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Journal of Chromatography B, 859 (2007) 74–83
JOURNAL OF CHROMATOGRAPHY B
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# Development and validation of a liquid chromatographic–tandem mass spectrometric method for determination of oseltamivir and its metabolite oseltamivir carboxylate in plasma, saliva and urine

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Received 30 July 2007; accepted 5 September 2007
Available online 19 September 2007

**Abstract**

A bioanalytical method for the analysis of oseltamivir (OP) and its metabolite oseltamivir carboxylate (OC) in human plasma, saliva and urine using off-line solid-phase extraction and liquid chromatography coupled to positive tandem mass spectroscopy has been developed and validated. OP and OC were analysed on a ZIC-HILIC column (50 mm × 2.1 mm) using a mobile phase gradient containing acetonitrile–ammonium acetate buffer (pH 3.5; 10 mM) at a flow rate of 500 μL/min. The method was validated according to published FDA guidelines and showed excellent performance. The lower limit of quantification for OP was determined to be 1, 1 and 5 ng/mL for plasma, saliva and urine, respectively and for OC was 10, 10 and 30 ng/mL for plasma, saliva and urine, respectively. The upper limit of quantification for OP was determined to be 600, 300 and 1500 ng/mL for plasma, saliva and urine, respectively and for OC was 10,000, 10,000 and 30,000 ng/mL for plasma, saliva and urine, respectively. The within-day and between-day precisions expressed as R.S.D., were lower than 5% at all tested concentrations for all matrices and below 12% at the lower limit of quantification. Validation of over-curve samples ensured that it would be possible with dilution if samples went outside the calibration range. Matrix effects were thoroughly evaluated both graphically and quantitatively. No matrix effects were detected for OP or OC in plasma or saliva. Residues from the urine matrix (most likely salts) caused some ion suppression for both OP and its deuterated internal standard but had no effect on OC or its deuterated internal standard. The suppression did not affect the quantification of OP.


*Keywords:* Avian influenza; Birdflu; High throughput; Ion suppression; Liquid chromatography/tandem mass spectrometry (LC/MS/MS); Oseltamivir; Stable isotope labeled (SIL) internal standard; Tamiflu

## 1. Introduction

Pandemic influenza is considered by many experts to be the most significant potential global public health emergency caused by a naturally occurring pathogen. Recent human cases of highly pathogenic strains of avian influenza (H5N1) have raised increased concerns about the imminence of this threat. The first documented outbreak of H5N1 infection in humans occurred in Hong Kong in 1997, with no further cases until 2003 when the disease quickly spread throughout South-East Asia. Avian influenza, especially H5N1, causes severe disease with high mortality (>70%) in humans. Oseltamivir is considered the leading currently available antiviral to counter a serious epidemic or pandemic outbreak of influenza [1,2]. Oseltamivir (OP) (Tamiflu®) is an ester prodrug which is rapidly and extensively hydrolysed *in vivo* to its active metabolite oseltamivir carboxylate (OC), a potent and selective inhibitor of influenza virus neuraminidase [3]. OP and OC are extensively excreted by glomerular filtration and renal tubular secretion with approximately 60–80% of an oral dose excreted in the urine as OC [4]. Human drug–drug interaction studies showed that probenecid

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doi:10.1016/j.jchromb.2007.09.018

inhibited the renal secretion of OC leading to approximately double the exposure (i.e. AUC) in terms of plasma concentrations. No interaction was observed with cimetidine or amoxicillin [3]. The prodrug OP has a short half-life and the metabolite OC an half-life of approximately 8–11 h [5]. There is only one published method for the determination of oseltamivir and its metabolite in EDTA plasma and urine using solid-phase extraction and LC–MS [6]. There is currently no method for determination in saliva. Recent data have shown that it is necessary to use an esterase inhibitor (e.g. fluoride/oxalate) to ensure sample integrity during clinical studies of OP [7,8]. There is therefore an urgent need to develop and validate an assay that permits determination of OP and OC in fluoride/oxalate stabilized plasma. The aim of the work described in this paper was to develop and validate an assay for determination of OP and OC in plasma, saliva and urine and to make it simple, robust and sensitive.

## 2. Experimental

### 2.1. Chemicals and materials

Oseltamivir (OP), oseltamivir carboxylate (OC) and their trideuterated internal standards (D-OP and D-OC) were obtained from F.Hoffmann-La Roche Ltd. (Basel, Switzerland). The structures are shown in Fig. 1. Acetonitrile (HPLC-grade), methanol (pro analysis) and HPLC-water were obtained from JT Baker (Phillipsburg, USA). Ammonium acetate (LC–MS grade) was from FLUKA (Sigma–Aldrich, St. Louis, USA). Ammonium acetate buffer solutions were prepared by dissolving appropriate amounts of ammonium acetate in HPLC-water and adjusting pH with acetic acid (Merck Darmstadt, Germany).

#### 2.1.1. Instrumentation—liquid chromatography–mass spectrometry

The LC system was an Agilent 1200 system consisting of a binary LC pump, a vacuum degasser, a temperature-controlled micro-well plate autosampler set at 20 °C and a thermostatted column compartment set at 20 °C (Agilent technologies, Santa Clara, USA). Data acquisition and quantification were performed using Analyst 1.4 (Applied Biosystems/MDS SCIEX, Foster City, USA). The compounds were analysed on a ZIC-HILIC (5 μm, 50 mm × 2.1 mm) column protected by a ZIC-HILIC guard column (16 mm × 1.0 mm) (Sequant, Umea, Sweden) at a flow rate of 500 μL/min. The ZIC-HILIC column has a sulfobetaine type zwitterionic stationary phase covalently attached to silica and is especially suitable for polar and hydrophilic analytes. The mobile phase should contain a high concentration of water miscible organic solvent (e.g. acetonitrile) to promote hydrophilic and weak electrostatic interactions between the analytes and the hydrophilic phase. Neutral and lipophilic compounds in general show poor retention on the column. The LC gradient programme is listed in Table 1.

