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Rapid quantification of quinine and its major metabolite (3S)-3-hydroxyquinine in diluted urine by UPLC–MS/MS

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ABSTRACT

A rapid UPLC–MS/MS quantitative assay for the quantification of quinine and (3S)-3-hydroxyquinine requiring minimal sample pre-treatment – dilute-and-shoot type approach – has been developed. The assay was run at 0.6 mL/min using gradient elution with (pH 10; 10 mM) ammonium bicarbonate and methanol with a total cycle time of 2.5 min on a 50 mm × 2.1 mm ID, 1.7 μm Acquity BEH column. Peak shapes were highly symmetrical allowing for accurate peak integration. Calibration curves for both analytes were constructed from 1.00 to 20.00 ng/mL, yielding R^2 values >0.995. Intra- and inter-batch assay precision and accuracy were evaluated using 6 injections of QC solutions on 3 separate days ($n = 18$) and were found to be within ±10% and 90–110% respectively. The method was shown to be suitable for quantitatively determining the ratio of quinine to (3S)-3-hydroxyquinine for a cohort of samples from an epidemiological study.

1. Introduction

Quinine can be mostly associated with the treatment of *Plasmodium falciparum* induced malaria and has been applied as such an agent since the 17th century. The human hepatic metabolism of quinine has been well studied and is known to be specifically due to the family of Cytochrome P450 (CYP) 3A enzymes [1–3]. CYP3A4 enzyme is the most abundant and important drug metabolising enzyme, thought to be responsible for the metabolism more than 50% of the most commonly prescribed medicines. It is thought that many of the profound adverse reactions and poor therapeutic responses to xenobiotics can be ascribed to atypical CYP3A4 activity [4]. The primary metabolite of quinine is (3S)-3-hydroxyquinine, which is the product of CYP3A4 enzyme monooxygenases activity. The ratio of (3S)-3-hydroxyquinine to quinine is of interest as this can be used to measure CYP3A4 activity phenotype across a sample population by simple oral administration of the drug and subsequent collections of urine and/or plasma.

The current methodology used to quantify quinine and (3S)-3-hydroxyquinine is performed by GC–MS [5], HPLC-fluorescence [6,7] and more recently a method using HPLC–UV [8]. The newer UPLC–MS/MS technique represents higher throughput, faster, and more accurate alternative. An earlier publication on the use of liquid chromatography interfaced with thermospray mass spectrometry [9] indicated the difficulty in obtaining suitable peak shapes for cinchona alkaloids in the context of drug development. McCalley et al. studied the chromatographic analysis of cinchona alkaloids, the family of compounds with which quinine is associated [10]. Quinine and several of its related metabolites possess two basic functional groups, hence it is of chromatographic importance to minimise interactions with acidic underivatised silanol groups associated with peak tailing. This can be achieved by running with an acidic mobile phase (~pH 2.0) thereby suppressing free silanol ionisation or by reducing the extent of the basic group protonation by analysis in highly basic media. Interactions between amino groups and underivatised silanols can be negated using ion-pairing reagents, however this is usually avoided when interfacing such separations with mass spectrometry due to ion suppression. The introduction of pH stable bridged-ethylene hybrid stationary phase materials enables analysis at high pH [11]. This has been evaluated by several groups [12–14] who noted improved sensitivity when compared to classical formic acid based mobile phases and dubbed "wrong-way-round ionisation" [15].

Since the methodology reported here is intended for the analysis of samples inherently concentrated with quinine and (3S)-3-

Abbreviations: UPLC, ultra performance liquid chromatography; MS, mass spectrometry; GC–MS, gas chromatography–mass spectrometry.

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hydroxyquinine, the sensitivity of the mass spectrometer can be taken advantage of for their quantification in heavily diluted urine by UPLC–MS/MS without the requirement for extensive sample pre-treatment such as solid-phase or liquid–liquid extraction methodologies. Although there are other known phase I and phase II metabolites of quinine the purpose of this study was to preserve and quantify only the primary drug and it's major metabolite (3S)-3-hydroxyquinine. With this in mind the simplest sample treatment was pursued so as not to liberate any O-conjugated metabolites.

