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Chiral on-line solid phase extraction coupled to liquid chromatography–tandem mass spectrometry assay for quantification of (R) and (S) enantiomers of methadone and its main metabolite in plasma

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ABSTRACT

The authors aimed at developing a liquid chromatography tandem mass spectrometry (LC-MS/MS) method with online extraction to determine (R)- and (S)- methadone enantiomers and its main metabolite 2-ethylidine-1,5-dimethyl-3,3 diphenylpyrrolidine (EDDP) in plasma.

The analysis combined straightforward sample preparation, consisting of protein precipitation with acetonitrile, and an online enrichment by a flush/back-flush cycle before the second dimension chromatography.

Using D_3-deuterated internal standards allows overcoming significant relative matrix effect. Our method was linear up to 2000 ng/mL. This simple sample preparation provides sensitive (the limit of quantitation is 25 ng/mL for (R,S)-methadone and EDDP and 12.5 ng/mL for (R)- and (S)- methadone), accurate and precise (the intra-day and inter-day imprecision and inaccuracy are lower than 15%) quantification of the plasma concentration of these drugs.

We have developed a reliable LC-MS/MS method for both routine therapeutic drug monitoring and pharmacokinetics studies and for toxicology analysis in the setting of methadone treatment or intoxication

1. Introduction

Methadone (MTD) is a synthetic opioid sold in France as a racemic (50/50) mixture of (R,S)–MTD. MTD is prescribed for opiate dependence, in accordance with guidelines of the health authorities used for opiate substitution treatment or maintenance treatment. Following administration, MTD is extensively metabolized. EDDP (2-ethylidene-1,5-dimethyl-3,3-diphenylpyrrolidine), an inactive metabolite, is the main product of this process. The main P450 cytochromes involved are: 1A2, 2B6, 3A4/5, 2C19, 2C9, 2E1 and 2D6 [1–6]. Some of these cytochromes display stereospecificity for one of the enantiomers. For example, CYP450 2B6

Abbreviations: EDDP, 2-ethylidene-1,5-dimethyl-3,3-diphenylpyrrolidine; MTD, methadone; LC-MS/MS, Liquid Chromatography tandem mass spectrometry; SPE, Solid Phase Extraction; ACN, Acetonitrile; QC, Quality Control; MRM, Multiple Reaction Monitoring; LLOQ, Lower Limit Of Quantification; CV, Coefficient of Variation; EMEA, European Medicines Agency; FDA, Food and Drug Administration; IS, Internal Sandard

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primarily metabolizes the (S)- form, CYP450 2C19 the (R)- form, while CYP450 3A4 not stereoselective metabolizes both enantiomers. The (R)- enantiomer is the active form of MTD, whose affinity is 10 times greater than that of the (S)- enantiomer for the μ1 and μ2 opioid receptors. The (S)- form is responsible for the poor cardiac tolerance to MTD (heart rhythm disorders such as QTc prolongation…) [7–9].

MTD is a molecule with a narrow therapeutic index that displays broad inter-individual variability, both in terms of pharmacokinetics and pharmacodynamics, which leads to considerable variability in terms of response to and tolerance of treatment [6–8]. Lot of studies purposed to measure plasma concentration of MTD by chromatography [10–24]. The majority of these methods used liquid-liquid extraction [7,10,15–19,24]. Several assays were proposed to measure plasma concentrations of (R)- and (S)- enantiomers of MTD and EDDP by liquid chromatography–tandem mass spectrometry assay (LC-MS/MS) [16,17]. We present the first liquid chromatography–tandem mass spectrometry method with a simple sample pre-treatment, which can be used for the simultaneous quantification of (R)- and (S)- enantiomers of MTD and its main metabolite EDDP.

2. Material and methods

2.1. Samples

All samples tested in this work derived from an ongoing drug-monitoring program and were reported in accordance with ethical guidelines. Informed consent was not required.

