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Sensitive determination of oseltamivir and oseltamivir carboxylate in plasma, urine, cerebrospinal fluid and brain by liquid chromatography–tandem mass spectrometry

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ABSTRACT

This manuscript describes the determination of oseltamivir (OP) and oseltamivir carboxylate (OC) in rat plasma, cerebrospinal fluid (CSF) and brain and in human plasma and urine using liquid chromatography coupled to tandem mass spectrometry. Threefold deuterated OP and OC served as internal standards. Protein precipitation with perchloric acid was followed by on-line solid-phase extraction and gradient separation on a reversed-phase column. After electrospray ionization, the compounds were detected in positive ion selected reaction monitoring (SRM) mode. Run time was 3.6 min. The lower limits of quantification (LLOQ) were 0.1 ng/mL in rat plasma and CSF, 0.5 ng/g in brain and 1 ng/mL in human plasma and urine. Inter-day and intra-day precisions and inaccuracies in rat matrices were below 10.2% and 13.9% (below 19.0% at LLOQ), respectively. Intra-assay precisions and inaccuracies in human matrices were below 11.7% and 8.9%, respectively. The recoveries were close to 100%, and no significant matrix effect was observed. The method was successfully applied to rat study samples.

1. Introduction

Oseltamivir phosphate (OP, Tamiflu®), an ethyl ester prodrug which is rapidly converted in vivo to the active neuraminidase inhibitor oseltamivir carboxylate (OC), is widely used for the treatment of influenza. There is a constant need to determine the concentrations of OP and OC in human and animal samples during post-marketing monitoring and research.

Liquid chromatography–tandem mass spectrometry (LC–MS/MS) has been routinely used to determine OP and OC in human and animal plasma and urine [1]. After off-line solid-phase extraction (SPE), separation was performed on a CN column within 7 min. The lower limits of quantification (LLOQ) for OP and OC were 1 and 10 ng/mL, respectively, in plasma and 10-fold higher in urine. An improved method for human plasma, urine and saliva using automated off-line SPE and chromatographic separation in hydrophilic interaction mode within 4 min resulted in higher throughput [2]. Detection on a very sensitive mass spectrometer facilitated the injection of low sample volumes and thus minimized matrix effects, while the same quantification limits as in the previous method were reported. An LC–UV method with 10 min run time and a quantitation limit of 15 ng/mL provided a cost-effective alternative to MS techniques for the determination of OC in human serum [3]. No validated methods for analysis of brain tissue and cerebrospinal fluid have been published. The original LC–MS/MS method [1] was used to analyze rat and mouse brain samples after homogenization with acetonitrile–water, achieving an LLOQ of 60 ng/g for OP and OC (unpublished method). LC–single MS with or without preceding SPE was employed to determine analyte levels below 30 ng/g in mouse and rat brain for exploratory investigations; the authors did not report quantitation limits or precision and accuracy data of their methods [4,5]. As the current methods still offer room for improvement regarding sample preparation efforts, overall analysis time, sensitivity or robustness, further method development was performed.

This manuscript describes the determination of OP and OC in rat plasma, cerebrospinal fluid (CSF) and brain as well as in human plasma and urine by LC–MS/MS. On-line solid-phase extraction using an in-house assembled column-switching system provided good selectivity and robustness, removing the need for elaborate off-line sample preparation. Superior sensitivity and short analysis time were obtained. The method was successfully validated according to regulatory requirements and employed for analysis of study samples.

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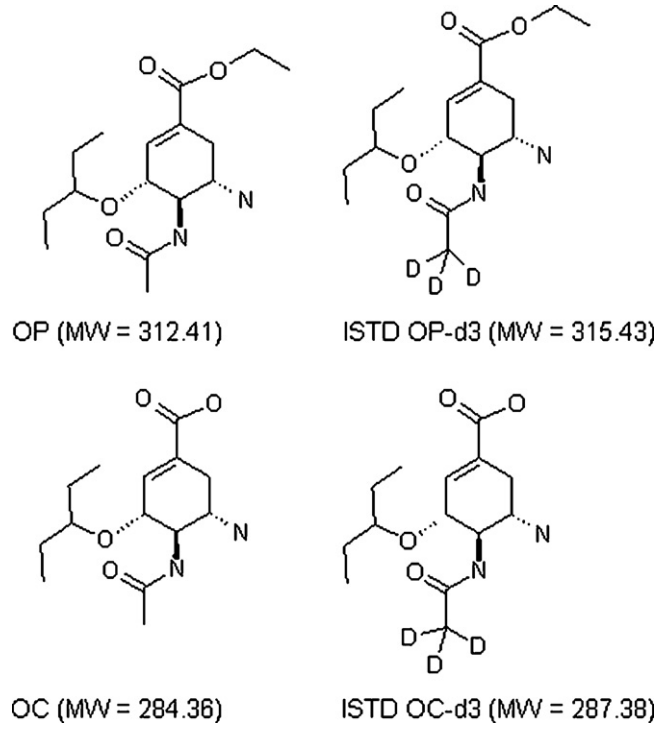

Fig. 1. Structures of analytes and internal standards.