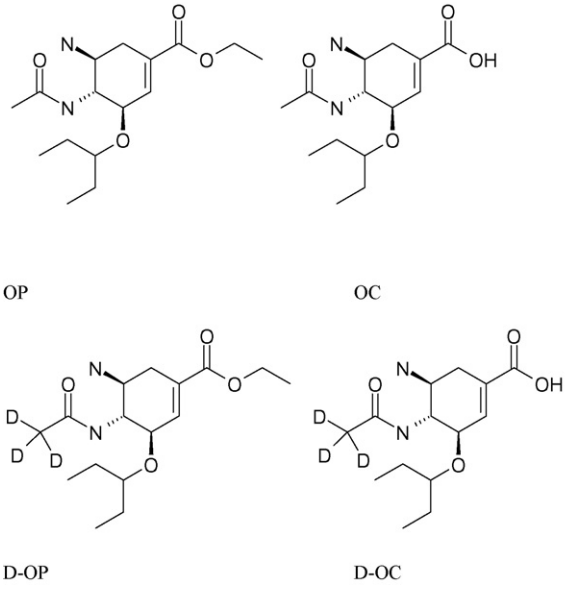


Fig. 1. Structure of OP, OC, D-OP and D-OC.

Table 1
LC gradient programme

| Time (min) | %Solvent B |
|---|---|
| 0 | 85 |
| 1.00 | 80 |
| 1.20 | 60 |
| 1.90 | 60 |
| 2.00 | 85 |
| 4.00 | 85 |

Solvent A: 10 mM ammonium acetate in water (+1% acetic acid, v/v), Solvent B: Acetonitrile.

An API 5000 triple quadrupole mass spectrometer (Applied Biosystems/MDS SCIEX, Foster City, USA), with a TurboV$^{TM}$ ionisation source (TIS) interface operated in the positive ion mode, was used for the multiple reaction monitoring (MRM) LC–MS/MS analysis. The mass spectrometric conditions were optimized for the compounds by infusing a 25 ng/mL standard solution in mobile phase at 10 μL/min using a Harvard infusion pump directly connected to the mass spectrometer. An additional tuning optimization was performed by continuously infusing the same standard solution at 10 μL/min via a "T" connector into the post-column mobile phase flow (500 μL/min). The TIS temperature was maintained at 575 °C and the TIS voltage was set at 5500 V. The curtain gas was set to 30.0 psi and the nebulizer (GS1) and TIS (GS2) gases at 50.0 and 45.0 psi, respectively. The CAD gas in the collision cell was set to 6 psi. Quantification was performed using selected reaction monitoring (SRM) for the transitions *m/z* 313–225 and 316–228 for OP and D-OP, respectively and 285–197 and 288–200 for OC and D-OC, respectively. Data were processed using Analyst 1.4 (Applied Biosystems/MDS SCIEX, Foster City, USA).

#### 2.1.2. Preparation of standards

Stock solutions of OP, OC, D-OP and D-OC (1–3 mg/mL) were prepared in water. Working solutions of OP and OC were prepared by serial dilution of the stock solutions in water.

Twenty microliters of the OP and OC working solutions were added to 1960 μL blank matrix (i.e. saliva, urine or fluoride/oxalate plasma) to yield spiked calibration standards. The calibration ranges, limit of detection and lower limit of

Table 2
Method settings for plasma, saliva and urine

| | Calibration range (ng/mL) | LLOQ (ng/mL) | LOD (ng/mL) |
|---|---|---|---|
| Plasma | | | |
| Oseltamivir | 1–600 | 1 | 0.25 |
| Oseltamivir carboxylate | 10–10,000 | 10 | 2.5 |
| Saliva | | | |
| Oseltamivir | 1–300 | 1 | 0.25 |
| Oseltamivir carboxylate | 10–10,000 | 10 | 2.5 |
| Urine | | | |
| Oseltamivir | 5–1500 | 5 | 1.25 |
| Oseltamivir carboxylate | 30–30,000 | 30 | 7.5 |

quantification for OP and OC in plasma, saliva and urine are summarised in Table 2. A calibration curve was constructed using 50 μL of each standard. Linear regression with peak-height ratio (analyte/internal standard) against concentration with 1/concentration$^2$ ($x^2$) weighting was used for quantification. Quality control (QC) samples for determination of accuracy and precision at three concentrations (LLOQ × 3, mid-range and upper-range) were prepared in the same manner as the calibration standards and stored at −86 °C until analysis. The calibration standards and QC samples were stored at −86 °C (in cryovials for long-term storage and as 50 μL aliquots in a capped 1 mL polypropylene 96-well plate for maximum 1 week) until analysis.

A combined working solution of D-OP and D-OC was stored in 1 mL aliquots at −86 °C until use when it was thawed and diluted 1:6 with water. The final concentration of D-OP/D-OC was 60/1000 ng/mL for plasma analysis, 30/1000 ng/mL for saliva analysis and 150/3000 ng/mL for urine analysis. The stock solutions of D-OP and D-OC were stored at −86 °C until use.

### 2.1.3. Analytical procedure

An eppendorf stream multistepper was used to add 50 μL internal standard solution to 50 μL sample in a 96-well plate. Ammonium acetate buffer 500 μL (pH 3.5; 5 mM) was added with a 12-channel pipette and the 96-well plate was gently mixed (500 rpm) on a Mixmate™ for about 10 min. The 96-well plate was centrifuged at 1100 × *g* for 10 min and the sample was loaded onto a conditioned MPC-SD standard well SPE

