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Simultaneous determination of OZ277, a synthetic 1,2,4-trioxolane antimalarial, and its polar metabolites in rat plasma using hydrophilic interaction chromatography

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ABSTRACT

OZ277 is a synthetic 1,2,4-trioxolane antimalarial currently being evaluated in clinical trails. Biotransformation of OZ277 in rats results in the generation of metabolites with large differences in polarity which complicates the development of a method for the simultaneous analysis of all species. A simple, sensitive and selective hydrophilic interaction liquid chromatography–mass spectroscopy (HILIC/MS) method for simultaneous determination of OZ277 and its major metabolites in rat plasma was developed and validated. The method involves protein precipitation with acetonitrile followed by separation on a Waters Atlantis™ HILIC Silica column using gradient elution. The analytes were monitored using a positive electrospray ionization interface in selected ion monitoring mode. The calibration range for all of the analytes was 5–10,000 ng/mL and the lower limit of quantitation was 5 ng/mL using a 50 μL rat plasma sample. The inter- and intra-day accuracy and precision was within 12%. The recovery of all analytes from rat plasma over a wide concentration range was 90% or greater. The method has been successfully used for quantifying OZ277 and its metabolites in plasma following intravenous administration to rats.

1. Introduction

Artemisinin and its semi-synthetic analogues are extensively utilised for the treatment of multi-drug resistant malaria [1]. Despite their proven clinical efficacy, their therapeutic utility is hampered by their poor biopharmaceutical properties and cost due to their semi-synthetic nature [2]. In addition, there is an increasing concern for the potential development of resistance to these agents [3,4]. These factors have stimulated interest in the discovery of novel and fully synthetic peroxide antimalarials, with the dispiro-1,2,4-trioxolane, OZ277 (also known as RBx11160, Fig. 1) being the first such agent to be tested in human clinical trials [2].

Previous research conducted with OZ277 and other structurally related 1,2,4-trioxolanes has shown that these agents are susceptible to Fe(II)-mediated degradation resulting in the formation of an adamantane lactone and the corresponding cyclohexanone [5] (NP15, Fig. 2). This reaction occurs due to the presence of the inherently reactive peroxide bond which is required for antimalarial activity. *In vivo*, the cyclohexanone can be further reduced to the corresponding alcohol (NP22, Fig. 2) by reductases expressed in blood and other vital organs. A recent report on the potential metabolites of OZ277 generated from studies in human liver microsomes also identified the potential for the formation of three hydroxylated metabolites, two of which occurred on the adamantane ring [6] (OZ381 and OZ397, Fig. 1) and one on the cyclohexyl ring.

Pharmacokinetic (PK) and metabolism studies in preclinical animal models are a critical part of the drug discovery and development process and rely upon the availability of suitable analytical methods for the quantification of drug and potential metabolites in blood, plasma and other relevant biological matrices. The purpose of the current investigation was to develop and validate an analytical method for the quantitation of OZ277 and its expected metabolites and *in vivo* degradation products in rat plasma.

In preliminary studies, a single set of conditions suitable for all the analytes could not be identified using typical reversed phase columns (C8 and C18) due to the large differences in polarity between these species. Recently, several new columns have become available for the analysis of small polar compounds, and one such column utilises hydrophilic interaction chromatography (HILIC) as the separation modality [7–12]. This separation method, which was first described by Alpert [13], has been in use for many years for the separation of sugars [14], carbohydrates [15,16], peptides [17,18] and natural product extracts [19]. Many different polar stationary phases can be utilised in HILIC including underivatised silica, aminopropyl, amide, poly(succinimide) and other bonded silicas,

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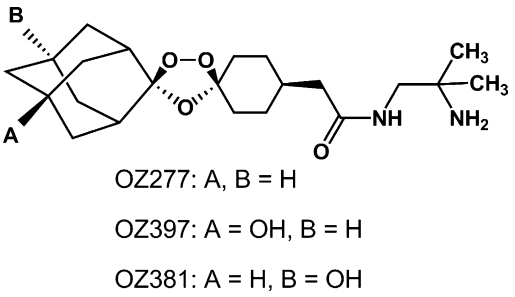

Fig. 1. Structures of OZ277 and its adamantane hydroxylated metabolite(s).

and polymer-based supports [20]. Mobile phases typically comprise a high percentage of organic solvent and a small percentage of water. HILIC requires an appropriate percentage of water (~5–15%) to maintain a stagnant enriched water layer on the surface of the polar stationary phase into which the polar analytes partition. Separation is then achieved by eluting the analytes with a strong organic mobile phase. The higher organic content in HILIC mobile phases provides low column back pressures and enhanced MS sensitivity due to lowering of the droplet surface tension during the MS electrospray process [21].

The current report focuses on the development and validation of a simple, reliable and sensitive HILIC/MS method using simple protein precipitation for quantitative analysis of OZ277 and its expected metabolites and *in vivo* degradation products in rat plasma within a single analytical run. The method has been successfully applied to the evaluation of the PK characteristics of OZ277 and its metabolites in rats following intravenous (IV) administration.

