, electrospray ionization; EtOH, ethanol; LC, liquid chromatography; LLE, liquid liquid extraction; MeOH, methanol; MS/MS, tandem mass spectrometry; MTD, methadone; PB, peripheral blood; PF, pericardial fluid; PrOH, propanol; QC, quality control; RT, retention time; SPE, solid phase extraction; SFC, supercritical fluid chromatography; S/N, signal to noise.

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metabolism [7], ratio between R-methadone and S-methadone in blood is shown to be significantly different between individuals and vary from 1 to 4 [8,9].

The first article describing the analytical enantiomeric separation of methadone in plasma has been published in 1991 by Beck et al. [10] using LC with an α1-acid glycoprotein (AGP) column coupled with an UV detector. Since then, several methods mainly using LC-UV have been proposed [11–14] with an off-line sample preparation performed by LLE or SPE. More recently, enantiomeric methadone separation was carried out with LC coupled with MS to quantify methadone enantiomers in human post-mortem samples, including fluids and tissues [8,9].

For almost 50 years, dried blood spots (DBS) have been used for collection, analyses and long-term storage of blood samples. In the last two decades, DBS also became a more discussed matrix in forensic toxicology analysis. For instance, Odoardi et al., [15] developed an LC-MS/MS method to analyse drugs of abuse using a DBS matrix. Recently, Metzger et al. [16], have quantified R- and S-methadone in DBS matrix from real human cases using an LC-MS/MS without a full description of the method validation results. Compared to the LC platforms, dried matrix spots have been scarcely employed in conjunction to SFC technique until now, and only a few studies were published in the literature [17,18]. Although chiral SFC is largely used in the pharmaceutical industry, its application in forensic analysis is limited [19,20], since LC and GC remain the most commonly used techniques for chiral separation [21].

Several studies comparing SFC and LC have been published [22–26], some of them evaluating SFC-MS/MS and LC-MS/MS performance in the field of bioanalysis [22,23,27]. For instance Borovcova et al. [27] described a systematic comparison between validation parameters results from the two different instrumental techniques used for the determination and the quantitation of 15 new psychoactive drugs. Hoke et al. [22] compared validation results and human plasma samples results obtained separating the ketoprofen enantiomers with the same column with both LC-MS/MS and SFC-MS/MS.

The present study describes two analytical methods for the separation and quantitation of methadone enantiomers, using both SFC-MS/MS and LC-MS/MS. These two bioanalytical methods were fully validated for chiral methadone quantitation and a series of 93 DMS obtained from real post-mortem cases were then successfully analysed with both methods.

2. Materials and methods

2.1. Reagents and standards

Reference solutions of R-/S-MTD, R-/S-EDDP at 1 mg/mL in methanol and R-/S-MTD-D9, R-/S-EDDP-D3 at 0.1 mg/mL in methanol were obtained from Lipomed (Lipomed, Switzerland). R-methadone was obtained from L-Polamidon medicament (Mundipharma Medical Company, Basel). Methanol, acetonitrile and isopropanol were obtained from Carlo Erba (Carlo Erba, Italy). Formic acid 98–100% LC-MS grade and ammonium acetate were purchased from Sigma (Sigma, Germany). H_2O was obtained from Milli-Q system from Millipore. All solvents and inorganic chemicals were of analytical grade. Whatman 903 Paper Saver Snap Apart cards used for dried matrix spots were obtained from Whatman (Whatman, United Kingdom). Pressurized carbon dioxide (CO2, 99.99%) was purchased from Carbagas (Lausanne, Switzerland) and was employed for the SFC measurements.

2.2. Preparation of calibration curves

Methadone and EDDP solutions were used to prepare calibration standards in blank bovine whole blood with concentrations of 20, 50, 100, 200, 500, 1000, 2000, 5000 ng/mL for each racemate. 10 µL of each calibration sample was spotted to the Whatman paper and dried for at least 3 h at room temperature before extraction.

2.3. Postmortem cases

Autopsy cases were selected in which methadone was revealed in femoral blood during toxicological routine analysis from January 2016 to July 2020. When possible, the following matrices were collected during autopsy for the present study: femoral whole blood, cardiac whole blood, vitreous humour and pericardial blood. All samples were stored at −20 °C until analysis. Femoral blood, cardiac blood and vitreous humour were stabilized with sodium fluoride. A total of 93 post-mortem samples from 35 post-mortem cases related to the methadone were analysed: 32 peripheral bloods, 29 cardiac bloods, 23 vitreous humour and 9 pericardial fluids.

2.4. Sample preparation

The same samples were used for LC-MS/MS and SFC-MS/MS analyses. 10 µL of calibration, QC and human post-mortem samples (i.e. whole blood, cardiac blood, vitreous humour, pericardial fluid) were added to the Whatman paper and dried for at least 3 h at room temperature prior extraction. Dried matrix spots were cut and added to an Eppendorf with 100 µL internal standard mix in methanol (40 ng/mL Methadone-d9 and 10 ng/mL EDDP-d3). Eppendorf were vortexed for 30 s and incubated at RT for 30 min. Then, extracts were transferred into vials and a volume of 0.5 µL was injected in both LC and SFC conditions.

2.5. Instrumentation and analytical method

2.5.1. LC-MS/MS

LC separations were performed on an Acquity UPLC *I-class* system (Waters, Milford MA, USA) composed of a binary solvent delivery pump, an autosampler with flow through needle (SM-FTN) injection system and a column oven equipped with an active preheater. The autosampler temperature was fixed at 15 °C and the column was heated at 25 °C. The Acquity separation module was coupled to a Xevo TQ-XS mass detector equipped with an ESI interface (Waters, Milford MA, USA). Chromatographic separation was achieved using AGP stationary phase (Chiral Technologies, France) (100 × 2.1 mm i.d., 5 µm particle size) with an AGP guard column (10 × 2.0 mm i.d., 5 µm particle size) at 25 °C. The mobile phase consisted of aqueous ammonium acetate 10 mM pH 5.8 (A) and isopropanol (B). The following gradient elution was used (runtime 14 min), starting with 6% B for 8 min, increased to 8% B at 8.10 min, held to 11 min, increased to 20% B at 11.20 min, held to 12 min, and returned to initial conditions of 6% B at 12.10 min and maintained until 14 min. The flow rate was assessed at 0.3 mL/min.

The ESI source was operated in the positive mode with the following conditions: source temperature and desolvation gas (nitrogen) temperature were set at 150 °C and 650 °C, respectively, the gas flow was delivered at 1000 L/h and the capillary voltage was set at 1.0 kV. Product ions were obtained by collision-induced fragmentation in the multiple reaction monitoring (MRM) mode. MRM transitions and conditions for measurement of methadone were: 310 m/z > 105 m/z, 310 m/z > 223 m/z, 310 m/z > 265 m/z (quantifier); cone voltage 35 V, collision energy 26 eV, 22 eV and 14 eV, respectively. Methadone-d9 are: 319 m/z > 105 m/z (quantifier), 319 m/z > 268 m/z; cone voltage 35 V, collision energy 30 eV and 15 eV, respectively. EDDP are: 278 m/z > 186 m/z, 278 m/z > 219 m/z, 278 m/z > 249 m/z (quantifier), cone voltage 30 V, collision energy 32 eV, 38 eV and 22 eV, respectively. EDDP-d3 are: 281 m/z > 234 m/z (quantifier), 281 m/z > 249 m/z; cone voltage 30 V, collision energy 30 eV and 24 eV, respectively. Waters MassLynx software Version 4.2 was used for instrument control and quantitation.

