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RESEARCH ARTICLE

A Novel, Quick Column Switching RP-HPLC Guided Metabolite Profiling of Albendazole-Praziquantel in Rat Plasma: Designing New Combination Dosage Regimen with Higher Therapeutic Window

Suddhasattya Dey[a], Shreya Shah[b], Manik Ghosh[a], Neha Karki[c], Souvik Basak[d*] and Nanda Gopal Sahoo[c*]

[a]*Birla Institute of Technology, Department of Pharmaceutical Analysis and Quality Assurance, Mesra-835215, Ranchi, Jharkhand, India;* [b]*Sigma Institute of Pharmacy, At-Bakrol, Woghodai, Near Ajwa-Nimata Road, Vadodara, Gujarat-390016, India;* [c]*Department of Chemistry, D.S.B. Campus, Kumaun University, Nainital, Uttrakhand, India;* [d]*Dr. B.C. Roy College of Pharmacy and Allied Health Sciences, Bidhannagar, West Bengal- 713206, India*

Abstract: ***Background:*** In order to combat neurocysticercosis, effective and novel fixed dose drug combinations (FDC) of combination dosage regimens have been always under trial; with Albendazole (ABZ) and Praziquantel (PZQ) combination being one of them. Such outcome is basically dependent on the pharmacokinetic profiling of their metabolites such as AlbendazoleSulphoxide (ABZSO, active), AlbendazoleSulphone ($ABZSO_2$, inactive) and PZQ-TRANS; followed by their back extrapolations to suggest a safe, however potent dosage regimen.

Objective: Development of a novel, rapid yet sensitive column switching RP-HPLC method for routine estimation of the aforementioned drugs and metabolites. The improved dosage version in combination is also targeted.

Methods: Pooling rat plasma, with column-switch, parent Albendazole-Praziquantel combination was estimated at 225nm using Enable C-18G column, whereas AlbendazoleSulphone and Sulphoxide were estimated using Phenomenex C-18 Luna column at 290nm.

Results: The method showed high accuracy recovering 92~113% of the metabolite or drugs with LOQ as low as 5~50 ng μL^{-1}. Moreover, administration of ABZ-PZQ combination revealed about 3 times slower elimination of active ABZSO than by solitary administration of ABZ. The former also achieved higher C_{max} (77.8078 $\mu g\ mL^{-1}$) compared to the latter (69.685 $\mu g\ mL^{-1}$). Also, ABZSO $t_{1/2}$ in the former has been estimated as 4.25 h compared to 1.38 h of the latter indicating slower ABZSO metabolism in combination therapy than the solo one.

Conclusion: The improved pharmacokinetics data could be attributed to the synergistic effect of ABZ and PZQ with each other. This has led to the designing of a novel safe ABZ-PZQ combination dosage regimen for therapeutic applications.

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1. INTRODUCTION

Neurocysticercosis is a potential helmintic disorder posing serious threat to common people all over the world especially Latin American, Asian and African countries [1-4]. Instead of ABZ alone, ABZ-PZQ combination therapy has started taking over the market for the last few years [4, 5] such as inhuman hydatid disease [6-9]. The main pathway of ABZ metabolism inside body is oxidation which converts ABZ into its active metabolite AlbendazoleSulphoxide (ABZSO). This is again oxidized to the terminal inactive metabolite AlbendazoleSulphone ($ABZSO_2$) [10]. For this metabolism, ABZ is often present in blood in trace amount and a sensitive analytical method is under demand to evaluate ABZ, ABZSO and $ABZSO_2$concentrations in blood [11-15]. PZQ is mainly metabolized by hydroxylation [16-18], with trans-4-hydroxyPraziquantel (TRANS) being an active metabolite [19]. High-performance liquid chromatography (HPLC) [19-20] and capillary electrophoresis (CE) [21-31] have been used for the estimation of these analytes. Furthermore, newer official regulations and practices are being tried to evaluate metabolites in human plasma [35-37]. However, the limitations of these methods are that the analyses of ABZ metabolites, PZQ and TRANS have been described only for the single drugs**;** thus protocol is always under demand to analyze these drugs with their metabolites in