2. Experimental

2.1. Compounds, reagents and solvents

OP (phosphate salt), OC (tartrate) and the internal standards OP-d3 and OC-d3 (threefold deuterated drugs) were synthesized at F. Hoffmann-La Roche Ltd. The structures are shown in Fig. 1. Ethanol and methanol (Lichrosolv for HPLC) were obtained from Merck (Darmstadt, Germany), and acetonitrile (HPLC grade S) from Rathburn (Walkerburn, U.K.). Formic acid (p.a.) was purchased from Fluka (Buchs, Switzerland). Perchloric acid 70% was purchased from Merck. Dichlorvos 98.8% was purchased from Sigma–Aldrich (St. Louis, MO, USA). The water used for the preparation of all solutions was obtained from a Milli-Q apparatus (Millipore, Billerica, MA, USA). Animal blank EDTA plasma, CSF and brain were supplied by internal laboratories. Human blank EDTA plasma was purchased from a blood bank (TRINA Bioreactives, Nänikon, Switzerland). Dichlorvos was added to blank plasma at 200 μg/mL to prevent the ex vivo hydrolysis of OP. This dichlorvos-treated plasma was used to prepare calibration standards and quality control samples.

2.2. Calibration, quality control samples and internal standards

Stock solutions of the analytes and internal standards were prepared by dissolving the appropriate amounts with water to obtain concentrations of 1 mg/mL free base. The OP and OC stock solutions were mixed and serially diluted with ethanol to obtain spiking solutions in the range 0.01–500 μg/mL. Aliquots of 10 μL were spiked to 990 μL of blank rat EDTA plasma to obtain nine calibration standards in the concentration range 0.1–500 ng/mL. Quality control (QC) samples were prepared at 0.1, 0.3, 30, 300 and 500 ng/mL in rat plasma, CSF or brain homogenate. A dilution QC sample at 3000 ng/mL was prepared in rat plasma. For human plasma and urine, calibration standards were prepared in the range 1–5000 ng/mL and QC samples at 1, 3, 300, 3000 and 5000 ng/mL.

The internal standard working solution was prepared by diluting the stock solutions with 0.5 M aqueous perchloric acid solution to obtain final concentrations of 10 ng/mL OP-d3 and 20 ng/mL OC-d3.

2.3. Sample preparation

2.3.1. Plasma, urine and CSF

To 50 μL of sample, which was pipetted into a 96 deep well rack (Milian Instruments, Geneva, Switzerland), 200 μL of internal standard working solution was added using the automated pipettor Tecan Genesis 100/4 (Tecan Schweiz AG, Männedorf, Switzerland). The samples were vortexed briefly using a MixMate (Eppendorf, Hamburg, Germany) and centrifuged for approximately 10 min at 5000 × *g* in a Multifuge 3 S-R (Heraeus, Osterode, Germany).

2.3.2. Brain

Aliquots (100–300 mg) were homogenized within 30 s after adding the fourfold amount of water, using a Heidolph RZR 2051 control stirrer with a glass piston and potter (GlasKeller, Basel, Switzerland). To 100 μL of brain homogenate, 400 μL of ISTD working solution was added, followed by mixing and centrifugation. The results for brain homogenate are corrected by the dilution factor 5 to obtain the concentrations per gram brain.

2.4. Liquid chromatography

The trapping column for on-line SPE was a Gemini C_{18} Mercury, 2.0 mm × 10 mm, 5 μm particle size from Phenomenex (Torrance, CA, USA) and the analytical column an Atlantis T3, 3.5 μm, 2.1 mm × 50 mm from Waters (San Jose, USA). Mobile phases were 0.2% aqueous formic acid (C_A), 0.2% formic acid in water/acetonitrile/ethanol 50/475/475 (v/v/v) (C_B), 0.1% formic acid in water/methanol 80/20 (v/v) (A), 0.1% formic acid in water/methanol 5/95 (v/v) (B).

A scheme of the LC–MS/MS system is shown in Fig. 2. The SIL-HTc autosampler with system controller (Shimadzu, Kyoto, Japan) was equipped with a 200 μL sample loop; needle and valve rinse was performed using water/ethanol 5/95 (v/v). A LC-10ATvp pump (Shimadzu) delivered mobile phases C_A or C_B, and the dilution pump (L-6000A, Merck-Hitachi, Tokyo, Japan) delivered mobile phase C_A onto the trapping column. A 6-port switching valve (H.S. valve 7000E V (LabSource, Reinach, Switzerland) directed the effluent of the trapping column either to waste or onto the analytical column. Two LC-10ADvp pumps (Shimadzu) delivered mobile phases A and B to the analytical column.

