Journal of Chromatography B, 877 (2009) 3083–3088

Contents lists available at ScienceDirect

Journal of Chromatography B

journal homepage: www.elsevier.com/locate/chromb

Enantioselective analysis of praziquantel and trans-4-hydroxypraziquantel in human plasma by chiral LC–MS/MS: Application to pharmacokinetics

Renata Monteiro Lima[a], Maria Augusta Drago Ferreira[b], Teresa Maria de Jesus Ponte[b], Maria Paula Marques[a], Osvaldo Massaiti Takayanagui[c], Hector Hugo Garcia[d], Eduardo Barbosa Coelho[c], Pierina Sueli Bonato[a], Vera Lucia Lanchote[a,*]

[a] Faculdade de Ciências Farmacêuticas de Ribeirão Preto, Universidade de São Paulo, Ribeirão Preto, Brazil
[b] Faculdade de Farmácia, Odontologia e Enfermagem, Universidade Federal do Ceará, Fortaleza, Brazil
[c] Faculdade de Medicina de Ribeirão Preto, Universidade de São Paulo, Ribeirão Preto, Brazil
[d] Instituto de Ciências Neurológicas, Lima, Peru

ARTICLE INFO

Article history:
Received 25 March 2009
Accepted 27 July 2009
Available online 3 August 2009

Keywords:
Praziquantel
Enantiomers
Pharmacokinetics
Metabolism

ABSTRACT

A simple enantioselective method for the determination of praziquantel (PZQ) and trans-4-hydroxypraziquantel (4-OHPZQ) in human plasma was developed and validated by high-performance liquid chromatography/mass spectrometry. The plasma samples were prepared by liquid–liquid extraction using a mixture of methyl-tert-butylether/dichloromethane (2:1, v/v) as extraction solvent. The direct resolution of PZQ and 4-OHPZQ enantiomers was performed on a Chiralpak AD column using hexane–isopropanol (75:25, v/v) as the mobile phase. Diazepam was used as internal standard. The method described here is simple and reproducible. The quantitation limit of 1.25 ng/ml for each PZQ enantiomer and of 12.5 ng/ml for each 4-OHPZQ enantiomer permits the use of the method in studies investigating the kinetic disposition of a single dose of 1.5 g racemic PZQ. Enantioselectivity in the kinetic disposition of PZQ and 4-OHPZQ was observed in the clinical study, with the demonstration of a higher proportion of the (+)-(S)-PZQ and (−)-(R)-4-OHPZQ enantiomers in plasma.

1. Introduction

The major asset of praziquantel (PZQ) is probably its broad spectrum of activity. PZQ is the drug of choice for the treatment of all forms of schistosomiasis and infections caused by other trematodes. Neurocysticercosis can also be treated with high doses of PZQ. After the administration of PZQ, side effects are observed in a relatively high percentage of patients (30–60%), but these effects are usually mild and transient and disappear within 24 h [1].

PZQ is a chiral compound marketed as the racemate (Fig. 1), although its (+)-(S) enantiomer has no proven pharmacological activity [2–4]. However, toxicity is the same for the two PZQ enantiomers [5]. Indeed, Wu et al. [4] found that a single 20 mg/kg (−)-(R)-PZQ was as efficacious as 40 mg/kg of the racemic praziquantel in patients with Schistosomiasis Japonica. Moreover, the authors observed that (−)-(R)-PZQ produced fewer side effects than racemic praziquantel.

Westhoff and Blaschke [6] observed higher plasma concentrations of (+)-(S)-PZQ in humans, i.e., the enantiomer with no pharmacological activity, and of the active metabolite (−)-(R)-trans-4-hydroxypraziquantel (4-OHPZQ), a relevant fact since the latter metabolite seems to possess antischistosomal activity [2].

Four HPLC methods using ultraviolet detection have been described for the enantioselective analysis of PZQ [7–9], and only one HPLC method using ultraviolet detection has been reported for the simultaneous enantioselective analysis of PZQ and 4-OHPZQ in plasma samples [6]. Two other capillary electrophoresis methods have been developed for the enantioselective analysis of PZQ and its main metabolite 4-OHPZQ in plasma samples [10,11].

