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Liquid chromatographic separation of praziquantel enantiomers in serum using a cellulose-based chiral stationary phase*

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Abstract: *R*(−) and *S*(+) praziquantel (PZQ) in human serum were resolved and quantified using a stereospecific LC method. Each enantiomer and the internal standard 2-methylamino-5-chlorobenzophenone were isolated from serum using a solid-phase extraction procedure on a cyanopropyl column. Recoveries of 98.8, 93.3 and 35.4% were obtained for *R*(−) PZQ, *S*(+) PZQ, and the internal standard, respectively. A cellulose-based chiral analytical column (Chiralcel OD) was used with a mobile phase consisting of hexane–2-propanol (70:30, v/v). Linear calibration curves were obtained in the concentration range 50–1000 ng ml^{-1} for each enantiomer in serum. The detection limit for each enantiomer in serum using UV detection at 212 nm was 5 ng ml^{-1} (*S/N* = 3). The limit of quantitation of each enantiomer was 25 ng ml^{-1}.

Keywords: *Liquid chromatography; praziquantel enantiomers; solid-phase extraction; serum; Chiralcel OD column.*

Introduction

Praziquantel, 12-cyclohexylcarbonyl-(1,2,3,6,7,11b) hexahydro-4H-pyrazine, (2, 1a) isoquinoline-4-one (PZQ) (Fig. 1) is an anthelmintic drug with activity against all known species of schistosoma, liver flukes, lung flukes and cestodes. The development of this drug was of particular significance for the treatment of *Schistosomiasis japonica* infections since there has previously been no safe and effective therapy [1, 2]. PZQ is a chiral compound which possesses an asymmetric center at carbon 11b (see Fig. 1). The anthelmintic activity is predominantly associated with the *R*(−) enantiomer [3]. Stereoselective differences in the pharmacodynamic, pharmacokinetic, and toxicologic properties of PZQ have been shown [4, 5]. *In vitro* results demonstrate the *R*(−) PZQ is 352 times more potent than racemic PZQ against *S. mansoni* [4]. The *S*(+) enantiomer has been shown to have only minimal biological potency at plasma levels that have been shown to cause some cardiotoxicity. Although both enantiomers give a bradycardia effect, only *S*(+) PZQ causes this effect at sub-therapeutic concentrations. Furthermore, the frequency of right atria arrhythmias in rabbits is greater with the *S*(+) enantiomer [6].

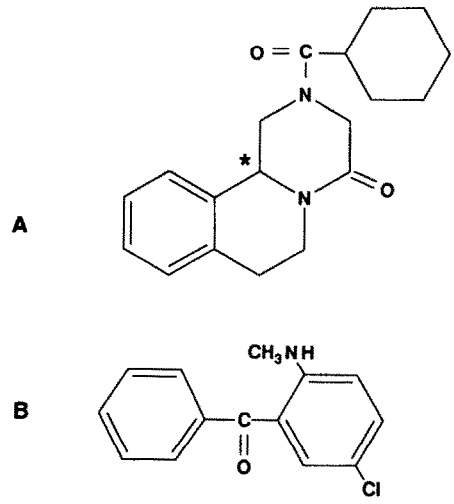

Figure 1
Chemical structures of praziquantel (A) and 2-methylamino-5-chlorobenzophenone (B). The asterisk denotes the chiral centre in praziquantel.

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Analytical methods for PZQ in the literature have mostly involved assay of racemic PZQ. Assays of the drug in biological samples have been reported using radiometry, fluorescence, GC and LC [7, 8]. Radiometry is limited in that the use of radioactive PZQ is not practical in routine clinical studies in patients. Fluorometric analysis involves a complex sample preparation and GC utilizes a chemical derivatization procedure prior to assay. The LC separation of PZQ and several metabolites of PZQ and determination of the enantiomeric ratio of the primary chiral metabolite, a *trans*-4-hydroxy PZQ, and PZQ were performed using tandem achiral/chiral columns [9]. Variable recoveries (77–93%) of PZQ and its metabolites were obtained from serum and urine using liquid–liquid extraction techniques.

Experimental

Reagents and chemicals

Praziquantel reference standard powder was obtained from the United States Pharmacopeial Convention (Rockville, MD). The $R(-)$ and $S(+)$ enantiomers were kindly supplied by Dr Y.H. Liu of Chongqing University of Medical Science (Chongqing, PR China). The internal standard 2-methylamino-5-chlorobenzophenone (see Fig. 1) was obtained from Aldrich (Milwaukee, WI). HPLC grade hexane, 2-propanol and acetonitrile were obtained from J.T. Baker (Phillipsburg, NJ). HPLC grade ethyl acetate was obtained from E.M. Science (Gibbstown, NJ). Drug-free human serum was obtained from Fisher Scientific (Pittsburgh, PA). Ethyl, octyl, octadecyl, phenyl and cyanopropyl solid-phase extraction columns (100 mg/1 cc size) and the VAC-Elut vacuum manifold were obtained from Analytichem (Sunnydale, CA).