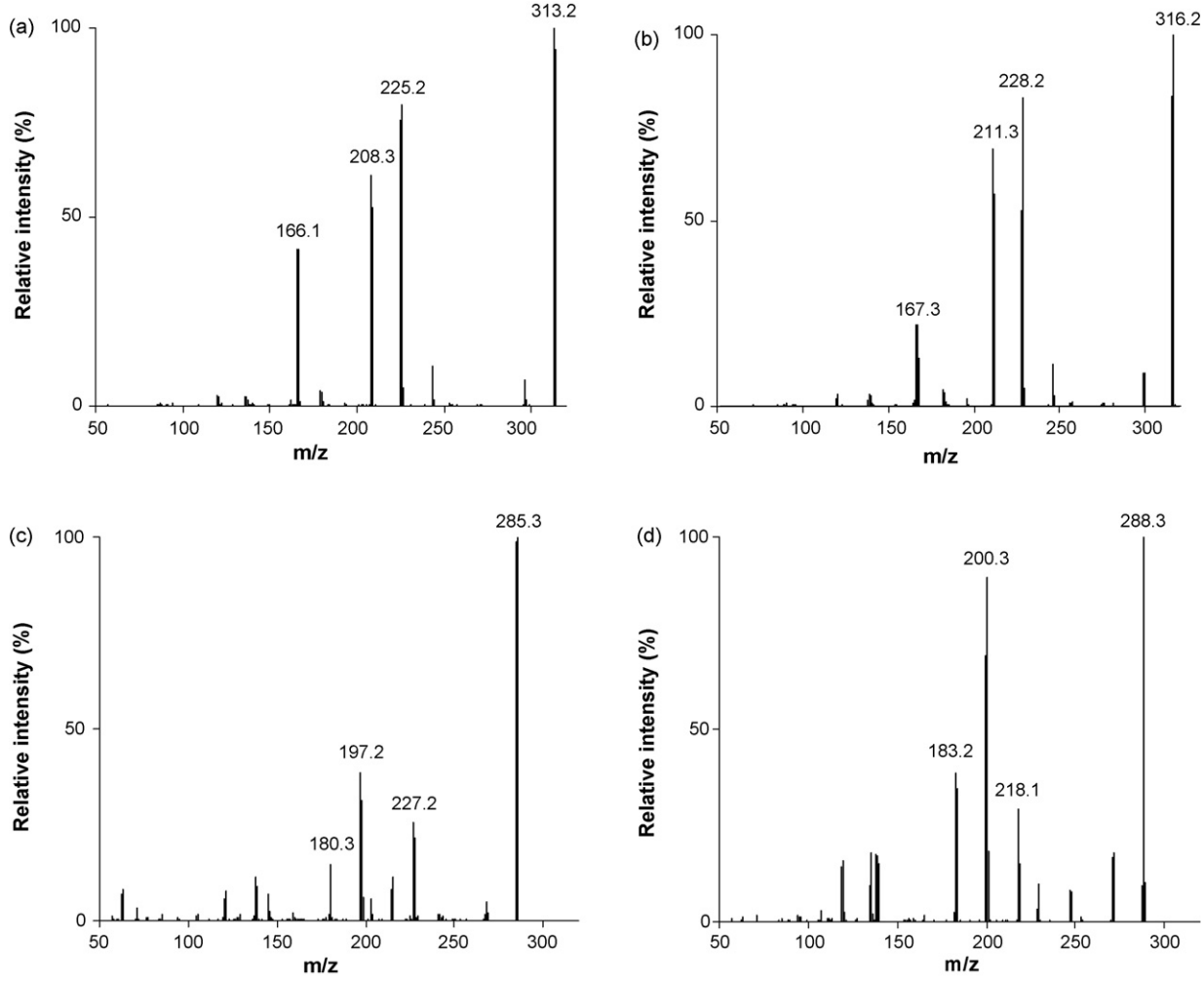


Fig. 2. Collision-induced dissociation mass spectra (*m/z* 50–320) for OP (a), D-OP (b), OC (C) and D-OC (d).

Table 3
Inter-, intra- and total-assay precision (ANOVA) for oseltamivir and oseltamivir carboxylate in human plasma

| Oseltamivir | Inter-assay CV (%) | Intra-assay CV (%) | Total-assay CV (%) | Oseltamivir carboxylate | Inter-assay CV (%) | Intra-assay CV (%) | Total-assay CV (%) |
|---|---|---|---|---|---|---|---|
| QC 1, 3 ng/mL | 4.20 | 3.95 | 3.99 | QC 1, 30 ng/mL | 3.84 | 2.60 | 2.83 |
| QC 2, 30 ng/mL | 1.80 | 1.36 | 1.44 | QC 2, 400 ng/mL | 1.90 | 1.19 | 1.33 |
| QC 3, 300 ng/mL | 1.11 | 1.27 | 1.24 | QC 3, 4000 ng/mL | 1.63 | 1.18 | 1.26 |

$n = 5$, $h = 4$.

Table 4
Inter-, intra- and total-assay precision (ANOVA) for oseltamivir and oseltamivir carboxylate in human saliva

| Oseltamivir | Inter-assay CV (%) | Intra-assay CV (%) | Total-assay CV (%) | Oseltamivir carboxylate | Inter-assay CV (%) | Intra-assay CV (%) | Total-assay CV (%) |
|---|---|---|---|---|---|---|---|
| QC 1, 3 ng/mL | 4.00 | 2.96 | 3.15 | QC 1, 30 ng/mL | 0.85 | 2.74 | 2.54 |
| QC 2, 20 ng/mL | 2.19 | 2.11 | 2.13 | QC 2, 400 ng/mL | 0.92 | 1.17 | 1.13 |
| QC 3, 150 ng/mL | 2.28 | 1.42 | 1.58 | QC 3, 4000 ng/mL | 1.35 | 1.20 | 1.22 |

$n = 5$, $h = 4$.

96-well plate. The whole sample volume (600 μL) was loaded for plasma and saliva analyses while only 200 μL of the total sample volume was loaded for urine analysis. All steps in the SPE procedure were conducted using a 12-channel pipette as follows: methanol 850 μL was added to each SPE well and vacuum at about 5–7 mm Hg was applied for about 15–20 s. Ammonium acetate buffer (pH 3.5; 5 mM) 400 μL was added to each SPE well and vacuum at about 5–7 mm Hg was applied for about 15–20 s. The samples were loaded onto the SPE plate and vacuum at about 1 mm Hg was applied for 2 min. The vacuum was thereafter increased by 1–2 mm Hg every 2 min until all samples had passed through the SPE wells. The SPE plate was washed with 500 μL water followed by 850 μL methanol and 850 μL methanol–water (90:10, v/v). Full vacuum was applied for about 30 min then the SPE column tips were wiped dry with paper. A 96-collection plate (1 mL) was inserted into the vacuum manifold and 900 μL methanol–ammonium acetate 50 mM (90:10, v/v) was added to each SPE well. Vacuum at about 1 mm Hg was applied for about 2 min and the vacuum was then increased about 1 mm Hg every minute until all elution solvent had passed through the SPE plate and into the collection plate. The collection plate was covered with a Nunc seal mat and mixed on a Mixmate™ at 600 rpm for 10 min. 2.5 μL was injected into the LC–MS/MS system.

