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QUANTITATIVE DETERMINATION OF PRAZIQUANTEL IN SERUM BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

A simple method for the quantitation of praziquantel by high-performance liquid chromatography is described. This method has a lower limit of sensitivity of 2.5 ng of drug per ml of human serum and a relative standard deviation of 2.6% at concentrations of 5 ng/ml.

INTRODUCTION

Praziquantel (PZQ), a pyrazino isoquinoline derivative with activity against all five species of schistosomes infecting man [1—5], is the current drug of choice for mass chemotherapy of schistosomiasis. Advantages of this agent include high efficacy after oral administration, low toxicity and a 1-day treatment regimen. Praziquantel also is effective against a variety of hermaphroditic flukes and cestodes of clinical importance to humans [6—8].

Clinical investigation of the pharmacokinetics and toxicity of such a clinically useful drug as PZQ requires the availability of a sensitive and specific assay for the routine measurement of drug levels in blood and other body fluids. Presently, radiometric [9], fluorometric [10], gas—liquid chromatographic [11], and bioassay techniques [12] are available for the measurement of this compound. However, the use of radioactive drug would not be suitable for routine clinical studies in patients. The other methods are limited by requirements for complex sample preparation or derivatization.

We now report the development of a simple, sensitive, and quantitative procedure to measure PZQ levels in serum by high-performance liquid chromatography (HPLC). This method is clinically applicable, readily validated, and eliminates the need for involved sample preparation or derivatization.

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MATERIALS AND METHODS

Standards and solvents

Praziquantel, 2-cyclohexylcarbonyl-4-oxo-1,2,3,6,7,11b-hexahydro-4H-pyrazino[2,1-*a*]isoquinoline, and the internal standard, 2-cycloheptylcarbonyl-4-oxo-1,2,3,6,7,11b-hexahydro-4H-pyrazino[2,1-*a*]isoquinoline, were provided by E. Merck (Darmstadt, G.F.R.). The structures of these compounds are shown in Fig. 1A. Acetonitrile (Burdick & Jackson Labs., Muskegon, MI, U.S.A.) and ethyl acetate (Fisher Scientific, Pittsburgh, PA, U.S.A.) were HPLC grade. The ethyl acetate was washed three times with equal volumes of distilled water before use.

Serum assay

Reagents. (1) Solvent, 38% aqueous acetonitrile; (2) water-saturated ethyl acetate; (3) praziquantel solution, 1 mg of praziquantel dissolved in 10 ml of 38% aqueous acetonitrile; (4) internal standard solution, 0.8 mg of internal standard dissolved in 10 ml of 38% aqueous acetonitrile.

Sample treatment. A 10-μl (0.8-μg) aliquot of internal standard solution is added to 1 ml of mouse, human, or fetal calf serum and mixed vigorously for 15—30 sec. The sample is extracted three times with 2 ml of water-saturated ethyl acetate. Each sample is mixed for 1 min after addition of organic solvent and phase separation is achieved by centrifugation at 1600 *g* for 5 min. The three ethyl acetate phases from each sample are combined and transferred to a 10-ml pear-shaped flask for removal of solvent by rotoevaporation (Buchler Rotovapor R110). Each sample residue is resuspended in 250 μl of 38% aqueous acetonitrile and aliquots of 25 μl are injected into the HPLC apparatus.

Apparatus

Analyses are carried out with a Model M-6000A high-performance liquid chromatograph (Waters Assoc., Milford, MA, U.S.A.) equipped with a Model SF-770M-A2 variable-wavelength UV detector (Schoeffel Instrument Company, Westwood, NJ, U.S.A.).

An octadecyl silica gel RP-18, 5 μm (Spheri-5), 10 cm × 4.6 mm MPLC™ analytical cartridge and an RP-18, 5 μm (Spheri-5), 3 cm × 4.6 mm precolumn both from Brownlee Labs. (Santa Clara, CA, U.S.A.) are used as the stationary phase. The mobile phase is 38% aqueous acetonitrile.

Operating conditions

A flow-rate of 1.5 ml/min and a detector wavelength of 210 nm at ambient temperature are employed throughout. After 15—20 analyses, the column is cleaned with 100% acetonitrile for 20 min, using a Waters Assoc. Model 660 solvent programmer (flow program No. 6).

Quantitation of PZQ

The formula for calculation of the PZQ concentration in serum is as follows: $C_2 = (A_2 \cdot C_1)/(A_1 \cdot 1.13)$, where C_1 = concentration of internal standard added to serum, C_2 = concentration of PZQ in serum, A_1 = area under the

internal standard peak in the HPLC chromatogram, and A_2 = area under the PZQ peak in the HPLC chromatogram.