2.5.2. SFC-MS/MS

The analyses have been performed using on a Waters Acquity UPC^2 system (Waters, Milford, MA, USA) equipped with a Binary Solvent Manager delivery pump, a Sample Manager autosampler which included

a 10 μL loop for partial loop injection, a column oven with active pre-heater, a PDA detector with an 8.4 μL flow-cell and a two-step (active and passive) backpressure regulator (pre-BPR). The chromatographic system was hyphenated to a Waters Xevo TQ-S mass detector equipped with an ESI interface via a double-T splitter interface from Waters (Waters, Milford MA, USA) [28]. Chromatographic separation was achieved using a Chiralpak IH-3 stationary phase (Chiral Technologies, France) (150 mm length × 3.0 i.d., 3 μm particle size). Elution solvents consisted of CO_2 (A) and Methanol:H_2O (98:2, v:v) containing ammonium acetate 20 mM (B). The following elution gradient was used (runtime 14 min), from 0 to 9 min 10% B, increased to 40% B at 9.5 min, held to 12 min, changed to 10% B at 12.20 min and maintained to the initial conditions till 14 min. The flow rate was 0.4 mL/min and the injected volume was 0.5 μL. The automated backpressure regulator (ABPR) was set at 150 bar, with the make-up flow set up at 0.5 mL/min and the column temperature was set at 30 °C.

The electrospray source was operated in the positive ionization mode (ESI+). The source temperature and desolvation gas (nitrogen) temperature were set at 150 °C and 450 °C, respectively. The flow gas was delivered at rate of 1000 L/h. The capillary voltage was set at 1.0 kV. MRM transitions and conditions for measurement of methadone are: 310 m/z > 77 m/z, 310 m/z > 105 m/z, 310 m/z > 265 m/z (quantifier); cone voltage 25 V, collision energy 48 eV, 29 eV and 15 eV respectively. Methadone-d9 are: 319 m/z > 105 m/z, 319 m/z > 268 m/z (quantifier); cone voltage 25 V, collision energy 29 eV and 15 eV respectively. EDDP are: 278 m/z > 186 m/z, 278 m/z > 234 m/z, 278 m/z > 249 m/z (quantifier), cone voltage 25 V, collision energy 35 eV, 30 eV and 25 eV respectively. EDDP are: 281 m/z > 234 m/z (quantifier), 281 m/z > 249 m/z; cone voltage 30 V, collision energy 30 eV and 23 eV respectively. Waters Mass-Lynx system software Version 4.2 was used for instrument control and quantitation.

2.6. Method validation

Validation for both LC-MS/MS and SFC-MS/MS methods was performed in agreement with the document: "Guideline on bioanalytical method validation" published by the European Medicines Agency (2016). The following parameters were assessed: calibration model, selectivity, specificity, accuracy, precision, carry-over, interferences, ionization suppression/enhancement, recovery, limit of detection (LOD), limit of quantitation (LOQ), uncertainty and stability.

Accuracy and precision were determined for each QCs (quality controls for enantiomer: LLOQ 10 ng/mL, low 30 ng/mL, medium: 1000 ng/mL and high 2000 ng/mL) in five replicates and in five independent analytical runs. Sensitivity was determined for the LLOQ in in six replicates and in five independent runs. Selectivity and specificity were determined by injecting 10 different human blood samples, which were fortified at the QC LLOQ, and injecting six different human blood samples containing the following drug groups: benzodiazepines, THC, cocaine, opioids, LSD, antidepressant and neuroleptics. Carry-over was evaluated in triplicate following injection of the 3xULOQ (ULOQ: upper level of quantitation) calibration standard. Recovery was assessed by comparing pre-spike samples with post-spike samples in triplicate for three different QCs (low, medium, high). Matrix effect was determined by comparing post-spike samples in matrix, with the post-spike samples without matrix in triplicate for three different QCs (low, medium, high). Limit of quantitation (LOQ) was defined to be the first calibration point and limit of detection was evaluated visually for signal-to-noise ratio S/N = 3. Stability of methadone and EDDP on DBS matrix was assessed using 3 different QCs levels (low, medium, high) stored for 5 months and analysed in triplicate using a freshly prepared calibration curve.

3. Results

3.1. Chromatographic enantiomeric separations

R-/S-methadone and R-/S-EDDP were resolved using LC-MS/MS platform equipped with the AGP column (see Fig. 1). This was in agreement with the results published by Beck et al. [10]. The widths of the peaks at baseline were the following: R-methadone 1.2 min, S-methadone 1.4 min, R-EDDP 0.7 min, S-EDDP 0.9 min.

To achieve a suitable separation in SFC, various mixtures of organic modifiers and CO2 were initially tested under isocratic conditions for the analysis of methadone only, including CO_2/MeOH (80:20), CO_2/EtOH (80:20), CO_2/2-PrOH (80:20) and CO_2/ACN (80:20). The proportions of CO_2 and organic modifiers in the mobile phase were further adjusted to obtain retention factors between 1.5 and 15 for methadone and EDDP and gradient elution (up to 40% MeOH) was used. Finally, some additives (2% water and ammonium acetate 20 mM) were added to the mobile phase to improve peak shapes for the basic drug and metabolite. In SFC, R-methadone was injected alone to know which chromatographic peak corresponds to which enantiomer. Fig. 2 shows the corresponding methadone enantiomeric separation, and the elution order of the two methadone enantiomers was the same in both LC and SFC. On the other hand, chiral separation of R-/S-EDDP was not achieved, due to the lower chiral selectivity of the Chiralpak IH-3 column compared to the AGP column. The peak widths at baseline were as follow: R-methadone 0.45 min, S-methadone 0.40 min, R-/S-EDDP 0.18 min.

Retention factor (k'), selectivity (α), column efficiency (N) and resolution (R_S) were calculated for both analytical separations and results are presented in Table 1. Both chromatographic methods showed a satisfactory enantiomeric methadone separation, with a greater differentiation obtained in LC. In details, selectivity (α) are better in LC compared to that obtained in SFC for both methadone and EDDP. However, column efficiency (N) is much higher in SFC compared to LC for both compounds. In the end, it appears that the resolution (R_S) of 3.61 obtained in LC for the methadone enantiomers was quite higher than the one obtained in SFC (R_S = 1.80). When comparing the two analytical conditions, the enantiomeric separation in LC was achieved using a protein coated stationary phase, which has shorter lifetime, a maximum tolerable of 40–50% organic concentration in mobile phase, a long-term storage recommended in the fridge and, unfortunately, a lower repeatability between batches. On the other hand, the polysaccharide coated column used in SFC allows a much better repeatability between batches, a long-term storage at room temperature, possibility to increase organic concentration in mobile phase and longer lifetime compared to the AGP column [29].