2.2. Chemicals, reagents and standard solutions

All solvents and reagents were HPLC-grade and were purchased from VWR International (Fontenay-sous-Bois, France). Racemic MTD, racemic MTD-D_3, EDDP and EDDP-D_3 solutions were purchased from LGC standards (Molsheim, France) (Fig. 1). Purified (S)–MTD, purchased from Alsachim (Illkirch, France), was only used to identify the (S)–MTD enantiomer peak. Racemic MTD-D_3 and EDDP-D_3 were used as internal standard (IS). In-house prepared multilevel plasma calibrator containing both MTD and EDDP were used for all LC-MS/MS assays. In house prepared quality control (QC) (two levels) and one level of commercial QC provided by Medichem Diagnostica (Steinenbronn, Germany) were used for all LC-MS/MS assays. Protein precipitation solution was a mixture of ACN containing IS (1 μg/mL) and stored at −20 °C. The different lots of drug-free plasma samples originated from our laboratory.

2.3. LC-MS/MS assay

2.3.1. Standard, quality controls and sample preparation

Working solutions were prepared as follows: (R, S)–MTD, EDDP, MTD-D_3 and EDDP-D_3 at a concentration of 10 μg/mL (methanol). A seven-point calibration curve and homemade quality controls were prepared for each analyte by diluting know volumes of the working solution in drug-free human plasma (collected with K_3EDTA as anticoagulant). For (R,S)–MTD and EDDP the calibration curve points were: 0, 100, 250, 500, 1000 and 2000 ng/mL, and the homemade quality control values were 200 and 800 ng/mL. The commercial QC value was 100 ng/ml for (R,S)–MTD and 28 ng/mL for EDDP.

Each sample was further treated as described. Protein precipitation was carried out in 1.5 mL polypropylene tubes (Eppendorf, Le Pecq, France). A volume of 100 μL of calibrator, QC or patient sample was mixed with 200 μL of precipitation solution. The mixture was vortex-mixed for 5 min, and centrifuged 10 min at 15300 g at 4 °C. Subsequently the supernatant was transferred in a polypropylene tube with pierceable membrane screw caps, and 20μl was injected in the chromatographic system.

2.3.2. Instrumentation setup

Instrument setup is shown in Fig. 2. The chromatographic system consists of Agilent 1200 Series components (Palo Alto, USA) including two binary pumps, column oven, and autosampler. The hardware configuration included a triple quadrupole mass spectrometer ABSciex API 3200 QTrap™ (Toronto, Canada) equipped with a turboionSpray ionization source. ESI voltage was set to 5000 V with positive ionization mode. Nitrogen was used both as nebulizing gas and drying gas. The source temperature was 600 °C. Positive ion electrospray, schedule MRM mode was used for analytes and IS (Table 1).

2.3.3. Two dimension chromatographic separation

The chromatographic conditions are presented in the Fig. 2. First dimension chromatography is an on-line enrichment performed by a perfusion column (Oasis HLB™ 2.1 × 20 mm, 5 μm, Waters, Saint-Quentin En Yvelines, France). The binary pump 1 A supplied water/ACN (95:5, v/v) delivered at a flow rate of 4 mL/min. After 1.2 min of enrichment of analytes and IS on the Oasis column, the valve was switched. The back-flush elution was performing by the mobile phase constituted of water/ACN (50:50, v/v) / triethylamine (0.04%)