2. Experimental

2.1. Reagents

The trioxolanes OZ277, OZ397 and OZ209 (Figs. 1 and 2) and authentic standards of NP15 and NP22 (Fig. 2) were synthesised [6] and generously provided by Prof. Jonathan Vennerstrom, University of Nebraska Medical Centre, Omaha. Isopropanol (IPA) and acetonitrile (ACN) were of HPLC grade and were obtained from Burdick and Jackson (Michigan, USA) and Merck (Darmstadt, Germany), respectively. Ammonium formate was purchased from Sigma–Aldrich (MO, USA) and formic acid was from Ajax Finechem (New South Wales, Australia).

2.2. Preparation of standard solutions

Primary stock solutions of OZ277, OZ397, NP15, NP22 and OZ209 (internal standard, IS) were prepared at a concentration of 1 mg/mL in ethanol. A working stock solution with a final concentration of 200 μg/mL of each analyte was prepared from the primary stock solutions and was diluted with ethanol to prepare calibration spiking solutions at concentrations of 0.05, 0.1, 0.5, 1, 5, 10, 50 and 100 μg/mL. The IS was prepared at a concentration of 0.2 μg/mL in ACN.

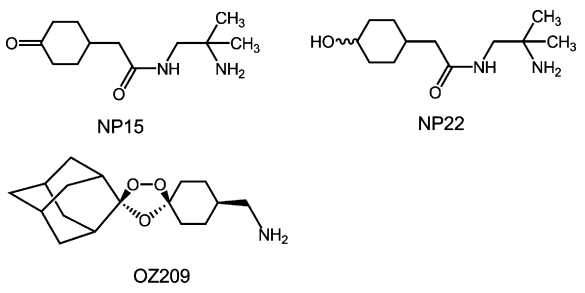

Fig. 2. Structures of NP15, NP22 and OZ209 [internal standard (IS)].

2.3. Preparation of calibration standards

Calibration standards were prepared fresh daily by spiking 50 μL aliquots of blank rat plasma with 5 μL of calibration spiking solution to produce calibration standards at concentrations of 5, 10, 50, 100, 500, 1000, 5000 and 10,000 ng/mL.

2.4. Sample processing

Rat plasma samples, calibration standards and the quality control (QC) samples were processed using a simple protein precipitation method. To each tube containing 50 μL of plasma, 300 μL of ACN containing the IS (0.2 μg/mL) was added. The contents of the tubes were vortex mixed for 1 min and centrifuged at 10,000 rpm for 3 min after which 200 μL of the supernatant was transferred into a clean HPLC autosampler vial. For each sample, a 10 μL aliquot was injected onto the HPLC column.

2.5. LC/MS instrument and conditions

Liquid chromatography was performed using a Waters 2795 Alliance™ HT separation module equipped with an autosampler and column oven. The HPLC system was attached to a Waters Micromass ZQ single quadrupole mass spectrometer with an electrospray ionization (ESI) interface. Data acquisition and processing were achieved using MassLynx (version 4.1) software. Chromatographic separation of OZ277 and its metabolites was achieved using a binary gradient method on a 2.1 mm × 50 mm, 5 μm Atlantis® HILIC Silica column (Waters Corp., Milford, MA, USA) fitted with a 2.1 mm × 10 mm, 5 μm Atlantis® HILIC Silica guard column. The column was maintained at 40 °C during analysis. Mobile phase A consisted of a mixture of 0.1 M ammonium formate (adjusted to a pH 3.0 with formic acid) and ACN in the ratio of 5:95 (v/v), while mobile phase B consisted of 0.1 M ammonium formate (adjusted to pH 3.0 with formic acid) and IPA in the ratio of 5:95 (v/v). The gradient program was as follows: 40–45% mobile phase B from 0 to 5 min, 45–80% mobile phase B from 5 to 5.5 min, maintaining 80% mobile phase B until 6 min, 80–40% mobile phase B from 6 to 6.5 min and maintaining 40% mobile phase B until 9 min. The flow rate was maintained at 0.5 mL/min.

MS conditions for OZ277 and its metabolites were optimised using the built in syringe pump in the instrument by infusing individual working stock solutions at a flow rate of 10 μL/min. The following MS conditions were identified: desolvation gas (nitrogen) 450 L/h, cone gas (nitrogen) 100 L/h, source temperature 90 °C, desolvation temperature 350 °C, probe capillary voltage 3.2 kV, cone voltage +20 kV and a dwell time 0.1 s.

Preliminary method development studies showed similar MS response factors for OZ381 and OZ397. Since these hydroxylated metabolites co-eluted under the present chromatographic conditions, their total concentration (OZ381 + OZ397) was quantified using OZ397 as the analytical standard. Initial pharmacokinetic studies in rats indicated that the ratio of plasma concentrations for OZ397 to OZ381 was >5:1 suggesting that OZ397 was the more predominant metabolite (unpublished data).

Data acquisition was performed using the ESI interface in positive ion mode. The protonated molecular ions $[M+H]^+$ of OZ277 (m/z 393.2), OZ397/OZ381 (m/z 409.3), NP15 (m/z 227.1), NP22 (m/z 229.1) and the IS (m/z 294.4) were monitored using selected ion recording (SIR).