Both, LC-MS/MS and SFC-MS/MS methods offer the same runtime of 14 min. Figs. 3 and 4 show the blank DBS and the lowest calibration point in DBS for both analytical methods using LC-MS/MS and SFC-MS/MS, respectively. Background noise in LC was higher compared to that observed in SFC.

3.2. Methods validation results

Comparison between methods validation parameters obtained in LC-MS/MS and SFC-MS/MS are shown in Tables 2, 3 and 4.

Mean determination coefficients of the calibration curves were calculated for five different analytical runs. Mean values for R-methadone in LC-MS/MS and SFC-MS/MS (r^2 value was equal to 0.9992) was the same (see Table 1). Mean correlation coefficient for S-methadone in LC-MS/MS and SFC-MS/MS were also totally comparable (R^2 of 0.9992 and 0.9991 in LC-MS/MS and SFC-MS/MS, respectively) (see Table 2). Results for accuracy and precision of R- and S- methadone enantiomers were obtained by analysing 4 QCs levels (LLOQ: 10 ng/mL, low: 30 ng/mL, med: 1000 ng/mL and high: 2000 ng/mL) in quintuplicate. Mean QCs accuracy for R- and S-methadone in LC was 3.93 and 3.85%,

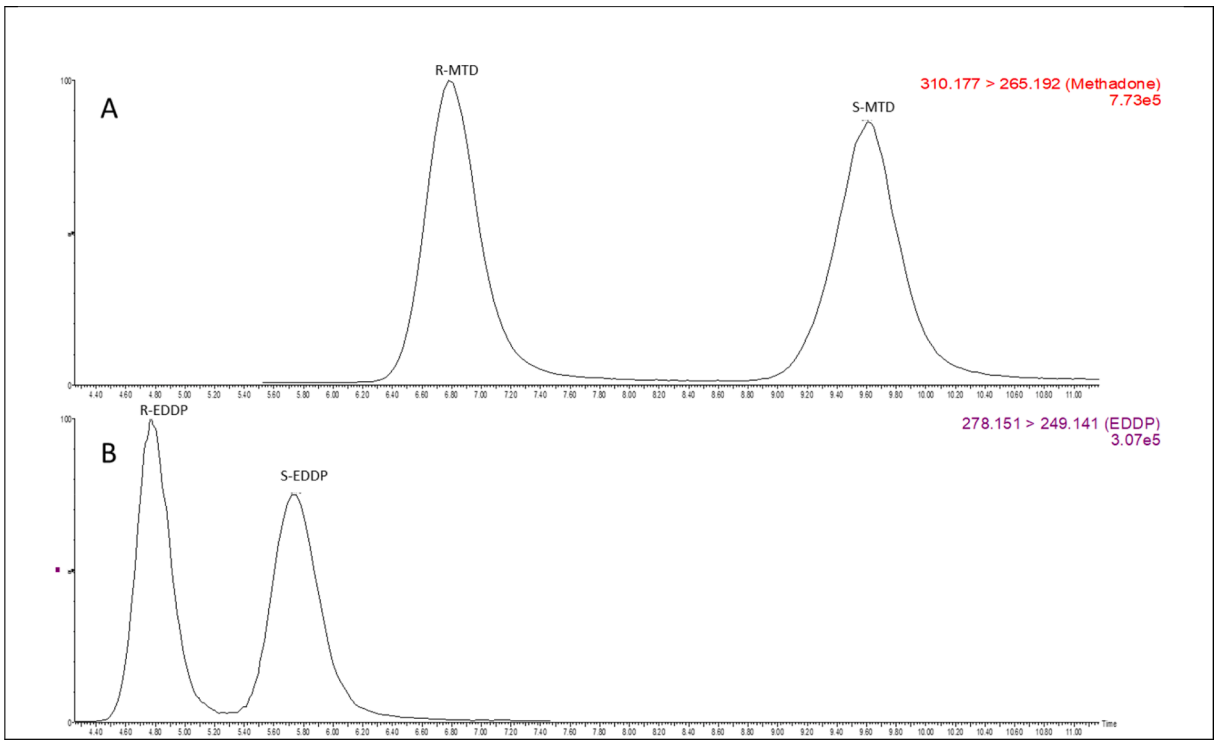

Fig. 1. Chiral LC separation using AGP column. Standards racemic mixtures were analysed at a concentration of 60 ng/mL. (A): R-methadone (RT: 6.78 min), S-methadone (RT: 9.61); (B): R-EDDP (RT: 4.77 min), S-EDDP (RT: 5.75 min).

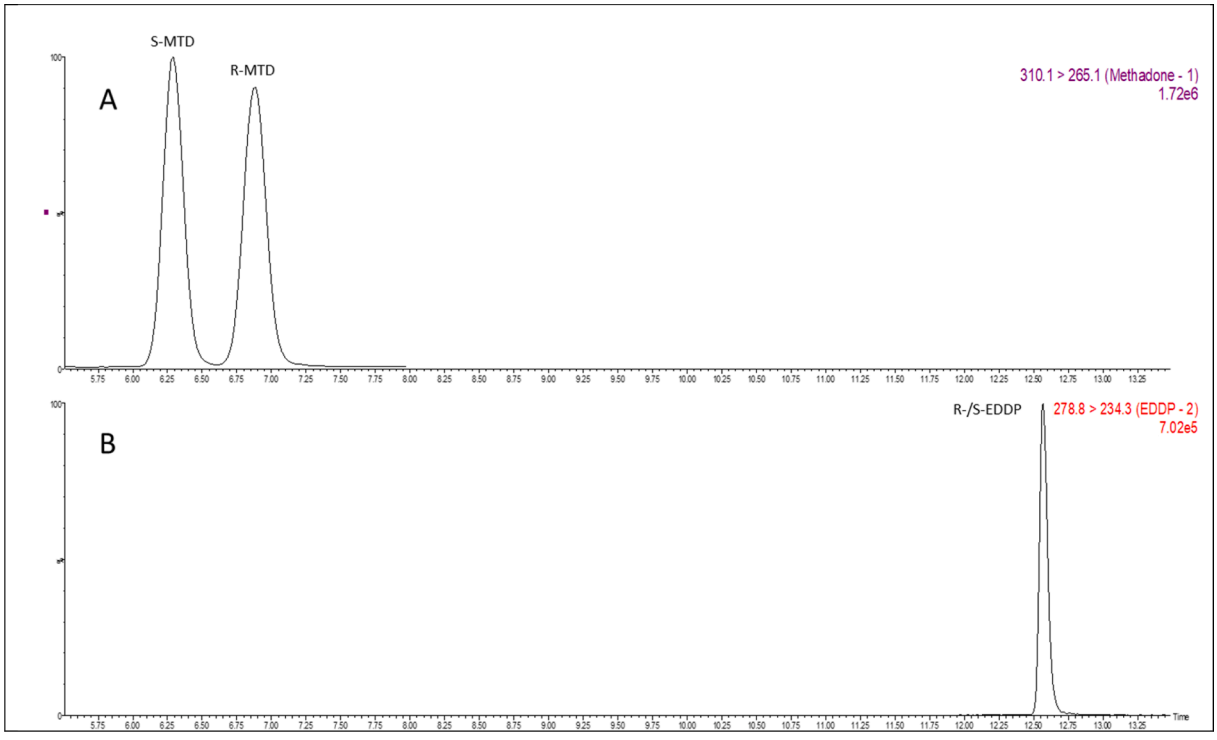

Fig. 2. Chiral SFC separation using Chiralpak IH-3 column. Standards racemic mixtures were analysed at a concentration of 60 ng/mL. (A): R-methadone (RT: 6.29 min), S-methadone (RT: 6.89); (B): R-/S-EDDP (RT: 12.57 min).