2. Experimental

2.1. Chemicals

Quinine free base (98% Pure) and quinine-D_3 (chemical purity 97%, isotopic purity 99%) were purchased from Toronto Research Chemicals Inc. The single diastereoisomer (3S)-3-hydroxyquinine (determined in-house to be 98% pure at King's College London by UPLC–UV) metabolite was not commercially available, however it was kindly donated by Prof. James M. Cook of the University of Wisconsin, Milwaukee, USA via his published synthetic route [16]. HPLC gradient grade methanol, acetonitrile and trifluoroacetic acid (TFA) were purchased from Fisher Scientific Ltd. (Loughborough, UK). Isopropyl alcohol (IPA), ammonium bicarbonate, ammonium hydroxide and formic acid (all LC–MS grade) were purchased from Sigma–Aldrich (Dorset, UK). High purity water (18.2 MΩ) was provided in-house using a Milli-Q purification system (Millipore, UK). All buffer solutions were vacuum filtered through a 0.22 μm membrane filter (Sigma–Aldrich).

2.2. Standard preparation

Preparations of stock and QC quinine and (3S)-3-hydroxyquinine standards were prepared in methanol at a concentration of 0.1 mg/mL in methanol. A stock solution of the internal standard quinine-D_3 was prepared at a concentration of 0.1 mg/mL in methanol and further diluted to a working concentration of 250 ng/mL. The working stock standard was diluted to 100 ng/mL inclusive of 250 ng/mL of the internal standard (quinine-D_3) and was further diluted to yield the following calibration standards on each day of analysis: 0, 1, 2, 5, 10 and 20 ng/mL, all inclusive of 5 ng/mL internal standard. Working QC solutions were prepared once from stock QC and diluted to concentrations of 1, 5 and 20 ng/mL, inclusive of 5 ng/mL internal standard, and used fresh for each analysis. All working standards were prepared in methanol–water (50:50, v/v). All solutions were stored at −20 °C and used within two months of preparation with no observed deviation from QC concentrations during this time period.

2.3. Sample treatment

Fasting urine samples were collected from a total of 315 monozygotic and dizygotic twins from the TwinsUK adult registry (www.twinsUK.ac.uk) between 14 and 16 h after administration of a 300 mg dose of quinine sulphate. Dilution of the samples was carried out using the following scheme: 50 μL of urine was added to 50 μL of internal standard followed by the addition of 900 μL methanol–water (50:50, v/v). Samples were then vortexed and centrifuged for 10 min at 9000 rpm and finally diluted by adding 50 μL of the supernatant to 950 μL methanol–water (50:50, v/v) ready for LC–MS analysis. Aliquots were collected and stored at −40 °C until analysed.

2.4. Chromatographic conditions

Liquid chromatography was carried out on a Waters Acquity Ultra Performance Liquid Chromatography system using a 50 mm × 2.1 mm ID column packed with Acquity BEH C_{18} 1.7 μm particles (Waters Corporation, Milford, MA). The column was thermostated at 40 °C and operated at a flow rate of 0.6 mL/min with a gradient program. During method development solutions of (pH 4.0; 10 mM) and (pH 8.0; 10 mM) ammonium bicarbonate were prepared by adjusting with formic acid. For the final method, mobile phase A consisted of (pH 10.0; 10 mM) ammonium bicarbonate adjusted with ammonium hydroxide solution. Mobile phase B was methanol. The gradient program was as follows: linear increase from 50% B to 100% B over 1 min then held for 0.2 min and re-equilibrated for 1.3 min at 50% B before the next injection. Full loop injection mode was employed using a 20 μL sample loop. Two separate [needle wash] programs were employed, a weak needle wash which was methanol–water (50:50, v/v) and a strong needle wash which was a mixture of isopropyl alcohol–acetonitrile–water–trifluoroacetic acid (60:30:10:0.1, v/v/v/v).

2.5. Mass spectrometry

The liquid chromatograph was interfaced to a Quattro Premier XE tandem mass spectrometer (Micromass, Manchester, UK). All analyses were performed in positive electrospray ionisation mode. Optimisation of multi-reaction monitoring (MRM) transitions for quinine and (3S)-3-hydroxyquinine was carried out by infusion of 1 μg/mL methanolic solutions in order to obtain the most intense fragment ions. Conditions for LC–MS/MS analyses were optimised manually by tee-union infusion against the mobile phase flow and using a solvent composition at which the analytes eluted from the chromatographic column. Nitrogen desolvation gas was set at 900 L/h and the cone gas at 50 L/h. The capillary voltage was set at 0.8 kV, the cone voltage for quinine and (3S)-3-hydroxyquinine was set at 40 eV. Source and desolvation temperatures were maintained at 120 °C and 375 °C respectively. Collision energies (CE) were optimised for multiple reaction monitoring (MRM) for quinine 325 > 160 (30 eV) and (3S)-3-hydroxyquinine 341 > 160 (35 eV), the acquisition mass window was set to 0.1 a.m.u. and the collision gas (Argon) was maintained at 3.5×10^{-3} mbar. The dwell time was set at 5 ms, and inter-channel and inter-scan delays were set at 10 ms.