2.6. Method validation

2.6.1. Matrix effect

Qualitative assessment of potential matrix effects was initially conducted by post-column infusion of OZ277, its metabolites and the IS during the chromatographic analysis of blank rat plasma extracts as previously described [22]. A standard solution containing each of the analytes in ACN at 1 μg/mL was infused at a flow rate of 10 μL/min during the chromatographic analysis of six blank rat plasma extracts from six different animals. The SIR of each analyte was examined to qualitatively assess the potential affects of plasma components on the ionization. Quantitative estimation of potential matrix effects was also undertaken to calculate the absolute matrix effect (ME). Six different lots of blank rat plasma were precipitated with ACN as described above and then the supernatant spiked with OZ277 and its metabolites at concentrations of 20 and 10,000 ng/mL. The corresponding peak areas of each of the analytes in the spiked plasma supernatant (i.e. post-precipitation) were then expressed as a ratio of the peak areas for the solvent standards prepared in ACN/water (6:1) at equivalent concentrations. This ratio of ACN/water in the solvent standard closely mimics the ACN/aqueous ratio in the plasma supernatant following protein precipitation. The same evaluation was performed for the IS at a concentration of 200 ng/mL. A value of 100% indicates that the response of OZ277 and its metabolites in rat plasma was unaffected by the matrix components. A value of >100% indicates ionization enhancement and <100% indicates ion suppression. To assess the variability in the matrix effect, the relative standard deviation (RSD) of the ME was calculated by direct comparison of the peak areas of individual analytes between different lots of plasma.

2.6.2. Selectivity

The selectivity of the method for each of the analytes in the presence of potentially interfering endogenous matrix components was tested by screening six different lots of analyte free heparinised rat plasma collected from six rats.

2.6.3. Absolute recovery

The absolute recovery was determined by comparing the peak area obtained from plasma samples spiked with OZ277 and its metabolites prior to protein precipitation with those from plasma samples spiked following the precipitation procedure. Recovery was determined for three replicates of plasma spiked with OZ277 and its metabolites at low (50 ng/mL), medium (500 ng/mL) and high (2000 ng/mL) concentrations.

2.6.4. Precision and accuracy

The intra-day precision and accuracy of the method was assessed using QC samples at six concentration levels (5, 20, 50, 500, 2000 and 10,000 ng/mL). The inter-day precision and accuracy was determined over four days at three concentration levels (50, 500 and 2000 ng/mL). The accuracy of the intra- and inter-day data was calculated as the percent deviation of the measured concentration (determined using the calibration curve run on the same day) from the nominal concentration.

2.6.5. Calibration range and lower limit of quantitation (LLOQ)

Calibration standards for OZ277 and its metabolites were prepared over the concentration range of 5–10,000 ng/mL using the calibration spiking solutions. A standard curve was constructed from the peak area ratios (peak area of analyte/peak area of the IS) versus nominal concentrations by fitting to a quadratic equation using a weighted (1/concentration) regression. Calibration curves were prepared fresh and included in each analytical run.

The LLOQ was determined by evaluation of the signal to noise ratio (S/N) and the precision and accuracy with six replicates for each of six samples. The LLOQ was defined as the lowest calibration standard which had a S/N ≥ 10, precision of <20% and accuracy within the range of ±20%.

2.6.6. Stability

In order to determine the short-term bench top stability of OZ277 and its metabolites, two sets of triplicate rat plasma samples were spiked at low (50 ng/mL), medium (500 ng/mL) and high (2000 ng/mL) concentrations of each analyte. One set was processed immediately and a duplicate set was allowed to sit at room temperature (~25 °C) for 3 h, which was the maximum time required to process a representative set of samples. All the samples were analysed by comparison to a freshly prepared calibration curve.

Stability of processed samples during storage within the autosampler was assessed for OZ277 and its metabolites over a period of ~24 h. A calibration curve (5–10,000 ng/mL) and six replicates of the samples for each concentration (50, 500 and 2000 ng/mL) were prepared. The calibration standards were first run followed by injections of the replicate samples at 6 h intervals over a period of 24 h. The peak area ratios of each of the analytes obtained over a 24 h period were compared to determine their stability under the autosampler conditions.

The stability of OZ277 and its metabolites at −80 °C was investigated at 0, 9 and 15 days using spiked rat plasma samples at three different concentrations (50, 500 and 2000 ng/mL). Six replicates at each concentration were thawed and analysed initially and on days 9 and 15 by comparison to a freshly prepared calibration curve.

2.7. Pharmacokinetic study in rats

Three male Sprague–Dawley rats weighing 290–310 g were selected for this study. All animal work was undertaken in accordance with the guidelines of the Australian and New Zealand Council for the Care and Use of Animals in Research and the protocol was approved by the Institutional Animal Experimentation Ethics Committee.

OZ277 (maleate salt) was dissolved in 5% glucose solution and administered intravenously via an indwelling jugular vein cannula. Blood samples (220 μL) were collected via an indwelling carotid artery cannula prior to dosing, at the end of the OZ277 infusion (0.08 h) and at 0.25, 0.5, 1, 2, 3, 5, 8, 11, 15 and 24 h post-dosing. Plasma was separated by immediate centrifugation of the collected blood samples at 4 °C. The plasma fractions were transferred to polypropylene tubes, snap frozen and stored at −80 °C until analysis (within 7–10 days of sample collection). On the day of analysis, plasma samples were thawed and processed using the described procedure.