respectively. Mean QCs accuracy for R- and S-methadone in SFC was 2.78 and 2.70%, respectively. Highest bias level calculated in LC was 6.9% (R-methadone QC LLOQ), whereas in SFC it was 5.5% (S-methadone QC high). Mean precision (between run) for R- and S-methadone in LC was 3.28 and 4.20%, respectively. Mean precision (between run) for R- and S-methadone in SFC was 6.20 and 6.35%, respectively. The worst precision level calculated in LC was 7.3% (within run, R-methadone QC LLOQ), while in SFC, it was 10.5% (between run, R-methadone QC LLOQ). Methadone recovery using a simple methanol extraction was close to 100%. The matrix effect using LC-MS/MS analysis was

Table 1
Results comparison between UHPSFC and UHPLC for the main chromatographic parameters.

		Retention time (RT)	Retention factor (k)	Selectivity (α)	Resolution (Rs)
LC					
	t_0	0.74			
R-MTD	t_{R1}	6.78	8.16		
S-MTD	t_{R2}	9.61	11.99	1.47	3.61
R-EDDP	t_{R1}	4.77	5.45		
S-EDDP	t_{R2}	5.75	6.77	1.24	1.81
SFC					
	t_0	2.02			
R-MTD	t_{R1}	6.29	2.12		
S-MTD	t_{R2}	6.89	2.41	1.14	1.80
R-/S-EDDP	t_{R1}	12.57	5.23		

negligible and a slight ion enhancement was observed in SFC-MS/MS. According to the validation design, LOQ in LC and SFC was defined at the first calibrator at 10 ng/mL for both methadone enantiomers. LOD (Calculated for S/N = 3) in LC was 2.5 ng/mL for both methadone enantiomers, whereas it was equal to 0.5 ng/mL in SFC. Sensitivity in SFC was about 5-times better than in LC for methadone enantiomers. No interferences were observed after the injection of six different human blood samples containing benzodiazepines, cocaine, amphetamines, opioids, antidepressants, neuroleptics and cannabinoids. Uncertainty was calculated using guidelines from ISO/IEC [30] and defined to be 2 times the highest SD. Uncertainty for R- and S- methadone quantitation in LC-MS/MS was lower compared to that calculated in SFC-MS/MS. In the case of methadone metabolite (EDDP), mean correlation coefficients of the calibration curves were calculated for five different analytical runs. The average values for R-EDDP and S-EDDP in LC and for the racemic R-/S-EDDP in SFC were the same, with an R^2 value of 0.9995 (see Table 3). EDDP recovery using a simple methanol extraction was

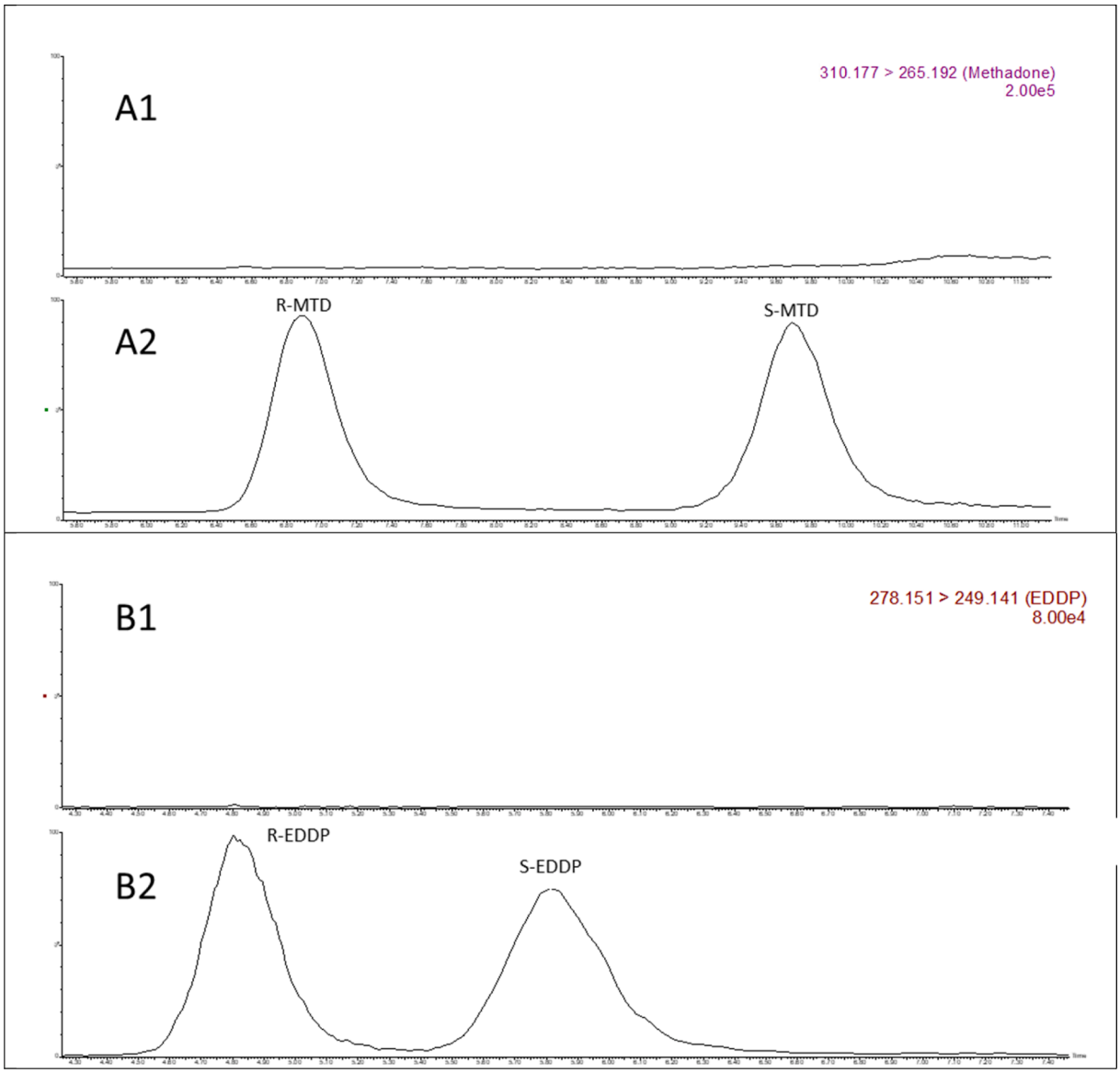

Fig. 3. R-/S-methadone and R-/S-EDDP enantiomeric separation in dried blood spot matrix obtained with UHPLC-MS/MS. Comparison between blank matrix and DBS fortified at the first point of the calibration curve (10 ng/mL for each enantiomer). (A1): Blank DBS matrix chromatogram compared to (A2) first calibration point at enantiomeric concentration of 10 ng/mL R- and S-methadone. (B1): Blank DBS matrix chromatogram compared to (B2) first calibration point at enantiomeric concentration of 10 ng/mL R- and S-EDDP.