*Address correspondence to these authors at the Dr. B.C. Roy College of Pharmacy and Allied Health Sciences, Bidhannagar, West Bengal- 713206, India; Tel: +91-9051226973; E-mail: souvik_basak1@yahoo.com
[*]Department of Chemistry, D.S.B. Campus, Kumaun University, Nainital, Uttrakhand, India; Tel: +91-05942235055; E-mail: ngsahoo@yahoo.co.in

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drugs with their metabolites in combination form [16-34]. There is only one method available till date for combined estimation of ABZ, PZQ and their metabolites by HPLC coupled with electrospray mass spectrometry [38] which is both a cost and resource intensive method. Actually, LC-MS or LC-MS-MS may provide output with significantly low LOD and LOQ; however application of the same for regular pathological analysis is a serious cost-intensive and tedious job. Thus, a simple, quick, robust and sensitive analytical method has been on demand for regular estimation of these analytes for pathological purposes. Another problem of combined therapy of ABZ-PZQ is higher dose treatment with both the drugs. Challenges remain in designing a proper dosage regimen because of the excessive higher dose of both such as 15mgkg^{-1} day^{-1} for ABZ and 50 mg kg^{-1} day^{-1}for PZQ [23, 39-41]. We felt that treatment with such kind of higher dose may cause systemic toxicity, thus a lower and safer dose of this drug combination is of utmost demand. Attempts have been made in this manuscript to solve this problem. This paper describes the simultaneous determination of ABZ, ABZSO, $ABZSO_2$ and PZQ in rat plasma by a simple, sensitive, column switching HPLC-UV method with significantly low LOD and LOQ. The authors made an attempt to validate the method with column switch rendering the method to be both time and cost effective. The column switching has been adopted because of the overlapping of metabolite retention times in single column. The method also proved to be more accurate, selective and specific for the analytes. Furthermore, we also tried several possible dose combinations of test drugs in rat model and finally proposed a better human dosage regimen for them with safe and high therapeutic window.

2. MATERIALS AND METHODS

2.1. Materials

Albendazole (Fig. **1A**) was obtained from Mercury Labs Ltd, Vadodara, India, Praziquantel (Fig. **1B**) was obtained from Micro Labs Ltd., Goa, India, AlbendazoleSulphoxide (Fig. **1C**) and AlbendazoleSulphone (Fig. **1D**) were synthesized in the laboratory. Diazepam (Fig. **1E**) and Oxfendazole (Fig. **1F**) were obtained from HetroPharma Ltd. Hyderabad India as gift samples. Acetonitrile, methanol and water (obtained from S. D. Fine Chemicals limited, Worli, Mumbai, India.) of HPLC grade were used. All the other reagents (perchloric acid, ortho phosphoric acid dihydrogen potassium phosphate buffer) used for the development of liquid chromatographic method for the determination of Albendazole and Praziquantel in rat plasma were of analytical grade obtained from Merck Specialties private limited, Worli, Mumbai, India.

2.2. Synthesis of AlbendazoleSulphoxide (ABZSO) and AlbendazoleSulphone ($ABZSO_2$)

Synthetically, ABZSO was acquiesced by oxidizing 1gm ABZ with 0.47 mL 30% H_2O_2 in 8 mL glacial acetic acid at 0ºC. Routine TLC analysis was performed to monitor the progress and completion of the reaction. After product formation had been completed, the pH was adjusted to 6. 0 using 10 (M)NaOH solution. The product precipitated at this pH, was subsequently filtered and stored for further use. The schematic diagram for the synthesis of ABZSO and $ABZSO_2$ from ABZ is given (Fig. **2**).

2.3. Equipment and Chromatographic Conditions

A high-performance liquid chromatography (Shimadzu, Kyoto, Japan) was used, composed of a LC-20AT Prominence solvent delivery module, a manual rheodyne injector with a 20-µL fixed loop and a SPD-20A Prominence UV–visible detector. Separation of Albendazole, PraziquantelandDiazepam(IS) was performed on Enable C-18G column (Column Length×i.d: 250mm×4.6mm; particle size 5µm; Enable) at 25ºC with mobile phase acetonitrile: water (60:40) at 1.0 mLmin^{-1} with UV detection at 225nm. Separation of Albendazole Sulphoxide, Albendazole Sulphone and Oxfendazole (IS) was performed on Phenomenex C18 Luna column (Column Length ×i.d: 250mm × 4.6mm) at same temperature with ace-