This paper describes for the first time a highly sensitive LC–MS/MS assay for the simultaneous determination of PZQ and 4-OHPZQ enantiomers in human plasma. The validated method was applied in a clinical study and proved to be suitable for pharmacokinetic investigation, presenting a quantitation limit lower than those reported in the literature.

* Corresponding author at: Faculdade de Ciências Farmacêuticas de Ribeirão Preto da Universidade de São Paulo, Departamento de Análises Clínicas, Toxicológicas e Bromatológicas, Avenida do Café s/n, Campus da USP, 14040-903, Ribeirão Preto, SP, Brazil. Tel.: +55 16 36024699; fax: +55 16 36024725.
E-mail address: lanchote@fcfrp.usp.br (V.L. Lanchote).

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doi:10.1016/j.jchromb.2009.07.036

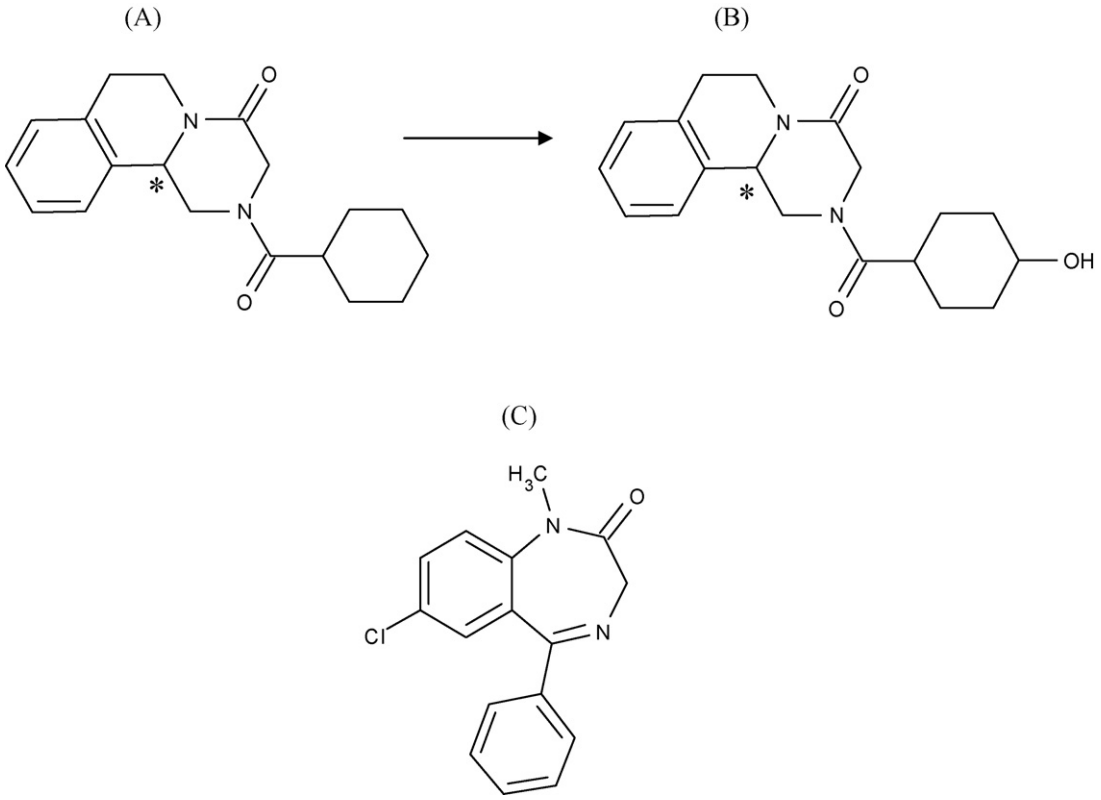

Fig. 1. Structures of praziquantel (A), 4-hydroxypraziquantel (B) and the internal standard diazepam (C). *The chiral center.

2. Experimental procedure

2.1. Chemicals and reagents

Standard solutions of *rac*-PZQ (Merck) and *rac*-4-OHPZQ (kindly supplied by Merck and by Dr. G. Blaschke and Dr. M.J. Surpili) were prepared in methanol at concentrations ranging from 0.1 to 100 μg *rac*-PZQ/ml and from 1 to 300 μg *rac*-4-OHPZQ/ml.