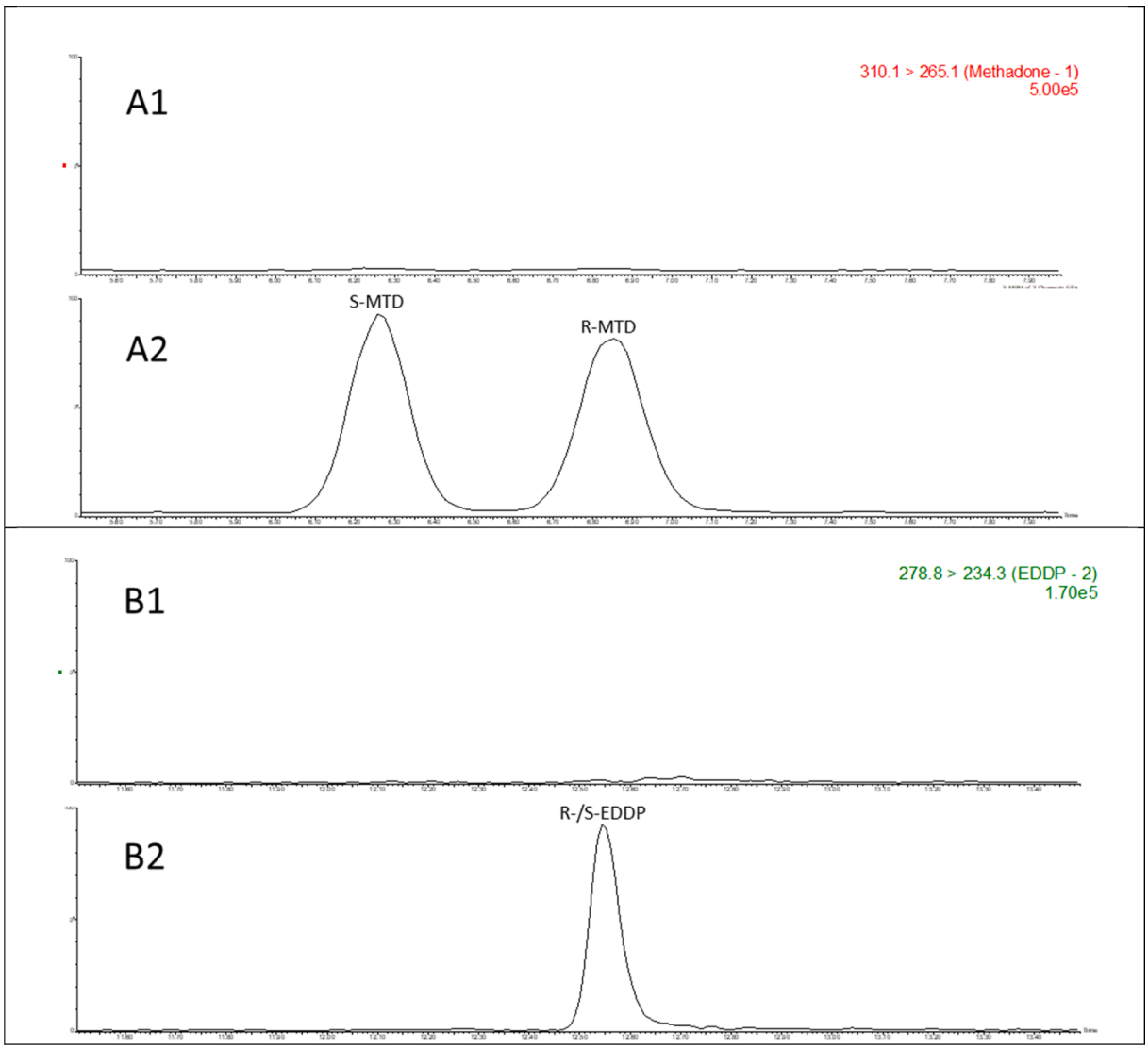

Fig. 4. R-/S-methadone enantiomeric separation and R-/S-EDDP chromatographic peak in dried blood spot matrix obtained with UHPSFC-MS/MS. Comparison between blank matrix and DBS fortified at the first point of the calibration curve (10 ng/mL for each enantiomer). (A1): Blank DBS matrix chromatogram compared to (A2) first calibration point at enantiomeric concentration of 10 ng/mL R- and S-methadone. (B1): Blank DBS matrix chromatogram compared to (B2) first calibration point at the concentration of 20 ng/mL R-/S-EDDP.

comprised between 85% and 91%. The matrix effects using SFC-MS/MS analysis were negligible and a slight ion suppression is observed using LC-MS/MS. No interferences were observed after the injection of six different human blood samples containing benzodiazepines, cocaine, amphetamines, opioids, antidepressants, neuroleptics and cannabinoids. Accuracy and precision for all samples used for stability assessment were in between ±15.0%. At least, methadone and EDDP on DBS were stable for 5 months when blood is stored as a dried blood spot at room temperature.

3.3. Human post-mortem samples: Results and comparison

Post-mortem dried matrix spots were analysed with both LC-MS/MS and SFC-MS/MS and results obtained for all samples were compared for the two bioanalytical methods and reported in Appendix 1. A significant spearman correlation is determined by comparing results obtained with the two methods for R-methadone, S-methadone (Appendix 1) and R-/S-EDDP (see Appendix 2) quantitation.

Median methadone concentration in cardiac blood was equal to 1217 ng/mL, while it was equal to 1038 ng/mL in peripheral blood (Table 5). R/S methadone ratio was found to be similar in all the four tested matrices and was in favour of the R- enantiomer form. Number (N°) of post-mortem samples for EDDP was not the same as for methadone, because samples results where EDDP was measured under the LOQ were not included. Total EDDP concentration in peripheral blood and cardiac blood was highly comparable (Table 5), with values of 285 ng/mL for the peripheral blood and 286 ng/mL for the cardiac blood, respectively. R- and S-EDDP ratio was similar in all four matrices and was in favour of the S-form.

R-methadone and S-methadone ratios between cardiac blood and peripheral blood showed a significant difference in favour of the cardiac blood (Table 6). R-methadone and S-methadone ratios between pericardial fluid and peripheral blood showed a significant difference in favour of the pericardial fluid (Table 6) for seven post-mortem cases where both fluids were available.