3. Results and discussion

3.1. Method development

The primary aim of these investigations was to develop a simple and robust chromatographic method for the simultaneous analysis of OZ277 and its metabolites and *in vivo* degradation products in rat plasma. Method development was initiated with the optimisation of the MS conditions. The next phase of method development included optimisation of the chromatographic conditions including the type of column and the mobile phase composition. Initial studies with conventional reversed phase columns (e.g. C8 and C18) revealed that OZ277 and OZ397 (and OZ381) were retained well on these packing materials, whereas the more polar analytes, NP15 and NP22, eluted in the void volume even with the use of a highly aqueous mobile phase (>98% aqueous). A cyanopropyl (CN)

bonded phase column was also evaluated for its ability to separate the polar analytes [23]. Using this packing material, OZ277 and its metabolites were well separated using a simple gradient method, however, a relatively low mobile phase pH was required for optimum peak shape. Unfortunately, the retention characteristics of the column deteriorated with time due to inherent stability issues of the packing material under the slightly acidic mobile phase conditions utilised [24].

During the early nineties, Alpert described a novel separation technique for polar solutes called HILIC [13], which employs low-aqueous/high organic mobile phases. Application of this separation technique to highly polar and ionic species has been widely reported in recent years [13,19,25–27], however this separation modality is not commonly used for the quantitation of both hydrophilic and hydrophobic solutes within the same sample. Under the current application, the hydrophilic analytes, NP15 and NP22, exhibited good retention characteristics on the HILIC column, and the more hydrophobic solutes, OZ277 and OZ397/OZ381, were also retained sufficiently to allow accurate quantitation.

Optimisation of the pH, mobile phase composition and flow rate were required to obtain optimum peak shapes for each of the analytes. An increase in the concentration of ammonium formate in the buffer resulted in sharpened peak shapes for each of the analytes with a concentration of 100 mM resulting in sharp peaks without compromising the MS sensitivity due to ion suppression. Use of an acidic pH (~3.0) resulted in good peak shapes with satisfactory retention times for all the analytes. A high initial percentage of the less polar mobile phase A (95%, v/v ACN in ammonium formate buffer) was required for optimum retention of OZ277 and OZ397/OZ381, whereas an increase in mobile phase polarity with time was required for the elution of NP15 and NP22. By employing 60% mobile phase A and 40% mobile phase B (95%, v/v IPA in ammonium formate buffer) as the initial conditions and increasing the concentration of phase B using a linear gradient, optimal separation of all the analytes was achieved within a short analytical run time (<10 min for the complete cycle).

The peak shapes of the late eluting peaks (NP15 and NP22) were also found to be sensitive to mobile phase flow rate. A flow rate of 0.5 mL/min was found to result in good separation of all peaks with optimum peak shapes for each of the analytes.

3.2. Optimisation of the sample preparation procedure

Two methods were evaluated for the separation of analytes from endogenous components present in rat plasma. Initial studies were conducted using liquid–liquid extraction (LLE) with either ethyl acetate, dichloromethane or methyl *t*-butyl ether, alone or in combination. Following LLE, the extraction efficiency of the more polar metabolites, NP15 and NP22, was found to be very poor. No significant increase was observed in their extraction efficiency by increasing the pH of the plasma samples to result in deprotonation of the amine functionality. These results indicated that a single LLE method was unlikely to efficiently extract these analytes from the plasma matrix.

Protein precipitation is considered to be one of the simplest methods for processing biological samples such as plasma. Numerous different precipitants can be utilised for this purpose, however ACN is often incorporated due to its extensive use as a component in HPLC mobile phases, and the fact that most analytes are stable in this solvent. At a volume ratio of 2:1 precipitant to plasma, ACN removes more than 92% of plasma proteins [28]. By increasing the volume ratio to 5:1 (ACN:plasma), the removal of plasma proteins is further increased and the peak shape was found to be good for each of the analytes on the HILIC column.

