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Validation and long-term evaluation of a modified on-line chiral analytical method for therapeutic drug monitoring of (*R*,*S*)-methadone in clinical samples☆

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ABSTRACT

Matrix effects, which represent an important issue in liquid chromatography coupled to mass spectrometry or tandem mass spectrometry detection, should be closely assessed during method development. In the case of quantitative analysis, the use of stable isotope-labelled internal standard with physico-chemical properties and ionization behaviour similar to the analyte is recommended. In this paper, an example of the choice of a co-eluting deuterated internal standard to compensate for short-term and long-term matrix effect in the case of chiral (*R*,*S*)-methadone plasma quantification is reported. The method was fully validated over a concentration range of 5–800 ng/mL for each methadone enantiomer with satisfactory relative bias (−1.0 to 1.0%), repeatability (0.9–4.9%) and intermediate precision (1.4–12.0%). From the results obtained during validation, a control chart process during 52 series of routine analysis was established using both intermediate precision standard deviation and FDA acceptance criteria. The results of routine quality control samples were generally included in the ±15% variability around the target value and mainly in the two standard deviation interval illustrating the long-term stability of the method. The intermediate precision variability estimated in method validation was found to be coherent with the routine use of the method. During this period, 257 trough concentration and 54 peak concentration plasma samples of patients undergoing (*R*,*S*)-methadone treatment were successfully analysed for routine therapeutic drug monitoring.

1. Introduction

Matrix effects could represent an important drawback when dealing with liquid chromatography (LC) coupled to mass spectrometry (MS) or tandem mass spectrometry (MS/MS). In addition to interferences that could be identified, matrix effects can affect trueness and precision, and therefore accuracy of the assay. Matrix effects can be due to short-term and/or long-term variations inside the MS interface or capillary transfer, which can result in signal suppression or enhancement [1]. Short-term matrix effects mainly result from co-elution of residual matrix components with analytes and/or internal standards (ISs). This can alter the ionization process of the compounds of interest, although these co-eluting species do not appear on the chromatographic trace when only selected ions of the target compounds are monitored [2]. Thus, the scan mode should be selected during the development step. As suggested by Enke's model, short-term matrix effects are based on an equilibrium competition between target analytes and ionized endogenous species for charged sites on the surface of droplets obtained during the electrospray ionization (ESI) process [3]. Long-term effects are difficult to be evidenced and could be related to the MS geometry. According to the commercially used set-up, various effects could appear. As an example, in the case of a MS transfer glass capillary, ions could be absorbed on the surface at high temperature, and then further released, leading to unpredictable signal suppressions and enhancements. Long-term effects can also result from variable electronic effects, with accumulation of some ions in the MS system, leading to large amplitude signal variations.

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These phenomena, particularly present when dealing with complex matrices, such as biological fluids are drastically reduced by an efficient sample preparation such as liquid–liquid extraction (LLE), off-line solid phase extraction (SPE) or column-switching procedures (on-line SPE), with or without sample treatment such as dilution or protein precipitation [4–6]. Furthermore, an adequate chromatographic separation with elution of the compounds outside the matrix effect time window is required, with matrix compounds generally observed at the beginning of the chromatogram [7]. Short-term matrix effects should be closely assessed during method development. Qualitative evaluation can be performed with the most currently implemented technique using the continuous post-column infusion of an analyte solution [8]. Quantitative assessment can also be performed by the comparison of the signal obtained with the analysis of pure standard solutions of the analytes and blank extracts spiked with the analytes before and after extraction as described previously [9].

When dealing with quantitative analysis, matrix effects can be corrected with the use of stable isotope-labelled IS, owing to similar physico-chemical properties and ionization behaviour of the matching analyte and the IS. This approach is recommended in a consensus document proposed by the American Association of Pharmaceutical Scientists (AAPS) and the Food and Drug Administration (FDA) [10]. However, in particular situations and according to the selected isotope-labelled IS, its retention time could be different from the target analyte, and insufficient to compensate for matrix effects when these are present only at the retention time of the IS or the analyte [11]. In these situations, it is mandatory to work with a co-eluting stable isotope-labelled IS. The IS concentration should be chosen at a relatively low concentration corresponding to about the first third of the calibration range, in order to minimize potential interferences on the analyte due to cross-contribution from potential impurities from the isotope-labelled IS.