In Fig. 5, the correlation between R-methadone and R-EDDP measured in all post-mortem dried matrix spots analysed is shown. In all samples, R-methadone was more concentrated than R-EDDP, which was present in low quantity. Although several samples showed a slightly higher S-EDDP concentration compared to the S-methadone (Fig. 6), S-

Table 2
Validation parameters comparison for R-methadone quantitation methods using LC-MS/MS and SFC-MS/MS.

Validation parameter	Validation data LC	Validation data SFC
Calibration model	Wheighted linear curve 1/x, R-methadone-d9 as internal standard.	
	Eight point calibration curves with levels: 10, 25, 50, 100, 250, 500, 1000, 2500 ng/mL	
	Mean correlation coefficient (r2): **0.9992**	Mean correlation coefficient (r2): **0.9992**
Bias	LLOQ (10 ng/mL): **93.1%**	LLOQ (10 ng/mL): **97.5%**
	low (30 ng/mL): **98.0%**	low (30 ng/mL): **97.9%**
	medium (1000 ng/mL): **104.4%**	medium (1000 ng/mL): **102.0%**
	high (2000 ng/mL): **97.6%**	high (2000 ng/mL): **95.5%**
Precision	**Inter-day CV:**	**Inter-day CV:**
	LLOQ (10 ng/mL): **3.3%**	LLOQ (10 ng/mL): **10.5%**
	low (30 ng/mL): **4.4%**	low (30 ng/mL): **4.3%**
	medium (1000 ng/mL): **2.5%**	medium (1000 ng/mL): **5.1%**
	high (2000 ng/mL): **2.9%**	high (2000 ng/mL): **4.9%**
	Intra-day CV:	**Intra-day CV:**
	LLOQ (10 ng/mL): **3.3%**	LLOQ (10 ng/mL): **4.4%**
	low (30 ng/mL): **7.1%**	low (30 ng/mL): **8.0%**
	medium (1000 ng/mL): **3.1%**	medium (1000 ng/mL): **2.1%**
	high (2000 ng/mL): **5.8%**	high (2000 ng/mL): **4.0%**
Carry over	**No carryover** was observed after 3xULOQ (7500 ng/mL) after three injection repetition	
Interference studies	**No interfering signal** from matrix, internal standard, common drugs of abuse and prescription medications from 10 samples taken from 10 human sources.	
Recovery	**95–100%**	
Matrix effect	**98–100%**	**103–117%**
Limit of quantification (LOQ)	**10 ng/mL**	**10 ng/mL**
Limit of detection (LOD)	**2.5 ng/mL**	**0.5 ng/mL**
Selectivity, specificity	**No interferences**	
Standard Uncertainly (SD)	**14.2%**	**21.0%**

Table 3
Validation parameters comparison for S-methadone quantitation methods using UHPLC-MS/MS and UHPSFC-MS/MS.

Validation parameter	Validation data LC	Validation data SFC
Calibration model	Wheighted linear curve 1/x, S-methadone-d9 as internal standard.	
	Eight point calibration curves with levels: 10, 25, 50, 100, 250, 500, 1000, 2500 ng/mL	
	Mean correlation coefficient (r2): **0.9992**	Mean correlation coefficient (r2): **0.9991**
Bias	LLOQ (10 ng/mL): **94.2%**	LLOQ (10 ng/mL): **97.4%**
	low (30 ng/mL): **98.2%**	low (30 ng/mL): **97.9%**
	medium (1000 ng/mL): **104.8%**	medium (1000 ng/mL): **101.6%**
	high (2000 ng/mL): **97.0%**	high (2000 ng/mL): **94.5%**
Precision	**Inter-day CV:**	**Inter-day CV:**
	LLOQ (10 ng/mL): **6.3%**	LLOQ (10 ng/mL): **10.0%**
	low (30 ng/mL): **4.3%**	low (30 ng/mL): **4.6%**
	medium (1000 ng/mL): **2.8%**	medium (1000 ng/mL): **5.6%**
	high (2000 ng/mL): **3.4%**	high (2000 ng/mL): **5.2%**
	Intra-day CV:	**Intra-day CV:**
	LLOQ (10 ng/mL): **4.0%**	LLOQ (10 ng/mL): **4.4%**
	low (30 ng/mL): **6.8%**	low (30 ng/mL): **8.5%**
	medium (1000 ng/mL): **3.1%**	medium (1000 ng/mL): **3.2%**
	high (2000 ng/mL): **5.8%**	high (2000 ng/mL): **5.2%**
Carry over	**No carryover** was observed after 3xULOQ (7500 ng/mL) after three injection repetition	
Interference studies	**No interfering signal** from matrix, internal standard, common drugs of abuse and prescription medications from 10 samples taken from 10 human sources.	
Recovery	**95–100%**	
Matrix effect	**98–100%**	**103–117%**
Limit of quantification (LOQ)	**5 ng/mL**	**1 ng/mL**
Limit of detection (LOD)	**2.5 ng/mL**	**0.5 ng/mL**
Selectivity, specificity	**No interferences**	
Standard Uncertainly (SD)	**13.6%**	**20.0%**

methadone concentration measured in the majority of the post-mortem samples was higher compared to the S-EDDP.

4. Discussion

In the present study, we compared two fully validated bioanalytical methods involving two different chromatographic separation modes, namely LC-MS/MS and SFC-MS/MS. The same volume of 0.5 µL was injected in both LC and SFC systems and the two methods have the same analysis time of 14 min. We compared the method validation parameters and the bioanalytical quantitation results obtained with both methods for a wide range of post-mortem samples. Both methods were validated following the same guidelines and results are summarized in Tables 2, 3 And 4. LC-MS/MS method has a lower uncertainty for both methadone enantiomers, while SFC-MS/MS gives a lower LOD. Chromatogram showing the chiral separation of methadone in SFC-MS/MS (Fig. 4) suggests a lower signal to noise in blank matrix compared to that obtained in LC (Fig. 3), even if the signal intensity produced by the MS/MS device was lower compared to LC-MS/MS. In addition, the SFC-MS/MS method presented a better accuracy for both enantiomeric methadone enantiomers, whereas LC-MS/MS showed a greater precision (Tables 1 and 2). Methadone matrix effect was negligible in LC-MS/MS and a slight ion enhancement was observed in SFC-MS/MS. On the contrary, EDDP did not show any matrix effect in SFC-MS/MS compared to the

Table 4
Validation parameters comparison for R-/S-EDDP quantitation methods using UHPLC-MS/MS and UHPSFC-MS/MS.