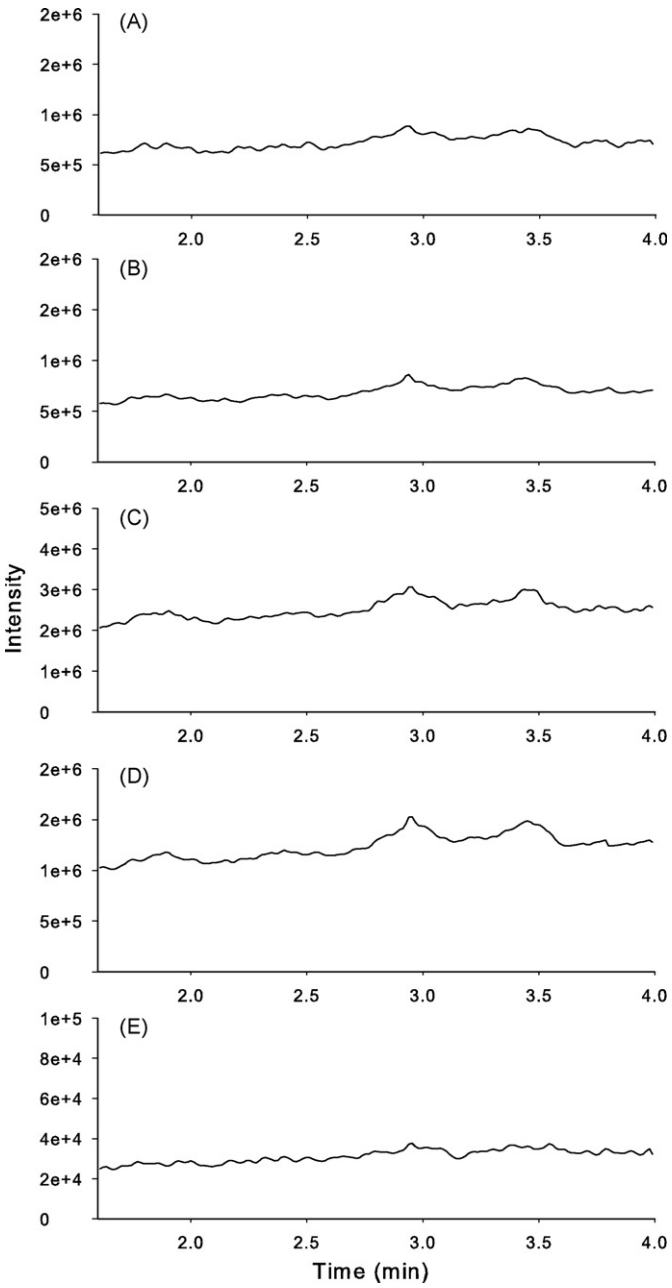

Fig. 3. Representative post-column analyte infusion LC/MS chromatograms (SIR) for [A] OZ277; [B] OZ397; [C] NP15; [D] NP22 and [E] IS obtained during the chromatographic analysis of precipitated blank rat plasma.

3.3. Method validation

3.3.1. Matrix effect

Matrix effects on the ionization of the analytes can occur due to co-eluting endogenous components of rat plasma. The results of the qualitative estimation of matrix effects using post-column infusion experiments (Fig. 3) showed the absence of any ion suppression or enhancement regions at the retention times of the analytes and the IS (Figs. 3 and 4).

Quantitative assessment of absolute matrix effects and the potential variability was assessed using six sources of rat plasma. The ME values were between approximately 87% and 119% for each of the analytes and the IS, indicating no major enhancement or suppression of the ionization (Table 1). The calculated RSD values for all

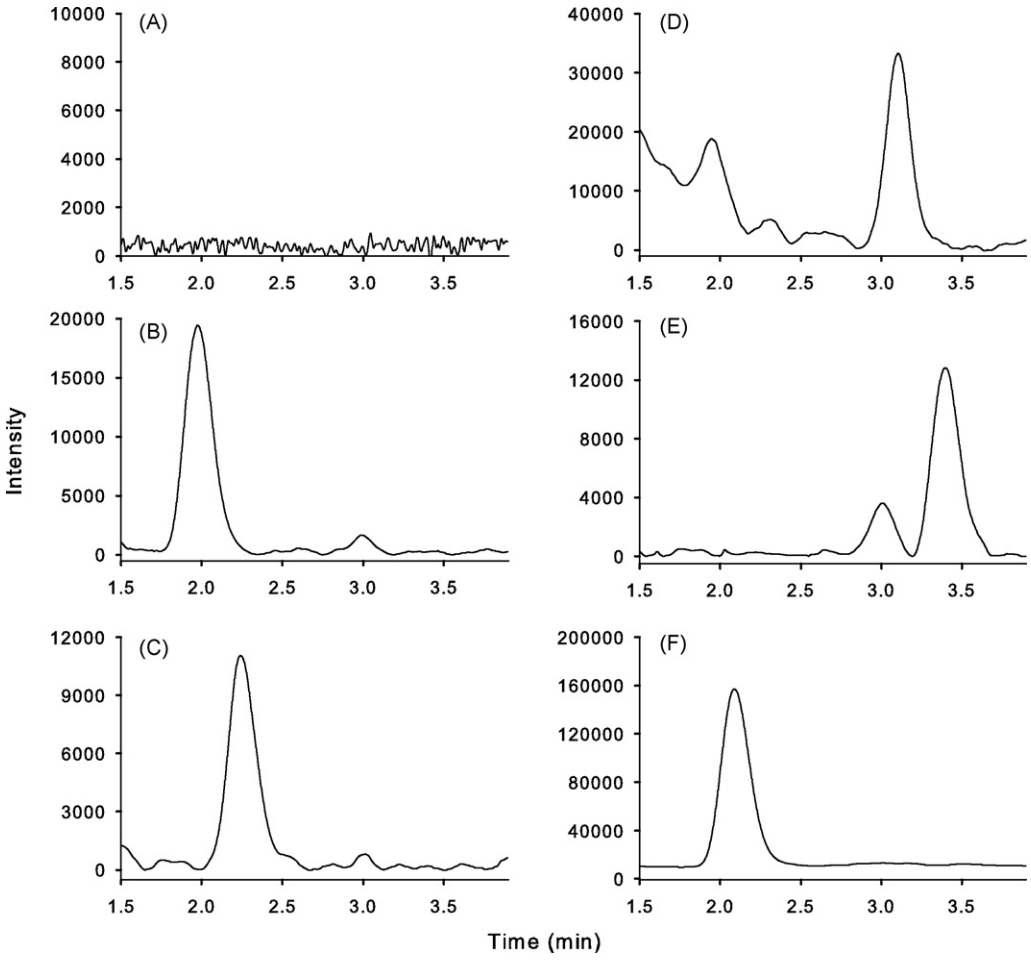

Fig. 4. Extracted SIR chromatograms of OZ277 and its metabolites spiked into rat plasma. (A) Blank rat plasma; (B) OZ277 (10 ng/mL, 1.97 min); (C) OZ397 (10 ng/mL, 2.25 min); (D) NP15 (10 ng/mL, 3.10 min); (E) NP22 (10 ng/mL, 3.40 min); and (F) OZ209 (200 ng/mL, 2.08 min). Note that additional peaks observed in (D) between ~1.8 and 2.5 min were due to insource fragmentation of OZ277 and OZ397. Also note that the peak observed in (E) at ~3.0 min is the C^{13} isotope of NP15, which has similar *m*/*z* as NP22.

the analytes across the different lots of rat plasma were also within the acceptable limits of variation (RSD values of <11%).