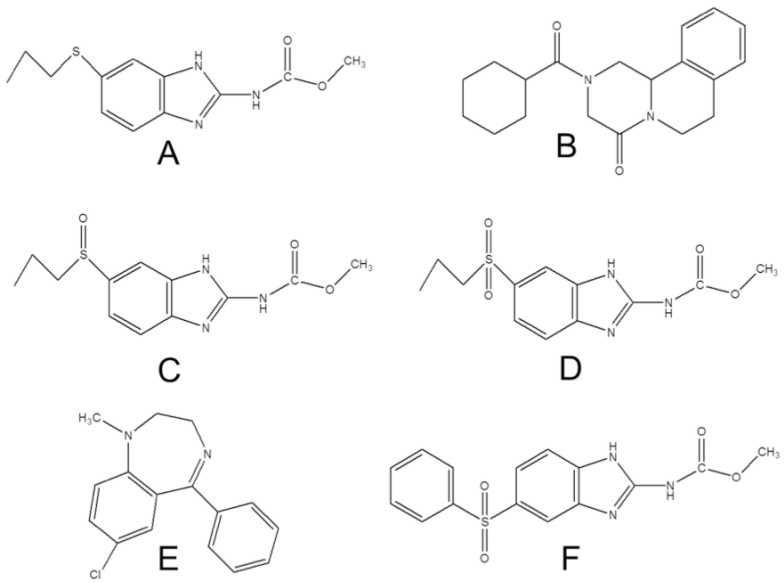

Fig. (1). Chemical structure of analytes.**A.** Albendazole (ABZ). **B.** Praziquantel (PQZ) **C.** AlbendazoleSulphoxide (ABZSO) **D.** Albendazole Sulphone ($ABZSO_2$) **E.** Diazepam (IS) **F.** Oxfendazole (IS); IS: Internal Standard.

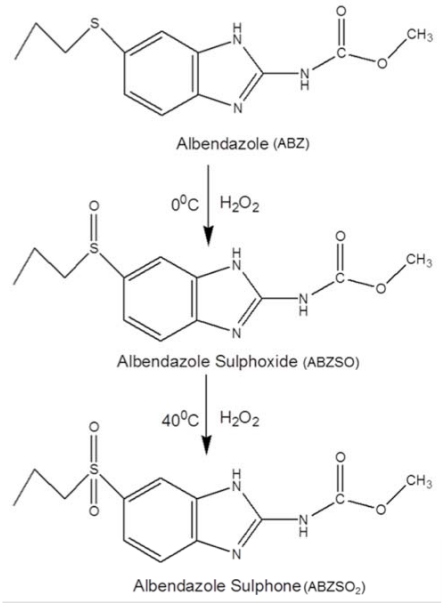

Fig. (2). Reaction scheme used for the synthesis of ABZSO and-$ABZSO_2$ from ABZ.

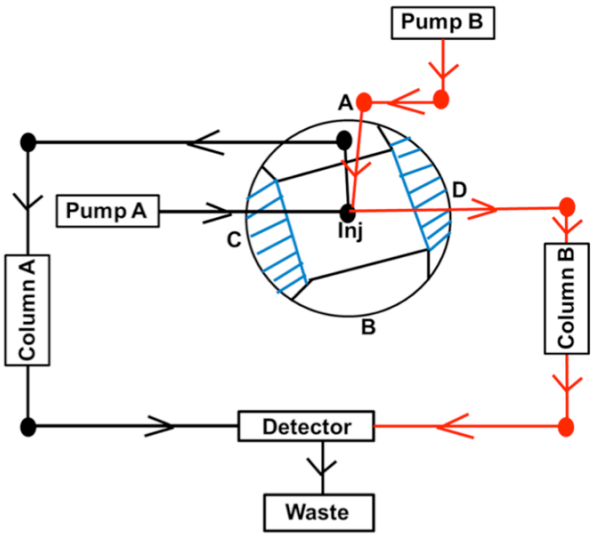

Fig. (3). Outline of Column Switch in HPLC system. A, B, C, D are four ports of the valve. A/B is one of the operating positions while C/D is the other switch position. Inj: the Injection port. Pump A and B are two operating pumps.

tonitrile: methanol: phosphate buffer ratio of 20: 25: 55, and pH 6.9 with the same flow rate at 290nm. The data acquisition was made by Spinchrom Chromatographic Station® CFR Version 2.4.195 (Spinchrom Pvt. Ltd., Chennai, India). As PZQ has no absorbance in 290nm, ABZSO and $ABZSO_2$ could effectively be determined at this wavelength. The data acquisition was made by Spinchrom Chromatographic Station® CFR Version 2.4.195 (Spinchrom Pvt. Ltd., Chennai, India).