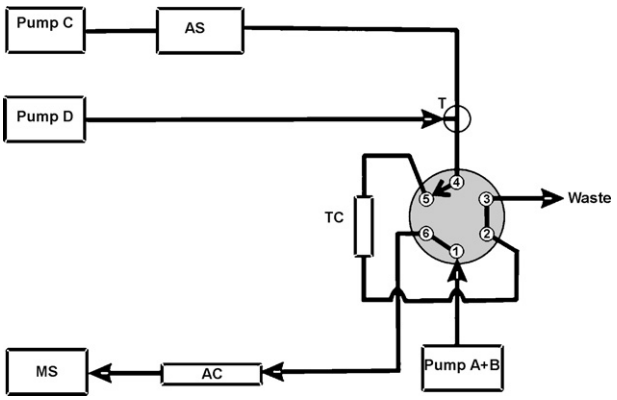

Fig. 2. Scheme of on-line SPE–LC–MS/MS system. AS, autosampler; TC, trapping column; AC, analytical column; pump C, trapping and flushing with phases C_A and C_B; pump D, on-line dilution; pump A + B, analytical separation with mobile phases A and B.

The sample solution (50 μL for rat or 5 μL for human matrices) was injected onto the trapping column by flushing mobile phase C_A through the autoinjector loop at 0.3 mL/min. Simultaneously, on-line dilution was performed via a T-piece at 1 mL/min. After 0.6 min the dilution flow was stopped, and the trapping column was rinsed with C_A for additional 0.2 min (2 mL/min). The valve was switched at 0.8 min to connect trapping and analytical column, and the analytes and internal standards were transferred to the analytical column in backflush mode (90% A and 10% B, 0.3 mL/min). After 1.4 min the trapping column was separated from the analytical column (valve switch) and washed with C_B for 0.8 min at flow rate of 2 mL/min, followed by re-conditioning with C_A. On the analytical column, the fraction of B was linearly increased to 80% within 1.5 min. After elution of the analytes, the column was rinsed with 100% B for 0.4 min and then re-equilibrated with 90% A/10% B. The software of the mass spectrometer controlled the Shimadzu autosampler, HPLC pumps and switching valve. The total run time was 3.6 min.

2.5. Mass spectrometry

A TSQ Quantum Ultra triple quadrupole mass spectrometer (Thermo, San Jose, CA, USA), equipped with an ESI (electrospray) source and operated in the positive ion mode, was used. Data acquisition was performed on a Dell Precision 380 computer with Xcalibur 2.0 software distributed by Thermo. The sprayer voltage was set at 4000 V, the source CID voltage at 3 V and the tube lens voltage at 45 V. The capillary temperature was maintained at 300 °C. Nitrogen was used as sheath, auxiliary and sweep gas with flow settings of 50, 10 and 5, respectively. Argon was used as collision gas at 1.5 mTorr, and the collision energy was set at 20 eV. Data acquisition was carried out in centroid mode at unit mass resolution (peak width for Q1 and Q3 set to 0.7 amu). The scan time was 50 ms and the scan width was 0.02 amu. Selected reaction monitoring (SRM) mode was performed at the transitions m/z 313.1 → 166.0 for OP, m/z 316.1 → 167.0 for OP-d3, m/z 285.1 → 138.0 for OC and m/z 288.1 → 139.0 for OC-d3. Product ion spectra for analytes are shown in Fig. 3.

2.6. Calibration and validation

The calibration curves ($y = a + bx$) were calculated by weighted linear least-squares regression (weighting factor $1/x^2$) of the measured peak area ratios analyte/ISTD (y) *versus* the nominal analyte concentration (x). The calibration curves were then used to calculate the concentrations of the analytes in QC and unknown samples from the measured peak area ratios. Method validation according to regulatory requirements [6] and departmental standard operating procedures was performed. Inter-day precision (defined as coefficient of variation of replicate analyses) and accuracy (defined as the degree of closeness of the determined value to the nominal value) were evaluated by analyzing QC samples (including the LLOQ and upper limit of quantification) in rat plasma against a calibration curve prepared in rat plasma on five independent occasions. Intra-day precision and accuracy were evaluated by analyzing QC samples (including the LLOQ and upper limit of quantification) prepared in plasma, CSF and brain in five replicates against one plasma calibration set. The selectivity of the methods was evaluated by analyzing blank samples for interference of endogenous compounds.

The recovery was investigated comparing the analyte response of spiked and extracted samples (QC samples) with extracted blank plasma samples where the nominal concentrations of OP and OC had been spiked into the supernatant (reference samples). The matrix effect was investigated comparing the analyte response in the reference samples with samples where the nominal concentration of the analyte had been spiked into the solvent used for extraction (matrix-free samples). The overall recovery resulted from comparing the QC samples with matrix-free samples. All data were based on peak areas; no corrections by the internal standard response were made. Three concentration levels in five aliquots were analyzed. To show matrix suppression qualitatively, post-column infusion of the analytes (100 pg/μL at 5 μL/min) into the effluent of the analytical column was performed [7].

