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High-performance liquid chromatography determination of praziquantel enantiomers in human serum using a reversed-phase cellulose-based chiral stationary phase and disc solid-phase extraction

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Abstract

A sensitive HPLC method for the quantification of praziquantel enantiomers in human serum is described. The method involves the use of a novel disc solid-phase extraction for sample clean-up prior to HPLC analysis and is also free of interference from *trans*-4-hydroxypraziquantel, the major metabolite of praziquantel. Chromatographic resolution of the enantiomers was performed on a reversed-phase cellulose-based chiral column (Chiralcel OJ-R) under isocratic conditions using a mobile phase consisting of 0.1 *M* sodium perchlorate–acetonitrile (66:34, v/v) at a flow-rate of 0.5 ml/min. Recoveries for *R*-(−)- and *S*-(+)-praziquantel enantiomers were in the range of 84–89% at 50–500 ng/ml levels. Intra-day and inter-day precisions calculated as R.S.D. were in the ranges of 3–8% and 1–8% for both enantiomers, respectively. Intra-day and inter-day accuracies calculated as percent error were in the 0.2–5% and 0.3–8% ranges for both enantiomers, respectively. Linear calibration curves were in the concentration range 10–600 ng/ml for each enantiomer in serum. The limit of quantification of each enantiomer was 10 ng/ml. The detection limit for each enantiomer in serum using a UV detector set at 210 nm was 5 ng/ml ($S/N=2$).

Keywords: Enantiomer separation; Praziquantel

1. Introduction

Praziquantel, 2-cyclohexylcarbonyl-1,2,3,6,7,11b-hexahydro-4H-pyrazino[2,1-*a*]iso-quinolin-4-one, is a synthetic heterocyclic anthelmintic agent. It is mainly used for the treatment of schistosomiasis caused by all *Schistosoma* species pathogenic to humans, clonorchiasis caused by *Clonorchis sinensis*, and opisthorchiasis caused by *Opisthorchis viverrini* [1–3]. Praziquantel is a chiral compound with an asymmetric center at the 11b position (Fig. 1) and usually used in an antischistosomal therapy as its racemate although its effect is attributed to the *R*-(−)-enantiomer with the *S*-(+)-enantiomer considered to be ineffective [4,5]. In addition, it was reported that there are differences in intrinsic clearance of *R*-(−)- and *S*-(+)-enantiomers [6].

There are several assays available for the determination of praziquantel in biological samples with the use of HPLC, GC–MS, radiometric and fluorimetric techniques [7–11]. Only two papers involved in the chiral separation and determination

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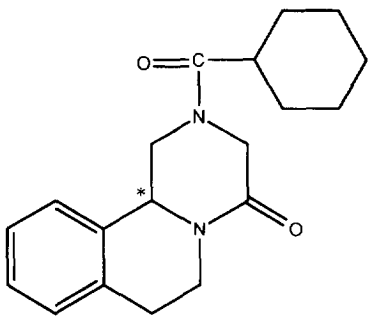
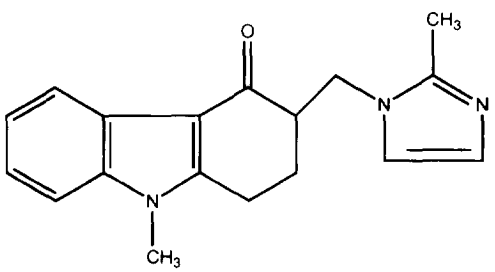


Praziquantel



Ondansetron (IS)

Fig. 1. Chemical structures of praziquantel and ondansetron (I.S.).

of praziquantel enantiomers have been reported in which a cellulose-based chiral stationary phase (Chiralcel OD) was used and operated in a normal-phase mode [12,13].

In this paper, we describe a sensitive and stereospecific assay for the quantification of praziquantel enantiomers in human serum using a reversed-phase cellulose-based chiral stationary phase (Chiralcel OJ-R) and disc solid-phase extraction. The method is linear over the range 10–600 ng/ml using a UV detector set at 210 nm. The detection limit of the method for each enantiomer was 5 ng/ml ($S/N=2$). The procedure is free of interference from *trans*-4-hydroxypraziquantel, the major metabolite of praziquantel.