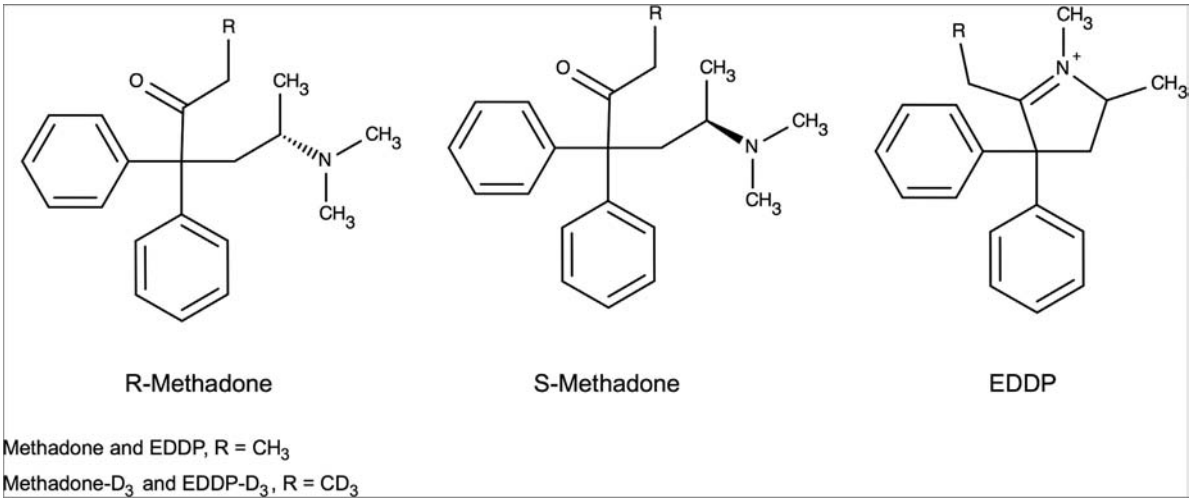

Fig. 1. Chemical structure of methadone, EDDP and internal standards.

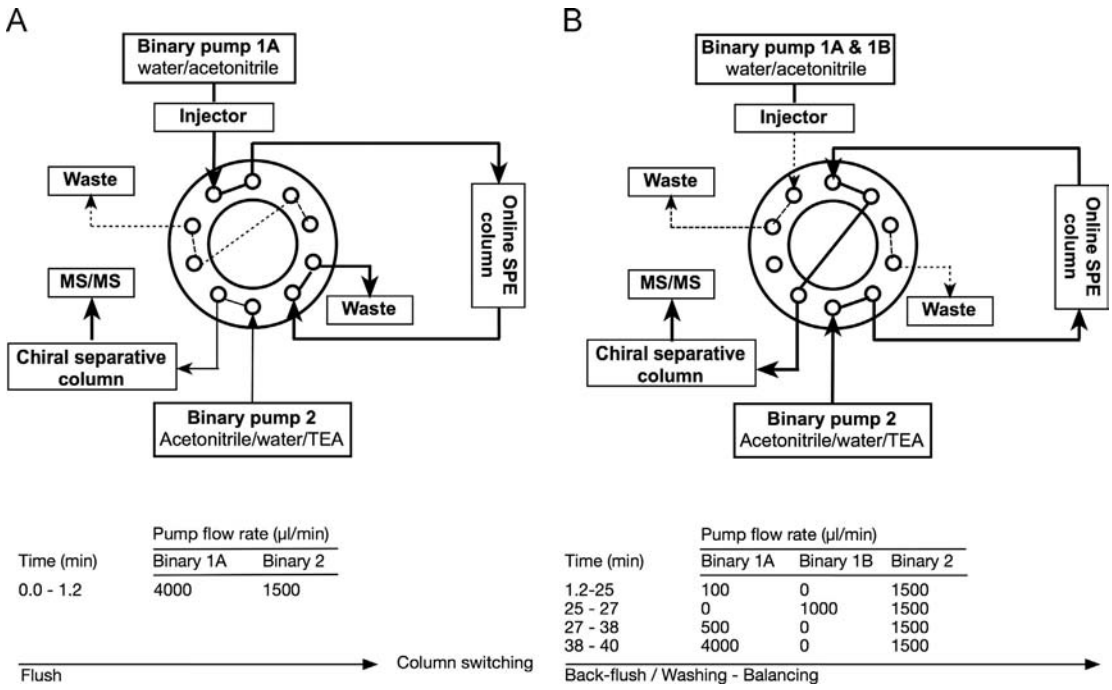

Fig. 2. Timetable of SPE and HPLC mobile phase flow rate and ten-port switching valve position programming. Connections and positions of the column-switching valve for on-line extraction step from 0.0 to 1.2 min (**A**). Analytes elution, transfer to HPLC column, analysis and SPE-Online washing step from 1.2 to 40 min (**B**).

Table 1
Instrument settings for mass/charge (m/z) transitions.