A solution of diazepam (internal standard, Sigma, St. Louis, MO, USA) was prepared at a concentration of 50 ng/ml methanol.

The solvents used as mobile phase and in the extraction procedure were of chromatographic grade and were purchased from Merck, J.T. Baker and Acros.

2.2. Chromatographic analysis

The HPLC system consisted of a Shimadzu chromatograph (Kyoto, Japan) equipped with an LC-10 AD pump and a CTO-10 AS oven. The PZQ and 4-OHPZQ enantiomers were resolved on a 250 mm × 4.6 mm Chiralpak® AD column (particle size 10 μm; Chiral Technologies, Inc., Exton, PA, USA) using a CN 4 mm × 4 mm pre-column (Merck). The mobile phase for elution of the enantiomers consisted of hexane–isopropanol (75:25, v/v). The enantiomers were eluted at a flow rate of 1.2 ml/min. The column was kept at 23 °C.

A Quattro Micro-triple quadrupole mass spectrometer (Micromass, Manchester, UK) equipped with an electrospray interface (ESI) was used. Analyses were carried out in the positive ESI mode. The capillary voltage of the ESI was 4.0 kV. Temperatures of the source and desolvation system were kept at 100 and 250 °C, respectively. Nitrogen was used as the nebulizer gas at a flow rate of 416 l/h. Argon was used as the collision gas at a pressure of $\sim 2.23 \times 10^{-3}$ mbar. The cone voltage was maintained at 30 V for PZQ and 4-OHPZQ and at 20 V for the internal standard. The collision energy was 20 eV for PZQ, 4-OHPZQ and internal standard.

The MS/MS conditions were optimized by direct infusion of standard solutions (30 μg PZQ/ml and 40 μg 4-OHPZQ/ml) prepared in the mobile phase and introduced with an infusion pump at a flow rate of 20 μl/min. Analysis was carried out in the multiple reaction monitoring mode. Protonated ions $[M+H]^+$ and their respective ion products were monitored at the following transitions: 313 > 203 for PZQ, 329 > 203 for 4-OHPZQ, and 285 > 154 for the internal standard. Data acquisition and sample quantification were performed with the MassLynx Program, version 3.5 (Micromass).

2.3. Sample preparation

Plasma aliquots of 1 ml were supplemented with 25 μl of the working internal standard (50 ng/ml diazepam). The samples were vortex mixed for 2–3 s and extracted with a mixture of 6 ml methyl-tert-butylether/dichloromethane (2:1, v/v). After mechanical tumbling for 30 min, the organic phases were separated by centrifugation at 4 °C (2000 × *g*) for 10 min. The organic phases (5 ml) were transferred to conical tubes, and the solvent was evaporated to dryness under an air flow at room temperature. The residues thus obtained were dissolved in 200 μl of the mobile phase and 130 μl of the solution was submitted to chromatographic analysis.

2.4. Calibration curves

Blank plasma samples were obtained from healthy volunteers (not treated with PZQ) recruited from the Blood Center of the local University Hospital.

The calibration curves were constructed using 1-ml samples of drug-free plasma spiked with 25 μl of each diluted standard solution of PZQ and 4-OHPZQ. The linear regression equations and the

correlation coefficients were obtained from the peak area ratios plotted against their respective concentrations (1.25, 12.5, 25, 50, 150 and 375 ng for each PZQ enantiomer/ml and 12.5, 125, 250, 500, 1500 and 3750 ng for each 4-OHPZQ enantiomer/ml). Samples were prepared as described in Section 2.3.

2.5. Matrix effect

The matrix effect was evaluated based on direct comparison of the peak areas of PZQ (500 ng/ml of each enantiomer), 4-OHPZQ (1500 ng/ml of each enantiomer) and internal standard (1.25 ng) injected directly into the mobile phase, and spiked post-extraction into extracts originating from six different sources of human plasma.

