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Investigation of Praziquantel metabolism in isolated rat hepatocytes

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Abstract

Isolated rat hepatocytes were used to elucidate the metabolism of praziquantel (PZQ). Our studies were designed to investigate mainly qualitative differences in the biotransformation of PZQ enantiomers. Additionally, the main metabolites *cis*- and *trans*-4-hydroxypraziquantel were determined semiquantitatively. For this purpose, racemic PZQ and both enantiomers were incubated with isolated rat hepatocytes. The incubation mixtures were investigated using high-performance liquid chromatography/mass spectrometry. Hepatocytes prepared from male Wistar rats were incubated in Krebs–Ringer buffer at 37°C for 4 h. Aliquots were withdrawn hourly throughout 4 h of incubation. We found that hepatocytes converted both enantiomers of PZQ to the major metabolites *cis*- and *trans*-4-hydroxypraziquantel. Additional metabolites were detected after incubating the *S*-(+)-enantiomer. These minor metabolites were identified by means of their mass/charge ratio as monohydroxypraziquantel metabolites of different, unknown structure.

Keywords: Praziquantel; Metabolism; HPLC/mass spectrometry; Rat hepatocytes

1. Introduction

Praziquantel (PZQ) (Fig. 1) is a broad-spectrum anthelmintic drug. The drug has proved useful especially in the treatment of schistosomiasis [1–3]. PZQ undergoes a pronounced first-pass metabolism in the liver, yielding preferentially monohydroxylated metabolites [4]. No unchanged drug is excreted in bile or urine. The drug is used in therapy as the racemate, although the anthelmintic activity is mainly associated with the *R*-(−)-enantiomer [5]. This paper describes the investigation of qualitative differences in the biotransformation of PZQ enantiomers. Additionally, the main metabolites *cis*- and *trans*-4-hydroxypraziquantel and non-metabolized praziquantel were determined semi-quantitatively by high-performance liquid chromatography (HPLC)/mass spectrometry after the incubation of both enantiomers and the racemate with isolated rat hepatocytes sepa-

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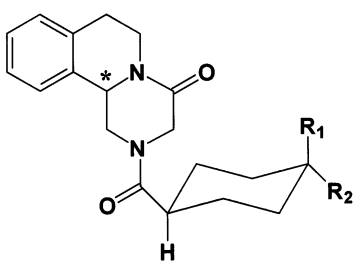
rately. In contrast to microsomes that had already been used for the investigation of praziquantel metabolism, hepatocytes represent an in vitro system with complete, uninterrupted, physiological levels of enzyme pathways and cofactors. The present consensus is that, for drug metabolism studies, hepatocytes in general behave more like the liver than other in vitro experimental systems [6]. Therefore, we used rat hepatocytes to investigate the stereoselectivity of praziquantel metabolism. The use of selective mass spectrometric detection allowed the identification of additional monohydroxypraziquantel metabolites of different, unknown structure.

2. Materials and methods

2.1. Chemicals and reagents

Racemic praziquantel was obtained from Merck (Darmstadt, Germany). *Trans*- and *cis*-4-hydroxypraziquantel were synthesized in our laboratory [7]. The enantiomers of praziquantel were separated by HPLC on a cellulose triacetate column. The optical purity of the enantiomers was determined to be $>99\%$ as described previously [8]. Bakerbond Octadecyl SPE columns were purchased from Mallinckrodt Baker (Griesheim, Germany). All chemicals were purchased from commercial sources as analytical or reagent grade, and were used without any additional treatment.



	R_1	R_2
praziquantel (1)	H	H
cis-4-hydroxypraziquantel (2)	OH	H
trans-4-hydroxypraziquantel (3)	H	OH

Fig. 1. Structure of praziquantel (1), *cis*-4-hydroxypraziquantel (2) and *trans*-4-hydroxypraziquantel (3).