Chromatographic system

The chromatograph consisted of a Beckman Model 110B pump (Fullerton, CA), a Rheodyne Injector Model 7125 (Cotati, CA) equipped with a 100 μl loop, a Kratos Spectroflow 757 variable wavelength UV detector (Ramsey, NJ) set at 212 nm, and a Hewlett–Packard Model 3392 integrator (Palo Alto, CA). The Chiralcel OD column (10 μm, 250 mm × 4.6 i.d.) equipped with a Chiralcel OD guard column (10 μm, 50 mm × 4.6 i.d.) were obtained from J.T. Baker (Phillipsburg, NJ). The mobile phase consisted of hexane–2-propanol (70:30, v/v) and was delivered at a flow rate of 1.0 ml min^{-1}. The solution was filtered (0.45 μm Nylon membrane) and sonicated prior to use. The chiral and guard columns were operated at ambient temperature (23 ± 1°C).

Preparation of standard solutions

Stock solutions (25 μg ml^{-1}) of $R(-)$ and $S(+)$ PZQ were prepared in absolute methanol and stored protected from light at ambient temperature (23 ± 1°C). A stock solution of 2-methylamino-5-chlorobenzophenone (12.8 μg ml^{-1}) in absolute methanol was also prepared and stored protected from light at ambient temperature.

Preparation of spiked human serum samples

Accurately measured aliquots (2, 5, 10, 20 and 40 μl) of the 25 μg ml^{-1} $R(-)$ and $S(+)$ PZQ standard solutions were each added to 1 ml volumetric tubes followed by the addition of 20 μl of internal solution. Blank human serum was added to volume to give standard solutions containing 50, 125, 250, 500 and 1000 ng ml^{-1} of each enantiomer.

Assay method

To a 1 ml human serum sample containing PZQ was added 20 μl of internal standard solution. After mixing for 10 s, the sample was transferred to a cyanopropyl solid-phase extraction column that had been pre-conditioned with one column volume of absolute methanol followed by one column volume of distilled water (N.B. do not allow sorbent to dry). After the entire serum sample had been aspirated through the column, the column was washed with one column volume of acetonitrile–water (10:90, v/v). The column was then dried under full vacuum for 10 min. The PZQ enantiomers and internal standard were eluted with 10–100 μl volumes of ethyl acetate. The ethyl acetate was evaporated to dryness under a nitrogen stream at 50°C. The residue was redissolved in 250 μl of mobile phase and duplicate 100 μl injections were made into the liquid chromatograph. Linear regression analysis of peak height ratios of each PZQ enantiomer to internal standard versus concentration of each enantiomer produced slope and intercept data which were used to calculate concentrations of $R(-)$ and $S(+)$ PZQ in each serum sample.

Results and Discussion

Initial studies in these laboratories concerning the separation of $R(-)$ and $S(+)$ PZQ enantiomers had found that a mobile phase of hexane–ethanol–2-propanol–acetonitrile (65:25:10:0.5, v/v/v/v) produced baseline separation (Rs = 1.5) of the enantiomers on a Chiralcel OD column with retention times of less than 10 min. The addition of both ethanol and acetonitrile to the mobile phase improved peak shape and greatly influenced k' of each analyte. As the assay development progressed, it became obvious that these polar modifiers were detrimental to the separation of the PZQ enantiomers and internal standard in serum extracts due to interfering peaks in the chromatograms. Finally, a mobile phase of hexane–2-propanol (70:30, v/v) was selected for the serum assay. This mobile phase, together with the Chiralcel OD column, provided a suitable separation of the PZQ enantiomers in a reasonable run time with sensitivity in the desired low ng ml^{-1} range.

System suitability for the PZQ enantiomers was based on the following chromatographic parameters and the performance of the method throughout validation. The retention times of $R(-)$ and $S(+)$ PZQ were 11.75 ± 0.14 and 14.16 ± 0.12 min, respectively (n = 4). Capacity factors (k') for the $R(-)$ and $S(+)$ enantiomers were 2.27 ± 0.04 and 2.93 ± 0.03, respectively (n = 4). The respective number of theoretical plates for the $R(-)$ and $S(+)$ enantiomers were 1524 ± 181 and 1533 ± 460 per 25 cm column (n = 4). Relative retention of $R(-)$ and $S(+)$ PZQ was expressed by the separation factor α, calculated to be 1.30 ± 0.02 (n = 4) from the ratio of the capacity factors. These data indicated that the method was suitable for analysis of the enantiomers in human serum.

The selection of an internal standard for use in this assay was difficult. Many amide-containing compounds were screened since PZQ also contained an amide group in its structure. Unfortunately, no suitable amide compound was found. Finally, 2-methylamino-5-chlorobenzophenone was selected as the internal standard based on its 8.12 ± 0.13 min retention time, k' of 1.26 ± 0.04, 2609 ± 108 theoretical plates on the 25 cm column, and an α of 1.80 ± 0.04 between the internal standard and the first eluting $R(-)$ PZQ enantiomer (n = 4). Quantitation was based on peak height ratios of each enantiomer to the internal standard.