2. Experimental

2.1. Reagents and chemicals

Racemic praziquantel reference standard was obtained from the United States Pharmacopeial Convention (Rockville, MD, USA). The *R*-(−)- and *S*-(+)-enantiomers were kindly supplied by Dr. Y.H. Liu of Chongqing University of Medical Science (Chongqing, P.R. China). The purity of the individual enantiomers was determined to be more than 99% (w/w) based on the USP 23 assay [14]. The internal standard ondansetron hydrochloride was obtained from Glaxo (Research Triangle Park, NC, USA). Racemic *trans*-4-hydroxypraziquantel was supplied by Dr. G. Blaschke of University of Münster Institute of Pharmaceutical Chemistry (Münster, Germany). HPLC-grade acetonitrile was purchased from J.T. Baker (Phillipsburg, NJ, USA). HPLC-grade sodium perchlorate was obtained from Fisher Scientific (Pittsburgh, PA, USA). Drug-free human serum was obtained from Biological Specialty Corporation (Colmar, PA, USA). C_{18}, C_8, cyanopropyl and phenyl solid-phase extraction columns (100 mg/1 cc size) were obtained from Varian Sample Preparation Products (Harbor City, CA, USA). C_{18}, C_{18} AR and C_8 solid-phase extraction discs (15 mg/3 cc size) were obtained from Ansys (Irvine, CA, USA). The Vac-Elut vacuum manifold was obtained from Analytichem International (Sunnyvale, CA, USA).

2.2. Chromatographic system

The HPLC system consisted of an Alcott Model 760 HPLC pump (Norcross, GA, USA), an Alcott Model 728 auto-sampler (Norcross, GA, USA) equipped with a 100-μl loop, a Kratos Model 757 variable-wavelength UV–Vis detector (Ramsey, NJ, USA) set at 210 nm and a Hewlett-Packard Model 3395 integrator (Avondale, PA, USA). The Chiralcel OJ-R column (5 μm, 150×4.6 mm I.D.) equipped with an opti-solv mini filter 2 μm (Optimize Technologies, Portland, OR, USA) was obtained from Chiral Technologies (Exton, PA, USA) and operated at ambient temperature. The mobile phase consisted of 0.1 *M* sodium perchlorate–acetonitrile (66:34,

v/v) and was delivered at a flow-rate of 0.5 ml/min. The solution was filtered through a 0.45-μm nylon membrane obtained from Alltech Associates (Deerfield, IL, USA) and sonicated prior to use.

2.3. Preparation of standard solutions

Stock solutions of 0.1 mg/ml of *R*-(−)- and *S*-(+)-praziquantel were prepared in 10-ml volumetric flasks by dissolving 1.0 mg of each enantiomer in 3 ml of acetonitrile followed by the addition of deionized water to volume and stored protected from light at 4°C. Stock solutions of 15 mg/ml of internal standard ondansetron hydrochloride calculated as the free base and 0.1 mg/ml of *trans*-4-hydroxypraziquantel were also prepared in the same way as stock solutions of praziquantel enantiomers and stored protected from light at 4°C. Appropriate dilutions of the *R*-(−)- and *S*-(+)-praziquantel stock solutions with deionized water gave 1, 10 and 20 μg/ml solutions which were used for spiking blank human serum.

2.4. Preparation of spiked human serum samples

Accurately measured aliquots (10 μl of the 1 μg/ml standard solution, 8 and 20 μl of the 10 μg/ml standard solutions, and 30 μl of the 20 μg/ml standard solution) of *R*-(−)- and *S*-(+)-praziquantel were each added into 1-ml volumetric tubes followed by the addition of 20 μl of 15 μg/ml of internal standard solution and 5 μl of 0.1 mg/ml of *trans*-4-hydroxypraziquantel. Drug-free human serum was added to volume and mixed well to give final concentrations of 10, 80, 200 and 600 ng/ml of each enantiomer.