After extraction into ethyl acetate, recoveries of four different known amounts of PZQ and internal standard added to 1 ml of human serum varied from 91.7 to 95.3% (average 93.1%) for PZQ and from 91.3 to 97.5% (average 93.2%) for the internal standard. Thus, no difference in the extraction efficiency of PZQ and internal standard was noted. However, a difference in the molar extinction coefficients at 210 nm was found for PZQ and the internal standard. The molar extinction coefficient of PZQ in 38% aqueous acetonitrile was 31,746 whereas the comparable value for the internal standard was 28,199. The calculated ratio of the two extraction coefficients at 210 nm is 1.13; this ratio is included in the formula for calculating PZQ concentrations.

UV spectroscopy

The UV spectra of PZQ and the internal standard (not shown), recorded on a Gilford 2400-2 spectrophotometer (Gilford Instruments, Oberlin, OH, U.S.A.), were identical except for the difference in extinction coefficients.

Mass spectrometry

Mass spectral analysis was performed with a Kratos MS-30 dual-beam mass spectrometer equipped with a Kratos DS-50 S computer system. Samples were introduced by direct probe and spectra obtained by electron impact at 4 kV accelerating voltage, an ionizing potential of 70 eV, and a resolving power of 3000.

Animals and pharmacokinetic studies

CF-1 female mice (22—25 g) were purchased from Jackson Laboratories (Bar Harbor, ME, U.S.A.) and maintained on rat chow and water ad libitum in the animal facilities at Case Western Reserve University School of Medicine. Non-fasted mice were given 200 mg/kg of PZQ dissolved in 0.4 ml of 2% Cremophor EL (Sigma, St. Louis, MO, U.S.A.) by gastric intubation. Blood samples were drawn from the retroorbital sinus. Pharmacokinetic studies of PZQ in mice were performed in CF-1 mice exposed to 120 cercariae of *Schistosoma mansoni* by tail immersion for 30 min [13].

RESULTS

Standards and recoveries

PZQ and the internal standard (Fig. 1A) were well resolved from each other under the chromatographic conditions selected (k' values of 6.0 and 9.3, respectively). Moreover, when human or mouse serum, free of added standards, was extracted with ethyl acetate and the extracts were analyzed, no extraneous peaks were found which might interfere with the analysis of either PZQ or the internal standard (Fig. 1B).

When 0.05, 0.1, 0.2, 0.4 and 0.8 μg of PZQ and internal standard were added to 1 ml of human serum, extracted, and aliquots analyzed by HPLC at 210 nm, the amounts of PZQ and internal standard detected were linear with

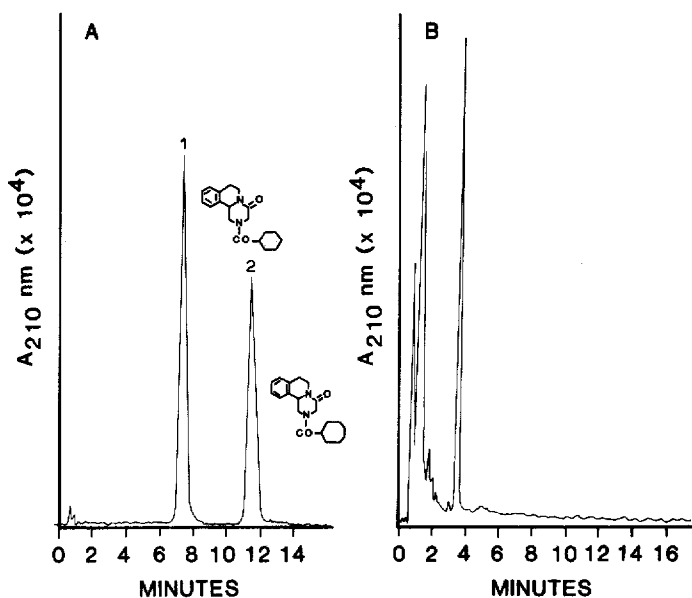

Fig. 1. (A) HPLC chromatogram of a mixture of praziquantel (PZQ), 1, and the internal standard (IS), 2. The concentration of each compound is 0.15 μg. The ratio of areas under the two peaks is 1.15 (PZQ:IS). (B) HPLC chromatogram of an ethyl acetate extract of normal human serum.

concentration. The linear regression equation of the line for the PZQ standard curve was $y = 1204.7x - 0.43$, correlation coefficient 0.99; for the internal standard $y = 976.7x + 1.24$, correlation coefficient 0.99. Identical results were obtained when the same amount of PZQ was added to either mouse serum or mouse plasma.

