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Development and validation of a liquid chromatographic-tandem mass spectrometric method for determination of piperaquine in plasma Stable isotope labeled internal standard does not always compensate for matrix effects

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Abstract

A bioanalytical method for the analysis of piperaquine in human plasma using off-line solid-phase extraction and liquid chromatography coupled to positive tandem mass spectroscopy has been developed and validated. It was found that a mobile phase with high pH (i.e. 10) led to better sensitivity than mobile phase combinations with low pH (i.e. 2.5–4.5) despite the use of positive electrospray and a basic analyte. The method was validated according to published FDA guidelines and showed excellent performance. The within-day and between-day precisions expressed as R.S.D., were lower than 7% at all tested concentrations (4.5, 20, 400 and 500 ng/mL) and below 10% at the lower limit of quantification (LLOQ) (1.5 ng/mL). The calibration range was 1.5–500 ng/mL with a limit of detection (LOD) at 0.38 ng/mL. 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. Matrix effects originating from the sample clean-up (i.e. solid-phase extraction) procedure rather than the plasma background were responsible for the ion suppression seen in this study. Salts remaining from the buffers used in the solid-phase extraction suppressed the signals for both piperaquine and its deuterated internal standard. This had no effect on the quantification of piperaquine. Triethylamine residues remaining after evaporation of the solid-phase extraction eluate were found to suppress the signals for piperaquine and its deuterated internal standard differently. It was found that this could lead to an underestimation of the true concentration with 50% despite the use of a deuterated internal standard.

Keywords: Antimalarial; Differential matrix effect; High throughput; Ion suppression; Liquid chromatography/tandem mass spectrometry (LC-MS/MS); Piperaquine; Stable isotope labeled (SIL) internal standard; Solid-phase extraction

1. Introduction

Malaria, caused by the mosquito-borne protozoan parasite *Plasmodium falciparum*, is the most important parasitic disease of man. Close to 500 million people are infected each year and up to 3 million die. Africa suffers the majority (>90%) of this mortality burden, affecting mostly children younger than 5 years [1,2]. Piperaquine (PQ), 1,3-bis-[4-(7-chloroquinolyl-4)-piperazinyl-1]-propane, is a bisquinoline antimalarial compound belonging to the 4-aminoquinoline group. PQ was first synthesized at Rhône-Poulenc in France in the 1950s but was not taken further into man until Shanghai Research Institute of Pharmaceutical Industry in China rediscovered PQ in the 1960s. PQ was used in China in very large quantities as a monotherapy and prophylactic drug from the 1970s until late 1980s when resistance started to emerge [3]. In recent years PQ has attracted renewed interest since it has been shown to be an effective partner to dihydroartemisinin (DHA) in the combination Artekin®. The combination has been used throughout South-east Asia for several years with good efficacy [4–9]. A partnership between Holleykin, Sigma-Tau, Oxford University and the Medicines for Malaria Venture (MMV) is currently trying to develop and register this combination for worldwide use [10]. Despite the extensive use of PQ since the 1960s in China, published pharmacokinetic data are still limited [7,11,12]. The

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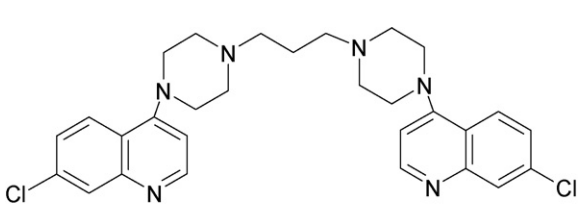
metabolism of PQ has not yet been studied in detail but PQ is thought to be eliminated mainly as unchanged drug. Five PQ metabolites in human urine were recently characterized using LC-MS and NMR [13]. A few methods that permit determination of PQ in biological fluids have been published. Three methods have been validated for the determination in plasma, one method for the determination in venous whole blood, one for the determination in capillary blood applied onto sampling paper and one for the determination in urine [14–19]. The most sensitive assay permits a lower limit of quantification (LLOQ) of 2.5 ng/mL using a sample volume of 1 mL plasma [16]. There is an urgent need for a more sensitive assay to adequately characterize the terminal elimination phase of PQ in malaria patients [20].

The aim of this work was to develop a sensitive robust high throughput LC-MS/MS method suitable for determination of PQ in plasma during clinical studies. An additional goal was to minimize the plasma volume needed to facilitate detailed pharmacokinetic studies with intense sampling schedules in children. The method has been validated according to published FDA-guidelines [21].

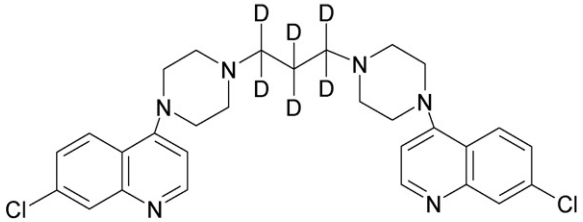
2. Experimental

2.1. Chemicals and materials

PQ was obtained from Guangzhou University of Traditional Chinese Medicine (Guangzhou, China). The stable isotope labeled (SIL) internal standard (D6-PQ) was obtained from Sigma-Tau (Pomezia, Italy). The structures are shown in Fig. 1. Acetonitrile (HPLC-grade), methanol (pro analysis) and HPLC-water were obtained from JT Baker (Phillipsburg, USA). Triethylamine (for synthesis) was obtained from BDH (Poole, UK). The phosphate buffer solutions were prepared by mixing appropriate amounts of sodium hydroxide (BDH) and ortho-phosphoric acid (Merck KGaA, Darmstadt, Germany) with HPLC-water. 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).



PQ



D6-PQ (IS)

Fig. 1. Structures of PQ and D6-PQ.