Stability data have been reported previously [1,2]. Further investigations were performed for low concentrations of OP and OC (0.3 ng/mL) in plasma and brain homogenate. Stability was examined by comparing the response of freshly prepared samples with that of spiked samples maintained at room temperature (injection solutions, plasma and brain homogenate) or at −20 °C (plasma and brain homogenate). Five aliquots for each condition were analyzed, and the stability was calculated according to a published procedure [8]. The compounds were considered to be stable if the concentration in stored samples was within ±15% of the concentration in the fresh samples.

Intra-day precision and accuracy as well as recovery and matrix effect were determined for human plasma and urine as described for rat matrices.

3. Results and discussion

Selective and sensitive analysis of complex biological materials often requires substantial efforts in sample preparation to achieve clean-up and enrichment of the analytes. Off-line extraction methods, although automated, add significantly to the overall analysis time. On-line solid-phase extraction is frequently employed for high-throughput quantitation of drugs. An in-house assembled column-switching system was successfully used for analysis of tissue samples [9] and therefore, a similar system was set up for the determination of oseltamivir and the carboxylate metabolite. The

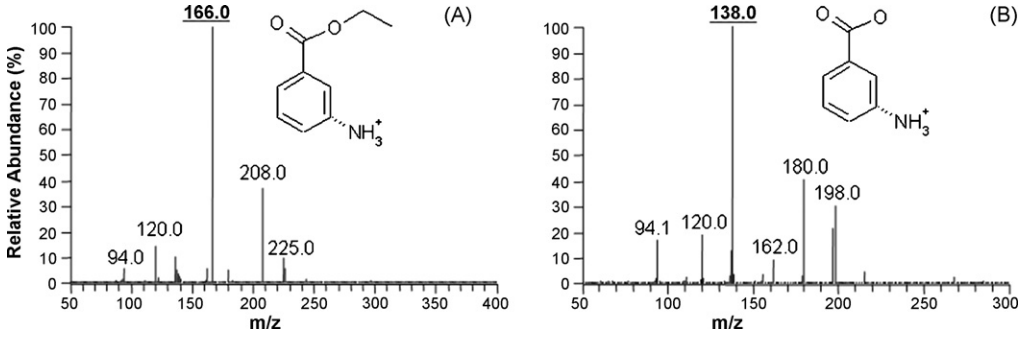

Fig. 3. Product ion spectra of OP (A) and OC (B).

method was first developed and validated for rat matrices and then adopted for human plasma and urine. To establish the final method parameters, we investigated different agents for protein precipitation, column materials for trapping and analytical column, mobile phases as well as MS settings. Detail are discussed in the following sections.

3.1. Sample preparation

Brain was homogenized with water to obtain a liquid matrix which could be handled in the same way as plasma, CSF or urine. Further off-line sample preparation consisted only of protein precipitation followed by centrifugation. As the analytes are highly soluble in water, aqueous perchloric acid was the best choice for protein precipitation because no compound loss during trapping occurred. Organic solvents (methanol, ethanol or acetonitrile) were not used for protein precipitation because breakthrough of OC on the trapping column was observed even using aqueous on-line dilution. Another advantage of perchloric acid precipitation was the removal of lipophilic endogenous compounds, which were insoluble in the supernatant and remained in the precipitate, thus not contributing to matrix effects during subsequent analysis.

3.2. On-line SPE

The aqueous injection solution provided good retention of OP and the more polar OC on the trapping column. The stationary phase was specified to be used with 100% aqueous solvents without phase collapsing and in pH range 1–12. On-line dilution was performed to reduce the perchloric acid concentration in the injection solution and therefore increase its pH before application onto the trapping column. The employed dilution factor (ratio of injection to dilution flow 0.3:1) raised the pH to approximately 1.2. The signal intensity increased linearly when injecting increasing sample volumes between 5 and 100 μL and therefore, no compound loss during the trapping step was expected when injecting 50-μL aliquots to achieve the required quantification limit in rat matrices. The column material was very robust and long-lasting, even when injecting supernatant from brain homogenate. The organic solvent fraction for elution from trapping onto analytical column was optimized at 28% methanol (corresponding to 10% B2). This allowed the transfer of both analytes in a narrow window to save time while permitting efficient subsequent gradient separation. If 100% mobile phase A2 was used the elution time for OP and thus overall analysis time was increased, whereas an increased fraction of organic solvent compromised the retention and peak shape of OC during analytical separation.

3.3. LC separation

During previous investigations we have experienced that HILIC columns, although well-suited to retain polar compounds, were less robust than RP columns, needed longer equilibration time and provided less separation efficiency. Therefore, we used RP material which usually provided excellent peak shape and separation. As analytical column, the Atlantis T3 was selected because it was recommended for superior retention of polar compounds and symmetric peak shape for basic drugs. The polar OC was sufficiently retained, and narrow, symmetric peaks were obtained for both analytes. Gradient elution, increasing the fraction of mobile phase B2 to 80% (corresponding to 76% methanol) was performed to separate isobaric endogenous compounds from the analytes.