### 2.1.4. Validation

Linearity and calibration models were evaluated using calibration curves obtained during 4 days. Precision and accuracy throughout the calibration range was evaluated by analysis of five replicates at three different concentrations daily for 4 days. Lower and upper limits of quantifications were evaluated by analysis of five replicates. Carry-over effects for OP, OC D-OP and D-OC were evaluated by injection of blank samples directly after injection of the highest point in the calibration curve. Over-curve dilution was evaluated by analysis of five replicates (diluted five times with blank matrix). Stability of OP and OC in human fluoride/oxalate plasma, saliva and urine was evaluated during three freeze/thaw cycles, at ambient temperature for 48 h and at 4 °C for 48 h. Bench-top stability of OP and OC as ready for extraction and in the autosampler was evaluated for 4 and 24 h, respectively. The concentrations were determined with $1/\text{amount}^2$ weighted linear regression using a calibration curve prepared each day. Intra-, inter- and total-assay precisions were calculated using analysis of variance (ANOVA). Selectivity was evaluated by analysis of blank matrix from six different donors. The potential interference of OP, OC, D-OP and D-OC on each other was also evaluated. Recovery was determined by comparing the peak area for extracted QC samples with the directly injected solution containing the same nominal concentrations of the analytes as the QC samples after extraction. Matrix effects were thoroughly evaluated using blank matrix from six different donors. A quantitative estimation of the matrix effects was obtained by comparing the peak area for QC samples spiked in elution solution with extracted blank matrix spiked with the same nominal concentration of the analytes. A qualitative visualization of the matrix effects was obtained through post-column infusion experiments as described by others [9,10]. Briefly, a

Table 5
Inter-, intra- and total-assay precision (ANOVA) for oseltamivir and oseltamivir carboxylate in human urine

| Oseltamivir | Inter-assay CV (%) | Intra-assay CV (%) | Total-assay CV (%) | Oseltamivir carboxylate | Inter-assay CV (%) | Intra-assay CV (%) | Total-assay CV (%) |
|---|---|---|---|---|---|---|---|
| QC 1, 15 ng/mL | 2.27 | 2.43 | 2.41 | QC 1, 90 ng/mL | 0.54 | 2.83 | 2.61 |
| QC 2, 100 ng/mL | 2.86 | 1.51 | 1.79 | QC 2, 1200 ng/mL | 3.14 | 1.03 | 1.56 |
| QC 3, 750 ng/mL | 2.96 | 1.42 | 1.76 | QC 3, 12,000 ng/mL | 2.88 | 0.84 | 1.38 |

$n = 5$, $h = 4$.

continuous post-column infusion of a 5–15 ng/mL OP/OC/D-OP/D-OC solution at 10 μL/min by a Harvard infusion pump through a T-connector was introduced to the mass spectrometer while samples to be tested were injected.

## 3. Results and discussion

OP is freely soluble in water and methanol and has one basic p$K_a$ estimated to about 7.75–8.80 [11,12]. OC is more hydrophilic than OP and has an additional acidic p$K_a$ at about 4.1 [12].

The starting point for the extraction method was the SPE procedure in the assay published previously by Wiltshire et al. [6]. The load, the wash, and the elution solutions were evaluated with respect to pH, ionic strength, counter-ion and solvent composition. It was necessary to use a buffer with low ionic strength during the load step to avoid loss resulting from competitive binding to the cation exchanger. It was advan-

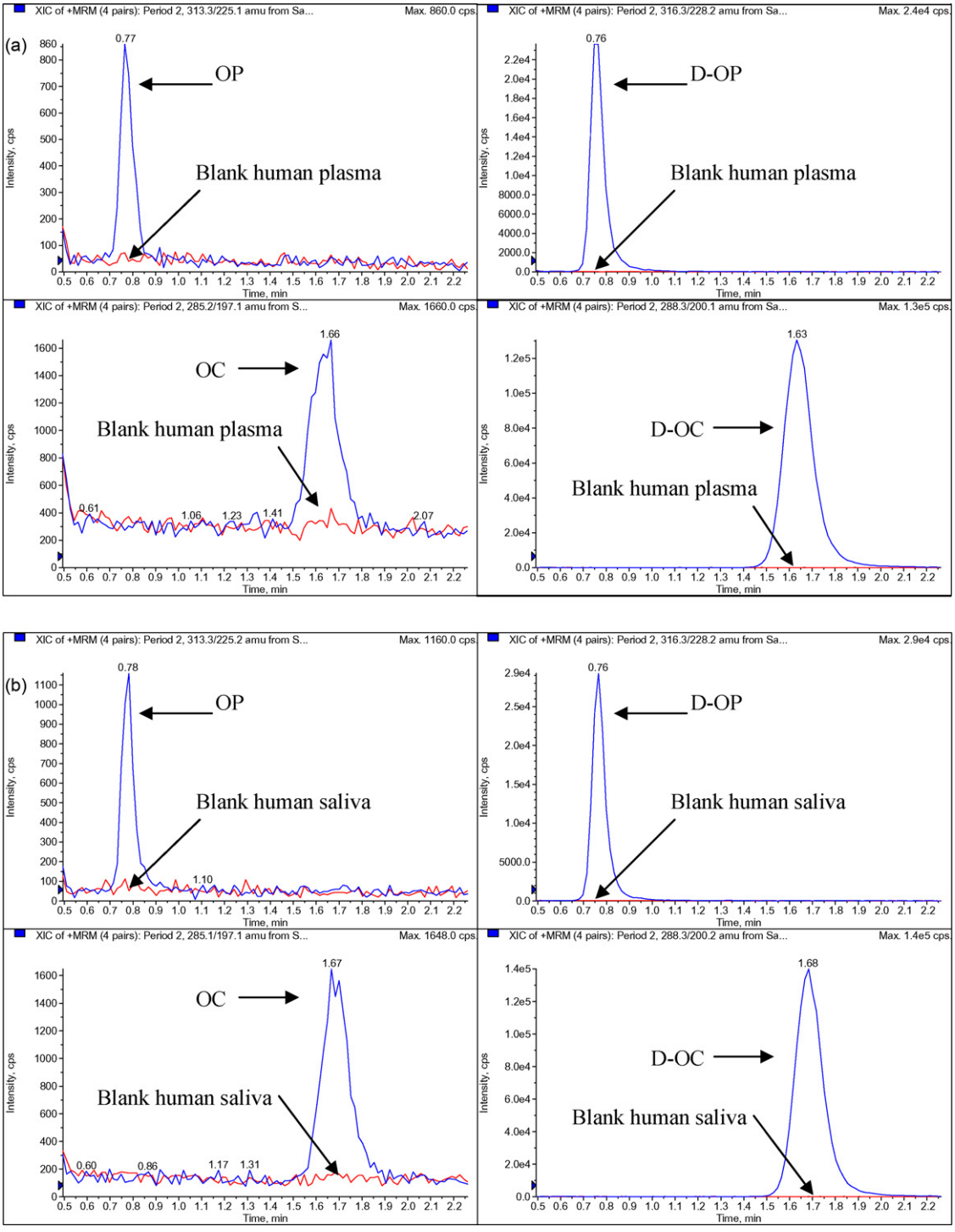


Fig. 3. Samples at LLOQ with overlay of blank matrix. Plasma (a), saliva (b) and urine (c).