2.2. 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 Gemini C18 (50 mm × 2.0 mm) column protected by a security guard column with a Gemini C18 (4.0 mm × 2.0 mm) guard cartridge (Phenomenex, Torrance, USA) under isocratic conditions using a mobile phase containing acetonitrile-ammonium bicarbonate 2.5 mM pH 10.0 (85:15, v/v) at a flow rate of 500 μL/min.

An API 5000 triple quadrupole mass spectrometer (Applied Biosystems/MDS SCIEX), 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 PQ and D6-PQ by infusing a 100 ng/mL standard solution in mobile phase at 10 μL/min using a Harvard infusion pump (Harvard Apparatus, Holliston, USA) connected directly 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 600 °C and the TIS voltage was set at 5500 V. The curtain gas was set to 35.0 psi, the declustering potential (DP) at 205.0 V, 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* 535–288 for PQ and 541–294 for D6-PQ.

2.3. Preparation of plasma standards

Stock solutions of PQ and D6-PQ (1000 μg/mL) were prepared in methanol-aqueous formic acid 1% (50:50, v/v). Working solutions of PQ ranging from 0.150 to 50 μg/mL were prepared by serial dilution of the stock solution in methanol-aqueous formic acid 1% (50:50, v/v). A working solution of D6-PQ (60 ng/mL) was stored in 1 mL aliquots at −86 °C until use when it was thawed and diluted with phosphate buffer to 10 ng/mL. The stock solution of D6-PQ was stored at −86 °C until use. Twenty microliters of the PQ working solutions were added to blank plasma (1980 μL) to yield spiked calibration standards at six different concentrations ranging from 1.5 to 500 ng/mL. A calibration curve was constructed using 50 μL plasma of each standard. Linear regression with peak-height

ratio (PQ/D6-PQ response) against PQ concentration with 1/concentration^2 (x^2) weighting was used for quantification. Quality control (QC) samples for determination of accuracy and precision in plasma at three concentrations (4.5, 20 and 400 ng/mL) were prepared in the same manner as the calibration standards and stored at −86 °C until analysis. The amount of stock solution in all spiked samples was kept at 1% of the total sample volume to minimize any systematic errors between real samples and standards. 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.

2.4. Analytical procedure

An eppendorf stream multistepper was used to add 50 μL phosphate buffer (pH 2.0; 0.05 M) containing D6-PQ (10 ng/mL) to 50 μL plasma in a 96-well plate. An additional volume of 700 μL phosphate buffer (pH 2.0; 0.05 M) was added with a 12-channel pipette and the 96-well plate was gently mixed (600 rpm) on a MIXMATE™ for about 10 min. The 96-well plate was centrifuged at 1100 × *g* for 10 min and 400 μL of the sample was loaded onto a conditioned MPC-SD standard well SPE 96-well plate. All steps in the SPE procedure were conducted using a 12-channel pipette as follows: methanol 950 μL was added to each SPE well and vacuum at about 5–7 mmHg was applied for about 15–20 s. Phosphate buffer (pH 2.0; 0.05 M) 500 μL was added to each SPE well and vacuum at about 5–7 mmHg was applied for about 15–20 s. Four hundred microliters of the samples were loaded onto the SPE plate and vacuum at about 1 mmHg was applied for 2 min. The vacuum was thereafter increased with 1–2 mmHg every 2 min until all samples had passed through the SPE wells. Methanol-phosphate buffer (pH 2.0; 0.05 M) (80:20, v/v) 950 μL was added to each SPE well and vacuum at about 3–5 mmHg was applied for 5 min. Full vacuum was applied for about 40 min where after the SPE column tips were wiped dry with paper. A 96-collection plate (1 mL) was inserted into the vacuum manifold and 950 μL methanol-triethylamine (98:2, v/v) was added to each SPE well. Vacuum at about 1 mmHg was applied for about 2 min and the vacuum was thereafter increased with about 1 mmHg every minute until all elution solvent had passed through the SPE plate and into the collection plate.

The SPE eluates were evaporated in the 96-well plate under a gentle stream of air in a water bath at 70 °C. When the samples visually looked dry they were left another hour in the water bath to make sure that all solvent was evaporated. The samples were reconstituted in 500 μL acetonitrile-ammonium bicarbonate (pH 10; 2.5 mM) (85:15, v/v) using a 12-channel pipette and mixed on a MIXMATE™ at 800 rpm for about 10 min. 5 μL was injected into the LC-MS/MS system.

2.5. Validation

Linearity and calibration model 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 three replicates daily for 4 days. Carry-over effects for PQ and D6-PQ 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 three replicates (2000 ng/mL PQ diluted 5 times with blank human plasma) daily for 4 days. Stability of PQ in human plasma was evaluated during 3 freeze/thaw cycles, at ambient temperature for 48 h, at 4 °C for 48 h and at 60 °C for 60 min. Bench-top stability of PQ before SPE and in the autosampler was evaluated for 4 and 24 h respectively. The concentrations were determined with 1/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 plasma from six different donors. The potential interference of PQ on D6-PQ and vice versa was also evaluated. Recovery was determined by comparing the peak area for extracted QC samples with direct injected solution containing the same nominal concentration of PQ and D6-PQ as the QC samples after SPE and reconstitution. Matrix effects were thoroughly evaluated using blank plasma from six different donors. A quantitative estimation of the matrix effects was obtained by comparing the peak area for samples spiked in elution solution with extracted blank matrix spiked with the same nominal concentration of PQ and D6-PQ. A qualitative visualization of the matrix effects was obtained through post-column infusion experiments as described by others [22,23]. Post-column infusion experiments were also used to evaluate ion suppression/enhancement effects caused by residues from solutions used in the SPE procedure. Briefly, a continuous post-column infusion of 1–2 ng/mL PQ/D6-PQ 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