2.2. Hepatocyte incubations

Hepatocytes were prepared from male Wistar rats using a two-step perfusion and collagenase digestion procedure as described elsewhere [9]. The viability of freshly isolated hepatocytes was determined by trypan blue exclusion and amounts to 84%. Because no experimental data was available, four different amounts of hepatocytes (2×10^6, 1×10^7, 2×10^7 and 1×10^8 cells/10 ml) were incubated in Krebs–Ringer buffer (pH 7.4) containing 5.5 mM glucose, 1 mM glutamine, 0.6% bovine serum albumen and 20 mM HEPES. Racemic praziquantel, *S*-(+)-praziquantel or *R*-(−)-praziquantel (2 mg/ml in methanol) was added to achieve a final concentration of 20 μg/ml. Hepatocytes were incubated for 4 h at 37°C. Aliquots (1000 μl) of the incubation mixture were withdrawn after 1, 2, 3 and 4 h of incubation. The samples were mixed with 1000 μl of acetonitrile to terminate the metabolism and stored at −20°C until analysis.

2.3. Liquid chromatography and mass spectrometry

The incubation samples were analyzed by HPLC/mass spectrometry. The chromatographic separation was performed on a 200×2.1 mm column packed with Superspher RP-18e (Merck) using a Waters 2690 separation module (Waters, Eschborn, Germany). The gradient system used for the chromatographic separation is presented in Table 1. The mobile phase was delivered at a flow rate of 0.2 ml min^{-1}. Samples were analyzed using an LCQ ion trap mass spectrometer (Thermoquest, San Jose, CA, USA). The liquid flow from the HPLC column was directed to the electrospray interface without a split. The mass spectrometer was operated in positive ion mode with the following key parameter settings: capillary temperature, 250°C; source voltage, 6.0 kV; full-scan mode ($m/z = 250$–600); sheath gas flow, 60 arbitrary units nitrogen; auxiliary gas flow, 20 arbitrary units nitrogen.

Table 1
HPLC gradient system used for the chromatographic separation of the PZQ metabolites

Time (min)	Acetonitrile (%)	Methanol (%)	0.01% Trifluoroacetic acid (v/v) (%)
0	10	12	78
10	12	12	76
20	14	12	74
21	15	5	80
60	50	5	45

2.4. Preparation of the samples

Prior to the analysis, solid-phase extraction (SPE) was performed. The cyclobutyl analogue of praziquantel (2-cyclobutylcarbonyl-1,2,3,6,7,11b-hexahydro-4*H*-pyrazino-[2,1-a]-isochinolin-4-on) was dissolved in methanol and used as internal standard (I.S.). Then, 50 μl I.S. solution was added to 800 μl incubation mixture. Acetonitrile had to be removed from the samples to ensure the retention of the analytes on the sorbent. Therefore, acetonitrile was removed under a steady stream of nitrogen. The remaining volume of the sample was acidified by adding 250 μl of 0.01% trifluoroacetic acid (TFA) (v/v). SPE was performed using 100 mg C_{18} cartridges to remove matrix components and to preconcentrate the metabolites. The cartridges were preconditioned with 3 ml methanol and 1 ml 0.01% TFA (v/v). A volume of 400 μl of each sample was slowly passed through the cartridges. The cartridges were rinsed with 400 μl 0.01% TFA (v/v) and the sorbent was dried for 10 min by applying a vacuum. In the next step, the analytes were eluted with 300 μl methanol and the extract was evaporated at ambient temperature under a steady stream of nitrogen. The residue was reconstituted in 50 μl of 0.01% TFA (v/v)/methanol (1:1), and 20 μl were analyzed by HPLC/mass spectrometry.

2.5. Calibration of the assay

Stock solutions were prepared by dissolving praziquantel, *cis*-4-hydroxypraziquantel and *trans*-4-hydroxypraziquantel in acetonitrile. The stability of the analytes in solution was investigated by HPLC/mass spectrometry analysis of quality control samples stored in a refrigerator at 4°C as well as at room temperature (21°C) every day for five consecutive days. Dilutions of the stock were made with acetonitrile/water (1:1) in order to yield working solutions at seven concentration levels, which were used for the preparation of calibration samples. Calibration samples were obtained by spiking the incubation buffer/acetonitrile (1:1) with working solutions in appropriate concentrations. Additionally, 50 μl of the I.S. stock solution was added. The concentration of the I.S. in the calibration samples was 294 μg/l. Each concentration level was analyzed three times. Linear regression analysis was performed by plotting the concentration versus the peak area ratios. The calibration curves were divided into two parts. The results of linear regression analysis are presented in Table 2.