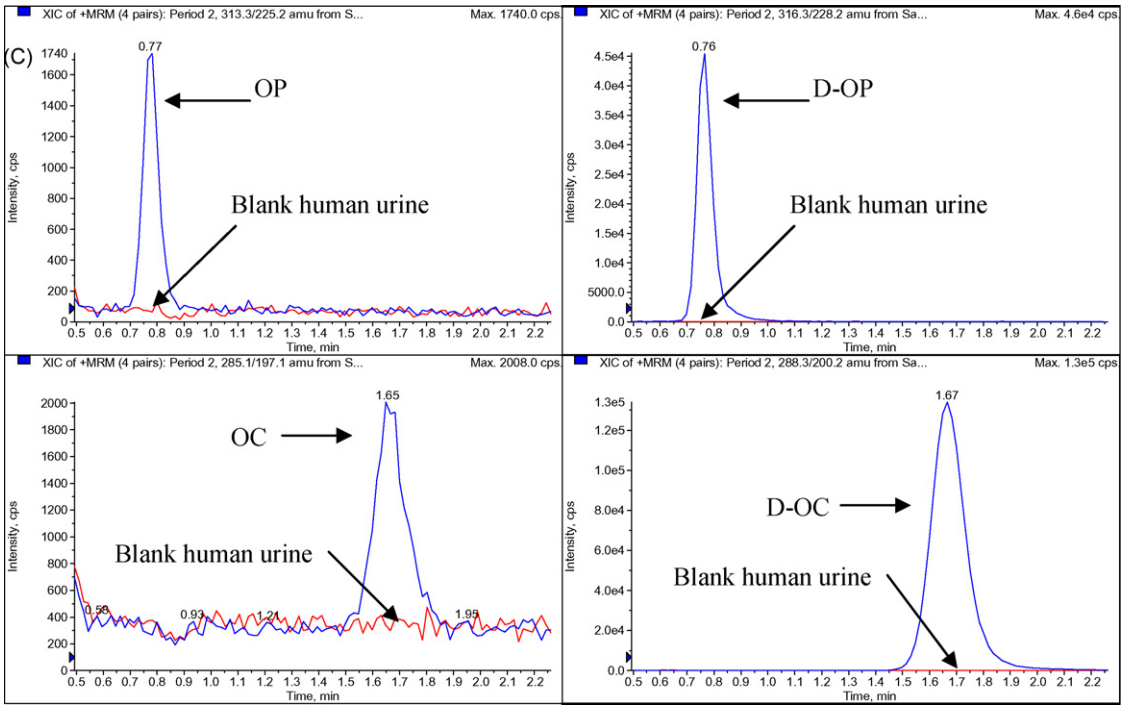


Fig. 3. *(Continued).*

tageous to wash the column with water and methanol–water combinations rather than ammonium acetate (pH 3.5; 5 mM) or methanol–ammonium acetate combinations for the same reason. A solution containing methanol–ammonium acetate 50 mM was adequate to elute OP and OC from the SPE column with high recovery. Unadjusted ammonium acetate was superior to pH adjusted ammonium acetate. It was important to avoid a basic pH throughout the method to avoid hydrolysis of OP.

The earlier LC–MS method for OP and OC utilized a CN column and a mobile phase containing 50% methanol [6]. An advantage of using HILIC LC coupled to MS for the analysis of polar compounds like OP and OC is that the amount of acetonitrile in the mobile phase is very high, in this case around 85%. The high amount of organic solvent in the mobile phase results in a more efficient electrospray ionisation and therefore higher sensitivity for the compound. Another advantage is that having more organic solvent in the sample compared to the mobile phase actually leads to band narrowing rather than the band broadening seen with conventional reversed phase LC. These features enabled the eluate (methanol–ammonium acetate 50 mM (90:10, v/v)) to be directly injected into the LC–MS/MS system without compromising sensitivity or reproducibility. This LC–MS/MS assay for quantification of OP/OC is approximately 500 times more sensitive than the previous published assay [6]. The limit of quantification for oseltamivir was 138 fg injected onto the column using an injection volume of 2.5 μL. The collision-induced dissociation (CID) mass spectra (*m/z* 50–320) for OP, OC, D-OP and D-OC are shown in Fig. 2a–d. The product ions at 225, 228, 197 and 200 *m/z* for OP, D-OP, OC and D-OC, respectively are consistent with a loss of the pentyloxy side chain.

### 3.1. Validation

The following concentrations of D-OP were chosen; 60, 30 and 150 ng/mL for plasma, saliva and urine analysis, respectively. These concentrations produced a low signal in the OP trace but contributed to less than 20% of a standard at LLOQ. The concentrations of D-OC chosen were 1000, 1000 and 3000 ng/mL for plasma, saliva and urine analysis, respectively. These concentrations produced a low signal in the OC trace but contributed to less than 20% of a standard at LLOQ. The highest concentrations of OP and OC did produce a low signal in the trace for their respective deuterated internal standards due to isotope interference. The signal contributed to less than 1% of the actual concentration used. Carry-over for OP and OC was less than 20% of a LLOQ sample and carry-over for D-OP and D-OC was not detectable with the chosen settings.