3.1. Method development

Earlier LC-methods for PQ have all utilized low pH phosphate buffers and many of them have also required different additives (such as trifluoroacetic acid or trichloroacetic acid) to produce acceptable peak shapes without severe tailing [18,19]. The Chromolith™ columns did not require additives but required low pH phosphate buffer to maintain reasonable peak shapes [15–17]. All attempts with short (speedrod) and micro-bore Chromolith™ columns and volatile mobile phase combinations resulted in severe tailing for the PQ peak. The Gemini column can endure high pH mobile phases for a long time without degradation of the silica. It has previously been shown that mobile phase combinations using a buffer with high pH often can be advantageous for the analysis of basic compounds using positive electrospray [24,25]. The MS response for PQ in this study was equivalent when infusing a working solution at a low pH and a high pH (i.e. pH 2.5 and 10). A mobile phase with high pH markedly improved the chromatography (less tailing) and increased the retention of PQ on the

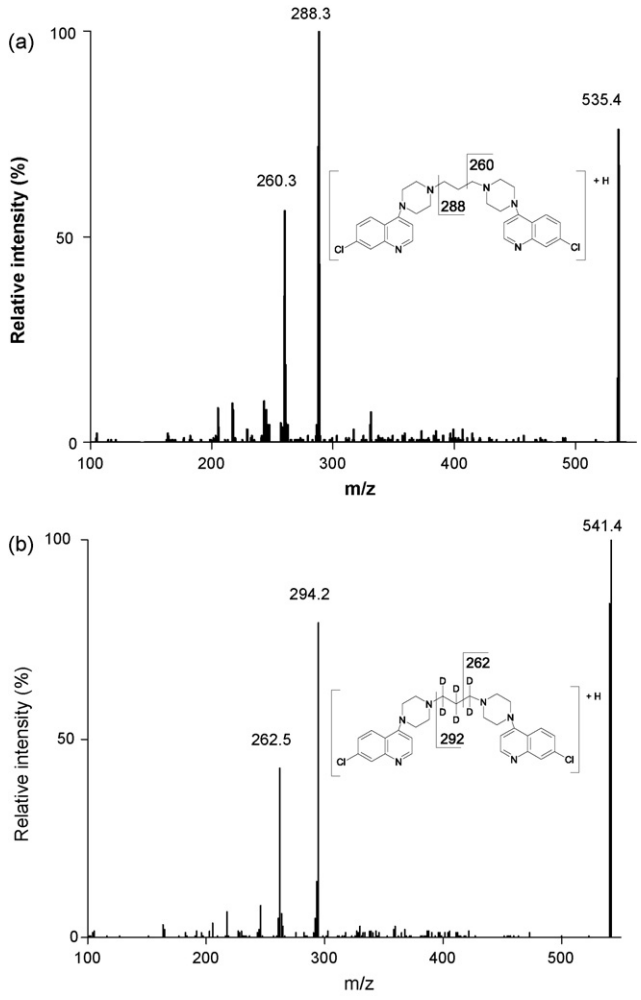


Fig. 2. Collision-induced dissociation mass spectra (*m/z* 100–550) for PQ and D6-PQ. For experimental conditions see Section 2.2.

column. PQ is very hydrophilic at low pH and very lipophilic at high pH (log *P* 6.2) and is considered as a weak base despite four pK_a values (at approximately 8.6, 8.6, 6.5 and 6.5) [26]. A mobile phase containing acetonitrile-ammonium bicarbonate 2.5 mM pH 10.0 (85:15, v/v) was finally chosen which could be compared with previous methods using low pH mobile phases and about 10% acetonitrile [16]. The high amount of organic modifier and the improved peak shape resulted in an approximate 50-fold increase in sensitivity compared to the experiments with low pH mobile phases. The collision-induced dissociation (CID) mass spectra (*m/z* 100–550) for PQ and D6-PQ are shown in Fig. 2a and b. The product ions at 288 and 294 *m/z* for PQ and D6-PQ respectively are consistent with a cleavage in the carbon bridge next to the aliphatic ring.

The starting point for the extraction method was to test and modify a high throughput 96-well plate solid-phase extraction (SPE) LC-UV assay published previously by Lindegardh et al. [16]. The idea was initially to change all the non-volatile phosphate buffers in the SPE steps from the original method to volatile acetate or formate buffer combinations. However, all attempts to modify the solutions resulted in a decreased recovery so the original SPE method remained unchanged.

3.2. Validation

The concentration of D6-PQ was chosen to 10 ng/mL as higher concentrations started to produce signals in the PQ trace which could influence the LLOQ. The highest concentration of PQ did not produce a signal in the trace for D6-PQ. The upper limit of quantification was set to 500 ng/mL since higher concentrations gave carry-over effects higher than 20% of the response for a LLOQ sample. Carry-over for PQ was less than 15% of a LLOQ sample and carry-over for D6-PQ 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. A calibration model using $1/x^2$ weighting was chosen as this generated an evenly distributed low error over the whole range as can be seen in Table 1. Precision and accuracy for the QC samples during the validation is shown in Table 2. The lower limit of quantification was determined to 1.5 ng/mL with a precision and accuracy well below 20% [21]. The limit of detection (LOD) was 0.38 ng/mL. The LOD was chosen as the lowest concentration that could be reliably distinguished from the background noise (i.e. ≥3 times the S.D. of a blank plasma sample) [21]. Precision and accuracy for LLOQ, upper limit of quantification and over-curve dilution samples are shown in Table 3. The presented LC-MS/MS assay use a much lower sample volume (i.e. 50 μL) and shows in general much lower variation and much better sensitivity (i.e. LLOQ) than previously published LC-UV methods for quantification of PQ in plasma [16,18,19]. The assay is approximately 3300 times more sensitive than the previous assay when comparing the amount of PQ injected on column at LLOQ [16].