2.4. Method of Column Switch

Method of column switch was followed as outlined in Fig. (**3**). Briefly, the whole mechanism operates *via* four port valve having ports A, B, C and D. Injected sample from the injector port of two pumps, pump A and pump B is channelized into respective analytical columns, column A and B using first and second mobile phases, respectively at constant flow rates. The valve operates at *two open-two close* mechanism. At position 1, port A and B remains open and pump A streamlines the sample injection through open port A into the analytical column A. Through detector, it ultimately goes to the waste. At position 2, port C and D remain open and pump B streamlines the sample injection through open port C into the analytical column B. Through detector, it also ultimately goes to waste.

2.5. Preparation of Stock and Standard Solutions

Stock solution (1mgmL^{-1}) of Albendazole, Albendazole-Sulphoxide, AlbendazoleSulphone, Praziquantel, Oxfendazole (internal standard) and Diazepam (internal standard) was prepared in methanol. Required dilutions were made by aliquoting appropriate volumes of stock solution and finally spiking them with required quanta of fresh methanol. The final standard concentrations of Albendazole and Praziquantel solutions were made as 50, 30, 20, 10, 1, 5, 0.5 and 0.05µg $mL^{-1}$with Diazepam (internal standard) as 0.5µg mL^{-1}, respectively. The similar standard dilutions for Albendazole-Sulphoxide and AlbendazoleSulphone were 800, 300, 100, 50, 10, 5, 1 and 0.5µg $mL^{-1}$with Oxfendazole (internal standard) as 0.5µg mL^{-1}, respectively. Stock solutions were refrigerated when not in use and replaced on a bi-weekly basis. Fresh standard solutions were prepared for each day of analysis or validation.

2.6. Calibration Curves

The calibration curves were prepared with concentrations based on rat plasma. Blank plasma was collected from untreated anesthetized animals. Clear supernatant was used for all the experiments after standard protein precipitation protocol followed with 8.25% perchloric acid. Plasma calibration solutions were prepared by spiking 180µL of plasma with 20µL of each ABZ, PZQ, ABZSO and $ABZSO_2$ standard solutions (mentioned before) and internal standards. The calibration concentrations of ABZ and PZQ were kept in the range of 0.005-5µg mL^{-1}; while for ABZSO and $ABZSO_2$, the same were maintained within 0.05-80µg mL^{-1}.

2.7. Validation of the Developed Method

The developed method was validated by estimating recovery, linearity, precision, accuracy, quantification limit and stability. The acceptable limit for the coefficient of variation and relative errors was set at 15%, whereas 20% variation was allowed for quantitation limit [Food and Drug Administration (FDA), ANVISA] [35-38].

2.8. Linearity

Linearity was determined using calibration standards prepared as described earlier. The regression co-efficient was estimated by partial least square (PLS) method using MS excel.

2.9. Accuracy and Precision

Accuracy and precision of the two above methods were determined by replicate analysis of five sets of samples

spiked with four different concentration levels of ABZ-PZQandABZSO-$ABZSO_2$ combinations (Table **2a** and **2b**).

2.10. Limit of Quantification

The limit of quantification (LOQ) for both the methods was determined from the lowest concentration of ABZ, PZQ, ABZSO and $ABZSO_2$ (in spiked plasma sample) that produced a peak height three times the baseline noise at a sensitivity of 0.005 AUFS (absorbance unit full scale) in a 200µl of plasma sample.