3. Results and discussion

3.1. LC–MS/MS method development

Initially, several pH conditions were evaluated in order to discriminate which pH would yield the optimum chromatographic peak shape as seen in Fig. 1. Due to the ion suppression of the basic quinuclidine center with mobile phases of higher pH, quinine becomes more hydrophobic and as such its retention increases. Furthermore, peak asymmetry improves allowing for accurate integration which is in turn beneficial to assay accuracy and precision.

The use of buffered pH manipulation for the separation of charged analytes is important for several reasons. Firstly, elution of analytes of interest away from problematic matrix components in neat or partially diluted biological fluids may be achieved by mobile phase pH manipulation, important with the use of electrospray ionisation [13,17]. Secondly, fine tuning of analyte resolution can be achieved by simply altering the pH of the mobile phase. As an example, the in-house synthesis of 3-hydroxyquinine yielded the (3S) and (3R) diastereoisomeric products, the separation of which

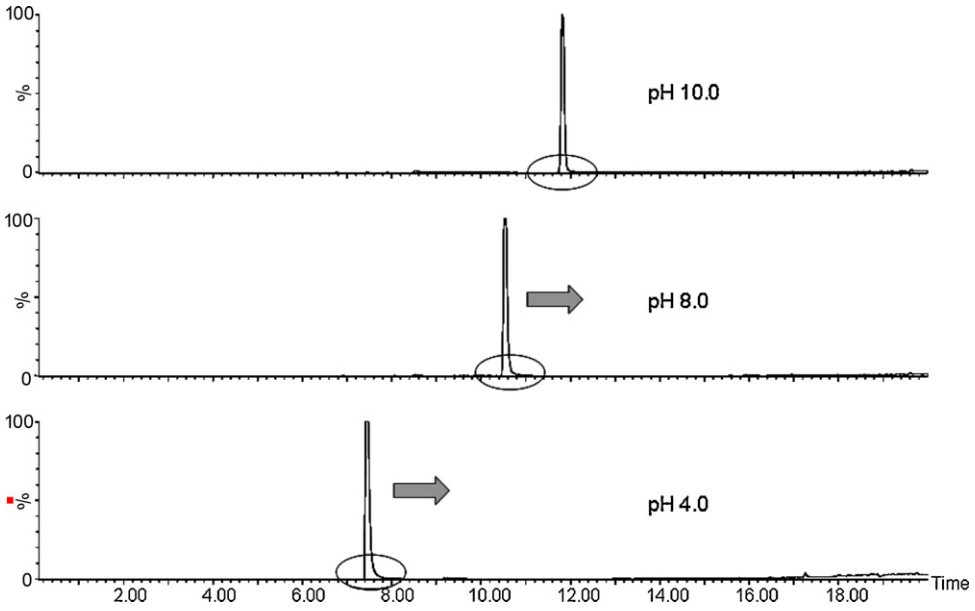

Fig. 1. Improvement in shape and increase in retention time as a function of pH for quinine. Chromatographic conditions: 20 min linear gradient from 0 to 100% methanol operated at 400 μL/min with a temperature of 60 °C using a 1.7 μm BEH C18 100 mm × 2.1 mm ID column.

could be affected by simple alteration of the mobile phase pH is shown in Fig. 2.

Due to the high flow rate and fast gradient applied to this analysis, optimal source conditions were required of 900 L/h and 50 L/h for desolvation and source gas respectively in order to assist electrospray. The capillary voltage was optimally determined to be 0.8 kV by manual adjustment, and the signal intensity was found to deteriorate at higher applied voltages (data not shown). Similarly, cone voltages were determined by infusion against the mobile phase flow rate used and at the solvent composition under which the analytes eluted. Source and desolvation temperatures were optimised and maintained at 120 °C and 375 °C respectively in order to compensate for the higher flow rates used.