3.4. MS detection

The analytes and internal standards ionized well with pneumatically assisted electrospray in the positive ion mode. The most abundant fragment ions at m/z 166 (OP) and m/z 138 (OC), see also Fig. 3, resulted from cleavage of the ethylpropoxy and acetylamino moieties. For the internal standards, additional fragment ions at m/z 167 (OP-d3) and m/z 139 (OC-d3) were observed as a result of deuterium migration during re-arrangement reactions. Optimum signal intensity was achieved using methanol and 0.1% formic acid in the analytical mobile phase. The methanol content in the mobile phase was sufficiently high to allow efficient evaporation and ionization (approximately 45% and 55% at elution of OC and OP, respectively). Acid contents above 0.1%, the addition of salts or the use of acetonitrile decreased the signal intensity. Our LC–MS/MS method using the TSQ Quantum mass spectrometer provided the same absolute sensitivity for OC as the previously reported method using an API 5000 [2], while a fivefold lower sensitivity was obtained for OP.

3.5. Validation results for rat matrices

3.5.1. Selectivity

No endogenous interferences co-eluting with OP or OC were observed in blank rat plasma, CSF and brain. A chromatogram of blank rat plasma is shown in Fig. 4A. The endogenous compound peaks at 2.43 min and, in brain only, at 2.8 min were well separated from the OP peak.

3.5.2. Calibration range and LLOQ

In contrast to previous methods, where the sensitivity for OC was 10-fold lower than for OP, our method showed only a twofold difference and therefore, the same calibration range could be validated for both analytes. Calibration curves were linear in the range 0.1–500 ng/mL; correlation coefficients of at least 0.997 were obtained. The back-calculated concentrations in standards deviated by less than 15% from the nominal concentrations. The lower limit of quantification (LLOQ) defined as the minimum concentration that can be measured routinely with a precision of <20% and an inaccuracy of <20% and a signal-to-noise ratio of at least five was 0.1 ng/mL for plasma, CSF and brain homogenate (corresponding to 0.5 ng/g for brain). A chromatogram of rat plasma spiked with OP and OC at the LLOQ is shown in Fig. 4B.

3.5.3. Precision and accuracy

The inter- and intra-day precisions and accuracies of the analytical method, evaluated for rat plasma, CSF and brain homogenate, met the pre-defined acceptance criteria (see Table 1). Although it is recommended to prepare calibration standards in the matrix to be analyzed, our data support that concentrations in CSF and brain homogenate could be calculated using a rat plasma calibration curve. Thus, the number of animals to be sacrificed for production of blank matrices was reduced.

3.5.4. Recovery and matrix effect

The recoveries for rat plasma were close to 100%, and the recoveries for CSF and brain calculated against spiked rat plasma supernatant were between 83.3% and 100.9%. The matrix effect was insignificant, resulting in excellent overall recoveries between 89.4% and 111.3% (see Table 2). The infusion experiments confirmed the absence of signal suppression at the retention times of the analytes; a slight signal enhancement was observed for OC in brain and for OP in plasma and brain compared to water (chromatograms not shown).

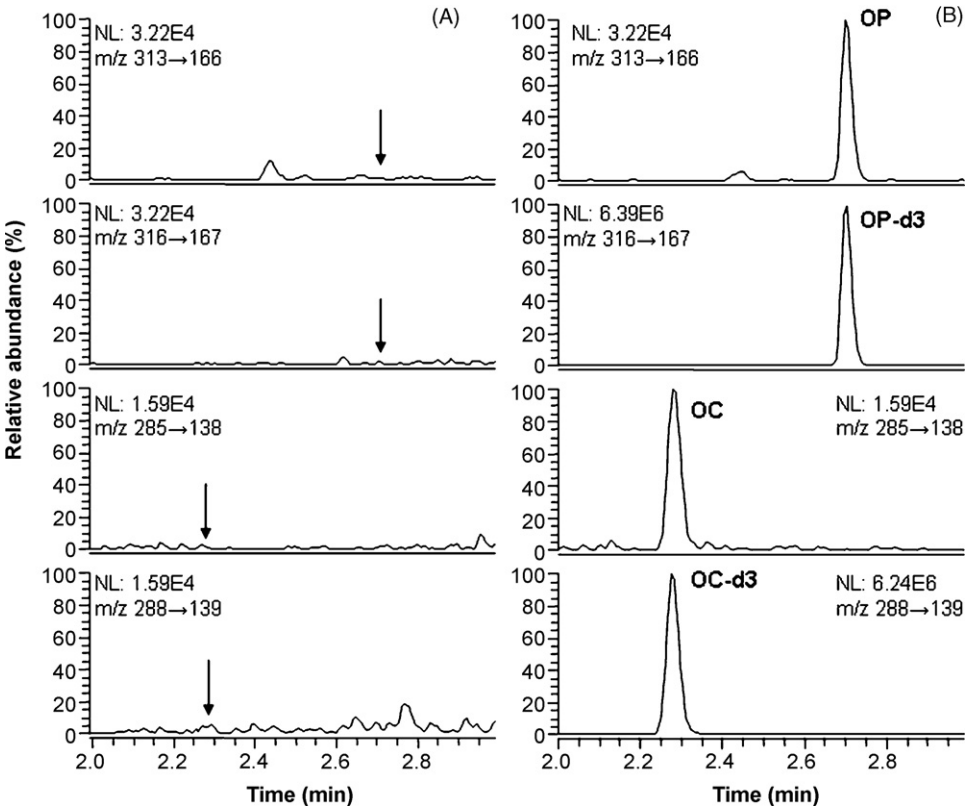

Fig. 4. SRM chromatograms of OP, OC and internal standards in blank rat plasma (A) and rat plasma spiked at LLOQ 0.1 ng/mL (B).