Methadone (MTD), a synthetic μ-opioid receptor agonist, is used for maintenance treatment in opioid-dependent patients and for pain relief [12]. This drug is mainly administered as a chiral mixture of (*R*,*S*)-MTD, but only (*R*)-MTD has a potent agonist action on the target receptor [13], while (*S*)-MTD has been associated with potential cardiac adverse events [14]. Due to a high interindividual variability in the stereoselective metabolism of (*R*,*S*)-MTD [15], analytical methods which allow quantification of each enantiomer separately are required for therapeutic drug monitoring of the patients to improve efficiency and safety of the treatment [16]. Progress has been made over the past few years for the chiral quantification of (*R*,*S*)-MTD in plasma. Several methods, using mainly HPLC-UV, have been proposed [17–20], with off-line sample preparation performed by LLE or SPE. More recently, we have proposed a LC–ESI-MS method using an on-line SPE with a column-switching system [21].

In this paper, an example of the choice of a co-eluting deuterated IS to compensate for short-term and long-term matrix effect was reported as well as the long-term stability of the method [21] used in routine analysis of clinical plasma samples of patients taking (*R*,*S*)-MTD.

2. Experimental

2.1. Chemicals

(*R*,*S*)-MTD was purchased from Hänseler AG (Herisau, Switzerland), (*R*,*S*)-MTD-D9 ((±)-6-di(trideuteromethyl)amino-4,4-diphenyl-1-trideuteroheptan-3-one) (isotopic purity, 100.0%) and (*R*,*S*)-MTD-D3 ((±)-6-dimethylamino-4,4-diphenyl-1-trideuteroheptan-3-one) (isotopic purity, 99.3%) from LGC Promochem (Molsheim, France), acetonitrile (ACN) from Stehelin P.H. and Cie AG (Basel, Switzerland), hydrochloric acid (HCl) from VWR International AG (Dietikon, Switzerland) and triethylamine (TEA) and formic acid from Sigma–Aldrich Chemie GmbH (Buchs, Switzerland).

2.2. Working solutions and spiked plasma

Working solutions of (*R*,*S*)-MTD at 1, 10 and 100 μg/mL in 0.01 M HCl were prepared by appropriately diluting a stock solution at 1000 μg/mL in 0.1 M HCl. Solutions of (*R*,*S*)-MTD-D9 and (*R*,*S*)-MTD-D3 at 0.5 μg/mL in ACN were prepared by diluting a stock solution at 1000 μg/mL in MeOH. Human blank plasma was obtained from the Regional Blood Transfusion Center (Lausanne, Switzerland). For the preparation of calibration and quality control (QC) samples, blank plasma samples were spiked with appropriate amount of (*R*,*S*)-MTD working solutions to reach a concentration range of 10–1600 ng/mL.

2.3. Sample preparation

Plasma samples (100 μL) were treated 1:2 (v/v) with the IS solution in ACN (200 μL) in order to precipitate the proteins. After vortexing, samples were centrifuged at 3300 × *g* for 11 min at 8 °C. One hundred microlitres of the supernatant was mixed with 150 μL H_2O and 50 μL of the mixture was injected into the LC–MS system.

2.4. LC–MS

The column-switching system comprised of an Agilent Series 1100 LC (Agilent Technologies, Waldbronn, Germany) equipped with an autosampler, a binary pump for sample loading and washing, a six-port switching valve and a mass spectrometer (see below). An additional Agilent Series 1100 LC isocratic pump was used to deliver the mobile phase for the chiral separation. The Chemstation software (Agilent Technologies) was used for instrument control, data acquisition and data handling. An Oasis HLB pre-column (20 mm × 2.1 mm I.D.; particle size 25 μm) from Waters Corp. (Milford, MA, USA) was used to extract MTD enantiomers from plasma samples. An in-line filter was placed prior to the pre-column to eliminate particles which could remain in the sample after precipitation and centrifugation steps. MTD enantiomers separation was performed on a Chiralcel OJ-R column (150 mm × 2.1 mm I.D., particle size 5 μm) from Daicel Chemical Industries (Tokyo, Japan). A guard column (10 mm × 4.6 mm I.D., particle size 5 μm) from Daicel Chemical Industries, packed with the same chiral stationary phase (CSP), was installed in front of the analytical column.

Fifty microlitres of the supernatant obtained from plasma after protein precipitation and centrifugation was injected onto the Oasis HLB pre-column with a mobile phase constituted of water–ACN (95:5, v/v) delivered at a flow rate of 4 mL/min. Simultaneously, the chiral column was conditioned with the mobile phase constituted of ACN–TEA 0.02% in water (65:35, v/v) delivered at a flow rate of 250 μL/min. After 1.2 min, the valve was switched and analytes were backflushed from the pre-column to the analytical column. After 3.5 min, the valve was switched to its original position for pre-column washing and reconditioning. The pre-column washing step was performed with a mobile phase constituted of water–ACN (10:90, v/v) supplemented with 0.1% formic acid for 2 min. Meanwhile, analytes were separated on the chiral column and detected by MS. All analyses were performed at 25 °C.