2.11. Stability

The stability was performed for all the analytes (ABZ, PZQ, ABZSO and $ABZSO_2$). The stability of ABZ and PZQ was assessed by allowing the spiked plasma samples to be stored at specific concentrations (Table **3**, Table **4**); the analyses were performed in triplicate by harboring the samples in a -20ºC freezer (Sanyo, Japan) for 6 months. For stability on storage, individual concentrations were sampled periodically for 1, 2, 4 and 6 months and assayed by HPLC to determine its residual concentration. The samples were allowed to freeze at -20ºC for 24h and then thawed at room temperature under ambient conditions, for freeze thaw stability. After thawing, the samples were refrozen at -20ºC for 24 h. This was repeated for three cycles and final concentrations were estimated subsequently.

2.12. Application of the Method to Biological Samples and Pharmacokinetics Study

The methods were applied to the investigation of the pharmacokinetics of Albendazole and Praziquantel along with their metabolites in twelve healthy wistar rats of three groups (weighing 150–250 gm) having equal proportion of male and female rats distributed in each group., the rats were housed one animal per cage at Animal House of Sigma Institute of Pharmacy, Baroda. Storage was performed at 22 ± 2°C at a relative humidity of 50 ± 1%. The animals were fed daily with standard food pellet and water *ad libitum*. The use of animals in this study was approved by GTU (Gujarat Technological University, Ahmadabad, Gujarat, India) and CPCSEA (Committee for the Purpose of Control and Supervision on Experimental Animals).

Venous blood samples (1.5 mL) were collected into heparinized-coated micro centrifuge plastic tubes (2 mL) from retro orbital plexus of wistar rats at the following time points: 0, 0.5, 1, 1.5, 2, 4, 6, 8, 18, 22, 26, 30, 46, 50, 72 and 96 h of dosing. The study was calibrated as per parallel design of bioequivalence study. Blood was collected from the retro orbital plexus, immediately centrifuged at 15000 rpm for 15 min and the plasma was collected carefully. The supernatant plasma layer was separated and stored at −20ºC until analyzed.

Each 200 µL of plasma was spiked with ABZ, PZQ, ABZSO and $ABZSO_2$ (different concentration), Diazepam and Oxfendazole (fixed concentration, internal standards) were taken in 1.5 mL micro-centrifuge tubes. Simultaneously, a blank (200 µL of plasma without analytes and internal standard) was also taken in 1.5 mL micro-centrifuge tube. 8.25% of perchloric acid was chosen as the protein precipitating agent throughout the whole bioanalytical study. With each 200 µL of plasma spiked sample, 45µL of 8.25% perchloric acid was mixed and allowed to stand for 300 seconds. 20µL of clear supernatant was passed through 0.45 µm syringe filter, transferred in Hamilton Syringe and subsequently injected through rheodyne injector for HPLC analysis. The analysis was followed after oral dose combination of ABZ-PZQ (50mg kg^{-1} for ABZ and40 mg $kg^{-1}$for PZQ) had been administered to selected animal/s. The study was approved by both GTU (Gujrat Technical University, Vadodora, Gujrat) and CPCSEA (Committee for the Purpose of Control and Supervision on Experimental Animals), New Delhi, India. The plasma bioanalyte concentrations were normalized based on the raw output of two biological replicates.

The pharmacokinetic parameters such as k_{el}, T_{max}, C_{max}, $t_{1/2}$, AUC, AUMC and MRT were calculated by using the software Phoenix(R) WinNolin. The maximum observed concentrations (C_{max}) and the time at which C_{max} was observed (T_{max}) were reported directly from the profile.

3. RESULTS AND DISCUSSION

3.1. Synthesis of AlbendazoleSulphoxide and AlbendazoleSulphone

H_2O_2 at 0ºC decomposes slowly and evolves stoichiometrically equivalent oxygen which leads to controlled oxidation of Albendazole, thus forming ABZSO. However, at 40ºC, the decomposition rate goes higher, resulting in the formation of copious oxygen. Oxygen then subsequently oxidizes ABZSO into $ABZSO_2$.

3.2. Characterization of synthetic compounds

The synthetic compounds (AlbendazoleSulphoxide and AlbendazoleSulphone) were characterized by IR, NMR, CHN analysis and melting point analysis (see supplementary information, Figs. **S1**~**S4**).