3. Results and discussion

Because of the complex nature of the samples, gradient elution was necessary to achieve a satisfactory chromatographic separation of the analytes. The separation of different monohydroxylated metabolites detected after the incubation of *S*-(+)-PZQ with rat hepatocytes was achieved only using the optimized gradient system containing acetonitrile, methanol and 0.01% trifluoroacetic acid (v/v). The investigation of the reconstructed ion chromatograms of the mass track $m/z = 329$, corresponding to the monohydroxypraziquantel metabolites, revealed differences in the metabolism of the enantiomers of PZQ. In Fig. 2, representative reconstructed ion chromatograms obtained after incubation of

S-(+)-PZQ and *R*-(−)-PZQ with rat hepatocytes are presented. The two most abundant signals observed after incubation of *R*-(−)-PZQ were identified as *trans*- and *cis*-4-hydroxypraziquantel by spiking the samples with the authentic compounds. After the incubation of the *S*-(+)-enantiomer, additional signals were observed (M1–M4). These results are in agreement with those obtained by Westhoff [10], who found that *R*-(−)-PZQ was mainly metabolized to *cis*- and *trans*-4-hydroxypraziquantel, whereas *S*-(+)-PZQ yielded additional monohydroxylated metabolites after the incubation with rat liver microsomes.

As described in Section 2.2, both enantiomers of praziquantel were incubated with hepatocytes and aliquots were withdrawn after 1, 2, 3 and 4 h of incubation. In Figs. 3 and 4, the concentration of *cis*- and *trans*-4-hydroxypraziquantel after the incubation of PZQ with 2×10^6 hepatocytes/10 ml for 1–4 h is presented. The concentration of

Table 2
Results of linear regression analysis of calibration data

	Part I (50–500 μg/l)	Part II (500–4000 μg/l)
Praziquantel		
Slope	0.0028	0.0023
$R.S.D._{slope}$ (%)	9.2785	0.9422
Intercept	0.0032	0.4579
$R.S.D._{intercept}$ (%)	28.3953	26.9853
Correlation coefficient (*r*)	0.9999	0.9990
Cis-*4-hydroxy-praziquantel*		
Slope	0.0034	0.0025
$R.S.D._{slope}$ (%)	5.5450	11.6165
Intercept	0.0032	0.5318
$R.S.D._{intercept}$ (%)	43.3865	44.4565
Correlation coefficient (*r*)	0.9996	0.9996
Trans-*4-hydroxy-praziquantel*		
Slope	0.0015	0.0013
$R.S.D._{slope}$ (%)	7.1698	7.6841
Intercept	0.0025	0.1453
$R.S.D._{intercept}$ (%)	42.7817	48.5467
Correlation coefficient (*r*)	0.9999	0.9998