The LC column-switching set-up was coupled to an Agilent Series 1100 MSD single quadrupole (Agilent Technologies) equipped with an orthogonal ESI source. Nitrogen was used both as nebulizing gas at a pressure of 25 psi and as a drying gas at a temperature of 350 °C with a flow rate of 7 L/min. ESI voltage

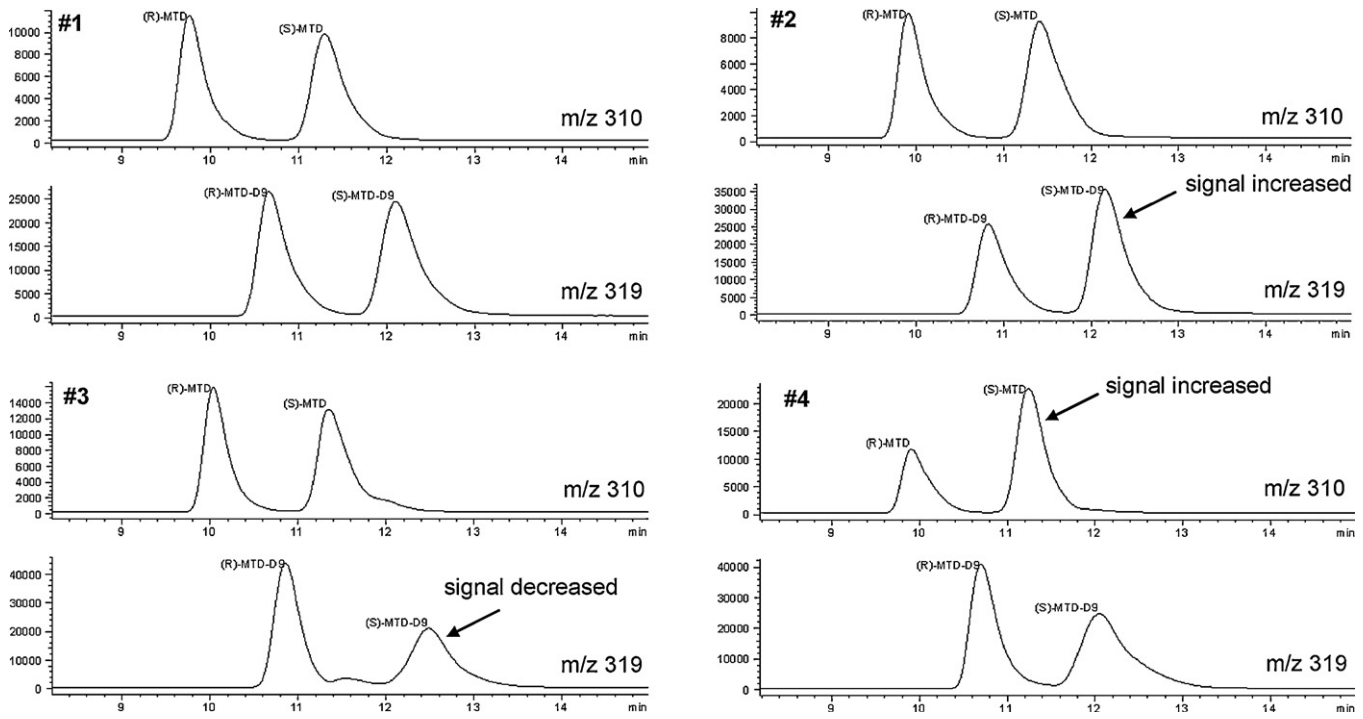

Fig. 1. Selected ion monitoring chromatograms showing normal situation (#1) and the different situations of signal modifications due to matrix effects (#2 to #4) observed with quality control samples, spiked at 300 ng/mL with (*R*,*S*)-MTD and using (*R*,*S*)-MTD-D9 (250 ng/mL) as internal standard.

was set to 5500 V (positive ionization mode). Selected ion monitoring (SIM) mode was chosen with a skimmer voltage optimized at 65 V for ions 310 *m*/*z* $[MTD+H]^+$, 313 *m*/*z* $[MTD\text{-}D3+H]^+$ and 319 *m*/*z* $[MTD\text{-}D9+H]^+$. Dwell time was 289 ms for each ion.