Linear calibration curves were generated by 1/amount$^2$ ($x^2$) weighted linear regression analysis. The back calculated concentrations for the calibration standards and the results for the precision samples were used to choose the regression model. Precision for the QC samples during the validation in plasma, saliva and urine are shown in Tables 3–5. Accuracy at all concentrations for all matrices was within ±5% of the nominal values. The lower limit of quantification (LLOQ) and limit of detection (LOD) for OP and OC in plasma, saliva and urine are summarised in Table 2. The precision and accuracy at LLOQ was well below 20% [13]. The LOD was chosen as the lowest concentration that could be distinguished reliably from the background noise (i.e. ≥3 times the S.D. of a blank plasma sample) [13]. The precision and accuracy at the upper limit of quantification and for over-curve dilution samples were within ±5% for all matrices.

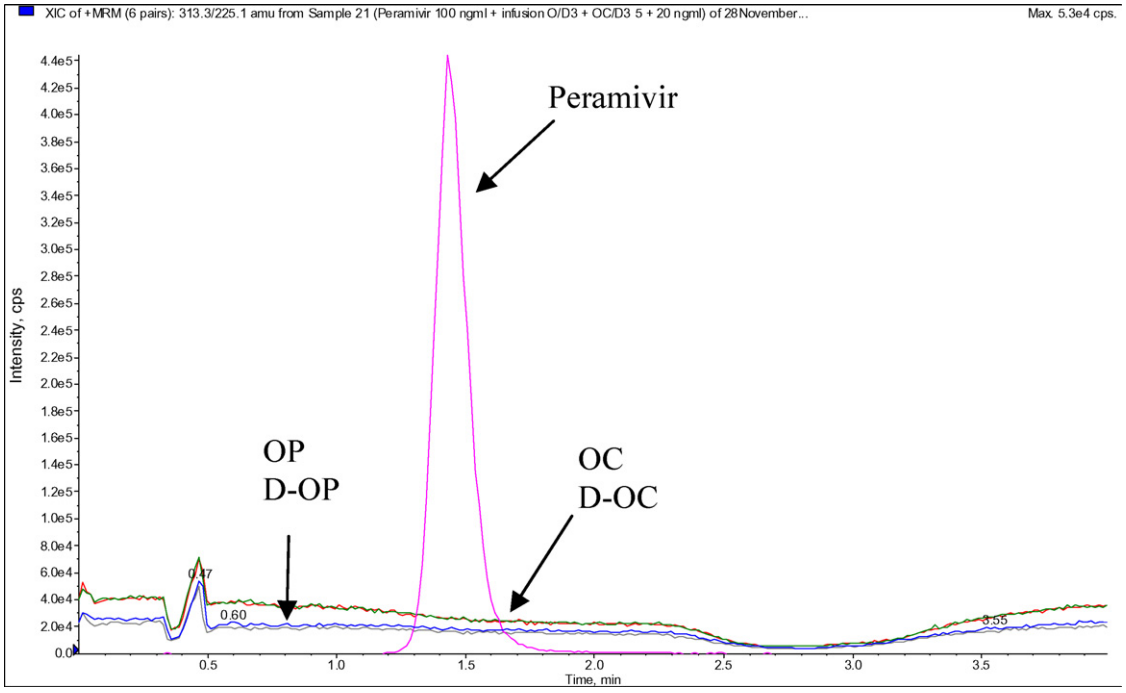


Fig. 4. Injection of peramivir 100 ng/mL during post-column infusion 10 μL/min of OP/D-OP 5 ng/mL and OC/D-OC 20 ng/mL.

None of the blank samples gave any signal that interfered with the peaks of OP, OC, D-OP or D-OC. An example of blank plasma, blank saliva and blank urine with an overlay of a LLOQ sample is shown in Fig. 3a–c. Due to the unsurpassed specificity of the MS/MS system, selectivity in terms of interfering signals is seldom a problem. However, co-administered drugs could still lead to suppression/enhancement effects if co-eluting or eluting close to the peaks of interest. Zanamivir and peramivir are two other potent neuraminidase inhibitors that potentially could be combined with OP during clinical studies. Post-column infusion experiments with injection of peramivir (100 ng/mL) and zanamivir (1000 ng/mL) confirmed that the responses of OP, OC, D-OP and D-OC were unaffected if either of these drugs were present in the samples (Figs. 4 and 5). OP and OC were stable in all matrices during three freeze/thaw cycles, at ambient temperature in all matrices for at least 48 h, and in all matrices at 4 °C

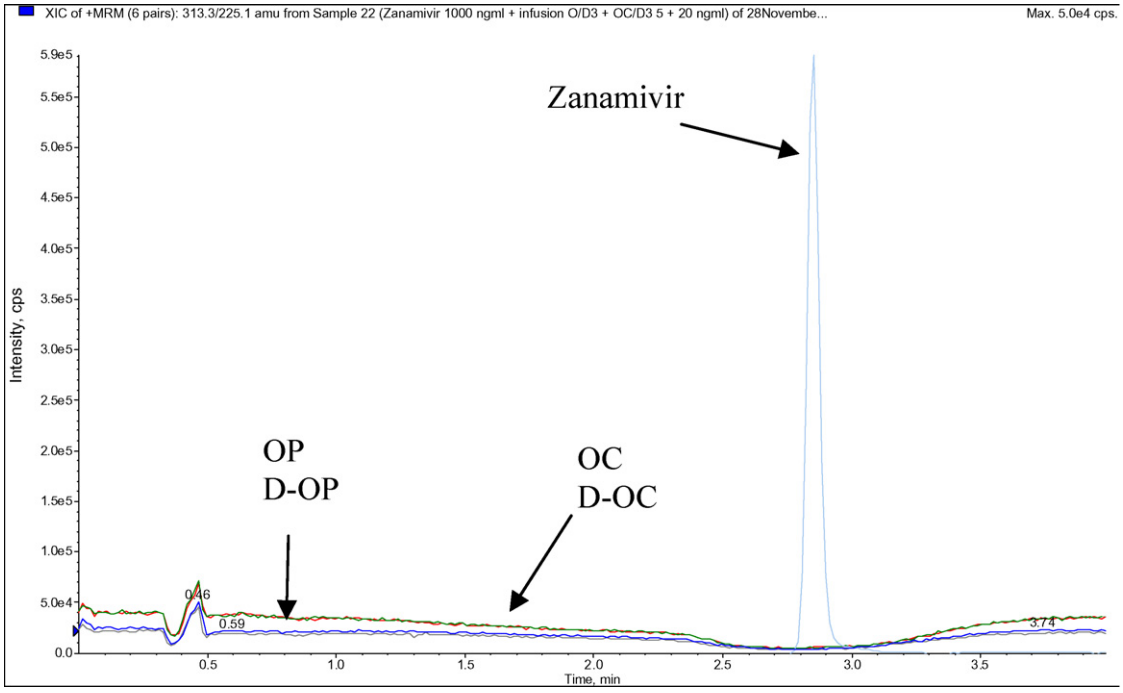


Fig. 5. Injection of zanamivir 1000 ng/mL during post-column infusion 10 μL/min of OP/D-OP 5 ng/mL and OC/D-OC 20 ng/mL.