None of the blank samples gave any signal that would interfere with the peaks of PQ or D6-PQ. 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. Post-column

Table 1
Back-calculated concentrations of standard curves for PQ in human plasma

Nominal concentration	1.5 ng/mL	4.5 ng/mL	15 ng/mL	50 ng/mL	200 ng/mL	500 ng/mL
Average (n = 8)	1.51	4.39	14.9	50.6	204	494
S.D.	0.05	0.13	0.30	1.67	5.89	16.93
CV (%)	3.62	2.98	2.04	3.29	2.89	3.43
Accuracy	100.4	97.6	99.6	101.1	101.8	98.7

Table 2
Inter-, intra- and total-assay precision (ANOVA) for PQ in human plasma

PQ	Inter-assay CV (%)	Intra-assay CV (%)	Total-assay CV (%)	Accuracy (%)
QC1 4.5 ng/mL	4.54	4.45	4.46	104.5
QC2 20 ng/mL	4.97	4.19	4.33	101.2
QC3 400 ng/mL	7.37	3.48	4.54	101.4

Table 3
Inter-, intra- and total-assay precision (ANOVA) for lower limit of quantification, upper limit of quantification and diluted over-curve samples for PQ in human plasma

PQ	Inter-assay CV (%)	Intra-assay CV (%)	Total-assay CV (%)	Accuracy (%)
LLOQ 1.5 ng/mL	9.97	6.67	7.72	105.9
ULOQ 500 ng/mL	4.31	1.80	2.73	100.0
Over-curve diluted to 400 ng/mL	5.66	3.44	4.17	100.1

infusion experiments with injection of 100 ng/mL DHA (the partner drug in the combination) confirmed that the responses of PQ and D6-PQ were unaffected if DHA was to be present in the samples. PQ was stable in human plasma during 3 freeze/thaw cycles, at ambient temperature in human plasma for at least 48 h, in human plasma at 4 °C for at least 48 h and in human plasma at 60 °C for at least 60 min. PQ was 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 a study in children with uncomplicated malaria in Kenya. Fig. 3 shows a chromatogram from a patient sample containing 1.68 ng/mL and a blank plasma sample. The patient sample was taken 63 days after a standard 3 days course with Artekin®.

3.3. Recovery and matrix effects

The recovery (unadjusted for matrix effects) of PQ was 67.9 ± 3.3 and 63.1 ± 1.5 (% ±S.D.) at 4.5 and 400 ng/mL respectively. The recovery (unadjusted for matrix effects) of D6-PQ (10 ng/mL) was 66.7 ± 2.6 and 64.5 ± 2.5 (% ±S.D.) when tested at PQ concentrations 4.5 and 400 ng/mL respectively and thus independent of PQ concentration. The recovery of the internal standard co-varied with the recovery of PQ thus

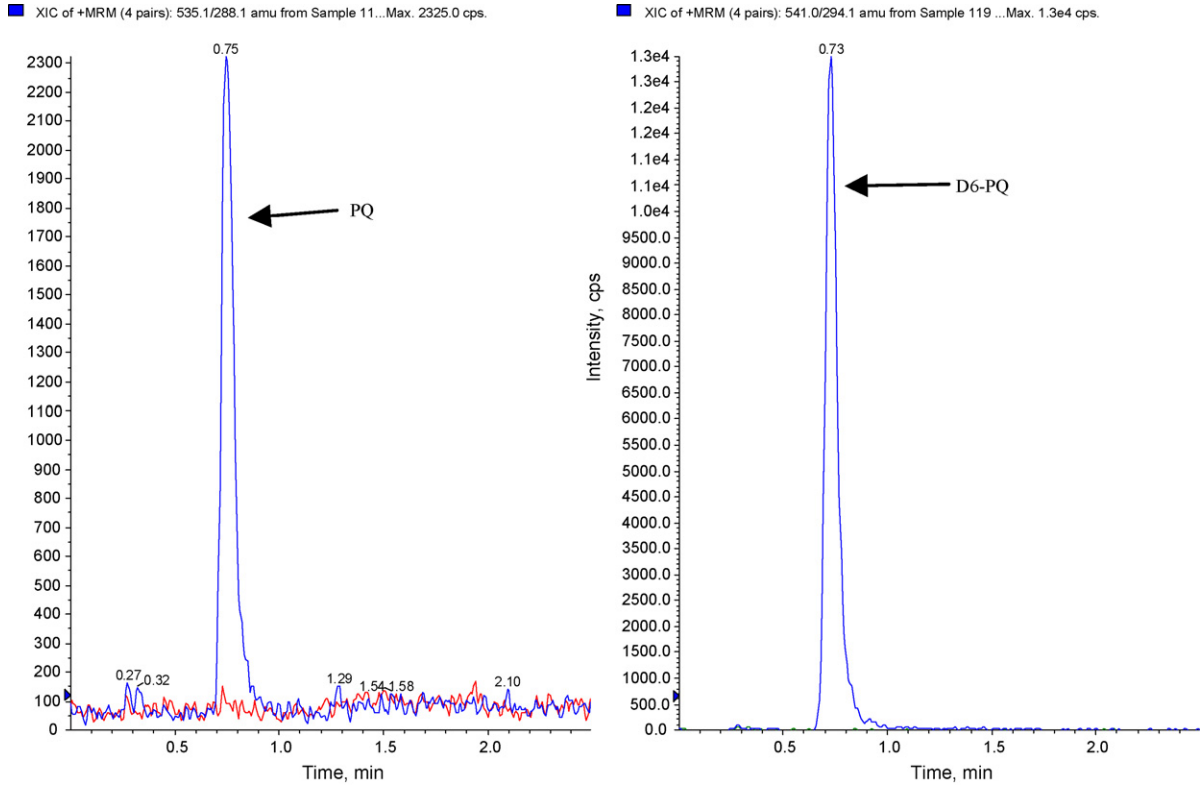


Fig. 3. Patient sample containing 1.68 ng/mL PQ. Overlay of blank plasma.