2.5. Method validation

The method was fully validated based on the approach proposed by the Société Française des Sciences et Techniques Pharmaceutiques (SFSTP) [22,23]. Method selectivity was assessed by analysing blank plasma samples ($n = 10$). For quantitative determination, validation experiments were repeated over 3 series ($j = 3$). For calibration, 6 levels ($k = 6$) (5, 50, 100, 200, 400 and 800 ng/mL for each MTD enantiomer) repeated 3 times ($n = 3$) were considered and for validation samples or QC samples, 4 levels ($k = 4$) (5, 200, 400, 600 ng/mL for each MTD enantiomer) repeated 4 times ($n = 4$) were analysed. To include analysis outside the calibration range, a supplementary level of QC sample at 2400 ng/mL for each MTD enantiomer was also included. This particular sample was diluted 1:4, v/v with blank plasma before the analysis.

Calibration curves were based on the peak area ratio of MTD to IS. The trueness, repeatability and intermediate precision were determined with recalculation of the QC samples with the daily response function established. Trueness was expressed as the ratio between theoretical and the average measured concentration. Repeatability was expressed as the relative standard deviation (R.S.D.) of the ratio of the squared root of the intra-day variance (intra-day standard deviation) on the theoretical value at each concentration level. Intermediate precision was expressed as the R.S.D. of the ratio of the squared root of the inter-day variance (inter-day standard deviation) on the theoretical value at each concentration level. Both variances were obtained using an analysis of variance (ANOVA) as described by Hubert et al. [22]. The LOQ was defined as the lower QC sample with an acceptable trueness, repeatability and intermediate precision. Stability tests were performed in plasma and after protein precipitation.

3. Results and discussion

3.1. Original method

We have previously developed and partially validated a LC–ESI-MS method for the enantioselective quantification of (*R*,*S*)-MTD in plasma [21]. During the development of this method, two deuterated (*R*,*S*)-MTD derivatives were evaluated as IS. The first was (*R*,*S*)-MTD-D3, for which (*R*)-MTD-D3 and (*S*)-MTD-D3 co-elute with (*R*)-MTD and (*S*)-MTD, respectively. The second was (*R*,*S*)-MTD-D9, where different retention times, compared to the corresponding (*R*,*S*)-MTD enantiomers, were observed. According to the particular ability to shape recognition displayed by the cellulose CSP, the nature of the substituent on the nitrogen group of MTD as well as its polarity appeared to be a critical point and the presence of $-CD_3$ moieties was found to significantly modify the interaction with the CSP.

With the former IS, a significant signal suppression was observed and (*R*)- and (*S*)-MTD-D3 signals decreased when concentrations of (*R*)- and (*S*)-MTD increased. On the other hand, these effects were not observed with (*R*,*S*)-MTD-D9 due to different retention times, and was therefore selected as IS [21]. After preliminary estimation of the quantitative performance, this method was then transferred to the destination site for complete validation and routine quantification of patient samples.

3.2. Signal modifications

Because the method has to deal with a considerable number of samples, complementary trials were performed with spiked plasma samples during method transfer. During this study, unexpected variability in peak detection occurred with QC samples. These effects were attributed to both short-term and long-term matrix effects. Whereas no signal modifications were observed in regular situations (Fig. 1, case #1), a non-negligible fraction of tested samples suddenly presented (*S*)-MTD-D9 enhanced (Fig. 1,

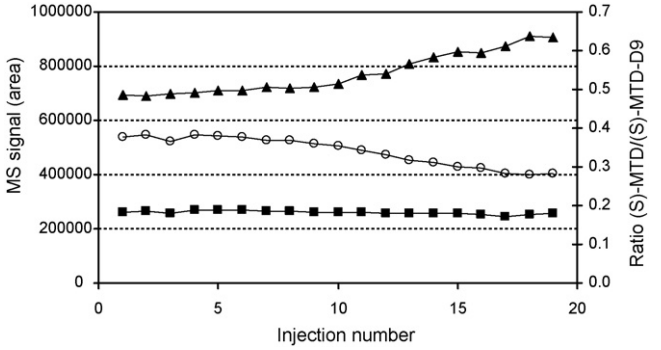

Fig. 2. Multiple injections of quality control plasma samples spiked at 400 ng/mL of (*R*,*S*)-MTD. The internal standard (*S*)-MTD-D9 signal (▲) increases within the series, suggesting the presence of long-term matrix effects. (*S*)-MTD signal (■) remains stable during the series, leading to a decrease in the (*S*)-MTD/(*S*)-MTD-D9 ratio (○).

case #2), or suppressed signals (Fig. 1, case #3), without a similar effect on (*S*)-MTD. Inversely, a (*S*)-MTD signal enhancement was also observed in some cases (Fig. 1, case #4), which was not corrected by (*S*)-MTD-D9. These signal modifications were generally observed after several sample injections within an analytical series, which corresponds generally to 2–3 h after the beginning of the analysis (Fig. 2). These random interferences monitored on QC results lead to an unacceptable high variability on the (*R*)-MTD/(*S*)-MTD ratio, which must be corrected before introducing the method for routine analysis of clinical samples.