Product ion spectra for quinine $[M+H]^+$ and (3S)-3-hydroxyquinine $[M+H]^+$ are shown in Fig. 3. Collision energies for quinine and (3S)-3-hydroxyquinine were optimised empirically by infusion until the optimal fragmentation was determined. The most intense fragment ion was chosen by which to perform quantification and was determined to be common for both analytes at 160 *m*/*z*.

One of the challenging aspects of developing this method was to eliminate analyte carryover. Due to the nature of the quinine in its hydrophobic state and the inherent sensitivity of the tandem mass spectrometer, significant carryover effects were observed in blank injections during method development. This can be analyte specific, i.e. the physicochemical nature of the analyte may imply an affinity for the chromatographic hardware. For instance, amines are known to chelate to metals. Weak and strong needle washes were implemented in the method however carryover could not be avoided. By changing the sample needle to a Teflon coated type, the effect was minimised and then further eliminated by introducing of isopropyl alcohol–acetonitrile–water–trifluoroacetic acid

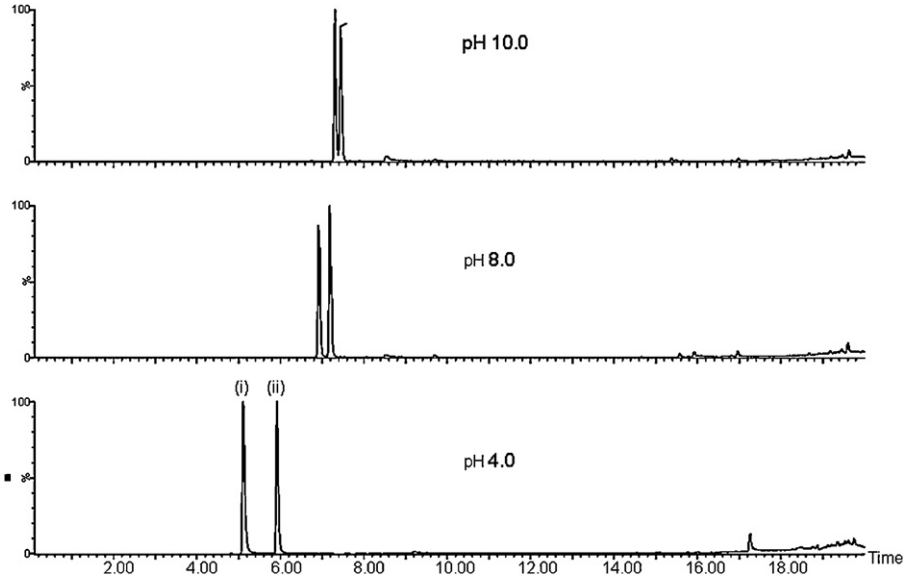

Fig. 2. Resolution and retention of and (3R)-3-hydroxyquinine (i) and (3S)-3-hydroxyquinine (ii) diastereoisomers as a function of pH. Conditions as in Fig. 1.