Compound	RT (min)	Precursor ion (m/z)	Product ion (m/z)	EP	CEP	CE	CXP
(R)–MTD	12.5	310.046	265.2	7.5	20	19	6
(S)–MTD	13.1						
(R)–MTD-D_3	12.4	313.046	268.2	7.5	21	19	6
(S)–MTD-D_3	13.0						
EDDP	34	278.088	234.1	8.5	18	31	4
EDDP-D_3	34.5	281.088	234.1	8.5	18	31	4

Retention times (RT), entrance potential (EP), cell entrance potential (CEP), collision energy (CE), cell exit potential (CXP) for API 3200. Dwell time was 100ms for each ion

at a constant flow rate of 1.5 mL/min during the total run-time. The second dimension chromatography was performed using a Phenomenex Lux 5 µm Cellulose-4, new column 150 × 4.6 mm (Torrance, USA) housed in oven at 25 °C. The Oasis column was washed with water (90%) / ACN (10%) / formic acid (0.1%) for 1 min with binary pump 1B.

2.4. LC-MS/MS validation procedures

2.4.1. Inaccuracy, imprecision and limits of quantitation and detection

Inaccuracy and imprecision were evaluated by analysing quality control samples at low, medium and high concentrations on five different days [25]. For intra-day validation, five samples of each quality control were analysed on the same day. For inter-day validation, concentrations of the quality control samples were determined on five separate days. Inaccuracy is defined as the percentage of deviation from the nominal level and imprecision as the coefficient of variation (%CV) within a single run (intra-assay) and between different days (inter-assay). The imprecision and the inaccuracy should not exceed 15%.

2.4.2. Limit of quantification

Triplicates of a dilution of the low QC determined the Lowest Limit of Quantification (LLOQ) of MTD and EDDP. The lowest concentration that can be measured with an imprecision and inaccuracy below 20% each, defines the LLOQ.

2.4.3. Matrix effects

The matrix effects were investigated according to the European Medicines Agency guidelines [26]. In the case of on-line sample preparation, the variability of the response from lot-to-lot should be assessed by analysing at least six lots of plasma matrix, spiked at three level of concentration: 100, 200 and 800 ng/mL for (R,S)–MTD and EDDP and 50, 100 and 400 ng/mL for (R)–MTD and (S)–MTD. The overall %CV calculated for the concentration should not be greater than 15%.

2.4.4. Carry-over effects

Carry-over effects were assessed by testing three high level plasma samples and three low level plasma samples in succession (L1, L2 and L3) [26]. This sequence was reproduced five times. There should not be found any statistical difference between L1 and L3 averages using a Student test. The level tested were respectively 100 and 800 ng/mL for (R,S)–MTD, 50 and 400 ng/mL for (R)- and (S)–MTD and 28 and 800 ng/mL for EDDP, according to the French committee of accreditation recommendations [27].

2.4.5. Extraction recovery

The recovery was determined by comparing the absolute peak area obtained from standard plasma extracted according the relevant procedure versus a blank human plasma extract spiked after extraction with the same amount of molecule (62.5, 125, 250, 500, 1000, 2000 and 4000 ng/ml) [25]. For the extraction recovery the plasma protein was precipitated by ACN alone without IS.

2.4.6. Stability

Stability of the analytes (aliquots of each level of quality control samples) was investigated in plasma stored at room temperature

and +4 °C for 3, 7 and 14 days, and at −20 °C for 3, 14 and 90 days. Stability of spiked whole blood stored at room temperature for 3, 7 and 14 days was verified. The mean concentration at each level should be within ±15% of the nominal concentration [26]. The stability of the stock solutions of drugs and IS stored at −20 °C was also evaluated.

2.4.7. Selectivity

Assessment of selectivity needs to be confirmed in the presence of in vivo metabolites of the analytes. Some metabolites may be converted to the parent drug during sample preparation and or undergo partial fragmentation in the ion sources at high temperatures giving the same molecular ion as for the parent drug [28,29]. The selectivity of the method was evaluated by monitoring all analytes and IS m/z transitions for MTD and EDDP free human plasma samples from six different sources to determine the presence or absence of endogenous peaks. The "cross-talk" between the MRM transition used for monitoring analytes and IS was evaluated by the analysis of five samples containing only one analyte and its IS.