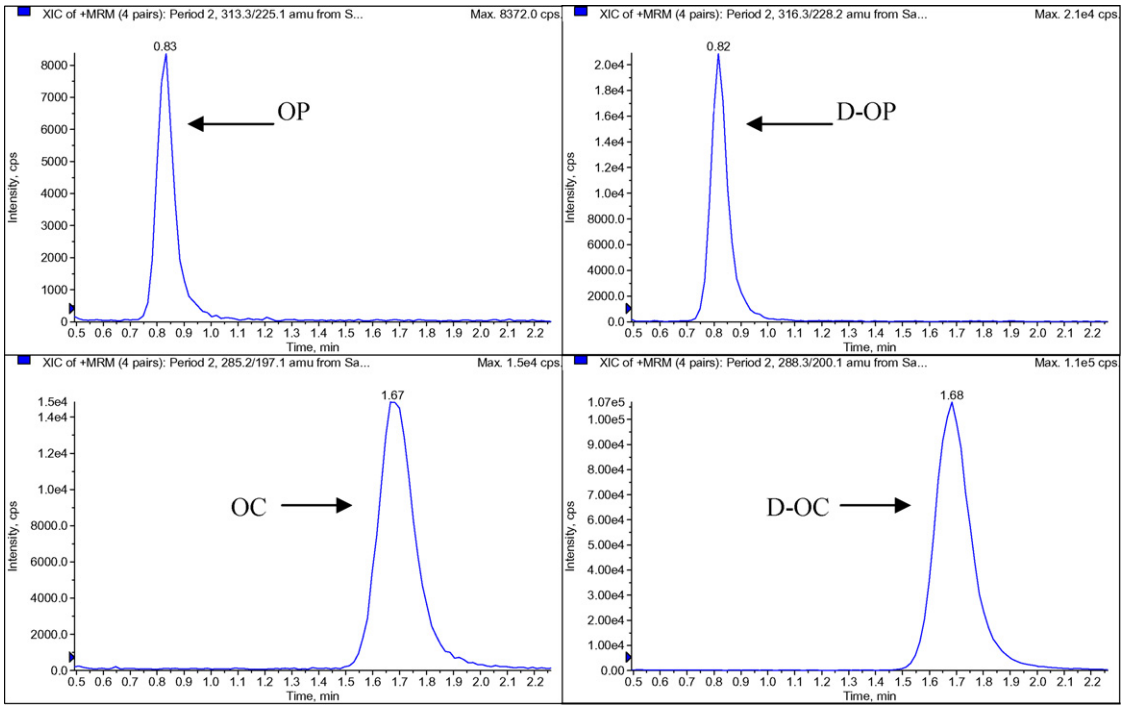


Fig. 6. Patient sample containing 6.73 ng/mL OP and 380 ng/mL OC.

for at least 48 h. We have previously shown that fluoride/oxalate plasma can prevent esterase mediated *ex vivo* conversion of OP to OC [7,8]. Both OP and OC were stable in fluoride/oxalate plasma during heat inactivation at 60 °C for at least 90 min [7]. OP and OC were stable as ready for extraction for at least 4 h and in the autosampler for at least 24 h. All results complied well with the generally accepted limits for R.S.D. and accuracy (<15%). The assay was implemented for the analysis of clinical samples from an interaction study in healthy volunteers. Fig. 6 shows a chromatogram from a patient sample taken 5 h after a single oral dose of 75 mg OP containing 6.73 ng/mL OP and 380 ng/mL OC.

### 3.2. Recovery and matrix effects

The recovery (unadjusted for matrix effects) of OP, OC, D-OP and D-OC in plasma and saliva was about 90% or higher at

Table 6
Matrix effects

| | Blank A | Blank B | Blank C | Blank D | Blank E | Blank F | Average | S.D. | CV (%) |
|---|---|---|---|---|---|---|---|---|---|
| Plasma | | | | | | | | | |
| OP 3 ng/mL | 104.9 | 103.0 | 98.1 | 104.5 | 103.5 | 102.1 | 102.7 | 2.4 | 2.4 |
| OP 300 ng/mL | 106.1 | 104.3 | 103.1 | 101.7 | 100.6 | 103.8 | 103.3 | 1.9 | 1.9 |
| D-OP 60 ng/mL | 104.5 | 105.3 | 104.7 | 102.3 | 99.7 | 105.2 | 103.6 | 2.2 | 2.1 |
| OC 30 ng/mL | 112.1 | 112.4 | 113.3 | 110.1 | 110.2 | 108.5 | 111.1 | 1.8 | 1.6 |
| OC 4000 ng/mL | 105.8 | 103.7 | 103.0 | 101.9 | 100.7 | 104.0 | 103.2 | 1.8 | 1.7 |
| D-OC 1000 ng/mL | 107.9 | 107.0 | 108.5 | 105.4 | 104.5 | 107.0 | 106.7 | 1.5 | 1.4 |
| Saliva | | | | | | | | | |
| OP 3 ng/mL | 93.3 | 86.5 | 98.2 | 95.3 | 89.8 | 102.5 | 94.3 | 5.8 | 6.1 |
| OP 150 ng/mL | 96.5 | 99.5 | 96.3 | 97.1 | 96.3 | 99.8 | 97.6 | 1.6 | 1.7 |
| D-OP 30 ng/mL | 94.8 | 94.1 | 93.7 | 93.7 | 91.0 | 92.6 | 93.3 | 1.3 | 1.4 |
| OC 30 ng/mL | 96.3 | 89.4 | 105.4 | 93.6 | 94.5 | 95.6 | 95.8 | 5.3 | 5.5 |
| OC 4000 ng/mL | 96.6 | 98.6 | 95.9 | 95.9 | 96.9 | 99.4 | 97.2 | 1.5 | 1.5 |
| D-OC 1000 ng/mL | 95.0 | 93.7 | 94.2 | 94.2 | 92.2 | 92.3 | 93.6 | 1.1 | 1.2 |
| Urine | | | | | | | | | |
| OP 15 ng/mL | 88.8 | 77.2 | 88.0 | 89.0 | 75.2 | 85.7 | 84.0 | 6.2 | 7.3 |
| OP 750 ng/mL | 88.3 | 79.4 | 85.8 | 88.0 | 78.1 | 84.8 | 84.1 | 4.3 | 5.1 |
| D-OP 150 ng/mL | 89.7 | 78.9 | 84.5 | 84.5 | 79.4 | 84.0 | 83.5 | 4.0 | 4.8 |
| OC 90 ng/mL | 97.1 | 96.6 | 100.4 | 97.8 | 95.2 | 94.2 | 96.9 | 2.2 | 2.2 |
| OC 12,000 ng/mL | 97.5 | 96.6 | 97.1 | 99.6 | 95.0 | 94.8 | 96.8 | 1.8 | 1.8 |
| D-OC 3000 ng/mL | 94.5 | 94.8 | 98.6 | 98.6 | 94.6 | 95.7 | 96.1 | 1.9 | 2.0 |