3.3.2. Selectivity

The selectivity of the method was evaluated by testing plasma collected from six rats for interfering matrix components, with each batch of plasma collected using heparin as the anti-coagulant. No interfering endogenous peaks were found for any of the different plasma lots. Representative chromatograms of processed blank rat plasma, and rat plasma spiked with OZ277 and its metabolites at 10 ng/mL are shown in Fig. 4.

3.3.3. Absolute recovery

The absolute recovery of OZ277 and its metabolites was evaluated by extracting rat plasma samples spiked at low, medium, and high concentrations. As shown in Table 1, average absolute

Table 1
Matrix effects (%) and absolute recovery (%) of OZ277 and its metabolites from rat plasma. Results represent the mean of $n = 6$ for matrix effect determinations and $n = 3$ for the recovery experiments.

Analyte	Matrix effects			Recovery		
	Nominal concentration (ng/mL)	Absolute matrix effect (mean %)	RSD (%)	Nominal concentration (ng/mL)	Recovery (mean %)	RSD (%)
OZ277	20	119	11.0	50	100	3.0
	10000	112	2.1	500	105	4.9
				2000	95	5.4
OZ397	20	90	11.0	50	108	2.3
	10000	96	7.6	500	108	4.7
				2000	100	8.7
NP15	20	96	7.1	50	103	5.7
	10000	87	0.9	500	106	1.6
				2000	93	3.5
NP22	20	93	8.9	50	105	11
	10000	93	6.7	500	107	5.2
				2000	96	5.4
IS	200	99	1.6			

Table 2
Precision and accuracy of OZ277 and its metabolites in rat plasma.

Intra-day						
Nominal concentration (ng/mL)	5	20	50	500	2000	10000
OZ277						
Mean ($n=6$)	5.0	21	56	565	2092	9846
RSD (%)	5.2	2.8	6.6	3.9	4.8	3.3
Accuracy (%)	−0.8	4.5	11	13	4.6	−1.5
OZ397						
Mean ($n=6$)	5	21	54	509	1940	9919
RSD (%)	7.6	4.6	8.7	3.7	5.2	2.8
Accuracy (%)	−0.5	5.3	7.0	1.8	−3.0	−0.8
NP15						
Mean ($n=6$)	5	21	54	525	1989	11176
RSD (%)	9.1	8.8	5.3	5.8	4.3	1.7
Accuracy (%)	2.6	7.2	7.2	5.1	−0.5	11.8
NP22						
Mean ($n=6$)	5	20	50	520	1936	10113
RSD (%)	9.3	2.2	6.7	4.0	3.9	3.4
Accuracy (%)	3.2	−0.3	0.3	3.7	−3.2	1.1

Inter-day			
Nominal concentration (ng/mL)	50	500	2000
OZ277			
Mean ($n=24$)	53	527	1978
RSD (%)	6.0	6.3	5.8
Accuracy (%)	6.1	5.3	−1.1
OZ397			
Mean ($n=24$)	53	518	2017
RSD (%)	7.4	6.1	7.6
Accuracy (%)	6.9	3.6	0.9
NP15			
Mean ($n=24$)	53	528	2064
RSD (%)	6.7	5.1	5.9
Accuracy (%)	5.0	5.6	3.2
NP22			
Mean ($n=24$)	51	518	2008
RSD (%)	7.3	4.6	5.7
Accuracy (%)	1.9	3.5	0.4

recoveries of each of the analytes were above 90% across the concentration range. The RSD of the recoveries over the concentration range was ≤11%, demonstrating consistency in the protein precipitation method.

3.3.4. Calibration range and LLOQ

The calibration range for OZ277 and its metabolites was established by analysing eight calibration standards over a concentration range of 5–10,000 ng/mL on four occasions. This range was selected to cover the concentration range likely to be encountered in samples arising from *in vivo* rat pharmacokinetic studies. The calibration curve was best fit using a quadratic equation weighted to 1/concentration and resulted in a correlation coefficient (r^2) > 0.998. The accuracy of the back-calculated concentrations for the calibration standards was within ±15% of the nominal concentration. The lowest calibration standard (5 ng/mL) was set as the LLOQ for OZ277 and its metabolites in rat plasma as it met each of the specified requirements (Table 2).

3.3.5. Precision and accuracy

The inter- and intra-day precision and accuracy was established from the analyses performed on four different days. The mean, precision and accuracy were calculated from the replicates over all four days ($n=24$) to provide inter-day values while intra-day values were calculated from the replicates on each day ($n=6$). The intra-day accuracy for OZ277 and its metabolites was within the range of −3% to 13% across the tested concentration range while the inter-day accuracy for all the analytes was within the range of −1% to 7%. Both intra- and inter-day precision values were less than 10% for each of the analytes (Table 2).