2.5. Assay method

Solid-phase extraction (SPE) discs (C_{18} AR) were attached to a vacuum manifold and conditioned with 2×1 ml of acetonitrile followed by 2×1 ml of distilled water (Note: do not allow sorbent to dry). Into the discs were transferred blank and spiked human serum samples and a small vacuum (2 kPa) was applied. After the entire serum sample had been aspirated through the disc, the disc was washed with 600 μl of acetonitrile–water (25:75, v/v), and then dried under full vacuum for a minimum of 5 min, and then the vacuum manifold was opened and collection tips were wiped. The praziquantel enantiomers and internal standard were eluted with 2×300 μl of acetonitrile. The eluent was evaporated to dryness under a nitrogen stream at ambient temperature. The residue was redissolved in 800 μl of mobile phase and triplicate 100-μl injections were made into the liquid chromatograph. Linear regression analysis of enantiomer concentration versus peak-area ratios of each praziquantel enantiomer to internal standard produced slope and intercept data which were used to calculate concentrations of *R*-(−)- and *S*-(+)-praziquantel in each serum sample.

3. Results and discussion

Reversed-phase cellulose-based chiral stationary phases employ the same chiral selectors as their normal-phase counterparts (e.g., Chiralcel OD-R vs. Chiralcel OD and Chiralcel OJ-R vs. Chiralcel OJ), but are designed for analytical applications using reversed-phase chromatography. Reversed-phase chromatographic techniques are preferable in pharmaceutical analysis to normal-phase chromatographic techniques since they involve the use of less organic solvents and do not use environmentally harmful solvents such as hexane, chloroform and methylene chloride. Besides, many drugs exist as salts which are water soluble and are easier to prepare samples for the reversed-phase chromatography. Praziquantel enantiomers were not resolved on the Chiralcel OD-R column although praziquantel enantiomers in serum were determined in our laboratories by a normal-phase HPLC method using a Chiralcel OD column [12]. However, a successful resolution was achieved in the reversed-phase mode on the Chiralcel OJ-R column. Initial separation of the enantiomers (R_s=2.4) with retention times of 18–24 min was obtained using a mobile phase consisting of 0.5 *M* aqueous sodium perchlorate–acetonitrile (70:30, v/v). The influences of buffer type, pH and concentration and amounts of acetonitrile in the mobile phase on resolution of the analytes of interest were investigated. The function of buffers in a column of this type is to suppress ionization of acidic and basic analytes since ionization of analytes

causes deformation and tailing of the analyte peaks. Since praziquantel is a chemically neutral compound, it was predicted that there would be no significant contributions of buffer type (phosphate and perchlorate), pH and concentration on the resolution of praziquantel enantiomers. The experiments performed in this laboratory confirmed those predictions. An increase in the concentration of acetonitrile in the mobile phase reduced retention times with loss of some resolution of the analytes of interest. In our case, whether a buffer or deionized water was used in the mobile phase was dependent on the internal standard used. If the internal standard was an acidic or basic compound, a mobile phase consisting of a buffer–acetonitrile instead of deionized water–acetonitrile as a mobile phase was necessary to prevent the internal standard peak from being deformed. The final mobile phase consisting of 0.1 *M* sodium perchlorate–acetonitrile (66:34, v/v) provided good peak shape for the internal standard, baseline separation of the two enantiomer peaks (R_s=2.7), suitable retention times (14–16 min for praziquantel enantiomers and 6.6 min for the internal standard), and sensitivity in the desired ng/ml range. Typical HPLC chromatograms for blank human serum and serum spiked with 80 ng/ml of each enantiomer and 300 ng/ml of internal standard are shown in Fig. 2.

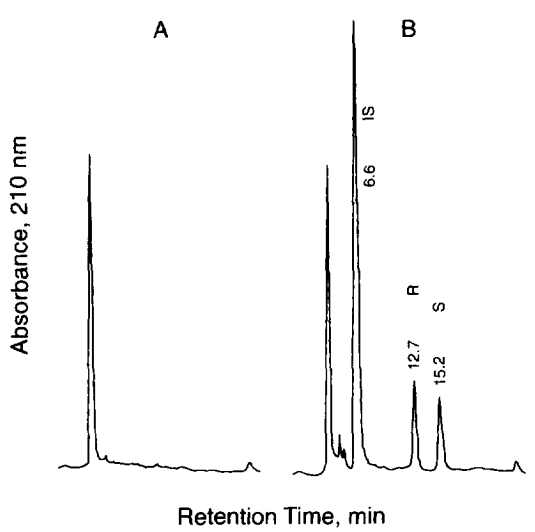


Fig. 2. Typical chromatograms of blank serum (A) and serum spiked with 80 ng/ml of each enantiomer and 300 ng/ml of internal standard (B). Peaks: R=*R*-(−)-praziquantel, S=*S*-(+)-praziquantel and I.S.=internal standard, ondansetron.