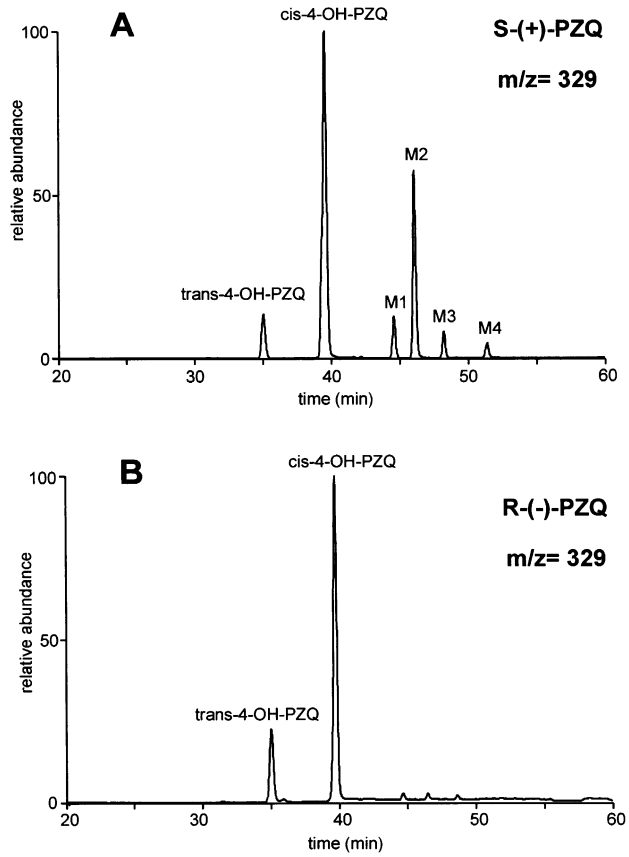


Fig. 2. Reconstructed ion chromatograms ($m/z = 329$) obtained after incubation of *S*-(+)-PZQ (A) and *R*-(−)-PZQ (B) using 2×10^7 hepatocytes/10 ml for 1 h.

cis-4-hydroxypraziquantel determined by HPLC/mass spectrometry was higher than the concentration of *trans*-4-hydroxypraziquantel for both enantiomers. Additionally, stereoselective differences were observed. The *R*-(−)-enantiomer of PZQ produced higher concentrations of *cis*- and *trans*-4-hydroxypraziquantel than the *S*-(+)-enantiomer (Figs. 3 and 4).

With increasing incubation time, the remaining concentration of non-metabolized *R*-(−)-PZQ decreased faster than the concentration of *S*-(+)-PZQ (Table 3). If 1×10^7 hepatocytes/10 ml were used in our in vitro experimental system, 2 h after starting the incubation non-metabolized *R*-(−)-PZQ was already no longer detectable (Table 3). These results confirm previous investigations of

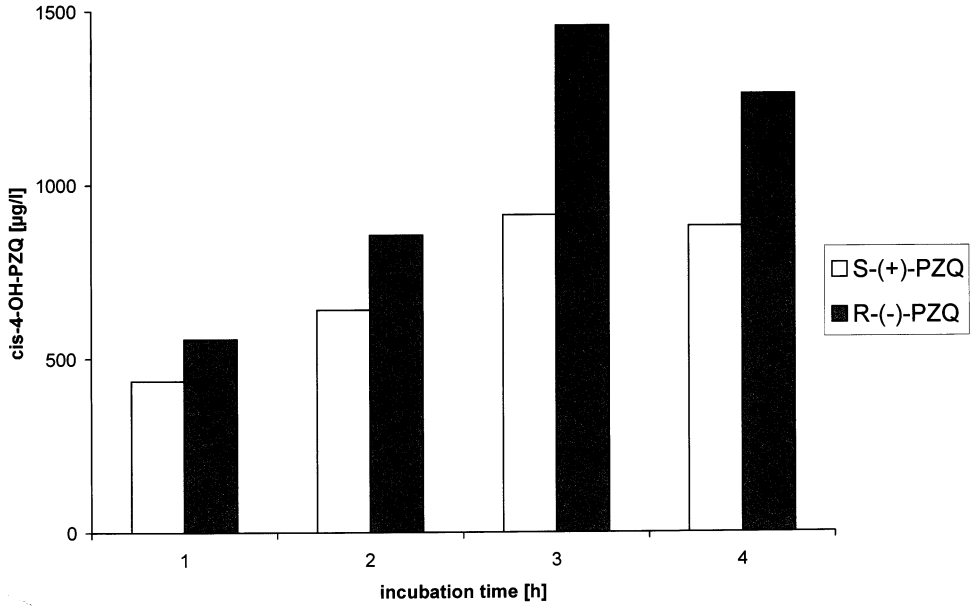


Fig. 3. Concentration of *cis*-4-hydroxypraziquantel obtained after incubation of *S*-(+)-PZQ and *R*-(−)-PZQ using 2×10^6 hepatocytes/10 ml for 1–4 h.