OP, OC, D-OP and D-OC spiked in extracted blank human plasma, saliva and urine vs. spiked in elution solution.

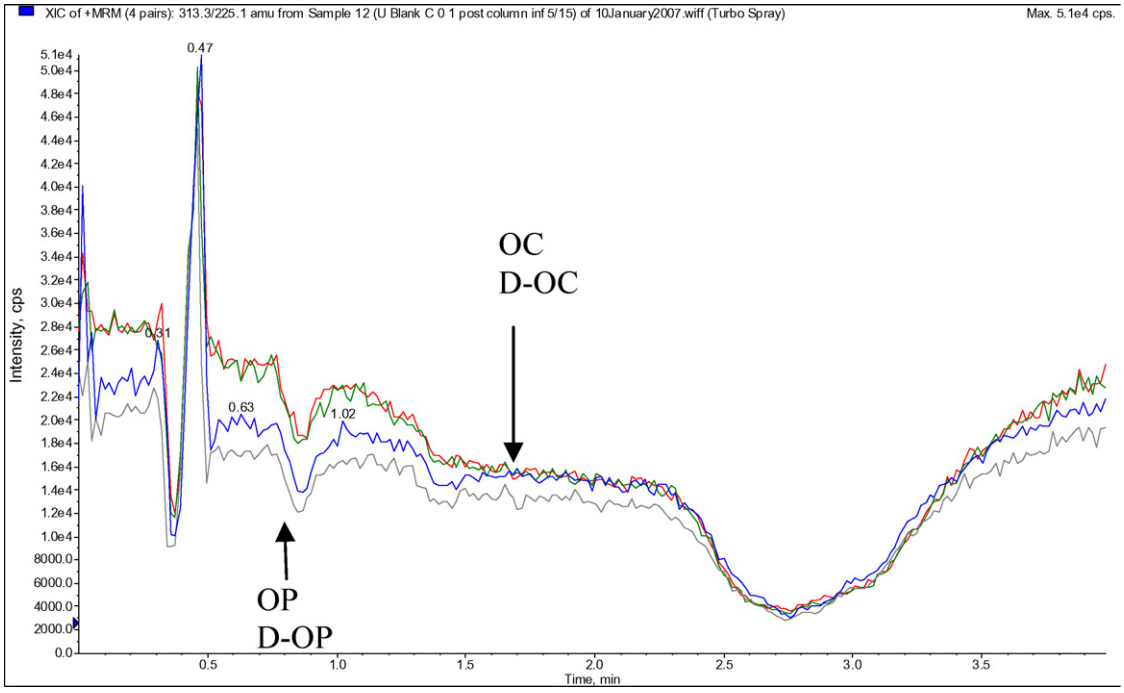


Fig. 7. Injection of extracted blank human urine during post-column infusion 10 μL/min of OP/D-OP 5 ng/mL and OC/D-OC 15 ng/mL.

all tested concentrations. The recovery (unadjusted for matrix effects) of OC and D-OC urine was also about 90% or higher at all tested concentrations but the recovery of OP and D-OP in urine only around 80%. The recovery of the internal standards co-varied with the recovery of OP and OC thus the normalized recovery (OP/D-OP and OC/D-OC) was close to 1 with low variation. The actual method recovery was high for all matrices and the findings for OP in urine are explained by a small amount of ion suppression caused by remaining salt residues from the matrix. An evaluation of the matrix effects for plasma, saliva and urine is shown in Table 6 where spiked samples in elution solution are compared with extracted blank matrix from six donors spiked with the same nominal concentration of the analytes. It is clear that OP suffers from a small amount of ion suppression in the urine matrix.

It can be seen in Table 6 that the normalized matrix effect (OP/D-OP) in urine is close to 1 with a low variation confirming that the internal standard compensates fully for the ion suppression.

Post-column infusion experiments where extracted blank urine was injected confirmed a region with ion suppression eluting around the retention time of OP and D-OP (Fig. 7).

## 4. Conclusion

A high throughput LC–MS/MS method for the determination of OP and OC in plasma, saliva and urine has been developed and validated. The LC–MS/MS assay presented here uses a smaller sample volume (i.e. 50 μL) and shows, in general, much lower variation than the previously published method for quantification of OP and OC in plasma and urine [6]. The new assay uses 96-well SPE instead of single cartridges and has a shorter LC run time enabling a much higher throughput than the previous assay. The eluate is directly injected into the LC–MS/MS system without compromising sensitivity or reproducibility. The internal standard compensated for matrix effects found for the urine matrix. This highly sensitive robust LC–MS assay should facilitate urgently needed pharmacokinetic studies on this important antiviral drug.

## Acknowledgements

We thank F.Hoffmann-La Roche Ltd. for providing oseltamivir standards. This study was part of the SE Asian Influenza Clinical Trials Network supported by the US National Institute of Allergy and Infectious Diseases (NIH N01-AO-00042), and the Wellcome Trust-Mahidol University-Oxford Tropical Medicine Research Programme (077166/Z/05/Z) supported by the Wellcome Trust of Great Britain.

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