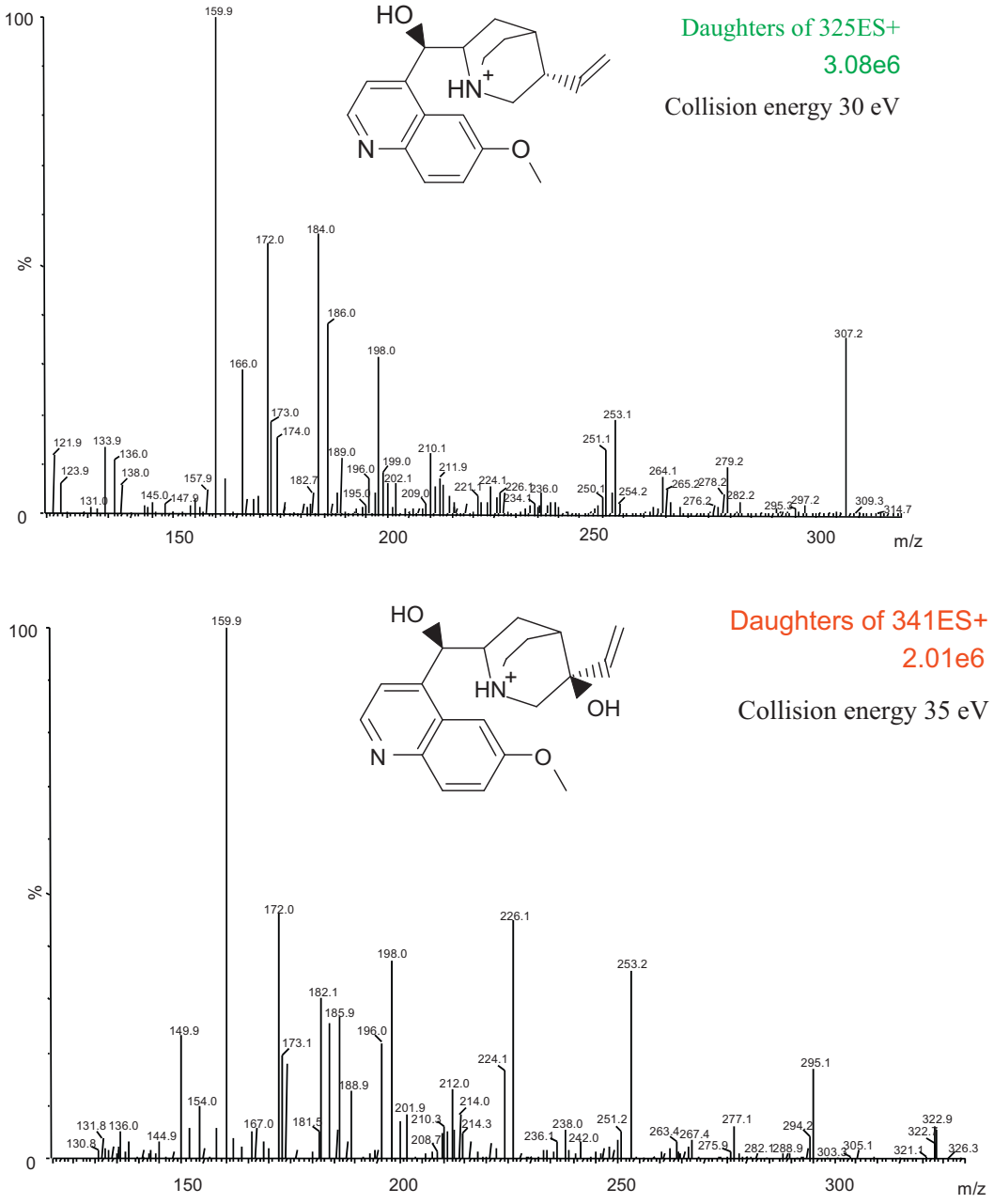

Fig. 3. ESI positive product ion spectra of quinine (top) and (3S)-3-hydroxyquinine (bottom).

(60:30:10:0.1, v/v/v/v) as the strong needle wash solution. An excellent treatment of carryover prevention for the analysis of peptides and proteins by nano LC–MS was outlined by Mitulovic et al. [18]. A possible explanation for sample carryover was that an ion-pairing mechanism, with TFA present in the strong needle wash altered quinine's strong affinity for surfaces within the fluidics of the liquid chromatograph thereby dramatically reducing carryover. It should also be pointed out that the initial gradient conditions can cause solubility issues between the sample plug and the mobile phase, resulting in smearing of the rotor stator upon turning from load to inject when it becomes in-line with the flowing eluent.

The approach of using a short column under fast gradient conditions has been demonstrated by other workers for the high throughput analyses of biological samples [19,20]. In the method reported here, short 50 mm × 2.1 mm ID columns packed with sub-2 μm materials gave the appropriate resolution and chromatographic performance required for rapid quantification using triple quadrupole mass spectrometry. Fig. 4 shows an LC–MS chromatogram from the final separation and ESI conditions from the MRM transitions 341 > 160 for (3S)-3-hydroxyquinine and 325 > 160 for quinine.

3.2. Performance of assay

The method was evaluated in terms of accuracy, precision, linearity (Table 1) and specificity for both analytes. Tables 2 and 3 show the precision and accuracy for quinine and (3S)-3-hydroxyquinine respectively from QC samples analysed at three concentration levels over three different days. No extraction was deemed necessary due to the elevated concentrations of quinine and (3S)-3-hydroxyquinine present in the samples. This allows for

Table 1
Linearity of quinine and (3S)-3-hydroxyquinine from 0 to 20 ng/mL performed over 3 days.