3.3. Method improvements

To avoid or minimize this problem, several improvements were evaluated, including MS setting adjustments, sample pre-treatment and column-switching times during the on-line extraction. An increase of the ACN volume used to precipitate proteins during the sample preparation step (ACN–plasma ratio 2:1 instead of 1:1) was selected, to decrease potential interferences due to contaminants as described by Polson et al. [24]. Modification of various washing steps in both extraction and analytical columns were achieved to enhance removal of potential residual contaminants. Finally, the initially used deuterated IS (*R*,*S*)-MTD-D9 was discarded and (*R*,*S*)-MTD-D3 was considered taking into account that short-term variation has to be mainly corrected. With these modifications, a decrease of matrix effects on analytes and IS was observed (Fig. 3, case #5), confirming that protein precipitation was a crucial parameter to adjust prior to on-line extraction as described elsewhere [7]. However, in some cases signal enhancements were still observed (Fig. 3, case #6), but due to the similar chromatographic behaviour of (*S*)-MTD and (*S*)-MTD-D3, corrected QC results were obtained. An extensive study was assessed to determine the reasons of this problem but signal enhancements were observed at different moments within a series, without any particular pattern.

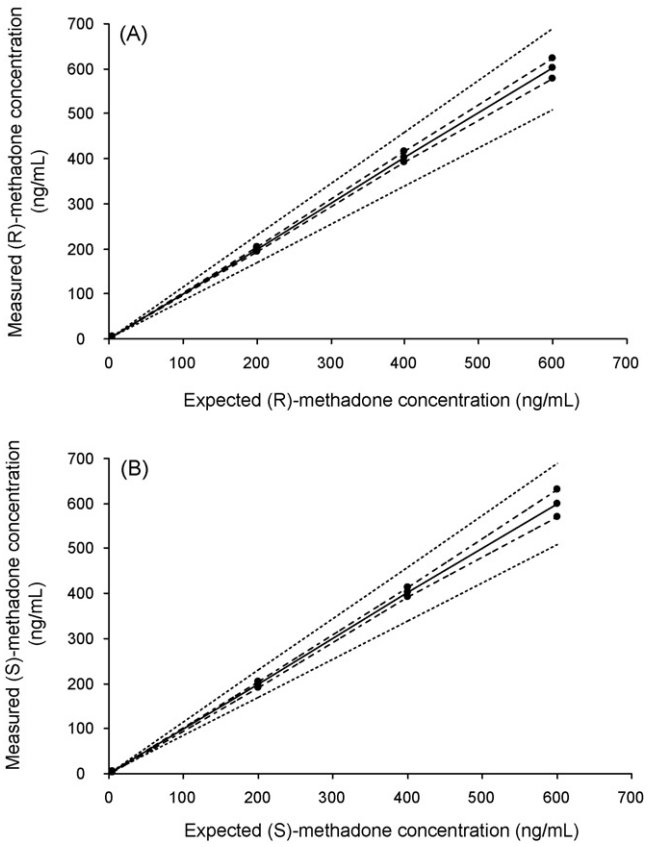

Fig. 4. Accuracy profiles for (*R*)-MTD (A) and (*S*)-MTD (B) obtained with QC plasma samples. The solid lines indicate the trueness, the dashed lines the accuracy and the dotted lines the acceptance limits of ±15%.

3.4. Method validation

After these modifications (sample preparation, choice of the IS), the method was fully validated. Selectivity was confirmed using 10 different blank plasma samples; no interferences from endogenous compounds were observed at the retention times of (*R*,*S*)-MTD and (*R*,*S*)-MTD-D3. No interferences were observed with the analysis of the following drugs at the 1000 ng/mL level each: amisulpride, amitriptyline, aripriprazole, atomoxetine, bupropion, 2-hydroxy-bupropion, chloropromazine, citalopram, clomipramine, clopenthixol, clozapine, cocaine, codeine, cotinine, 3-hyroxy-cotinine, amphetamine, methamphetamine, donepezil, duloxetine,