2.5. Data analysis, interpretation and statistics

Racemic MTD was used in all experiment, for (R)–MTD or (S)–MTD or (R,S)–MTD validation, only corresponding peaks were integrated and used for calculation. Chromatographic data processing was performed using the Analyst 1.5.2 software package (ABSciex, Foster City, USA). Linear regression analyses and statistical analyses were performed with Prism software (Graphpad Software, La Jolla, USA).

3. Results

3.1. LC-MS/MS method validation

The method was validated in human plasma over the concentration range of 100 to 2000 ng/mL for MTD and EDDP and 50 to 1000 ng/mL for (R)- and (S)- MTD. The calibration curves were satisfactorily fitted by linear regression (1/x weighting). Deviations of the back calculated concentrations were within 85% and 115% of the nominal concentrations (80% and 120% for the lower level which is the limit of quantitation) and the correlation coefficients for all calibration curves were above 0.990 [25–27]. Inaccuracy and imprecision, determined for both intra- and inter-runs, are summarized in Table 2. Intra-day and inter-day imprecision and inaccuracy were below 15% for (R,S)–MTD, (R)–MTD, (S)–MTD and EDDP. The LLOQ were respectively 25 ng/mL for (R,S)–MTD and EDDP, and 12.5 ng/mL for (R)–MTD and (S)–MTD. Figs. 3B and C shows representative chromatogram obtained from standard. Because no external quality control were available, the bias of our

Table 2
Method inaccuracy and precision assessment.

Analyte	QC concentration (ng/mL)	Intra-day ($n=5$)		Inter-day ($n=5$)	
		CV (%)	Mean inaccuracy (%)	CV (%)	Mean inaccuracy (%)
(R,S)-MTD	25(LLOQ)	5.95	7.72	1.41	−0.88
	100	3.49	−4.2	4.11	3.94
	200	8.47	−1.9	4.74	−5.2
	800	7.79	−5.28	7.87	−2.75
(R)–MTD	12.5 (LLOQ)	10.15	11.28	4.78	−4.16
	50	4.45	0.4	4.75	3.24
	100	9.55	1.76	4.50	−5.8
	400	11.85	−6.45	6.84	−4.82
(S)–MTD	12.5 (LLOQ)	13.77	4.32	3.55	−0.16
	50	7.31	−10.36	3.81	5.89
	100	5.16	2.4	4.91	8.88
	400	5.70	−3.75	7.59	0.42
EDDP	25 (LLOQ)	9.23	−2.16	11.22	13.92
	28	6.22	5.64	4.52	−2.71
	200	4.66	2.5	8.62	8.22
	800	3.55	0.55	10.19	9.58

The following abreviations were used: QC: quality control; LLOQ: lower limit of quantification; CV: coefficient of variation.