Validation parameter	Validation data LC R-EDDP	Validation data LC S-EDDP	Validation data SFC R-/S-EDDP
Calibration model	Wheighted linear curve 1/x, R-/S-EDDP-d3 as internal standard.		
	Eight point calibration curves with levels: 10, 25, 50, 100L, 250, 500, 1000, 2500 ng/mL		Eight point calibration curves with levels: 20, 50, 100, 200, 500, 1000, 2000, 5000 ng/mL
	Mean correlation coefficient (r2): **0.9995**	Mean correlation coefficient (r2): **0.9995**	Mean correlation coefficient (r2): **0.9995**
Bias	LLOQ (10 ng/mL): **104.2%**	LLOQ (10 ng/mL): **104.4%**	LLOQ (20 ng/mL): **109.0%**
	low (30 ng/mL): **91.7%**	low (30 ng/mL): **92.0%**	low (60 ng/mL): **93.7%**
	medium (1000 ng/mL): **100.6%**	medium (1000 ng/mL): **100.4%**	medium (2000 ng/mL): **96.8%**
	high (2000 ng/mL): **92.8%**	high (2000 ng/mL): **92.9%**	high (4000 ng/mL): **91.8%**
Precision	**Inter-day CV:**	**Inter-day CV:**	**Inter-day CV:**
	LLOQ (10 ng/mL): **3.8%**	LLOQ (10 ng/mL): **4.5%**	LLOQ (20 ng/mL): **5.3%**
	low (300 ng/mL): **3.9%**	low (300 ng/mL): **3.9%**	low (60 ng/mL): **3.2%**
	medium (1000 ng/mL): **3.8%**	medium (1000 ng/mL): **3.7%**	medium (2000 ng/mL): **4.1%**
	high (2000 ng/mL): **3.5%**	high (2000 ng/mL): **3.6%**	high (4000 ng/mL): **3.7%**
	Intra-day CV:	**Intra-day CV:**	**Intra-day CV:**
	LLOQ (10 ng/mL): **4.2%**	LLOQ (10 ng/mL): **4.0%**	LLOQ (20 ng/mL): **3.9%**
	low (300 ng/mL): **3.4%**	low (300 ng/mL): **3.7%**	low (60 ng/mL): **3.0%**
	medium (1000 ng/mL): **2.9%**	medium (1000 ng/mL): **2.6%**	medium (2000 ng/mL): **4.1%**
	high (2000 ng/mL): **3.4%**	high (2000 ng/mL): **3.5%**	high (4000 ng/mL): **3.0%**
Carry over	**No carryover** was observed after 3xULOQ (7500 ng/mL) after three injection repetition		
Interference studies	**No interfering signal** from matrix, internal standard, common drugs of abuse and prescription medications from 10 samples taken from 10 human sources.		
Recovery	**85–91%**		
Matrix effect	**91–93%**		**97–103%**
Limit of quantification (LOQ)	**10 ng/mL**		**20 ng/mL**
Limit of detection (LOD)	**1.5 ng/mL**		**0.5 ng/mL**
Selectivity, specificity	**No interferences**		
Standard Uncertainly (SD)	**16.6%**	**16.0%**	**18.0%**

slight ion suppression observed in LC-MS/MS. Though both methods separated the chiral methadone completely, the enantiomeric methadone separation in LC-MS/MS (Table 1) showed a greater resolution compared to that obtained in SFC-MS/MS.

From our knowledge, although a very limited number of publications [22–25] compared validation parameters between LC and SFC for bioanalytical methods on achiral and chiral compounds, results from real human samples were never compared until now. The present study provides the comparison between results obtained from 93 samples containing methadone. In Appendix 1, a very high correlation coefficient was obtained when comparing results obtained with the two methods, $r_s = 0.9977$ for R-methadone and $r_s = 0.9978$ for S-methadone. Interestingly, results are consistent for the three tested matrices and from low to high concentration levels, confirming the excellent linearity and accuracy of both methods. As highlighted in this work, although the enantiomeric EDDP separation was not achieved in SFC-MS/MS, the supercritical chromatography could be considered as an alternative to LC-MS/MS for the quantitative analysis of methadone enantiomers in biological fluids. Underlying the results obtained in our study, SFC-MS/MS should also be considered in other forensic routine applications, as a valid instrumentation for biological samples analysis.

Until now, only four studies were published describing the enantiomeric methadone separation in post-mortem samples, such as blood and tissues [8,9,31,32]. These studies used the same LC-MS/MS conditions for the enantiomeric separation and detection. This study provides the first R- and S-methadone quantitation using an SFC-MS/MS approach with dried matrix spot. Median R-/S-methadone ratio of 1.60 (0.79 – 4.23) and R-/S-EDDP of 0.84 (0.45 – 1.32) measured in peripheral blood are in agreement with previously published data [8,31]. Pharmacokinetics studies demonstrated the stereoselective CYP450 metabolism offered a longer half-life and a larger volume of distribution for R-methadone compared to the S-methadone [33–35]. Considering the higher cardiotoxicity of the S-methadone compared to the R-methadone [5], a novel compound could be developed to increase S-methadone metabolism by improving the S-stereoselective CYP2B6 enzyme activity [2].

In Table 6, we showed that ratio between pericardial fluid and peripheral blood was in favour of the former, with a significant correlation. Since scientists [36–38] revealed that pericardial fluid is a quite isolated compartment for different substances, similar to vitreous humour, this property could be verified for R-/S-methadone, by comparing pericardial fluid results with other isolated compartments, such as

Table 6
Ratio between cardiac blood and peripheral blood (CB/PB) with significant spearman correlation coefficient (R-methadone: $r_s = 0.7962$; $p < 0.0001$), (S-methadone: $r_s = 0.7655$; $p < 0.0001$). Ratio between pericardial fluid and peripheral blood (PF/PB) with significant spearman correlation (R-methadone: $r_s = 0.8929$; $p = 0.006$), (S-methadone: $r_s = 0.7143$; $p = 0.04$).

	N 27	CB/PB	N 7	PF/PB
R-MTD		**1.31**		**1.49**
S-MTD		**1.36**		**1.91**
Total MTD		**1.33**		**1.64**

Table 5
Median and ranges for total methadone and EDDP as well as R/S ratio found in the four examined matrices.

	N	Total MTD [ng/mL]	R/S-MTD ratio	N	Total EDDP [ng/mL]	R/S-EDDP ratio
Femoral blood	32	**1038** (32–5000)	**1.60** (0.79–4.23)	27	**285** (34–1421)	**0.84** (0.45–1.32)
Cardiac blood	29	**1217** (38–6900)	**1.63** (1.03–4.36)	28	**284** (32–1493)	**0.76** (0.56–1.07)
Pericardiac Fluid	9	**851** (89–3420)	**1.79** (0.81–4.22)	7	**257** (36–679)	**0.68** (0.52–1.02)
Vitreus	23	**145** (17–671)	**1.88** (1.11–4.67)	4	**65** (35–122)	**0.77** (0.68–0.84)