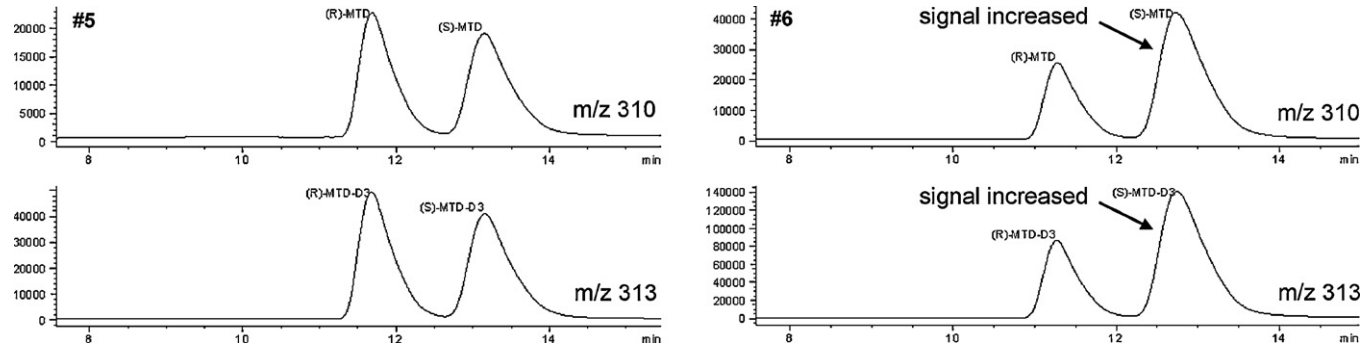

Fig. 3. Selected ion monitoring chromatograms showing normal situation (#5) and signal modifications due to matrix effects (#6) observed with quality control plasma samples spiked at 300 ng/mL with (*R*,*S*)-MTD and using co-eluting (*R*,*S*)-MTD-D3 (333 ng/mL) as internal standard.

Table 1
Validation results of (*R*,*S*)-methadone ($j = 3$; $k = 4$; $n = 4$).

Validation criterion	(*R*)-MTD	(*S*)-MTD
Trueness		
Relative bias (%)		
5 ng/mL	−0.5	0.3
200 ng/mL	−0.5	−1.0
400 ng/mL	1.0	0.8
600 ng/mL	0.2	0.1
Precision		
Repeatability/intermediate precision (R.S.D., %)		
5 ng/mL	3.4/9.0	4.9/12.0
200 ng/mL	0.9/1.4	1.2/1.7
400 ng/mL	1.1/1.8	1.4/1.5
600 ng/mL	0.9/2.0	1.3/2.7
Accuracy		
Lower and upper confidence limits of the total error (%)		
5 ng/mL	[−16.9; 15.9]	[−21.8; 22.4]
200 ng/mL	[−3.0; 2.0]	[−4.0; 2.0]
400 ng/mL	[−2.2; 4.2]	[−1.9; 3.5]
600 ng/mL	[−3.5; 3.9]	[−4.9; 5.0]
Linearity		
Range (ng/mL)	[5; 600]	[5; 600]
Slope	1.0045	1.0031
Intercept	−0.2766	−0.5462
r^2	0.9992	0.9988
LOQ (ng/mL)	5	5

j: number of series; *k*: number of levels; *n*: number of repetitions per level.

fluoxetine, flupenthixol, fluvoxamine, galantamine, haloperidol, heroin, imipramine, maprotiline, memantine, mianserine, midazolam, 1-hydroxy-midazolam, 4-hydroxy-midazolam, mirtazapine, moclobemide, morphine, nicotine, norclozapine, norfluoxetine, norsertindole, nortriptyline, olanzapine, paroxetine, quetiapine, reboxetine, remoxipride, risperidone, 9-hydroxy-risperidone, rivastigmine, sertindole, sertraline, trazodone, trimipramine, varenicline, venlafaxine, *O*-desmethyl-venlafaxine, ziprasidone.

Built on a signal to noise approach, the limit of detection was determined to be 1 ng/mL for each enantiomer. Through the use of accuracy profiles (Fig. 4), various regression models were compared for validation and the best model was selected as a compromise between mathematical complexity and accuracy of the results obtained. Among the evaluated mathematical relationship an ordinary least-squared regression was finally selected as the calibration model. Mean calibration curve equations of the three validation series were $y = 0.0964 \times -0.0038$ (determination coefficient, $r^2 = 0.9998$) and $y = 0.0995 \times -0.0049$ ($r^2 = 0.9997$) for (*R*)- and (*S*)-MTD, respectively. In accordance with the most recent regulatory recommendations, the acceptance limits were fixed at ±15% and the overall profile was completely included into the fixed limits. The LOQ at 5 ng/mL for each MTD enantiomer was confirmed. On the evaluated assay range, satisfactory relative bias (−1.0 to 1.0%), repeatability (0.9–4.9%) and intermediate precision (1.4–12.0%) were obtained for both (*R*)- and (*S*)-MTD as presented in Table 1. Sample stability was verified at low, medium and high (*R*,*S*)-MTD concentrations in quadruplicate at −20 °C for 16 months in plasma, as well as at 25 °C for 24 h, at 4 °C for 4 months and at −20 °C for 4 months after protein precipitation. After complete quantification of the stability samples, a decreased concentration of less than 2% was observed at each concentration level.