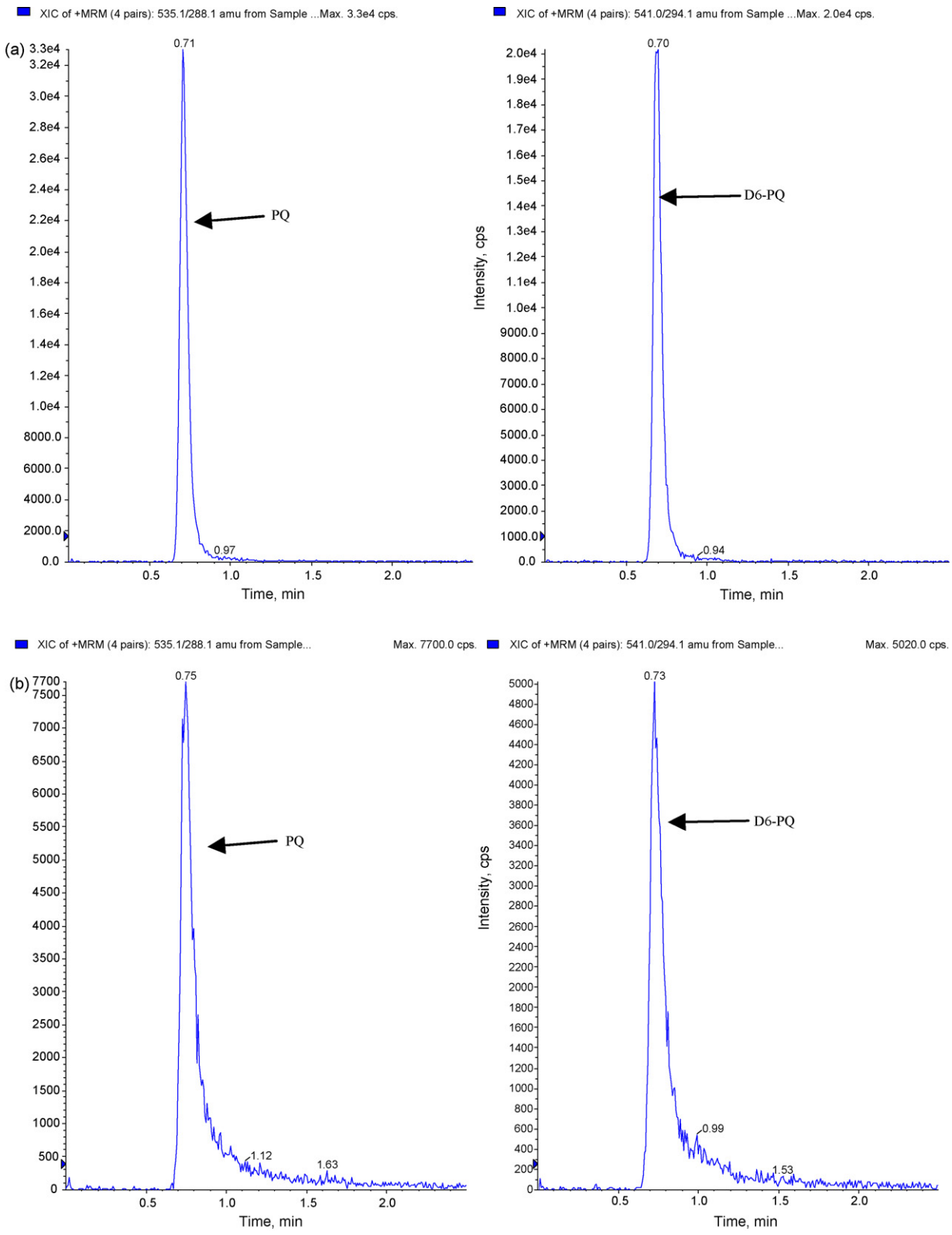


Fig. 4. Extracted spiked sample (20 ng/mL PQ) (a) and extracted spiked sample (20 ng/mL PQ) to which 50 μL phosphate buffer pH 2.0; 50 mM has been added prior reconstitution (b).

the normalized recovery (PQ/D6-PQ) was close to 1 with a low variation. The actual method recovery was higher and the findings above explained by a small amount of method specific ion suppression.

In theory a SIL internal standard will co-elute with the analyte and thus compensate for any matrix effects. It is however well known that partial separation between the SIL internal standard and the analyte often occur and is thought to be due to a

Table 4
Matrix effects

PQ	Blank A	Blank B	Blank C	Blank D	Blank E	Blank F	Average	S.D.	CV (%)
QC1 4.5 ng/mL	75.4	78.5	68.0	70.7	78.6	78.4	74.9	4.6	6.1
QC3 400 ng/mL	81.7	80.2	74.6	75.9	78.9	79.7	78.5	2.7	3.5
IS 10 ng/mL at PQ QC1 concentration	76.1	75.5	69.5	70.0	75.4	78.8	74.2	3.7	5.0
IS 10 ng/mL at PQ QC3 concentration	78.1	77.0	69.0	76.6	79.9	77.1	76.3	3.8	4.9

Recovery of PQ and D6-PQ in extracted blank human plasma vs. injection solution.

small change in lipophilicity when exchanging hydrogen for deuterium [27]. How noticeable the effect is depends on factors such as the number of labels, the size of the molecule, the efficiency of the column, retention mechanism and retention time of the compounds. Wang et al. recently showed that the response for carvedilol was significantly more affected than the response for the SIL carvedilol when they co-eluted with a region with severe ion suppression [28]. The consequence was that accuracy and precision of the method were severely affected.