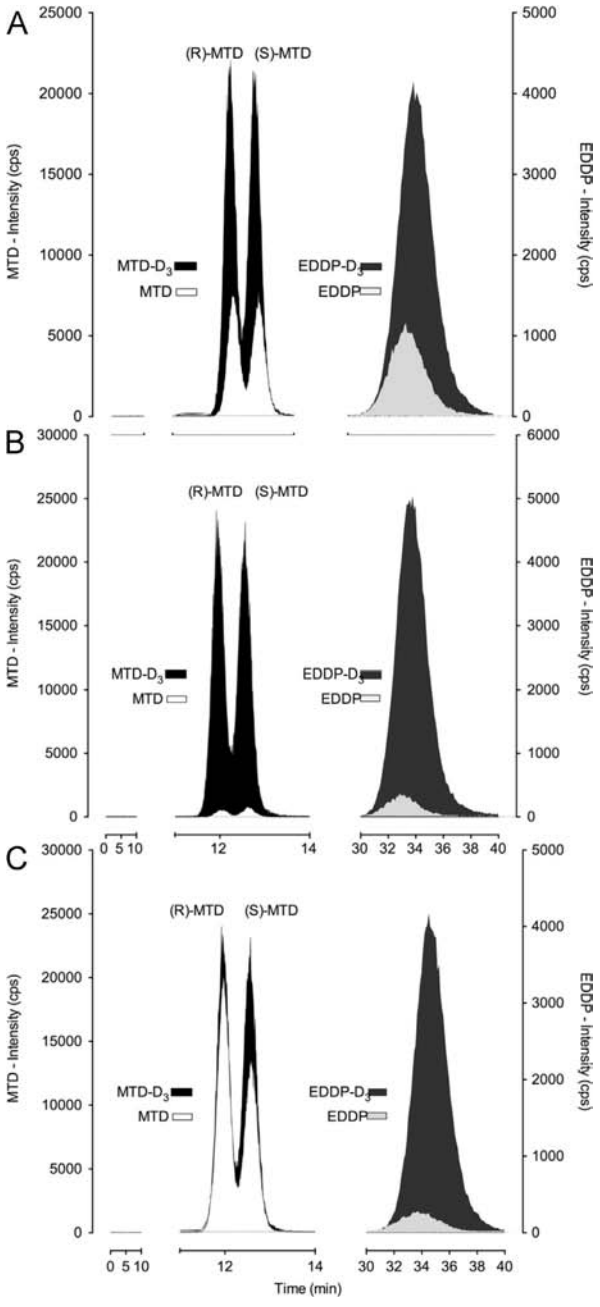

Fig. 3. Representative chromatogram. Example of extracted ion chromatograms obtained by schedule MRM for MTD, EDDP and IS. Chromatogram of plasma extract spiked at 100 ng/mL for MTD and EDDP (**A**). Chromatogram of plasma extracts spiked at 50 ng/mL (LLOQ) for (R,S)–MTD and EDDP (**B**). Chromatogram obtained from a patient treated with 80 mg of (R,S)–MTD (**C**). As indicated, the left and the right peak of MTD are respectively the (R)- and the (S)- enantiomers. For each chromatogram, MTD-D_3 and EDDP-D_3 were used at 1000 ng/mL. Black chromatogram: MTD-D_3, white chromatogram: MTD; dark gray: EDDP-D_3; light grey: EDDP.

method was evaluated based on the inaccuracy as purposed by the French committee of accreditation [27].

3.2. Carry-over and matrix effect

Carry-over effects proved to be moderate and acceptable and any statistical difference between L1 and L3 could not be established using a Student test. The exact carry-over values were respectively −0.78%, 0.24%, −1.26% and −1.22% for (R,S)–MTD, (R)–MTD, (S)–MTD and EDDP. No critical matrix effect was observed as the overall CV for the concentrations in the spiked plasma sample was below 15% (Table 3).

3.3. Stability

The stability of MTD and EDDP was verified in plasma stored at −20 °C for three months or stored at +4 °C and at room temperature for 15 days. To be considered as stable, the drug concentrations had to be within the ±15% of the nominal concentration. Additionally, stability of extracts at +4 °C for 72 h was demonstrated. −20 °C frozen MTD and EDDP quality controls and patient samples remained stable over for months. MTD and EDDP spiked in whole blood sample remain stable for 14 days at room temperature.

3.4. Extraction recovery

Extraction recovery was evaluated for all analytes using standards spiked at the concentrations mentioned in the Table 3. For all drug and metabolite, the mean recovery was 25% (see Table 4). Extraction of analytes was consistent over the entire range of the standard curve used.

3.5. Selectivity

No interference was found with the retention times of MTD and EDDP and IS (data not shown).

Table 3
Matrix effects investigation.

	%CV		
Analytes	L (n=6)	M (n=6)	H (n=6)
(R-S)–MTD	7.04	10.9	11.83
(R)–MTD	8.99	9.89	12.38
(S)–MTD	6.62	11.35	12.24
EDDP	5.93	7.48	14.28

CQQC values are respectively 200, 400 and 800 ng/mL for (R-S)–MTD and EDDP and 100, 200, 400 ng/mL for (R)–MTD and (S)–MTD. L, M and H were used respectively for low, middle and high concentration QC.