2.6. Validation

Recovery of PZQ and 4-OHPZQ was evaluated by comparing the peak areas obtained after plasma extraction with the peak areas obtained after injection of the standard solutions. Plasma samples spiked with PZQ (3, 500 and 1000 ng/ml of each enantiomer) and 4-OHPZQ (30, 1500 and 2850 ng/ml of each enantiomer) were prepared as described above. Standard solutions (25 μl) were evaporated to dryness, the residues obtained were dissolved in 200 μl of the mobile phase and shaken for 5 s, and 130 μl was injected into the chromatographic system.

The quantitation limit was determined by the analysis, in quintuplicate, of plasma samples spiked with PZQ or 4-OHPZQ at concentrations as low as 1.25 and 12.5 ng/ml of each PZQ and 4-OHPZQ enantiomer, respectively. The quantitation limit was defined as the lowest plasma concentration of each analyte obtained with an error of 20% or less.

Linearity was evaluated by the analysis of plasma samples spiked with increasing analyte concentrations in relation to those employed for construction of the calibration curve (1.25–1250 ng of each PZQ enantiomer/ml and 12.5–3750 ng of each 4-OHPZQ enantiomer/ml). Sample preparation and chromatographic conditions were the same as described above. The method was considered to be linear up to the highest concentration studied, presenting a linear relationship with the detector response.

Precision and accuracy were evaluated at concentrations of 3, 500 and 1000 ng of each PZQ enantiomer/ml and 30, 1500 and 2850 ng of each 4-OHPZQ enantiomer/ml. For the evaluation of intra-assay precision, five aliquots of each sample were analyzed using a single calibration curve. For interassay precision, aliquots of the samples were analyzed in duplicate on 5 consecutive days.