3.3. AlbendazoleSulphoxide: $C_{12}H_{15}N_3O_3S$

IR (max, KBr, cm^{-1}): 1026.7. ^{1}H NMR (400MHz, CF_3COOD, ı): 1.19 (t, 3H); 1.86 (sext, 2H); 3.30 (m, 2H); 4.1 (s, 3H); 7.92 (dd, 1H); 8.01 (d, 1H) and 8.32 (d, 1H). ^{13}C NMR (400MHz; CF_3COOD, ı): 13.5; 18.4; 57.1; 59.9; 113.7; 117.4; 125.4; 131.1; 133.5; 139.2; 148.2 and 156.0. Calculated for $C_{12}H_{15}N_3O_3S$: C, 51.19%; H, 5.33%; N, 14.93%; S, 11.37%. Found: C, 51.25%; H, 4.82%; N, 14.65%; S, 12.29%; mp 315–316 ºC.

3.4. AlbendazoleSulphone: $C_{12}H_{15}N_3O_4S$

IR (max, KBr, cm^{-1}): 1133.6; 1256.0. ^{1}H NMR (400MHz, CF_3COOD, ı): 1.10 (t, 3H); 1.87 (sext, 2H); 3.43 (t, 2H); 4.13 (s, 3H); 8.19 (dd, 1H); 8.03 (d, 1H) and 8.47 (d, 1H). ^{13}C NMR (400MHz; CF_3COOD, ı): 13.8; 18.9; 57.7; 61.0; 117.3; 117.6; 128.6; 131.2; 135.2; 138.0; 149.6 and 156.3. Calculated for $C_{12}H_{15}N_3O_4S$: C, 48.47%; H, 5.08%; N, 14.13%; S, 10.78%. Found: C, 48.24%; H, 5.41%; N, 13.46%; S, 10.89%; mp 292–293 ºC.

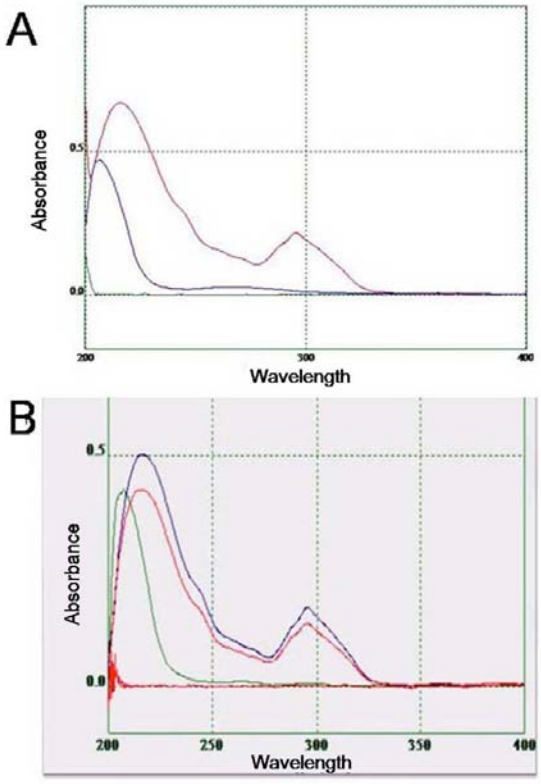

Fig. (4). Selection of detection wavelength **A.** ABZ and PZQ at 225 nm **B.** ABZSO and $ABZSO_2$ at 290 nm.

3.5. Chromatographic Separation

From thepeak overlap, the wavelength of detection for ABZ and PZQ was set at 225 nm (Fig. **4A**) while for ABZSO and $ABZSO_2$, it was set at 290 nm (Fig. **4B**). At this condition, the retention times for ABZ, PZQ and Diazepam (IS) were 3.7, 6.5 and 8.5 minutes, respectively (Fig. **5A**) using Enable C-18 column. The retention times for ABZSO, $ABZSO_2$ and Oxfendazole (IS) were 5.5, 7.0 and 8.2 minutes, respectively (Fig. **5B**) using Phenomenex C-18 Luna column. The chromatograms showed a good baseline separation for both the mobile phases.

3.6. Method Validation

3.6.1. Linearity

The coefficient of determination (r^2) for plasma was 0.998, 0.998, 0.999 and 0.999 for ABZ, PZQ, ABZSO and $ABZSO_2$, respectively indicating a strong linear relationship between the variables as summarized below (Table **1**).