3.5. Long-term evaluation of the method

The validated method was released for routine analysis of the stereoselective therapeutic drug monitoring of (*R*,*S*)-MTD in plasma. Owing to the results obtained during validation, a control chart process was established using both intermediate precision standard deviation (s_R) and FDA acceptance criteria. At the beginning of each analytical series and after some system suitability experiments, a new calibration was built and three QC levels at

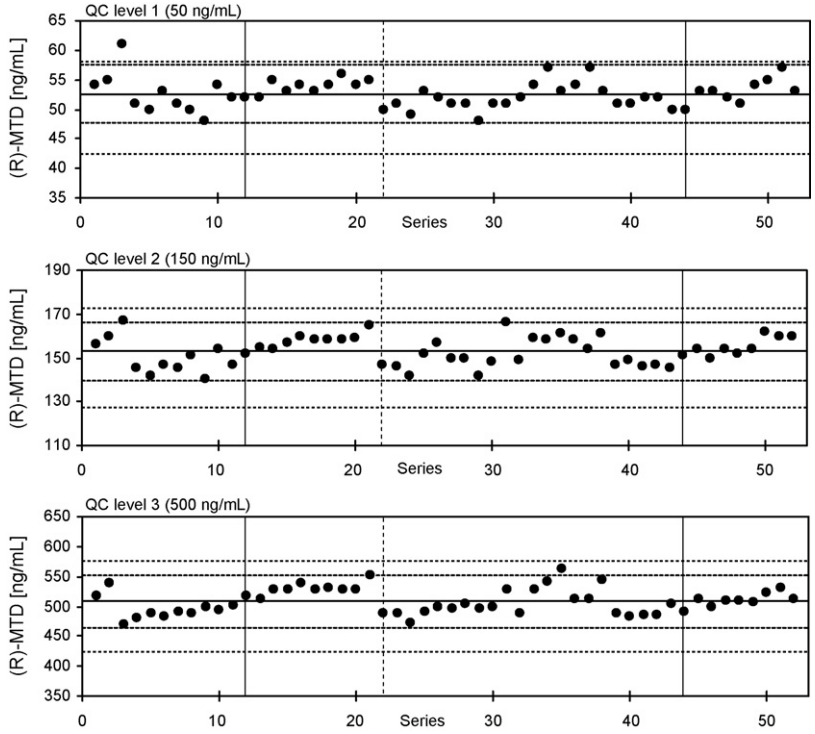

Fig. 5. One-year follow up (52 series × 3 levels) of quality control (QC) plasma samples spiked at 50, 150 and 500 ng/mL with each MTD enantiomer. Only data with (*R*)-MTD are shown, similar profiles were obtained with (*S*)-MTD. The horizontal solid lines indicate the mean values, the horizontal dashed lines the limit of ±2 standard deviations and the horizontal dotted lines the FDA acceptance limits of ±15%. The vertical solid lines indicate a new QC stock solution and the vertical dashed line a new calibration stock solution.