Table 4
Extraction recovery percentage.

Concentrations (ng/mL)	(R,S)–MTD	(R)–MTD	(S)–MTD	EDDP
4000	22.47	–	–	21.38
2000	22.00	20.90	24.19	22.50
1000	25.91	21.95	22.52	25.85
500	25.94	26.12	25.61	26.45
250	27.23	25.77	25.76	27.48
125	26.87	26.82	26.68	26.40
62.5	29.55	26.61	26.38	27.07
31.25	–	30.92	26.88	-
Mean ± sd	25.71 ± 2.67	25.58 ± 3.33	25.43 ± 1.57	25.30 ± 2.38

3.6. Application

Fig. 3C shows the chromatogram of a patient treated with 80 mg per day of MTD. We can show asymmetry of the two enantiomers peaks. The plasma concentrations measured for this patient are: 840 ng/mL for (R,S)–MTD, 483 ng/mL for (R)–MTD, 357 ng/mL for (S)–MTD and 176 ng/mL for EDDP.

4. Discussion

We describe the performance of an LC-MS/MS chiral method for simultaneous quantification of plasma (R)–MTD, (S)–MTD and EDDP concentrations. The main objective of this article was to proceed to a complete validation of our method in order to be able to monitor both MTD enantiomers and its main metabolite EDDP.

Assessment of intra- and inter-day variability was < 12% for all concentrations tested and < 14% for LLOQ (Table 2). Method accuracy was found to be within ±15% for intra-run and inter-run. No “cross-talk” from metabolites or endogenous compounds was observed. For (R,S)–MTD, (R)–MTD, (S)–MTD and EDDP, no signal at the retention time of the analytes of interest were observed in blank plasma. Overall performance of our method achieves a LLOQ of 50 ng/mL for racemic MTD and EDDP, and 25 ng/mL for the (R)- and (S)- enantiomers with an initial plasma sample volume of 100 μL. These performances are consistent with therapeutic drug monitoring, enantiomeric pharmacokinetics studies of MTD and toxicology.

We choose the one-line solid phase extraction before liquid chromatography–tandem mass spectrometry to reduce the matrix effect interferences [30]. Compared with a direct liquid chromatography–tandem mass spectrometry assay, the one-line solid phase extraction before liquid chromatography–tandem mass spectrometry prevents the introduction of endogenous compounds into the mass spectrometer to limit the matrix effects as previously demonstrated. Moreover, the use of MTD-D_3 and EDDP-D_3 as IS that co-elutes with analytes attenuates matrix effect interferences.

For all analytes, recovery is 25% and provides sufficient signal for the lower limit of quantitation. Although extraction recovery is not required according to the EMEA guidelines, the FDA guidelines indicates that recovery of the analyte need not to be 100%, but the extent of recovery of an analyte should be consistent, precise, and reproductible, as it is the case here [25,26]. We try to reduce the chromatographic run time, but enantiomeric separation need quite strict and rigorous conditions. Few assays have been reported in the literature to measure concentration of (R)- and (S)- enantiomers of MTD *plus* EDDP by LC-MS/MS [16,17,21,31]. only two methods were publish in serum [16,17]. We describe the first one with on-line setup developed to determine plasma concentration of EDDP *plus* (R)- and (S)- enantiomers of MTD.

5. Conclusion

For the first time, LC/MS-MS has been successfully applied for the quantitative determination of (R)–MTD, (S)–MTD and EDDP concentrations in plasma using D_3-deuterated IS and on-line extraction. The results of our validation indicate that this method is sensitive, accurate, precise without matrix effect and can be used for therapeutic drug monitoring, pharmacokinetics studies or toxicology analysis. Due to the importance and the difficulties of individualizing MTD treatment, data of enantio-selective variability of the pharmacokinetics of the MTD have to be validated in clinical studies and require a reliable and validated analytical method.

Conflict of interest

authors report no conflict of interest

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