3.6.2. Precision

Inter-day as well as intra-day replicates of ABZ, PZQ, ABZSO and $ABZSO_2$ gave a %R.S.D. within 5.27, 8.98, 7.75 and 11.35, respectively (should be less than 15 according to CDER guidelines for Bio-analytical Method Validation), revealing that the proposed methods are highly precise. Little variation of ABZ, PZQ, ABZSO and $ABZSO_2$ assays was observed; relative standard deviation (R.S.D.) for all four different concentrations of ABZ, PZQ, ABZSO and $ABZSO_2$ was below 15%. The intra-assay (within-day) and inter-assay (day-to-day) variations have also been summarized (Table **2a**, **2b**).

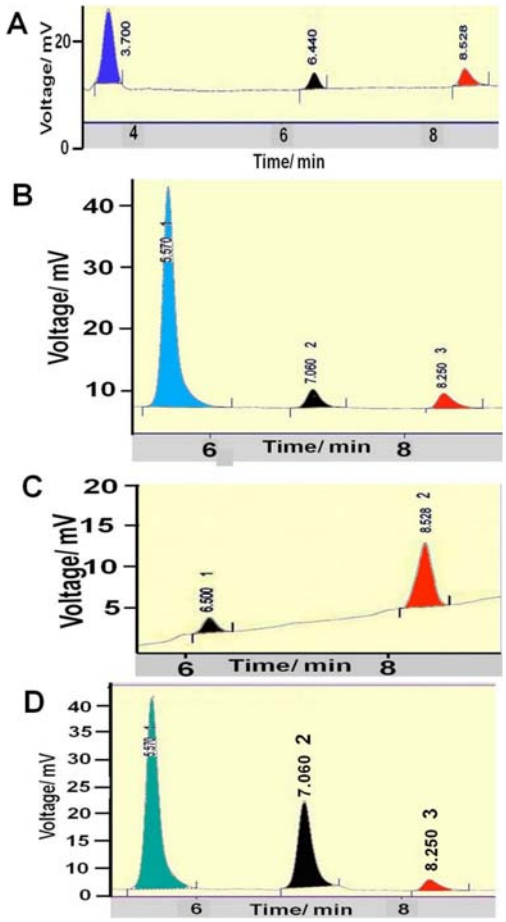

Fig. (5). HPLC Chromatograms.**A.** standard solution of ABZ, PZQ (2µg mL^{-1}) and Diazepam (0.5µg mL^{-1}) with retention times 3.7, 6.4 and 8.5 minutes **B.** standard solution of ABZSO, $ABZSO_2$ (80µg mL^{-1}) and Oxfendazole (0.5µg mL^{-1}) with retention times 5.5, 7.0 and 8.2 minutes **C.** Chromatogram of rat plasma after 2h of oral administration of ABZ and PZQ (50+40) $mgkg^{-1}$ body weight with retention times of 6.4 minutes for PZQ**D.** Chromatogram of rat plasma after 6h of oral administration of ABZ and PZQ (50+40) mg kg^{-1} body weight with retention times of 5.5 and 7.0 minutes for ABZSO and $ABZSO_2$ respectively.

3.6.3. Accuracy and Recovery

The inter-day and intra-day accuracy data in the present study ranged from 92.05-~115.5543% for drugs and the metabolites indicated that there was no interference from endogenous plasma components (Table **2a** and **2b**). The mean recoveries were found to be greater than 90%.

3.6.4. Selectivity

Selectivity was ascertained from the non-overlapping of consecutive peaks in HPLC chromatograms. Figs. (**6A** and **6B**) illustrate typical chromatograms for blank plasma in both mobile phases. However, for describing selectivity of the bioanalytes, we present here two sample chromatograms, PZQ at rat plasma (2h observation, Fig. **5C**); and ABZSO with $ABZSO_2$ (6h observation, Fig. **5D**). By column switching methods, PZQ was eluted at 6.5 min, whereas ABZSO with $ABZSO_2$ were eluted at 5.5 min and 7.0 min, respectively. Thus, the method proves to be highly selective for each of the analytes.