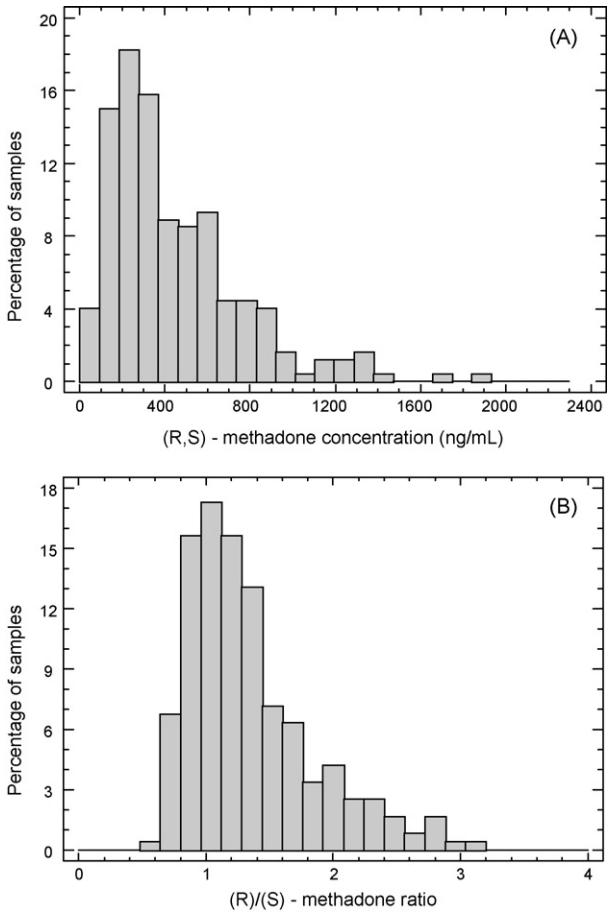

Fig. 6. (*R*,*S*)-MTD trough plasma concentrations (A) and (*R*)/(*S*)-MTD ratios (B) obtained with plasma samples of 257 patients taking (*R*,*S*)-MTD for a 1-year analysis period.

50, 150 and 500 ng/mL for each enantiomer (corresponding to a low, medium and high concentration of the investigated concentration range, respectively) were randomly included in the real sample analysis batch. As shown in Fig. 5, 52 different series with 3 levels of QC for both (*R*)-MTD and (*S*)-MTD were monitored, illustrating the long-term use of the method for the analysis of clinical samples. The results of these routine QC samples, analysed on each working day were generally included in the ±15% variability around the target value and mainly in the $2s_R$ interval around the trueness value, which illustrates the good performance of the method. It was found remarkable that on a 1-year follow up of this chiral therapeutic drug determination method, the intermediate precision variability estimated during the validation was coherent with the QC variability during the routine use. The three QC concentration levels used during routine use were selected to assess method uncertainty for the most often encountered patient's concentrations.

In this period, the mean relative bias (95% CI) was 5% (−4 to 15%), 2% (−7 to 11%) and 2% (−7 to 10%) for (*R*)-MTD QC samples at 50, 150 and 500 ng/mL, respectively. It was 6% (−6 to 17%), 2% (−7 to 11%) and 1% (−8 to 10%) for (*S*)-MTD QC samples at 50, 150 and 500 ng/mL, respectively. Considering the 3 levels together, only 4/156 (2.6%) and 9/156 (5.8%) QC samples were outside $\pm 2s_R$ for (*R*)-MTD and (*S*)-MTD, respectively, including only 1 sample outside $\pm 3s_R$ for (*R*)-MTD. In addition, only 1/156 (0.6%) and 2/156 (1.3%) QC samples were outside FDA acceptance limits of ±15% for (*R*)-MTD and (*S*)-MTD, respectively. Based on the quantitative results obtained, the previously reported ion suppression effect observed on (*R*,*S*)-MTD by its IS (*R*,*S*)-MTD-D3 did not decrease result performance in the recalculation process of the QC samples during validation, as well as in the overall analysis of 52 series during routine use. This suppression effect due to the co-elution of both analyte and IS could therefore be considered as a systematic error corrected by the use of an external calibration procedure.

During these 52 series, 257 trough concentration (prior drug administration) and 54 peak concentration plasma samples of patients under (*R*,*S*)-MTD treatment were successfully analysed in routine therapeutic drug monitoring. The analysed (*R*,*S*)-MTD concentration ranged from 20 to 1800 ng/mL. The mean concentration was 440 ng/mL while the median was at 349 ng/mL demonstrating the non-Gaussian distribution of (*R*,*S*)-MTD concentration as presented in Fig. 6A. Only two cases needed a dilution since their concentration was above 1600 ng/mL. The (*R*)/(*S*)-MTD trough plasma concentration ratios obtained with the collective of patients analysed are shown in Fig. 6B. A large variability in the proportion of (*R*)- and (*S*)-MTD plasma levels was observed with (*R*)/(*S*)-MTD ratios ranging from 0.6 to 3.1, due in part to the high interindividual differences in the stereoselective metabolism of each enantiomer [12,15,25].

4. Conclusions

This paper presents an illustration of potential interferences due to short-term or long-term matrix effects that can be observed in LC–ESI-MS. The choice of an isotope-labelled IS that co-elutes with the target analyte appeared useful for therapeutic drug monitoring in such cases. Using this approach, potential signal modifications should be the same for the analyte and the IS and should not compromise the quantitative result. The sample pretreatment with protein precipitation before analytes extraction is also an important parameter in the case of on-line SPE to minimize interferences due to potential contaminants. Using such approaches, the long-term accuracy of the assay during routine use is shown.

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