Table 1
Precision and accuracy data (%) for rat plasma, CSF and brain.

	Concentration (ng/mL)	OP		OC	
		Precision	Accuracy	Precision	Accuracy
Inter-day[a]					
Rat plasma	0.1	4.1	102.7	7.5	105.8
	0.3	5.8	97.5	7.6	98.2
	30	3.1	99.2	1.5	96.4
	300	3.7	105.8	3.5	102.1
	500	2.9	103.3	1.8	100.3
Intra-day[b]					
Rat plasma	0.1	4.4	108.4	5.5	111.5
	0.3	6.1	97.4	4.6	108.7
	30	5.4	104.2	4.5	104.5
	300	3.1	106.1	3.8	104.7
	500	1.8	108.1	3.5	108.7
	3000[c]	0.4	114.7	5.7	109.3
Rat brain homogenate	0.1	8.1	105.9	3.2	115.4
	0.3	10.2	104.8	7.6	109.8
	30	7.1	104.1	6.5	102.8
	300	5.8	99.4	5.9	105.0
	500	6.0	95.1	4.6	93.1
Rat CSF	0.1	5.9	119.0	5.9	112.6
	0.3	3.4	110.4	6.0	108.2
	30	0.9	113.9	0.9	110.7
	300	0.9	106.7	2.0	108.0
	500	1.3	104.5	1.8	105.3

[a] $n = 5$ from five independent runs.
[b] $n = 5$ from one single run.
[c] Dilution factor 100.

Table 2
Recovery and matrix effect (%) in rat plasma, CSF and brain ($n = 5$).

Matrix	Concentration (ng/mL)	OP			OC		
		Recovery	Matrix effect	Overall recovery	Recovery	Matrix effect	Overall recovery
Rat plasma	0.3	98.3	114.0	111.3	97.8	107.2	105.5
	30	97.3	111.6	106.2	95.1	103.6	100.8
	300	95.5	100.3	97.5	97.2	99.8	95.3
Rat CSF	0.3	83.3[a]		109.3	90.8[a]		89.4
	30	89.5[a]		108.8	97.5[a]		92.7
	300	93.4[a]		101.1	100.9[a]		93.2
Rat brain homogenate	0.3	95.3[a]		106.3	93.4[a]		102.2
	30	90.9[a]		103.1	92.4[a]		94.2
	300	95.7[a]		94.6	94.3[a]		95.5

[a] Calculated against spiked plasma supernatant.

3.5.5. Stability

OP and OC were stable at all investigated conditions (injection solutions for 24 h at room temperature (RT), plasma at least for 4 h at RT, brain homogenate for 24 h at RT, plasma and brain homogenate for 4 weeks at −20 °C and after three freeze-thaw cycles).

3.6. Analysis of study samples

Plasma, CSF and brain samples from various rat studies with different administration routes and dosages were analyzed. Inter-day precisions and inaccuracies of QC samples, which were measured together with the study samples, were below 15%. The sensitivity of the method permitted the detection of low concentration levels. Fig. 5 shows examples for SRM chromatograms of OP and OC obtained from rat brain samples.

3.7. Suitability for human plasma and urine

For the analysis of plasma and urine samples from humans after administration of efficacious doses (usually twice daily 75 mg), low limits of quantification are not required as the exposures of OP and OC are usually in the mid to high ng/mL range (OC: approximately 500 ng/mL at C_{max} and 150 ng/mL 12 h after dosing [10]). Therefore, the calibration standard concentrations were shifted to 1–5000 ng/mL, injecting only 5 μL of sample solution. No ionization suppression or enhancement was observed during infusion experiments of blank plasma or urine (data not presented). The analytes were well separated from isobaric endogenous compound peaks, and the peaks at LLOQ showed at least a signal-to-noise ratio of 5. Fig. 6 shows chromatograms obtained from human urine. Human plasma chromatograms were similar as those from rat plasma and are therefore not shown here. Excellent intra-day precisions and