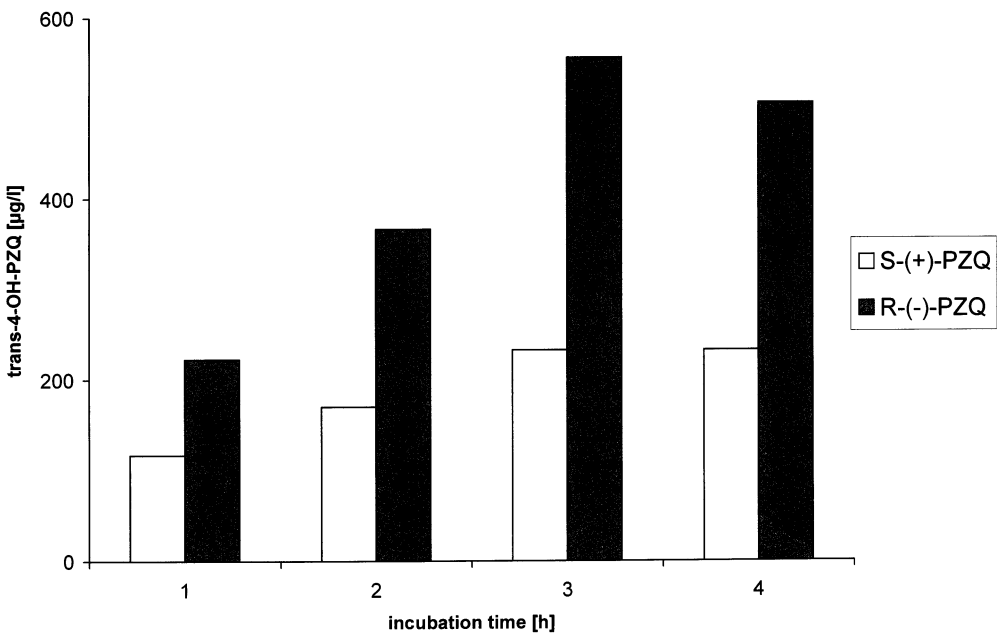


Fig. 4. Concentration of *trans*-4-hydroxypraziquantel obtained after incubation of *S*-(+)-PZQ and *R*-(−)-PZQ with 2×10^6 hepatocytes/10 ml for 1–4 h.

Table 3
Concentration of non-metabolized *S*-(+)-PZQ and *R*-(−)-PZQ after incubation using 2×10^6 hepatocytes/10 ml and 1×10^7 hepatocytes/10 ml for 1–4 h[a]

	Incubation time (h)	*R*-(−)-PZQ (μg/l)	*S*-(+)-PZQ (μg/l)
2×10^6 hepatocytes/10 ml	1	>3999.983*	>3999.983*
	2	3608.262	>3999.983*
	3	3049.789	>3999.983*
	4	2354.773	3749.250
1×10^7 hepatocytes/10 ml	1	640.822	2797.960
	2	Not detectable	577.816
	3	Not detectable	79.904
	4	Not detectable	<50.003*

[a] The results marked with an asterisk (*) were beyond the calibrated range of the assay.