No interferences were observed in blank human serum at the retention times of $R(-)$ and $S(+)$ PZQ. Figure 2 shows typical chromatograms for blank human serum, and serum spiked with 250 ng ml^{-1} of each enantiomer.

Various solid-phase extraction columns (C_{18}, C_8, C_2, phenyl, cyanopropyl) were investigated for serum clean-up prior to the LC assay. Absolute recoveries of the enantiomers on the C_{18} column were good (82.4 and 82.0% for $R(-)$ and $S(+)$ PZQ), but there was substantial interference with the internal standard peak by endogenous serum components. Recoveries of the enantiomers on C8, C2 and phenyl could not be calculated due to serum interferences. The cyanopropyl column was found to provide the best recoveries of both enantiomers and there was little or no interference with the internal standard peak by serum components. The absolute recoveries of $R(-)$ and $S(+)$ PZQ from human serum using the cyanopropyl column were 98.8 ± 0.7 and 93.3 ± 1.4%, respectively, at 800 ng ml^{-1} levels (n = 4). The absolute recovery of 2-methylamino-5-chlorobenzophenone from serum was 35.4 ± 6.6% (n = 4). The recoveries of the enantiomers and internal standard were calculated by a comparison of peak heights of extracted to unextracted analyte.

Linear calibration curves were obtained in the 50–1000 ng ml^{-1} range for each enantio-

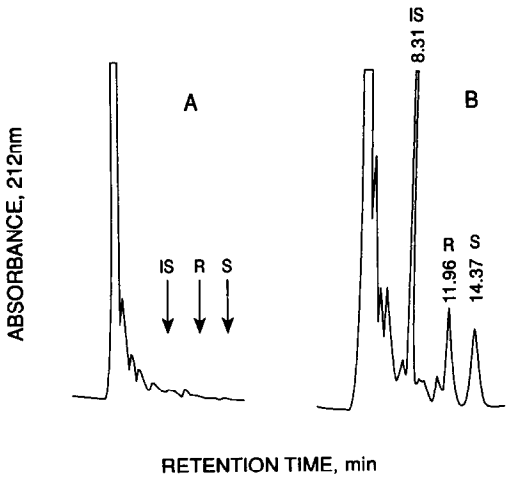

Figure 2
Typical chromatograms of (A) blank serum and (B) serum spiked with 250 ng ml^{-1} each of $R(-)$ and $S(+)$ praziquantel at 212 nm. Internal standard (IS) concentration was 256 ng ml^{-1}. See Experimental section for chromatographic conditions.

Table 1
Accuracy and precision data for the assay of PZQ enantiomers in spiked serum samples

Analyte	Conc. added (ng ml^{-1})	Conc. found (ng ml^{-1})	Error (%)	RSD (%)
R(−) PZQ	100	99.72 ± 2.42*	0.28	2.42
	750	763.66 ± 54.50	1.79	7.14
S(+) PZQ	100	100.01 ± 1.63	0.01	1.63
	750	763.33 ± 33.50	1.82	4.55

*Based on $n = 4$.

mer. Standard curves were fitted to a linear equation, $y = ax + b$, where y is the ratio of drug/internal standard peak heights, a and b are constants, and x is PZQ concentration. Typical regression parameters of a (slope), b (y-intercept) and correlation coefficient were calculated to be 0.00137, −0.01285 and 0.9992 for *R*(−) PZQ and 0.00086, 0.19823 and 0.9962 for *S*(+) PZQ, respectively. The precision and accuracy (per cent error) of the method was ascertained using spiked serum samples at 100 and 750 ng ml^{-1} levels. The data shown in Table 1 indicate that precision was in the 1.6–7.1% range and accuracy in the 0.01–1.8% range for both PZQ enantiomers.

Long-term stability of PZQ in human serum or aqueous solution was not determined in this study. The minimum detectable concentration of each enantiomer was determined to be 5 ng ml^{-1} ($S/N = 3$). The lowest quantifiable level was found to be 25 ng ml^{-1} for each enantiomer (*R*(−), 0.3% RSD, 1.6% error; *S*(+), 8.3% RSD, 2.4% error).

In conclusion, an LC method has been developed and validated for the assay of *R*(−) and *S*(+) PZQ in human serum. The method is suitable for the separation and quantification of each enantiomer over a 50–1000 ng ml^{-1} range and should be applicable to bioavailability and pharmacokinetic studies. Since the major PZQ metabolite, *trans*-4-hydroxypraziquantel, was not available for this study, separation of the metabolite using solid-phase extraction and resolution of the drug and metabolite on the Chiralcel column were not attempted. However, a previous literature reference on the chiral separation of PZQ and its hydroxymetabolite would indicate that both compounds would be easily separated if they were suitably extracted on the cyanopropyl solid-phase extraction column [9].

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