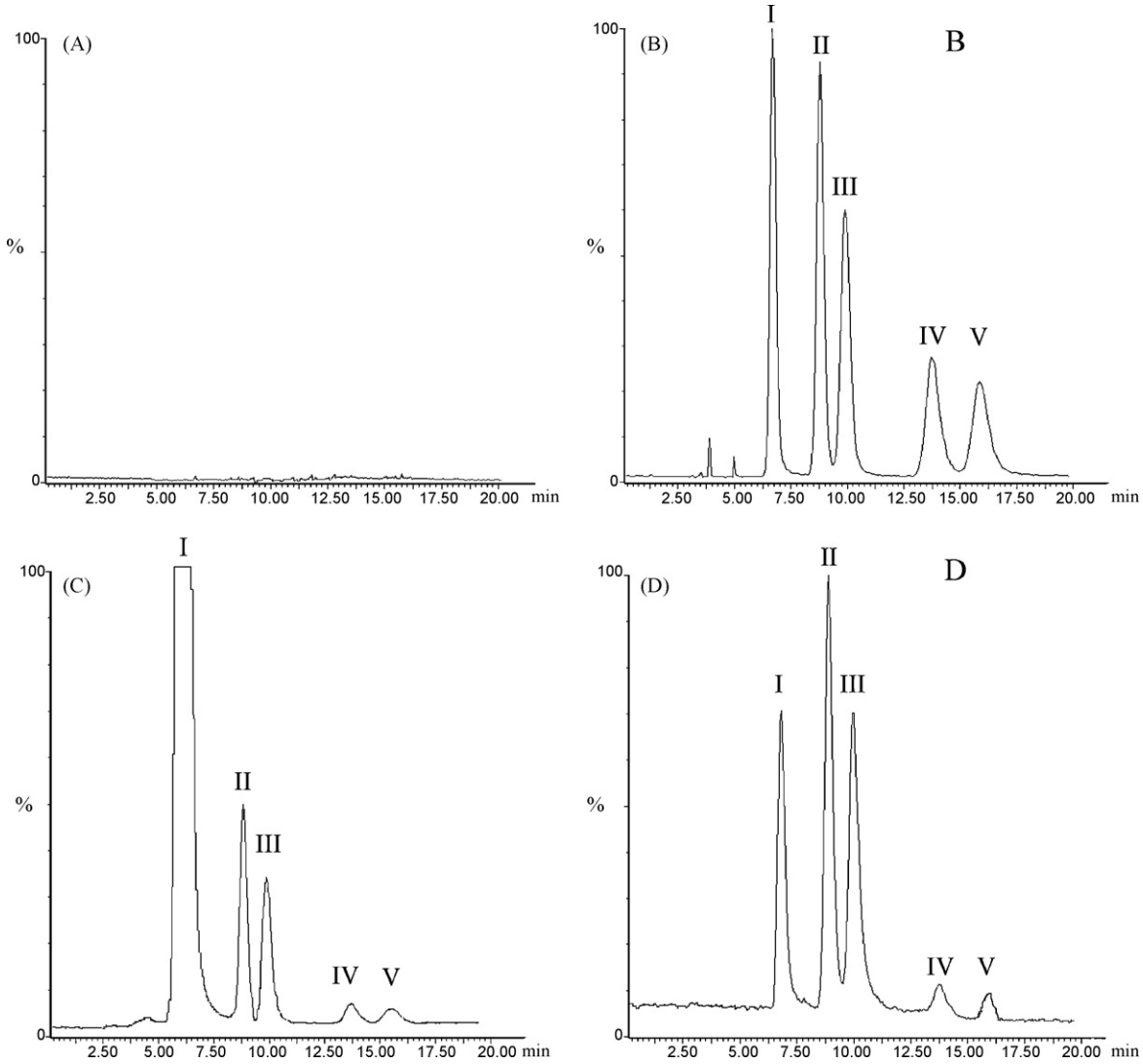

Fig. 2. Chromatograms obtained for the analysis of PZQ and 4-OHPZQ enantiomers in human plasma. (A) Blank plasma. (B) Plasma spiked with 50 and 250 ng/ml of each PZQ and 4-OHPZQ enantiomer, respectively: (I) internal standard; (II) (+)-PZQ; (III) (−)-PZQ; (IV) (−)-4-OHPZQ; (V) (+)-4-OHPZ. (C) Chromatograms obtained for the analysis of PZQ and 4-OHPZQ enantiomers in human plasma at their limit of quantification. (D) Plasma obtained 3 h after PZQ administration.

Freeze–thaw cycle (three cycles of 12 h, −20 °C) and short-term room temperature (room temperature, 23 ± 2 °C, for 6 h) stability tests were performed for samples containing concentrations of 3 and 1000 ng/ml of each PZQ enantiomer and of 30 and 2850 ng/ml of each 4-OHPZQ enantiomer. The peak areas obtained for the two stability tests were compared to the peak areas obtained with freshly prepared samples. The results are expressed as relative errors.

2.7. Application

The study was approved by the Ethics Committee of the University Hospital, Faculty of Medicine of Ribeirão Preto, University of São Paulo, Brazil. The volunteer received detailed explanations about the procedures and was included in the study after giving written informed consent. After a 12-h fast, the volunteer received a single dose of 1500 mg racemic PZQ (Cisticid, Merck, Brazil) with 200 ml water. Blood samples (5 ml) were collected through a catheter inserted into the antecubital vein into heparin-containing tubes at times zero, 0.5, 1, 1.5, 2, 2.5, 3, 4, 6, 8 and 10 h after drug administration. Blood samples were immediately centrifuged for plasma separation. Plasma samples were then stored at −20 °C until the time of analysis.

The pharmacokinetic parameters were calculated based on the plasma enantiomer concentration versus time curves using the WinNonlin program, version 4.0 (Pharsight Corporation, Mountain View, CA, USA). The parameters were calculated using first-order kinetics and a monocompartmental model including lag time.

3. Results and discussion

A simple method for the determination of PZQ and 4-OHPZQ enantiomers in human plasma was developed and validated by high-performance liquid chromatography/mass spectrometry (LC–MS/MS). The method was validated by evaluating linearity, recovery, quantitation limit, stability, precision, accuracy, and ionization suppression effects. Coefficients of variation and relative errors of less than 15% were considered to be acceptable, except for quantitation limit for which a value of less than 20% was established as recommended in the literature.

PZQ and 4-OHPZQ enantiomers were resolved on a Chiralpak AD chiral phase column using a mobile phase of hexane–isopropanol (75:25, v/v) at a run time of ∼20 min. Fig. 2 shows the chromatograms obtained for blank plasma, plasma enriched with PZQ and 4-OHPZQ standards corresponding to concentrations of 50 ng/ml of each PZQ enantiomer and 250 ng/ml of each 4-OHPZQ enantiomer, respectively, and plasma collected from a healthy volunteer 3 h after the administration of 1500 mg racemic PZQ.