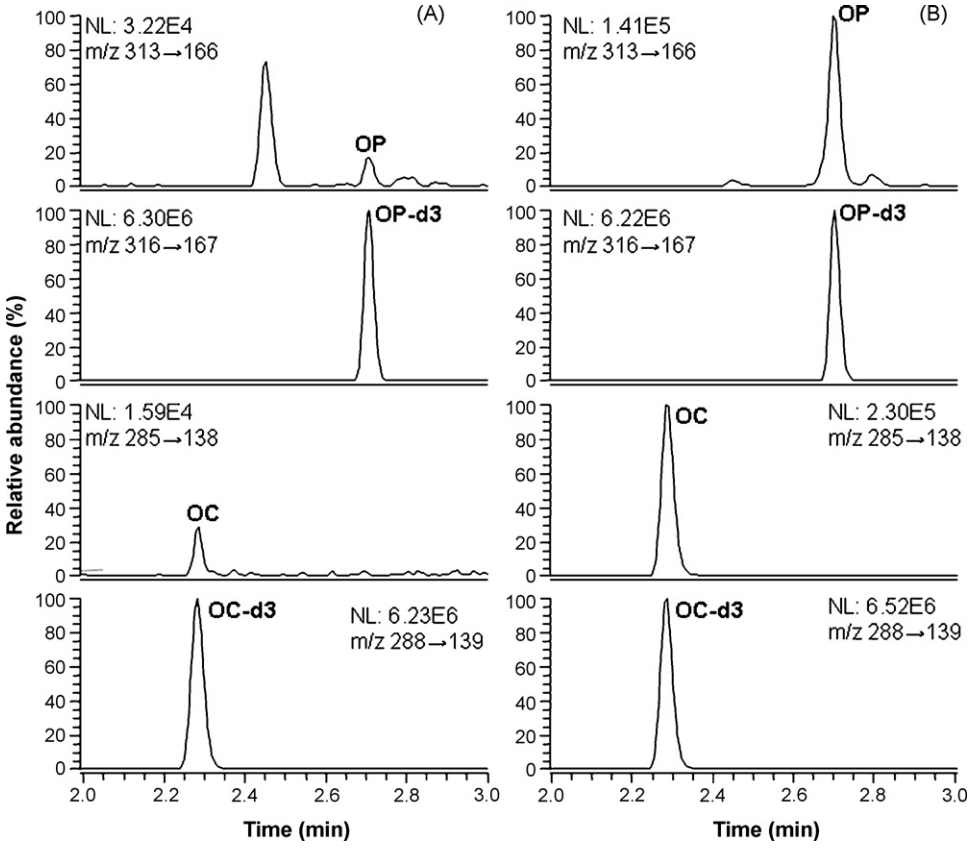

Fig. 5. SRM chromatograms of OP, OC and internal standards in rat brain (olfactory bulb) from a control animal (A) and from an animal dosed with OP (B). The signals correspond to concentration values of 2.96 ng/g OP and 9.6 ng/g OC.