No interferences were observed in blank human serum at the retention times of *R*-(−)- and *S*-(+)-praziquantel peaks.

The selection of an internal standard was difficult. When the mobile phase consisted of deionized water–acetonitrile (66:34, v/v), neutral praziquantel enantiomers were separated well, and a neutral compound could be selected as an internal standard. Some neutral compounds with an amide group in their structures were tested, but only lorazepam had a suitable retention time (11 min). However, the recovery for lorazepam from serum was too low to be used as an internal standard. Finally, ondansetron was selected as internal standard based on its suitable retention time (6.6 min) and a separation factor (α) of 3.14 between ondansetron and the first eluting *R*-(−)-praziquantel enantiomer using a mobile phase of 0.1 *M* sodium perchlorate–acetonitrile (66:34, v/v). Quantification was based on the plot of concentration of each enantiomer versus peak-area ratios of each praziquantel enantiomer to the internal standard.

The suitability of the system for the separation of the praziquantel enantiomers is shown in Table 1. The retention times of *R*-(−)- and *S*-(+)-praziquantel and internal standard ondansetron were 12.69±0.011, 15.22±0.012 and 6.62±0.024 min, respectively (n=6). Capacity factors (k') for *R*-(−)- and *S*-(+)-praziquantel enantiomers and internal standard were 2.32±0.002, 2.98±0.004 and 0.73±0.005, respectively (n=6). The calculated theoretical plates for *R*-(−)- and *S*-(+)-praziquantel enantiomers were 3183±5 and 3707±6 per 15 cm column (n=6). Relative retention of the *R*-(−)- and *S*-(+)-praziquantel enantiomers, as expressed by the separation factor α, was calculated to be 1.29. Resolution (R_s) of the internal standard and first eluting *R*-(−)-enantiomer peaks and of the *R*-(−)- and *S*-(+)-praziquantel enantiomer peaks were 6.7 and 2.7, respectively.

The cartridge solid-phase extraction technique was first introduced in the late 1970s and is still widely used for biological sample clean-up. However, clean-up effect of this technique is sometimes affected severely by its limitations such as restricted flow-rates, channeling and the excessive bed mass which increases the amount of nonspecific material introduced into the analytical instrument [15]. Compared with the solid-phase cartridge technique, the disc

Table 1
Chromatographic parameter data for praziquantel enantiomers and internal standard in spiked human serum

Analyte	R_s	α[a]	k'[b]	t_R (min)[b]	N[b,c]
Ondansetron (I.S.)			0.73±0.005	6.62±0.02	–[d]
	6.7	3.14			
R-(−)-Praziquantel			2.32±0.002	12.69±0.01	3183±5
	2.7	1.29			
S-(+)-Praziquantel			2.98±0.004	15.22±0.01	3707±6

[a] Separation factor, calculated as k'_2/k'_1.
[b] Mean±S.D., $n=6$.
[c] Theoretical plates, calculated as $N=16\ (t_R/w)^2$.
[d] Not calculated.

solid-phase technique is relatively new with advantages of reduced bed mass (less capacity of retaining impurities), no channeling, large flow area and rigid structure. In this study, four solid-phase extraction cartridges (C_{18}, C_8, phenyl and cyanopropyl) and three solid-phase extraction discs (C_{18}, C_{18} AR, and C_8) were investigated for serum sample clean-up prior to the HPLC assay. All tested cartridges showed interfering endogenous serum peaks at the retention times for praziquantel enantiomers, the C_{18} and C_8 discs showed less amount of impurities from serum, but were still not acceptable for sample clean-up. The C_{18} AR disc provided the best results in terms of clean-up and recoveries of *R*-(−)- and *S*-(+)-praziquantel enantiomers. The concentration of acetonitrile in washing solutions during solid-phase extraction process was critical in terms of recovery and clean-up. When 0.6 ml of deionized water–acetonitrile (75:25, v/v) was used, the best clean-up was obtained with reasonable and reproducible recoveries for analytes of interest. If the concentration of acetonitrile in washing solutions was less than 20% or more than 30%, the clean-up was either not good enough to be used for assay, especially in the lower concentrations of praziquantel enantiomers due to co-elution of impurities from serum or there was almost complete loss of the analytes of interest.