Analysis of blank human plasma (free of PZQ) did not reveal interference of endogenous components with the PZQ or 4-OHPZQ enantiomers. Table 1 shows that the matrix effect of the PZQ enantiomers and of the internal standard diazepam was practically absent. (+)-(S)-4-OHPZQ and (−)-(R)-4-OHPZQ had respectively 90.90% and 84.56% of the original sign, which may be significantly suppressed, although analytically acceptable.

Table 1
Matrix effect of PZQ, 4-OHPZQ and internal standard in six different sources of human plasma.

	Matrix effect (% of the original signal)
Internal standard	93.34
(+)-(S)-PZQ (500 ng/ml)	98.51
(−)-(R)-PZQ (500 ng/ml)	94.47
(−)-(R)-4-OHPZQ (1500 ng/ml)	84.56
(+)-(S)-4-OHPZQ (1500 ng/ml)	90.90

Values are the mean.

Table 2
Validation parameters obtained for the analysis of PZQ enantiomers in human plasma.

	(+)-(S)-PZQ	(−)-(R)-PZQ
Recovery (%)		
3 ng/ml	86.99	83.33
500 ng/ml	98.51	94.47
1000 ng/ml	97.33	90.61
Linearity (ng/ml)	1.25–1250	1.25–1250
r^2	0.9964	0.9943
Quantitation limit (ng/ml)	1.25	1.25
Precision (RSD %, $n = 5$)	11.24	7.01
Accuracy (% bias)	94.72	91.50
Interassay precision ($n = 5$, RSD %)		
3 ng/ml	3.25	4.69
500 ng/ml	4.05	11.40
1000 ng/ml	9.97	7.42
Intra-assay precision (RSD %)		
3 ng/ml ($n = 5$)	8.46	8.94
500 ng/ml ($n = 5$)	12.88	4.78
1000 ng/ml ($n = 5$)	8.90	9.49
Interassay accuracy (% bias)		
3 ng/ml ($n = 5$)	92.53	95.26
500 ng/ml ($n = 5$)	108.58	93.87
1000 ng/ml ($n = 5$)	103.08	105.05
Intra-assay accuracy (% bias)		
3 ng/ml ($n = 5$)	105.40	99.86
500 ng/ml ($n = 5$)	102.35	90.60
1000 ng/ml ($n = 5$)	107.81	96.66
Stability (% bias)		
Freeze–thaw cycles (−20 to 25 °C)		
3 ng/ml	8.20	13.81
1000 ng/ml	2.24	10.91
Room temperature for 6 h		
3 ng/ml	2.76	4.00
1000 ng/ml	8.82	9.61

n = number of replicates.

The plasma samples were prepared by liquid–liquid extraction using a mixture of methyl-tert-butylether/dichloromethane (2:1, v/v) as extraction solvent (Tables 2 and 3). The mean recovery rates were 91.9% for PZQ and 87.6% for 4-OHPZQ. Westhoff and Blaschke [6] reported recovery rates of 81.5% for PZQ and 76.5% for 4-OHPZQ in plasma samples using acetonitrile–toluene (4:6, v/v) as extraction solvent. Jabor and Bonato [10] used toluene supplemented with NaCl and obtained recovery of 96% for PZQ and of 87.6% for 4-OHPZQ in plasma samples.

The linearity of the method using the LC–MS/MS system ranged from 1.25 to 1250 ng/ml of each PZQ enantiomer and from 12.5 to 3750 ng/ml of each 4-OHPZQ enantiomer, with coefficients of correlation higher than 0.99 (Tables 2 and 3). The quantitation limit was 1.25 ng/ml for each PZQ enantiomer and 12.5 ng/ml for each 4-OHPZQ enantiomer (Tables 1 and 2). Westhoff and Blaschke [6] reported a detection limit of 5 ng/ml for the PZQ and 4-OHPZQ enantiomers using 0.5 ml serum and an HPLC system with UV detection set at 210 nm. Jabor and Bonato [10] determined PZQ and 4-OHPZQ in human plasma by cyclodextrin-modified micellar electrokinetic chromatography and reported quantitation limits of 50 ng/ml for the PZQ enantiomers and of 62.5 ng/ml for the 4-OHPZQ enantiomers, using 1 ml human plasma.