The ion suppression in our study was constant with very low variation between six different sources of blank plasma (Table 4). The normalized matrix effects (PQ/D6-PQ) were close to 1 with a low variation confirming that the labeled internal standard did compensate fully for this type of ion suppression. The true method recovery adjusted for matrix effect was high (i.e. around 80–90%). The ion suppression was probably a result of salt residues (i.e. phosphate salts) remaining from the solid-phase extraction step. The hypothesis that the labeled internal standard compensated fully for this type of ion suppression was tested by comparing an extracted QC2 sample (20 ng/mL PQ) and an extracted QC2 sample (20 ng/mL PQ) to which 50 μL phosphate buffer pH 2.0; 50 mM had been added prior reconstitution (Fig. 4a and b). The latter sample would represent a worst-case scenario. The phosphate salts resulted in severe suppression of both the PQ and the internal standard signal and a slight increase in retention time for both peaks (Fig. 4b). Both signals dropped with approximately 75% but the predicted concentration (i.e. 20.0 ng/mL) was still right on target compared to the nominal value (i.e. 20 ng/mL). Hence any ion suppression resulting from phosphate salts remaining from the solid-phase extraction step will not bias the quantification. Post-column infusion and injection of an extracted blank sample to which 50 μL phosphate buffer pH 2.0; 50 mM had been added prior reconstitution confirmed a region with severe ion suppression eluting prior to PQ (Fig. 5). Another type of method specific ion suppression can occur if residues of triethylamine (remaining from the solid-phase extraction elution solution) are present in the samples (i.e. it can occur if the eluates are not evaporated to complete dryness). Three extracted QC2 samples (20 ng/mL PQ) reconstituted as soon as they visually looked dry demonstrates this phenomenon. The first sample contains very little triethylamine and the quantification of PQ is unaffected (predicted concen-

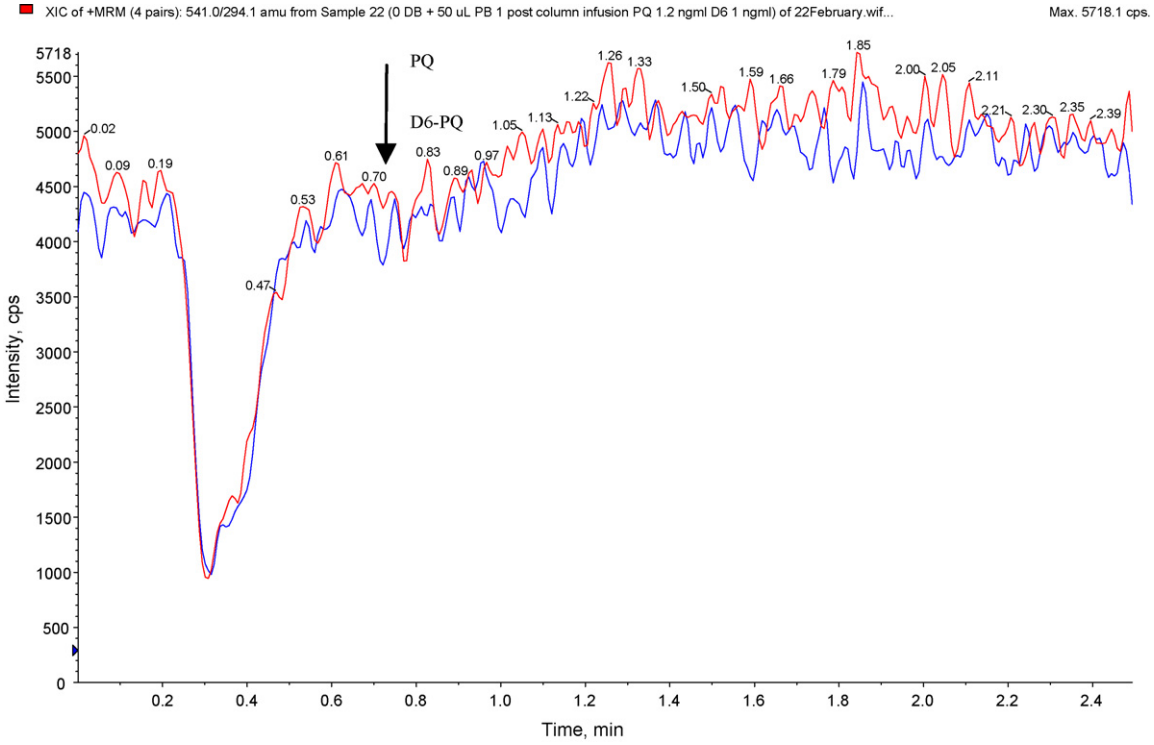


Fig. 5. Injection of extracted blank human plasma (to which 50 μL phosphate buffer pH 2.0; 50 mM has been added prior reconstitution) during post-column infusion 10 μL/min of PQ/D6-PQ 1.2 ng/mL.