Table 3
Short-term bench top stability of OZ277 and its metabolites in rat plasma. The results represent the mean of six measurements.

Analyte	Nominal concentration (ng/mL)	0 h (set I)		3 h (set II)	
		Mean	RSD	Mean	RSD
OZ277	50	52	3.8	52	5.5
	500	514	4.4	525	3.3
	2000	2099	4.9	2067	5.6
OZ397	50	51	8.7	48	2.4
	500	478	2.8	474	4.5
	2000	1850	4.1	1862	3.5
NP15	50	52	5.3	52	6.1
	500	523	4.4	527	2.2
	2000	2001	3.2	2132	5.8
NP22	50	51	4.7	51	4.7
	500	508	2.3	527	5.2
	2000	1965	2.4	2052	5.6

3.3.6. Short-term bench top stability

The short-term bench top stability of OZ277 and its metabolites was determined by comparing an immediately processed set of spiked samples (set I) with a set of samples that was processed after exposure to ambient conditions for 3 h (set II). No significant difference ($p < 0.05$) was observed in the calculated mean values between the two sets of samples (Table 3), establishing the stability of all the analytes in rat plasma under these conditions.

3.3.7. Autosampler stability

The stability of OZ227 and its metabolites in processed samples stored within the autosampler indicated that the analytes were stable under these conditions. The RSD for each of the analytes was within the range of 3–9% and the calculated concentrations were within 2–13% of the nominal concentrations. During this experiment, the RSD for the peak area of the IS was <6.0%. These results demonstrate the stability of each of the analytes and the IS following processing under autosampler conditions for a period of at least 24 h.

3.3.8. Storage stability

The stability of OZ277 and its metabolites in rat plasma at −80 °C was evaluated to determine acceptable storage conditions for the PK samples. Following plasma sample storage at −80 °C for 15 days, there was no discernable difference in the assayed concentrations compared to freshly analysed plasma samples. The RSD and accuracy values for each of the analytes ranged from 2% to 11% and −7% to 12%, respectively (Table 4).

4. Application of the analytical method

Rat plasma samples generated following IV administration of OZ277 were analysed by the described analytical method along with QC samples. The concentrations of OZ277 and its metabolites were within the calibration range and all of the QCs were found to meet the acceptance criteria (data not shown). The sensitivity and specificity of the analytical method were found to be sufficient for accurately characterizing the plasma pharmacokinetics of OZ277 and its metabolites. Fig. 5 represents the profile of OZ277 and its metabolites in rat plasma following IV administration of OZ277 at a dose of 10 mg/kg.

Table 4
Storage stability of OZ277 and its metabolites at −80 °C in rat plasma.

	9 days			15 days		
	Nominal concentration (ng/mL)					
	50	500	2000	50	500	2000
OZ277						
Mean (n = 6)	52	527	2008	52	488	1860
RSD (%)	3.9	2.4	4.3	5.5	2.8	3.5
Accuracy (%)	4.3	5.4	0.4	3.0	−2.4	−7.0
OZ397						
Mean (n = 6)	52	548	2158	56	537	2122
RSD (%)	6.0	3.0	4.5	5.4	2.6	3.3
Accuracy (%)	6.4	9.5	7.9	11	7.5	6.1
NP15						
Mean (n = 6)	50	555	2220	54	509	2044
RSD (%)	9.0	3.1	5.0	6.0	3.5	2.6
Accuracy (%)	0.2	11	11	7.6	1.9	2.2
NP22						
Mean (n = 6)	52	547	2169	51	496	1963
RSD (%)	7.3	2.8	4.2	10	2.6	2.9
Accuracy (%)	4.2	9.3	8.4	1.6	−0.8	−1.9

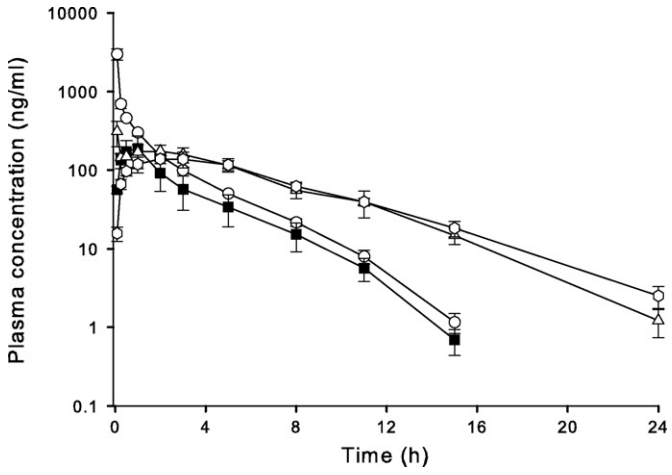

Fig. 5. Plasma concentration versus time profiles of OZ277 (open circles), OZ397 (closed square), NP15 (open triangle) and NP22 (open hexagon) after intravenous administration of OZ277 to male Sprague–Dawley rats at 10 mg/kg dose (mean ± SD, n = 3).