The lower limit of sensitivity for PZQ detection in either human or mouse serum is 2.5 ng/ml. The reproducibility of the method was checked by adding 5 ng of PZQ to three separate 1-ml serum samples, resuspending the residues in 100 μl of column solvent and injecting 50 μl into the HPLC apparatus. The variation in peak areas between these determinations was 2.6%. Repetitive injection of a sample from a human serum extract containing 5 ng of PZQ showed a variation of 2.9% ($n = 5$) in the calculated area (mm^2) of the PZQ peak.

Stability

The stability of PZQ in serum samples during storage was tested by adding 0.1 μg of the drug to five separate 1-ml serum samples obtained from two different people. One sample from each person was analyzed immediately. The others were frozen at −20°C and analyzed after 1, 2, 3, and 4 weeks of storage. The same concentrations were found after storage for up to 4 weeks.

Clinical application

Previous work [14] indicated that after oral administration of PZQ to schistosome infected mice, peak serum levels of PZQ were reached at 30 min and by 240 min most of the PZQ had disappeared from the serum. In order

to test the validity of the method, four mice, after oral administration of PZQ at a dose of 200 mg/kg, were bled at each of these time points and samples of approximately equal volume were pooled. The blood was allowed to clot at room temperature and the serum removed after centrifugation at 1600 *g* for 10 min. Aliquots of 1 ml of serum were processed and analyzed (see Materials and Methods).

The serum PZQ concentration was found to be 12.3 μg/ml in the 30-min sample and 0.2 μg/ml in the 240-min sample (Fig. 2A and B). In order to confirm that the absorbance peak designated as PZQ in Fig. 2A was due to PZQ, the fraction corresponding to this peak was collected and analyzed by mass spectroscopy after evaporation of the eluting solvent. The mass spectrum of the material in the peak fraction was found to be identical to the mass spectrum of authentic PZQ.

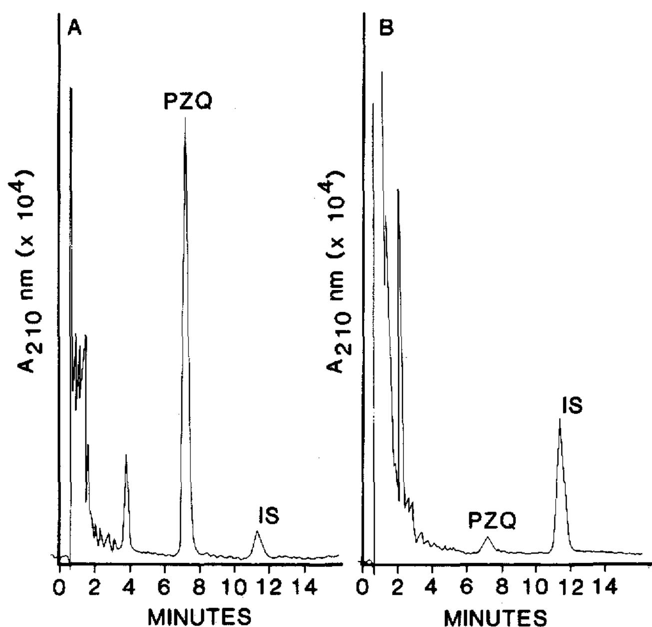

Fig. 2. PZQ concentration in serum of *S. mansoni* infected mice after oral administration of a single drug dose of 200 mg/kg. (A) At 30 min after drug administration. Due to the high concentration of PZQ present in serum, the injection volume was decreased to 5 μl which contained 0.016 μg of internal standard (IS). (B) at 240 min after drug administration. The injection volume was 25 μl which contained 0.08 μg internal standard.

DISCUSSION

The HPLC method described here combines simplicity of sample preparation and analysis with the selectivity and sensitivity required for clinical pharmacokinetic studies. Excellent separation of PZQ from internal standard was achieved and no extraneous peaks from serum components were noted in the HPLC chromatogram. Although PZQ is known to be metabolized extensively, predominantly to hydroxylated derivatives [15], more polar metab-

olites should either elute before the parent compound or remain in the aqueous phase. We found no mass spectral evidence for extraneous substances such as PZQ metabolites in the PZQ region of the HPLC trace of sera from PZQ treated mice.

This HPLC method is sufficiently sensitive to permit pharmacokinetic studies of PZQ in humans. Previously published data indicate that a peak level of 1 μg of PZQ per ml of serum was reached within 70—120 min after oral administration of 46 mg of PZQ per kg body weight [9]. This level is about 200-fold higher than that which can be determined with good accuracy by the present method.

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