The method was shown to be free of interference from spiked human serum samples containing *trans*-4-hydroxypraziquantel, the major metabolite of praziquantel. The metabolite had a retention time of 4.6 min and was well separated from the praziquantel enantiomers (t_R=14–16 min) and internal standard (t_R=6.6 min). In addition, there was no recovery of *trans*-4-hydroxypraziquantel from the solid-phase extraction procedures. If an analyst wanted to determine the amount of the hydroxy-metabolite of praziquantel in serum, the solid-phase extraction procedures would have to be significantly modified to obtain a reasonable recovery of this metabolite.

The recoveries of *R*-(−)- and *S*-(+)-praziquantel enantiomers from human serum were assessed by using spiked samples at several concentration levels. The absolute recoveries of *R*-(−)- and *S*-(+)-praziquantel were determined by a comparison of the extracted analyte peak area with the unextracted analyte peak area. The results are shown in Table 2.

Linear calibration curves were obtained in the 10–600 ng/ml range for each enantiomer of praziquantel. Standard curves were fitted to a first degree polynomial, $y=ax+b$, where y is the concentration of praziquantel enantiomer, x is the ratio of drug/internal standard peak areas, and a and b are constants. Typical values for the regression parameters a (slope), b (intercept) and correlation coefficient were calculated to be 429.9571, 3.345613 and 0.9995 for *R*-(−)-enantiomer, and 427.0736, 2.980158 and 0.9994 for *S*-(+)-enantiomer, respectively (n=8). The precision and accuracy (percent error) of the method were determined by using human serum samples spiked at 50, 150 and 500 ng/ml levels (Table 3). The data indicate that intra-day precision was in the 3–8% range (n=3) and intra-day accuracy in the 0.2–5% range (n=3) for both praziquantel enantiomers and that inter-day precision was in the 1–8% range (n=6) and inter-day accuracy in the 0.3–8% range (n=6) for both praziquantel enantiomers.

The minimum detectable concentration of each enantiomer of praziquantel was determined to be 5

Table 2
Absolute recovery data for praziquantel enantiomers in human serum

Analyte	Concentration level (ng/ml)	Recovery[a] (mean±S.D., $n=6$) (%)	R.S.D. (%)
R-(−)-Praziquantel	50	88.69±6.12	6.9
	150	84.18±4.25	5.1
	500	85.92±3.12	3.6
S-(+)-Praziquantel	50	85.03±4.42	5.2
	150	84.64±3.12	3.7
	500	87.08±3.05	3.5

[a] Recoveries were calculated by a comparison of the extracted analyte peak area to the unextracted analyte peak area.

Table 3
Accuracy and precision data for praziquantel enantiomers in human serum

Analyte	Concentration added (ng/ml)	Concentration found[a] (ng/ml)	Error (%)	R.S.D. (%)
Intra-day				
R-(−)-Praziquantel	50	44.76±1.48	10.5	3.3
	150	152.98±5.04	2.0	3.3
	500	494.17±20.09	1.2	4.1
S-(+)-Praziquantel	50	47.78±3.75	4.4	7.8
	150	149.78±5.21	0.2	3.5
	500	504.69±36.03	0.9	7.1
Inter-day				
R-(−)-Praziquantel	50	47.80±3.00	4.4	6.2
	150	153.24±6.55	2.2	4.3
	500	496.74±6.66	0.7	1.3
S-(+)-Praziquantel	50	54.04±3.47	8.1	6.4
	150	153.16±7.74	2.1	5.1
	500	502.65±15.09	0.5	3.0

[a] Based on $n=3$ for the intra-day study and $n=6$ for the inter-day study.

ng/ml ($S/N=2$). The lowest quantifiable level was found to be 10 ng/ml for each enantiomer: *R*-(−), 6.2% R.S.D., 9.7% error; *S*-(+), 0.4% R.S.D., 8.5% error.

In summary, an HPLC method has been developed and validated for the assay of *R*-(−)- and *S*-(+)-praziquantel enantiomers in human serum using a Chiralcel OJ-R column operated in the reversed-phase mode. The method utilizes a C_{18} AR disc solid-phase extraction for sample clean-up. The procedure is free of interference from *trans*-4-hydroxypraziquantel, the major metabolite of praziquantel, and is suitable for the separation and quantification of each enantiomer of praziquantel in 10–600 ng/ml range.

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