Analyte	Calibration equation[a]	r^2[b]	%RSD[b]
Quinine	$y = 0.738(0.0160)x - 0.0415(0.00478)$	0.9980	(±0.178)
3(S)-3-hydroxyquinine	$y = 0.187(0.00861)x - 0.0142(0.00441)$	0.9977	(±0.137)

[a] $n = 3$, data are mean (SD).
[b] $n = 3$, data are mean (%RSD).

Table 2
Accuracy and precision for quinine ($n = 3$ day, six replicate injections per day).

Theoretical conc. of quinine (ng/mL)	Intra batch			Inter batch
	Day 1	Day 2	Day 3	
1.0 (LLOQ)				
%CV (6 injections)	7.20	4.29	8.35	6.61
%Accuracy	99.50%	106.57%	96.93%	101.00%
5.0				
%CV (6 injections)	4.31	9.90	2.83	5.68
%Accuracy	102.49%	103.31%	97.43%	101.08%
20 (ULOQ)				
%CV (6 injections)	2.95	4.74	3.89	3.86
%Accuracy	102.01%	103.23%	100.65%	101.97%

matrix effects to be heavily diluted out in the devised dilution scheme removing the necessity for solid-phase or liquid–liquid extraction. Calibration curves were constructed based on the respective peak area ratios of quinine and (3S)-3-hydroxyquinine to quinine-D_3 versus their known concentrations providing a 1/concentration linear regression.

Calibration curves were constructed from 0 to 20 ng/mL for quinine and (3S)-3-hydroxyquinine in order to assess the reproducibility and accuracy of the LC–MS method for quantitative purposes. Pooled QC solutions were prepared separately at 1 (LLOQ), 5 and 20 (ULOQ) ng/mL and assessed for deviation from the daily preparations of calibration curve performed over 3 days with 6 injections of each control injected per day. All solutions contained 5 ng/mL quinine-D_3 internal standard. Intra and inter batch accuracy and precision were found to be ≤10% for both quinine and (3S)-3-hydroxyquinine, and the data is summarised in Tables 2 and 3 respectively. Linearity coefficients (r^2) were found to be ≥0.995 over the order of concentration required, as shown in Table 1. Also illustrated in Table 1 are mean intercept and slope data determined from 3 separate preparations of the calibration curve, notably intercepts for both analytes were very close to zero indicating carry was indeed eliminated. Fig. 4(e) and (f) shows a blank injection determined after the highest calibrant. All quoted LOQ data relate to diluted urinary sample concentrations. The method presented here provides rapid quantification of quinine and it's major metabolite (3S)-3-hydroxyquinine accomplishing a sample throughput of 25 per hour. This is a remarkable improvement to previously reported methodologies, which have run times of 22 min [6] and 20 min [8] respectively, affording measurements from a large cohort of samples to be realised in a short time frame.

Table 4
Retention time robustness metrics based on QC injections.

Analyte	Retention time (min)[a]	%RSD
Quinine	0.835	(±0.254)
(3S)-3-hydroxyquinine	0.487	(±0.219)
Relative retention[b]	1.715	(±0.289)

[a] $n = 58$, data are mean (RSD).
[b] $n = 58$, data are mean (RSD) as (3S)-3-hydroxyquinine/quinine retention times.

Table 5
Summary of 10 volunteers post-administration diluted urinary concentrations.

	Quinine conc. (ng/mL)	(3S)-3-hydroxyquinine conc. (ng/mL)
Volunteer 1	3.0	3.4
Volunteer 2	6.6	8.3
Volunteer 3	6.3	6.6
Volunteer 4	3.1	3.1
Volunteer 5	4.4	3.8
Volunteer 6	4.3	15.1
Volunteer 7	6.3	11.9
Volunteer 8	2.4	5.1
Volunteer 9	7.7	10.7
Volunteer 10	3.9	4.7

Chromatographic robustness was achieved throughout the course of running this method for a large number of samples from a phenotyping study. Table 4 highlights the retention time precision of each analyte determined from QC injections, the relative retention times are also shown as ratios of quinine/(3S)-3-hydroxyquinine. Reproducibility was found to be <±0.5% RSD based on 58 injections for both retention time and relative retention of the analytes. Using a mobile phase buffer at pH 10.0 and a slightly elevated column temperature of 40 °C, approximately 1700 injections have been made on the same column without any appreciable shift in retention time or deterioration in peak shape.

The method described here can be utilised to quantitatively assess the transformation of quinine to (3S)-3-hydroxyquinine as a specific marker for CYP 3A4 enzyme activity on a large sample size epidemiological study. A summary of diluted urinary concentrations determined from post-administration of quinine in volunteers is shown in Table 5.