The precision and accuracy of the method were tested by within-day and between-day analysis using plasma samples spiked with three concentrations of each PZQ enantiomer (3, 500 and 1000 ng/ml) and three concentrations of each 4-OHPZQ enantiomer

Table 3
Validation parameters obtained for the analysis of 4-OHPZQ enantiomers in human plasma.

	(−)-(R)-4-OHPZQ	(+)-(S)-4-OHPZQ
Recovery (%)		
30 ng/ml	78.26	85.96
1500 ng/ml	84.56	90.90
2850 ng/ml	92.97	92.74
Linearity (ng/ml)	12.5–3750	12.5–3750
r^2	0.9995	0.9995
Quantitation limit (ng/ml)	12.5	12.5
Precision (RSD %, $n = 5$)	5.84	12.32
Accuracy (% bias)	90.34	92.96
Interassay precision ($n = 5$, RSD %)		
30 ng/ml	4.78	4.52
1500 ng/ml	6.5	8.31
2850 ng/ml	5.83	2.96
Intra-assay precision (RSD %)		
30 ng/ml	8.84	10.30
1500 ng/ml	14.77	6.07
2850 ng/ml	10.25	9.13
Interassay accuracy (% bias)		
30 ng/ml	92.10	87
1500 ng/ml	94.11	93.93
2850 ng/ml	109.91	112.28
Intra-assay accuracy (% bias)		
30 ng/ml ($n = 5$)	107.47	84.91
1500 ng/ml ($n = 5$)	97.39	92.53
2850 ng/ml ($n = 5$)	110.74	107.92
Stability (% bias)		
Freeze–thaw cycles (−20 to 25 °C)		
30 ng/ml	11.42	9.80
2850 ng/ml	1.20	5.05
Room temperature for 6 h		
30 ng/ml	9.63	10.81
2850 ng/ml	2.41	11.50

n = number of replicates.

(30, 1500 and 2850 ng/ml). Relative standard deviations and relative errors of less than 15% were obtained for all samples analyzed (Tables 1 and 2). Stability testing after three freeze–thaw cycles and after storage at room temperature for 6 h revealed deviations of less than 15%, thus guaranteeing the stability of samples stored at −20 °C.

The method developed and validated in the present study was applied to the investigation of enantioselectivity in the kinetic disposition of PZQ and its metabolite 4-OHPZQ in a healthy volunteer treated with a single oral dose of 1500 mg racemic PZQ.