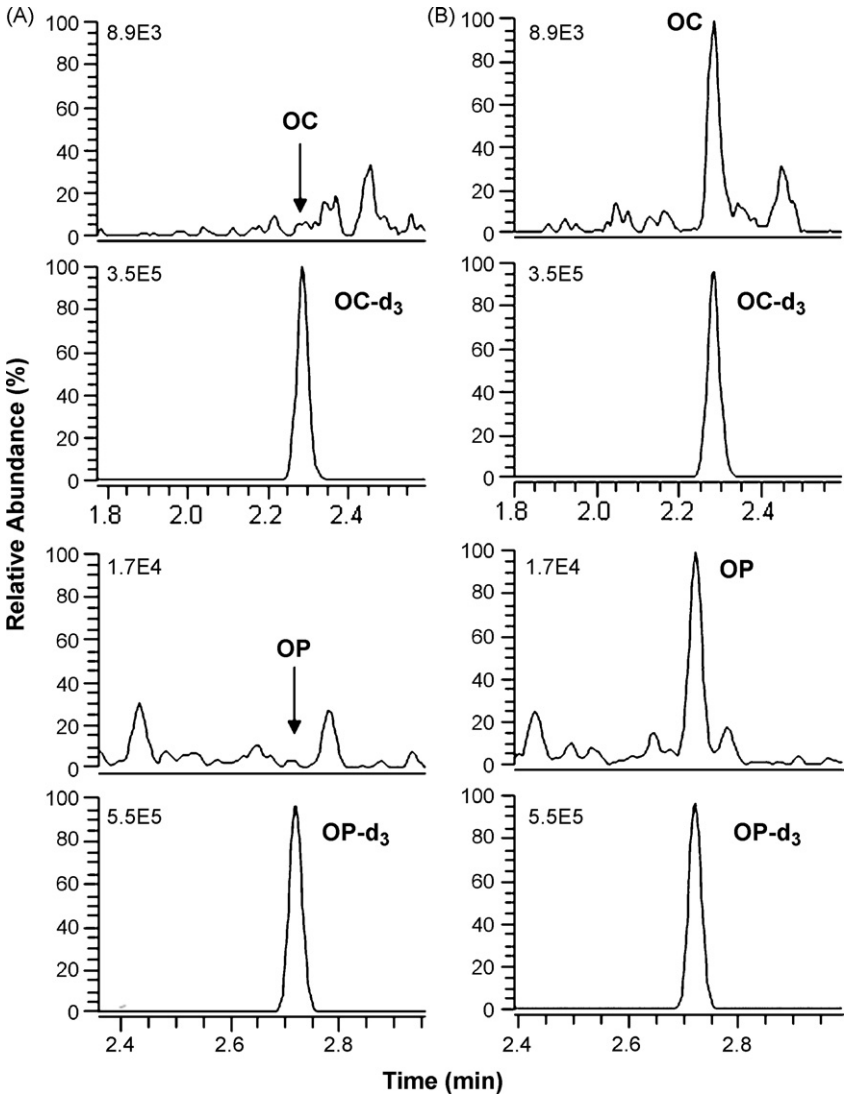

Fig. 6. SRM chromatograms of OP, OC and internal standards in blank human urine (A) and urine spiked at LLOQ 1 ng/mL (B). Arrows indicate the retention time of analytes in blanks.

accuracies were achieved (Table 3). Recoveries from human plasma and urine were close to 100%, and no matrix effect was detected. These data indicate that the method can be fully validated for human plasma and urine and subsequently be employed for sample analysis from clinical trials. Particularly when analyzing large sample numbers, on-line SPE is a very cost-effective procedure, because up to 1000 samples could be injected onto the same cartridge. In contrast to previous methods our assay did not suffer from low recoveries [1] or concentration dependent matrix suppression in urine [2]. OC could be determined with a quantitation

Table 3
Intra-day precision, accuracy, recovery and matrix effect (%) in human plasma and urine ($n = 5$).

Matrix	Concentration (ng/mL)	OP					OC				
		Precision	Accuracy	Recovery	Matrix effect	Overall recovery	Precision	Accuracy	Recovery	Matrix effect	Overall recovery
Human plasma	1	10.7	103.8				11.7	91.8			
	3	6.6	99.3	93.4	105.2	98.2	6.0	97.0	101.8	100.8	102.6
	300	3.4	102.4	93.9	104.0	97.7	4.0	102.6	96.8	101.5	98.3
	3000	5.2	98.3	95.3	101.4	96.6	1.2	102.3	97.8	101.2	99.0
	5000	2.6	97.3				2.3	95.5			
Human urine	1	6.9	107.7				8.4	102.6			
	3	3.3	108.9	102.2	104.8	107.1	7.3	103.3	103.3	96.3	99.3
	300	3.2	105.6	99.0	104.6	103.6	4.7	105.4	98.9	100.7	99.6
	3000	2.1	97.4	91.8	101.1	92.8	3.3	99.3	92.6	102.5	95.0
	5000	1.2	94.0				1.4	95.5			

limit of 1 ng/mL. Hence, even the use of a 10-times less sensitive MS instrument than the TSQ Quantum used here could result in the same quantitation limit of 10 ng/mL as described by other authors [1,2].

4. Conclusions

On-line solid-phase extraction presented a fast, simple and robust procedure for the determination of OP and OC by LC–MS/MS. It omitted the need for elaborate off-line sample preparation, minimized matrix effects, and provided excellent recoveries, and was consequently applied to different species and matrices. Variable injection volumes allowed for different sensitivity needs. Currently, this method presents the only validated procedure for brain analysis and for quantitation limits in the pg/mL range, supporting regulatory rat brain distribution studies at low doses. The high efficiency and low costs are valuable for large clinical trials and rapid turnaround.

References

[1] H. Wiltshire, B. Wiltshire, A. Citron, T. Clarke, C. Serpe, D. Gray, W. Herron, J. Chromatogr. B 745 (2000) 373–388.
[2] N. Lindegårdh, W. Hanpithakpong, Y. Wattanagoon, P. Singhasivanon, N.J. White, N.P.J. Day, J. Chromatogr. B 859 (2007) 74–83.
[3] G. Bahrami, B. Mohammadi, A. Kiani, J. Chromatogr. B 864 (2008) 38–42.
[4] K. Morimoto, M. Nakakariya, Y. Shirasaka, C. Kakinuma, T. Fujita, I. Tamai, T. Ogihara, Drug Metab. Dispos. 36 (2008) 6–9.
[5] A. Ose, H. Kusuhara, K. Yamatsugu, M. Kanai, M. Shibasaki, T. Fujita, A. Yamamoto, Y. Sugiyama, Drug Metab. Dispos. 36 (2008) 427–434.
[6] FDA Guidance for Industry, Bioanalytical Method Validation, May 2001.
[7] R. Bonfiglio, R.C. King, T.V. Olah, K. Merkle, Rapid Commun. Mass Spectrom. 13 (1999) 1175.
[8] U. Timm, M. Wall, D. Dell, J. Pharm. Sci. 74 (1985) 972–977.
[9] K. Heinig, F. Bucheli, J. Chromatogr. B 769 (2002) 9–26.
[10] G. He, J. Massarella, P. Ward, Clin. Pharmacokinet. 37 (1999) 471–484.