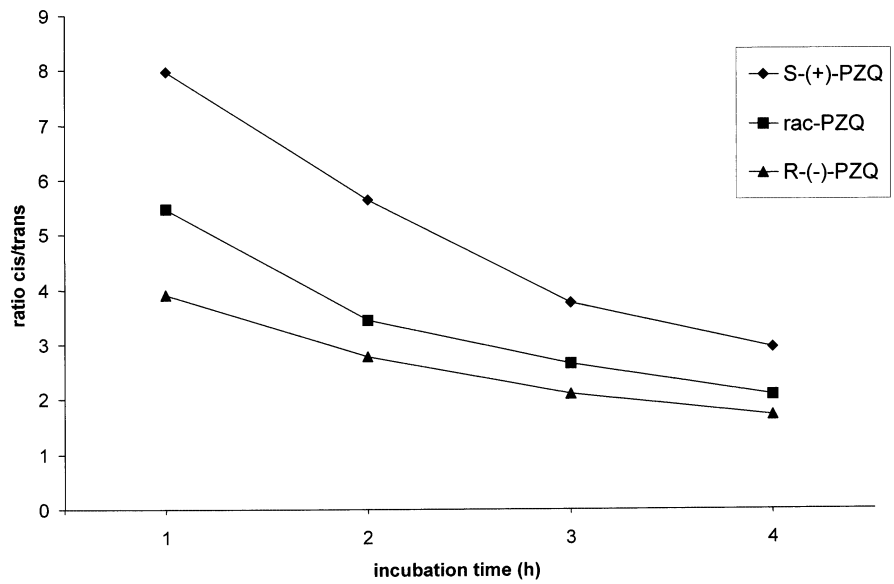


Fig. 5. Peak area ratio of *cis*-/*trans*-4-hydroxypraziquantel obtained after incubation of *S*-(+)-PZQ, racemic PZQ and *R*-(−)-PZQ using 2×10^7 hepatocytes/10 ml for 1–4 h.

Kaup [11], who found lower plasma levels of PZQ after application of *R*-(−)-PZQ than after the application of *S*-(+)-PZQ to rats.

Because *cis*- and *trans*-4-hydroxypraziquantel were further functionalized and/or conjugated with endogenous compounds and non-metabolized PZQ was no longer present in the incubation mixture at the same time, the concentration of *cis*- and *trans*-4-hydroxypraziquantel decreased with increasing incubation time if 1×10^7 hepatocytes/10 ml or more were used. In Fig. 5, the peak area ratio of *cis*- and *trans*-4-hydroxypraziquantel is plotted versus the incubation time using 2×10^7 hepacytes/10 ml. It is obvious that the concentration of *cis*-4-hydroxypraziquantel decreased faster than the concentration of *trans*-4-hydroxypraziquantel with increasing incubation time. This may be explained by a more stable behaviour of *cis*-4-hydroxypraziquantel in contrast to *trans*-4-hydroxypraziquantel towards additional metabolic changes.

4. Conclusions

The results of our investigations emphasize the stereoselective metabolism of praziquantel. In our studies, hepatocytes were used that behave more like the liver than any other in vitro experimental system. Qualitative as well as quantitative differences in the metabolism of the enantiomers of PZQ were observed. *R*-(−)-PZQ produced *cis*- and *trans*-4-hydroxypraziquantel, whereas *S*-(+)-PZQ produced additional monohydroxypraziquantel metabolites. Additionally, we found that *R*-(−)-PZQ in comparison with *S*-(+)-PZQ produced higher concentrations of *cis*- and *trans*-4-hydroxypraziquantel after incubation with rat hepatocytes.

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References

[1] P. Andrews, H. Thomas, R. Pohlke, J. Seubert, Med. Res. Rev. 2 (1983) 147.
[2] C.A. Redman, A. Robertson, P.G. Fallon, J. Modha, J.R. Kusel, M.J. Doenhoff, R.J. Martin, Parasitol. Today 12 (1996) 14.
[3] D. Cioli, L. Pica-Mattoccia, S. Archer, Pharmacol. Ther. 68 (1995) 35.
[4] K.U. Bühring, H.W. Diekmann, H. Müller, A. Garbe, H. Nowak, Eur. J. Drug Metab. Pharm. 3 (1978) 179.
[5] U. Staudt, G. Schmahl, G. Blaschke, H. Mehlhorn, Parasitol. Res. 78 (1992) 392.
[6] A.P. Li, P.D. Gorycki, J.G. Hengstler, G.L. Kedderis, H.G. Koebe, R. Rahmani, G. de Sousas, J.M. Silva, P. Skett, Chem. Biol. Interact. 121 (1999) 117.
[7] K. Kiec-Kononowicz, Z.S. Farghaly, G. Blaschke, Arch. Pharm. (Weinheim) 324 (1991) 235.
[8] J. Liu, J.T. Stewart, J. Chromatogr. B 692 (1997) 141.
[9] P. Moldeus, J. Högberg, S. Orrenius, Methods Enzymol. 52 (1978) 60.
[10] F. Westhoff, G. Blaschke, J. Chromatogr. 578 (1992) 265.
[11] A. Kaup, Ph.D. Thesis, University of Münster, Münster, 1989.