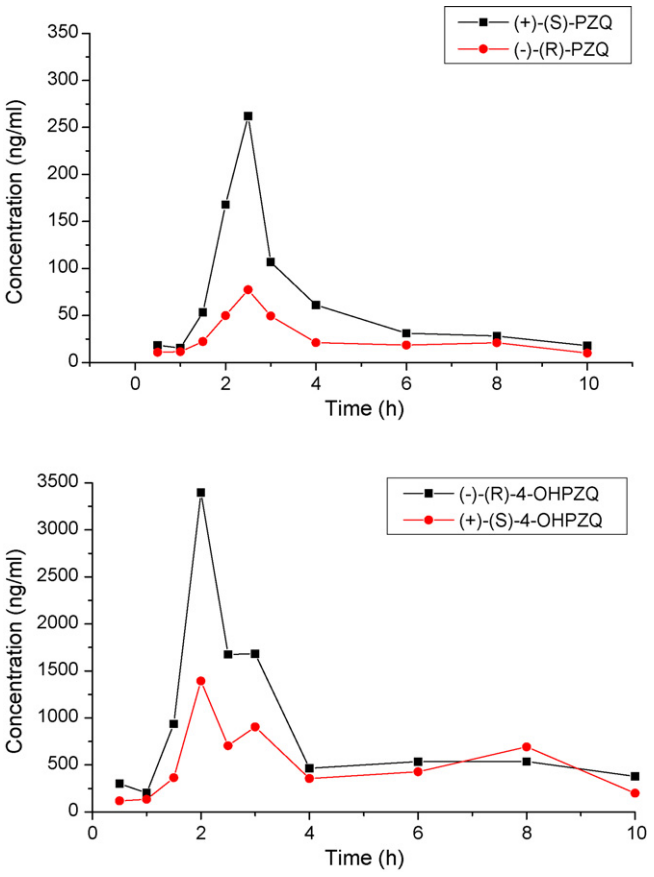

Fig. 3. Plasma concentration versus time curves of PZQ and 4-OHPZQ enantiomers obtained for a healthy volunteer after administration of a single oral dose of 1500 mg racemic PZQ.

Enantioselectivity in the kinetic disposition of PZQ and 4-OHPZQ was observed in the clinical study, with the demonstration of a higher proportion of the (+)-(S)-PZQ and (−)-(R)-4-OHPZQ enantiomers in plasma (Fig. 3). The enantiomer ratios of PZQ and its metabolite 4-OHPZQ are of special interest because only the (−)-(R) enantiomers have been shown to be effective in *in vitro* studies [2]. The AUC value for (−)-(R)-4-OHPZQ was 38 times higher than the AUC obtained for (−)-(R)-PZQ (Table 4). The $AUC_{R/S}$ ratios were 0.39 for PZQ and 1.67 for 4-OHPZQ (Table 4). Westhoff and Blaschke [6] reported R/S plasma concentration ratios ranging from 0.54 to 0.33 for PZQ and from 2.6 to 1.79 for 4-OHPZQ in five healthy Caucasian volunteers. The authors suggested that these differences might be due to an enantioselective first-pass metabolism of PZQ in the liver.

Table 4
Enantioselective kinetic disposition of PZQ and 4-OHPZQ in a healthy volunteer after oral administration of 1500 mg racemic PZQ.

Parameter	(+)-(S)-PZQ	(−)-(R)-PZQ	(+)-(S)-4-OHPZQ	(−)-(R)-4-OHPZQ
C_{max} (ng/ml)	164.91	51.38	1169.33	2175.58
t_{max} (h)	2.33	2.73	3.32	2.56
$t_{1/2}$ a (h)	0.59	0.23	5.33	8.66
K_a or K_f (h^{-1})	1.17	2.96	0.13	0.08
$t_{1/2}$ (h)	1.33	1.68	1.68	1.51
kel (h^{-1})	0.52	0.41	0.41	0.46
Vd/f (l/kg)	29.78	95.82	–	–
$AUC^{0-\infty}$ (ng h/ml)	857.13	337.90	7717.43	12,864.46
Cl/f(l/h/kg)	15.49	39.28	–	–
$AUC_{(R)}/AUC_{(S)}$	0.39		1.67	

C_{max}, maximum plasma concentration; t_{max}, time to reach C_{max}; $t_{1/2}$ a, absorption or formation half-life; K_a, absorption or formation rate constant; $t_{1/2}$, elimination half-life; kel, elimination rate constant; Vd/f, apparent distribution volume; $AUC^{0-\infty}$ area under the plasma concentration versus time curve; Cl/f, apparent total clearance.

4. Conclusions

We report for the first time a method for the direct determination of PZQ and 4-OHPZQ enantiomers in human plasma by LC–MS/MS. The method is simple and reproducible. The quantitation limit of 1.25 ng/ml for each PZQ enantiomer and of 12.5 ng/ml for each 4-OHPZQ enantiomer permits the use of the method in studies investigating the kinetic disposition of a single dose of 1500 mg PZQ.

Considering the CYP3A is the major enzyme involved in PZQ metabolism and considering that many drugs are CYP3A inducers (carbamazepine, dexamethasone) or inhibitors (cimetidine, ketoconazole) [12] the present method can also be used to optimize praziquantel treatment.

Acknowledgments

The authors are grateful to FAPESP, CNPq, FINEP and FAEPA for financial support, and to CAPES for granting research fellowships. We also thank Dr. G. Blaschke and Dr. M.J. Surpili for kindly providing trans-4-hydroxypraziquantel.

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