Table 3
Accuracy and precision for (3S)-3-hydroxyquinine (n=3 day, six replicate injections per day).

Theoretical conc. of (3S)-3-hydroxyquinine (ng/mL)	Intra batch			Inter batch
	Day 1	Day 2	Day 3	
1.0 (LLOQ)				
%CV (6 injections)	6.50	8.57	5.14	6.74
%Accuracy	100.17%	98.68%	93.60%	97.48%
5.0				
%CV (6 injections)	7.09	6.92	6.08	6.70
%Accuracy	98.55%	98.17%	97.75%	98.16%
20.0 (ULOQ)				
%CV (6 injections)	2.95	5.72	4.03	4.23
%Accuracy	101.79%	95.80%	101.54%	99.71%

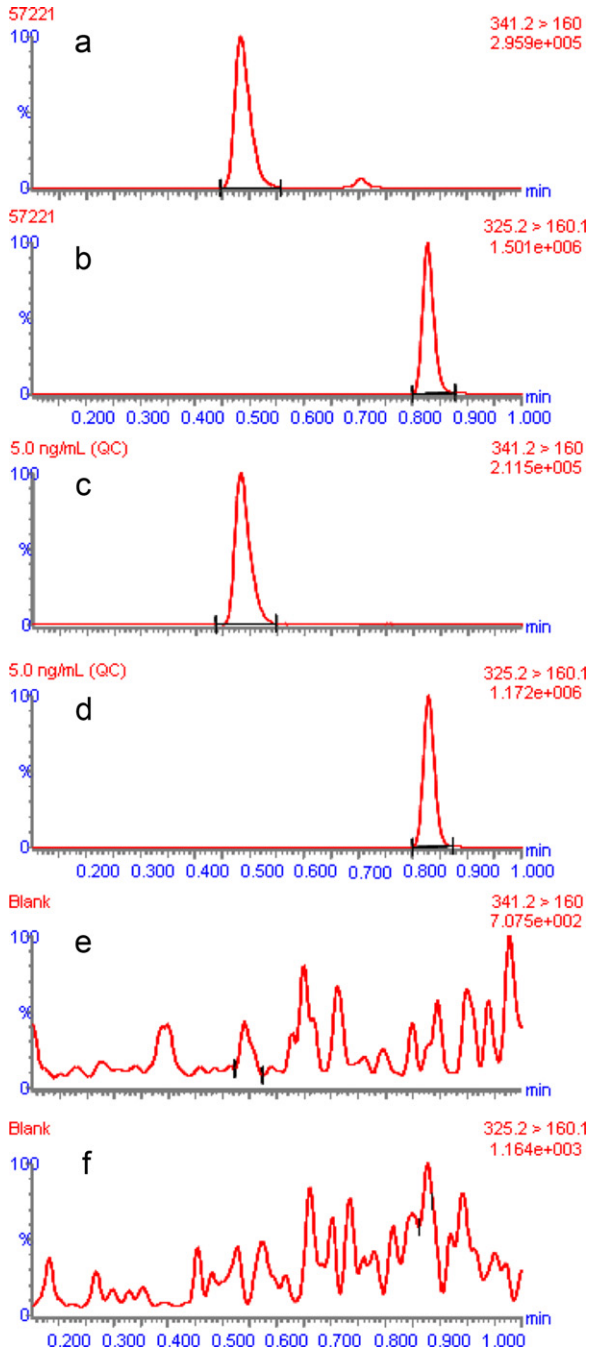

Fig. 4. Final UPLC–MS/MS separation in: (a) volunteer quinine, (b) volunteer (3S)-3-hydroxyquinine, (c) 5 ng/mL QC quinine, (d) 5 ng/mL (3S)-3-hydroxyquinine, (e) blank quinine trace and (f) blank (3S)-3-hydroxyquinine trace.

4. Conclusion

A rapid and simple method was developed for the analysis of urine samples collected post-dose from the administration of the drug quinine. Quantification of quinine and (3S)-3-hydroxyquinine could be realised using a mobile phase at pH 10.0, resulting in highly symmetrical peak shapes. Heavy dilution of urine samples removed the necessity for sample clean-up or concentration by either liquid–liquid or solid-phase extraction. Accuracy, precision and linearity were all found to be acceptable.

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