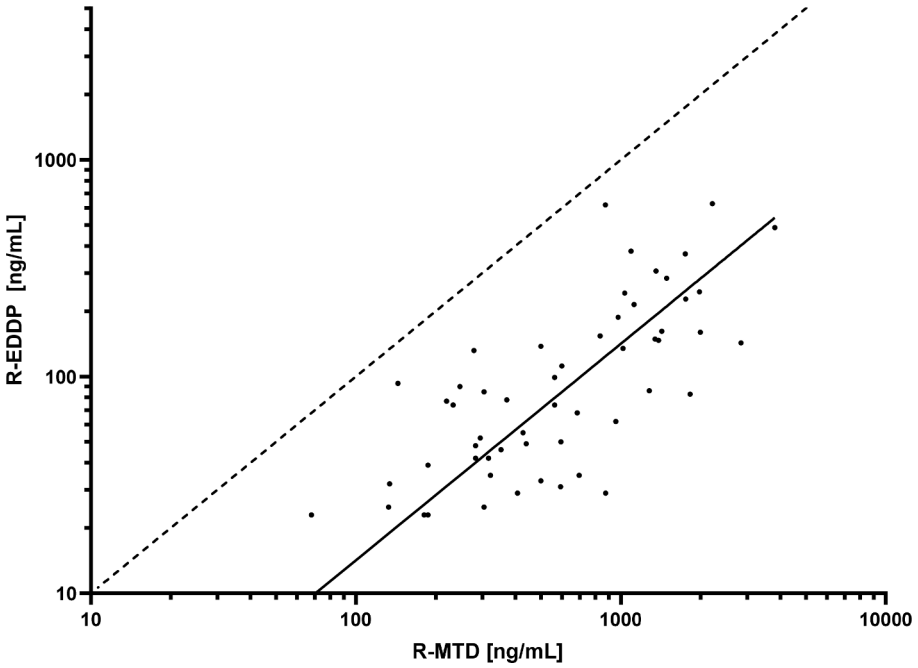

Fig. 5. Correlation between R-methadone and R-EDDP in all post-mortem analysis. Spearman correlation coefficient (r_s) was found to be significant with a $p < 0.0001$ and $r_s = 0.7308$.

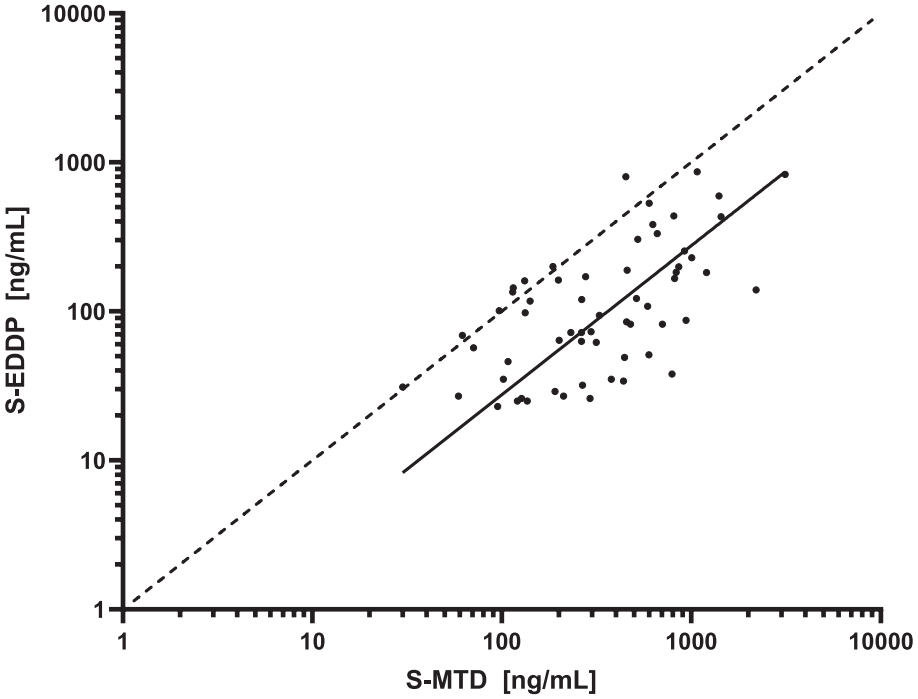

Fig. 6. Correlation between S-methadone and S-EDDP in all post-mortem analysis. Spearman correlation coefficient (r_s) was found to be significant with a $p < 0.0001$ and $r_s = 0.6196$.

cerebrospinal fluid [39], or by following the methadone distribution in pericardial fluid over time in post-mortem cases [38]. In vitreous humour, methadone and EDDP concentrations were lower than in the others analysed matrices (Table 5), probably due to the high hydrophobic property of both substances. Median of methadone concentration in vitreous humor of 145 ng/mL (N = 23) was in agreement with those obtained by Fernandez et al. [40] at 110 ng/mL (N = 5), while EDDP concentration totally disagree from the two studies. Median EDDP concentration obtained by Fernandez et al. [40] was 680 ng/mL (N = 5), whereas in our study it was only 65 ng/mL (N = 4) and, furthermore, results are probably lower because 19 samples presented EDDP concentration lower than the LOQ and were not included in the results. Our results for EDDP concentration in vitreous humor are in agreement with the results obtained in routine analysis on post mortem cases and with the relationship between methadone and EDDP in other human matrices [8,9,31,32].

Ratio between R-methadone and its main metabolite R-EDDP are shown to be in favour of the first one (Fig. 5). Although less significant,

ratio between S-methadone and S-EDDP showed the same behaviour (Fig. 6). R-methadone has a greater half-life time compared to S-methadone and probably, both have a longer half-life time compared to their main enantiomers metabolites, resulting in a higher concentration in almost all analysed matrices [2]. Besides polymorphic genetics in CYP450 enzymes, scientist have shown correlations between EDDP elimination and urine pH [41]. They have highlighted that as the urinary pH increases, the proportion of excreted EDDP increases.

5. Conclusion

This study described the development of LC-MS/MS and SFC-MS/MS enantiomeric methadone quantitation methods and compared their quantitative performance in real post-mortem dried matrix spots from blood, vitreous humor and pericardial fluid. Methods validation comparison and correlations graphs between results obtained with both methods confirms that SFC-MS/MS could be taken in consideration as an alternative to the widely used LC-MS/MS for bioanalytical methods development and validation. Both methods presented in this study can be easily and quickly used in toxicological routine analysis for the methadone quantitation in human fluids matrices, but the polysaccharide coated column IH-3 used in SFC does not allow the enantiomeric EDDP separation.

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CRediT authorship contribution statement

F. Mueller: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Project administration, Resources, Software, Validation, Visualization, Writing - original draft, Writing - review & editing. **G.L. Losacco:** Data curation, Investigation, Software, Visualization, Writing - review & editing. **R. Nicoli:** Investigation, Resources, Supervision, Visualization, Writing - review & editing. **D. Guillarme:** Conceptualization, Formal analysis, Investigation, Methodology, Project administration, Resources, Supervision, Validation, Visualization, Writing - review & editing. **A. Thomas:** Conceptualization, Formal analysis, Investigation, Methodology, Project administration, Resources, Supervision, Validation, Visualization, Writing - review & editing. **E. Grata:** Conceptualization, Formal analysis, Investigation, Methodology, Project administration, Resources, Supervision, Visualization, Writing - review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jchromb.2021.122755.

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