5. Conclusion

A sensitive HILIC/MS method was developed for the simultaneous determination of OZ277 and its metabolites in rat plasma. Although the HILIC stationary phase has been predominantly utilised for the analysis of highly polar, ionic solutes, this work demonstrates the utility of the stationary phase for the simultaneous separation of hydrophilic and hydrophobic solutes from complex matrices. The precipitation method was found to be fast and simple, thus saving time and money over solid phase extraction and LLE techniques. The method was sensitive and selective, and resulted in good accuracy and precision across a wide calibration range in rat plasma.

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References

[1] G.A. Biagini, P.M. O'Neill, P.G. Bray, S.A. Ward, Curr. Opin. Pharmacol. 5 (2005) 473.
[2] J.L. Vennerstrom, S. Arbe-Barnes, R. Brun, S.A. Charman, F.C. Chiu, J. Chollet, Y. Dong, A. Dorn, D. Hunziker, H. Matile, K. McIntosh, M. Padmanilayam, J. Santo Tomas, C. Scheurer, B. Scorneaux, Y. Tang, H. Urwyler, S. Wittlin, W.N. Charman, Nature 430 (2004) 900.
[3] A. Afonso, P. Hunt, S. Cheesman, A.C. Alves, C.V. Cunha, V. do Rosario, P. Cravo, Antimicrob. Agents Chemother. 50 (2006) 480.
[4] S. Kamchonwongpaisan, G. Chandra-ngam, M.A. Avery, Y. Yuthavong, J. Clin. Invest. 93 (1994) 467.
[5] D.J. Creek, W.N. Charman, F.C.K. Chiu, R.J. Prankerd, K.J. McCullough, Y. Dong, J.L. Vennerstrom, S.A. Charman, J. Pharm. Sci. 96 (2007) 2945.
[6] L. Zhou, A. Alker, A. Ruf, X. Wang, F.C.K. Chiu, J. Morizzi, S.A. Charman, W.N. Charman, C. Scheurer, S. Wittlin, Y. Dong, D. Hunziker, J.L. Vennerstrom, Bioorg. Med. Chem. Lett. 18 (2008) 1555.
[7] H.Y. Ji, D.W. Jeong, Y.H. Kim, H.H. Kim, Y.S. Yoon, K.C. Lee, H.S. Lee, Rapid Commun. Mass Spectrom. 20 (2006) 2127.
[8] J.C. Yunsheng Hsieh, Rapid Commun. Mass Spectrom. 19 (2005) 3031.
[9] H. Idborg, L. Zamani, P.-O. Edlund, I. Schuppe-Koistinen, S.P. Jacobsson, J. Chromatogr. B 828 (2005) 9.
[10] D.C.D. Amy, D. Delinsky, S. Muralidhara, Jeffrey W. Fisher, James V. Bruckner, Michael G. Bartlett, Rapid Commun. Mass Spectrom. 19 (2005) 1075.
[11] I.B. Paek, Y. Moon, H.Y. Ji, H.-H. Kim, H.W. Lee, Y.-B. Lee, H.S. Lee, J. Chromatogr. B 809 (2004) 345.
[12] C.A.W. Stacy, D. Brown, Michael G. Bartlett, Rapid Commun. Mass Spectrom. 16 (2002) 1871.
[13] A.J. Alpert, J. Chromatogr. A 499 (1990) 177.
[14] S.C. Churms, J. Chromatogr. A 500 (1990) 555.
[15] S.C. Churms, J. Chromatogr. A 720 (1996) 75.
[16] A.J. Alpert, M. Shukla, A.K. Shukla, L.R. Zieske, S.W. Yuen, M.A.J. Ferguson, A. Mehlert, M. Pauly, R. Orlando, J. Chromatogr. A 676 (1994) 191.
[17] T. Yoshida, Anal. Chem. 69 (1997) 3038.
[18] A.R. Oyler, B.L. Armstrong, J.Y. Cha, M.X. Zhou, Q. Yang, R.I. Robinson, R. Dunphy, D.J. Burinsky, J. Chromatogr. A 724 (1996) 378.
[19] M.A. Strege, Anal. Chem. 70 (1998) 2439.
[20] P. Hemstrom, K. Irgum, J. Sep. Sci. 29 (2006) 1784.
[21] K.A.S. Hien, P. Nguyen, J. Sep. Sci. 31 (2008) 1465.
[22] R. King, R. Bonfiglio, C. Fernandez-Metzler, C. Miller-Stein, T. Olah, J. Am. Soc. Mass Spectrom. 11 (2000) 942.
[23] K. Okusa, H. Tanaka, M. Ohira, J. Chromatogr. A 869 (2000) 143.
[24] A. HoubenovÃ¡, H.A. Claessens, J.W. de Haan, C.A. Cramers, K. StulÃk, J. Liq. Chromatogr. Relat. Technol. 17 (1994) 49.
[25] Vladimir V. Tolstikov, O. Fiehn, Anal. Biochem. 301 (2002) 298.
[26] H. Schlichtherle-Cerny, M. Affolter, C. Cerny, Anal. Chem. 75 (2003) 2349.
[27] H.L. Koh, A.J. Lau, E.C.Y. Chan, Rapid Commun. Mass Spectrom. 19 (2005) 1237.
[28] C. Polson, P. Sarkar, B. Incledon, V. Raguvaran, R. Grant, J. Chromatogr. B 785 (2003) 263.