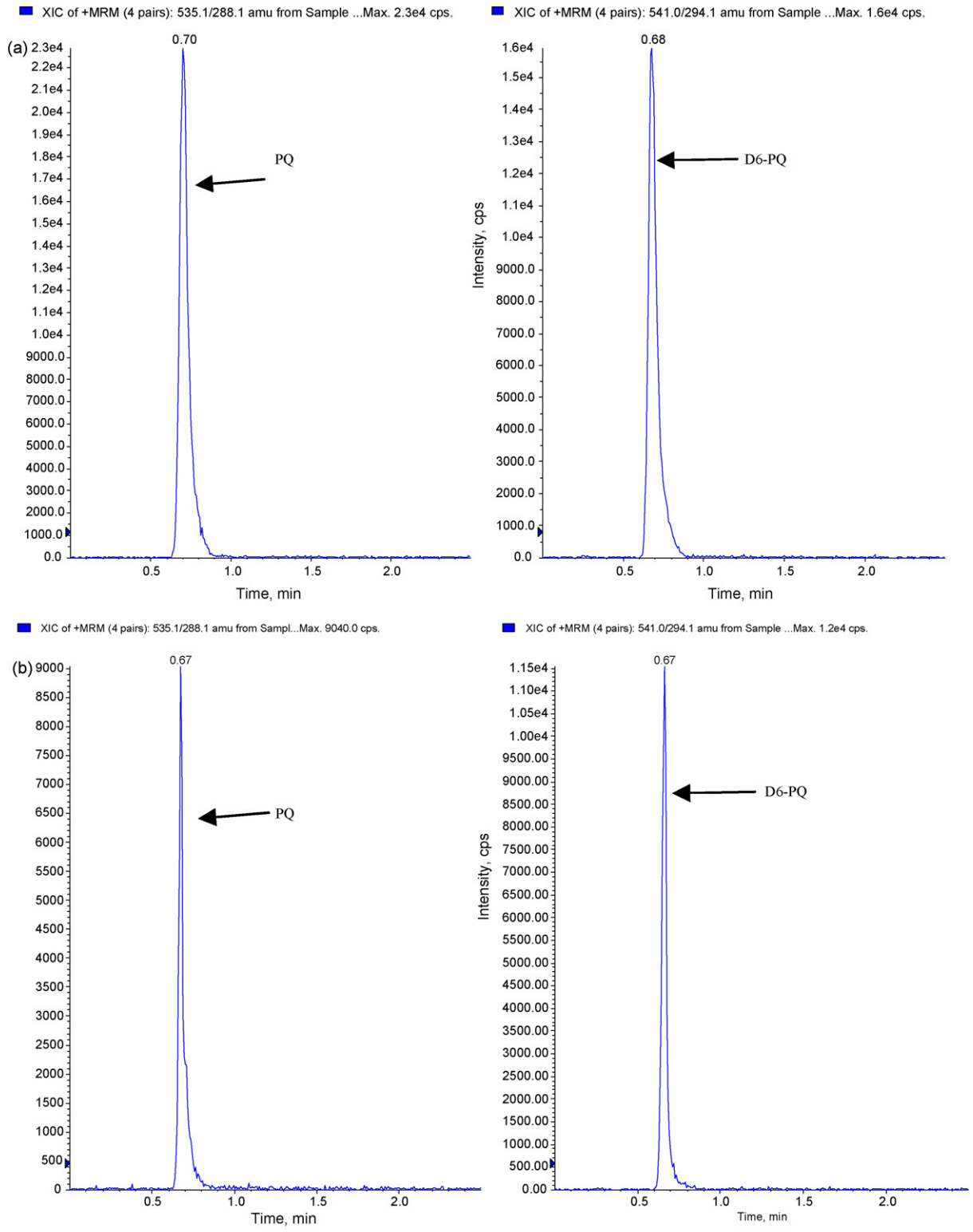


Fig. 6. Extracted spiked samples (nominal concentration 20 ng/mL PQ). Sample with little matrix effect, predicted concentration 18.8 ng/mL (a) and sample with severe matrix effect, predicted concentration 10.2 ng/mL (b).

tration 18.8 ng/mL, nominal 20.0 ng/mL) (Fig. 6a). It can be seen when comparing Fig. 6a with Fig. 4a that the tails of both the PQ and the internal standard peaks are suppressed, though this has no effect on the quantification. The second sample contains more triethylamine and the quantification of PQ is severely affected despite the use of a stable isotope labeled internal standard (predicted concentration 10.2 ng/mL, nominal 20.0 ng/mL) (Fig. 6b).

Our hypothesis that triethylamine could cause ion suppression which could severely affect quantification was further

confirmed by evaluation of extracted blank plasma and QC2 samples (20 ng/mL PQ) to which elution solution was added prior to reconstitution. The first sample to which 10 μL elution solution (representing 0.2 μL triethylamine) had been added prior reconstitution could still be accurately quantified (predicted concentration 20.4 ng/mL, nominal 20.0 ng/mL). Once again only the tail of the PQ and the internal standard peak was suppressed without effect on the quantification. The second sample to which 30 μL elution solution (representing 0.6 μL triethylamine) had been added prior reconstitution could not any longer be accurately quantified (predicted concentration 12.0 ng/mL, nominal 20.0 ng/mL).

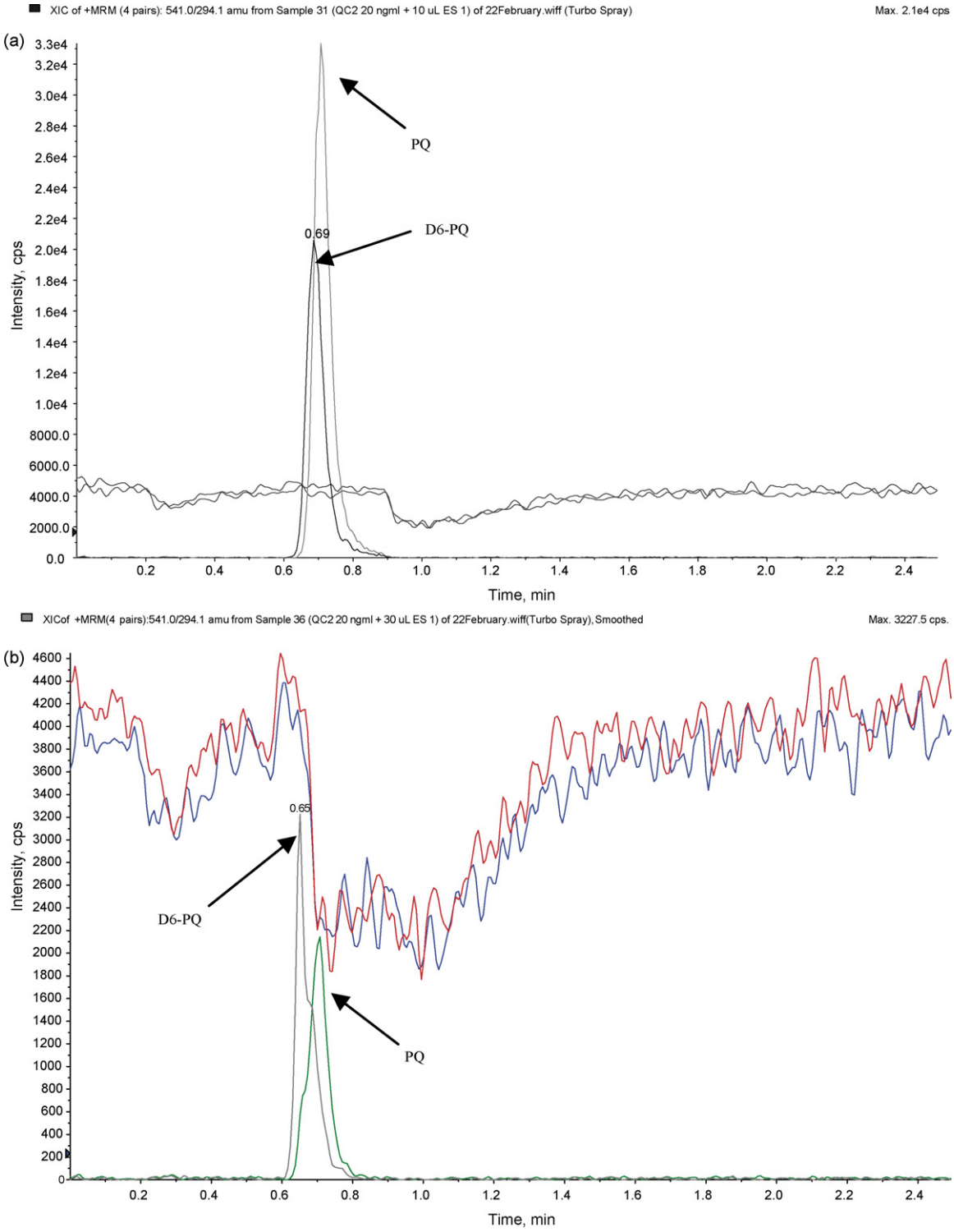


Fig. 7. Injection of extracted blank human plasma (+0.2 μL triethylamine) with overlay of QC2 sample (+0.2 μL triethylamine) (a) and extracted blank human plasma (+0.6 μL triethylamine) with overlay of QC2 sample (+0.6 μL triethylamine) (b) during post-column infusion 10 μL/min of piperaquine and internal standard 1.2 ng/mL.

Table 5
Matrix effects

PQ	Blank	Blank (0.025% TEA)	Blank (0.05% TEA)
QC1 4.5 ng/mL	63.8	64.6	17.2
QC3 400 ng/mL	71.0	66.7	19.5
IS 10 ng/mL at PQ QC1 concentration	65.9	68.4	25.1
IS 10 ng/mL at PQ QC3 concentration	68.6	68.0	30.4
Ratio QC1/IS	0.96	0.94	0.69
Ratio QC3/IS	1.03	0.98	0.64

Recovery of PQ and D6-PQ in extracted blank human plasma with and without TEA vs. injection solution.

Post-column infusion and injection of extracted blank samples to which 10 μL elution solution and 30 μL elution solution had been added prior reconstitution confirmed a region with severe ion suppression eluting directly after PQ (Fig. 7a and b). When the amount of triethylamine in the sample is low (Fig. 7a) the region of ion suppression co-elutes with the tails of the PQ and D6-PQ peak. When the amount of triethylamine in the sample is higher (Fig. 7b) the region of ion suppression co-elutes at or around peak maxima of the analytes. The deuterated internal standard D6-PQ is less lipophilic than the parent compound PQ thus eluting slightly earlier in the chromatogram. As a result, PQ eluted closer to the peak of ion suppression than D6-PQ. The amount of ion suppression at the peak maxima of the analytes decreased by as much as 50% from PQ to D6-PQ leading to lower analyte-to-IS ratio and incorrect quantification. The suppression of PQ and D6-PQ as a function of triethylamine in the samples is shown in Table 5.

4. Conclusion

A high throughput LC-MS/MS method for the determination of PQ in plasma has been developed and validated. The assay has been proven sensitive and reproducible and uses only 50 μL of plasma. The total analysis time for one batch (96 samples) is only around 6 h, easily enabling analysis of 192–288 samples a day. The assay will be a very valuable tool for analyzing samples from detailed pharmacokinetic studies in children. It was shown that the SIL internal standard compensated for some of the matrix effects resulting from the sample clean-up procedure i.e. remaining salt residues. The SIL internal standard failed to compensate if triethylamine was present in the sample and could lead to underestimation of the true concentration with 50%. The latter matrix effect is eliminated if care is taken to make sure that the eluates are completely dry before reconstitution.

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