Table 1. Spectral and statistical data for determination of ABZ, PZQ, ABZSO and $ABZSO_2$ by proposed HPLC method (Y= mX + C, where X is the concentration (µg mL^{-1})).

Parameters	Value for ABZ	Value for PZQ	Value for ABZSO	Value for $ABZSO_2$
Absorption maxima, λmax (nm)	290 nm	225 nm	290 nm	290 nm
Linearity range (µg mL^{-1})	0.005-5	0.005-5	0.05-80	0.05-80
Coefficient of determination (r^2)	0.998	0.998	0.999	0.999
Correlation coefficient (r)	0.999	0.999	0.999	0.999
Regression equation (Y)	Y=115.2x+12.03	Y = 16.80x + 2.510	Y=3.996x+1.564	Y=1.913x+0.575
Slope (b)	115.2	16.8	3.996	1.913
Intercept (a)	12.03	2.51	1.564	0.575
Limit of detection, LOD (µg mL^{-1})	0.0707	0.0707	0.224	0.224
Limit of quantitation, LOQ (µg/mL)	0.005	0.005	0.05	0.05

Table 2a. Summary of inter-day (*n* = 5) and intra-day (*n* = 5) precision and accuracy of the method in rat plasma for ABZ and PZQ ([a]Average of three and six determinations at three concentration levels for inter-day and intra-day respectively; [b]All the mean accuracies were calculated against their nominal concentrations).

Nominal Conc. (µg mL^{-1})	Mean Conc. Found[a] (µg mL^{-1}) ABZ	Mean Conc. Found[a] (µg mL^{-1}) PZQ	S.D. ABZ	S.D. PZQ	Precision (%R.S.D.) ABZ	Precision (%R.S.D.) PZQ	Mean Accuracy [b] (%) ABZ	Mean Accuracy [b] (%) PZQ
Inter-day (n=5)								
5	5.1841	5.0988	0.0282	0.0906	0.5453	1.7720	103.6828	101.9763
1	0.9243	1.0101	0.0127	0.0259	1.3730	2.5697	92.4268	101.0050
0.5	0.4784	0.5138	0.0069	0.0204	1.4408	3.9653	95.6727	102.7575
0.005	0.0050	0.0051	0.0002	0.0004	3.8004	7.3456	99.1686	101.9000
Intra-day (n=5)								
5	5.1970	5.1441	0.0054	0.0355	0.1039	0.6899	103.9391	102.8821
1	0.9206	0.9515	0.0078	0.0251	0.8428	2.6407	92.0552	95.1536
0.5	0.4952	0.5680	0.0018	0.0025	0.3549	0.4400	99.0322	113.6024
0.005	0.0050	0.0054	0.0001	0.0003	1.3762	5.7709	100.2778	108.5714

Table 2b. Summary of inter-day (*n* = 5) and intra-day (*n* = 5) precision and accuracy of the method in rat plasma for ABZSO and-$ABZSO_2$ ([a]Average of three and six determinations at three concentration levels for inter-day and intra-day respectively. [b] All the mean accuracies were calculated against their nominal concentrations).

Nominal Conc. (µg/mL)	Mean Conc. Found[a] (µg/mL) ABZSO	Mean Conc. Found[a] (µg/mL) $ABZSO_2$	S.D. ABZSO	S.D. $ABZSO_2$	Precision (%R.S.D.) ABZSO	Precision (%R.S.D.) $ABZSO_2$	Mean Accuracy [b] (%) ABZSO	Mean Accuracy [b] (%) $ABZSO_2$
Inter-day (n=5)								
80	80.398	81.491	0.326	0.669	0.406	0.821	100.4976	101.8638
5	5.035	4.912	0.144	0.065	2.855	1.320	100.6914	98.2499
1	1.101	1.157	0.065	0.025	5.945	2.155	110.0622	115.6543
0.05	0.050	0.050	0.000	0.004	0.825	7.722	99.78312	99.0493
Intra-day (n=5)								
80	80.214	81.611	0.268	0.290	0.334	0.355	100.2678	102.0142
5	5.055	4.956	0.122	0.029	2.415	0.594	101.0901	99.1113
1	1.121	1.139	0.049	0.015	4.395	1.308	112.1121	113.9362
0.05	0.049	0.050	0.001	0.004	1.238	8.567	98.97898	99.7177

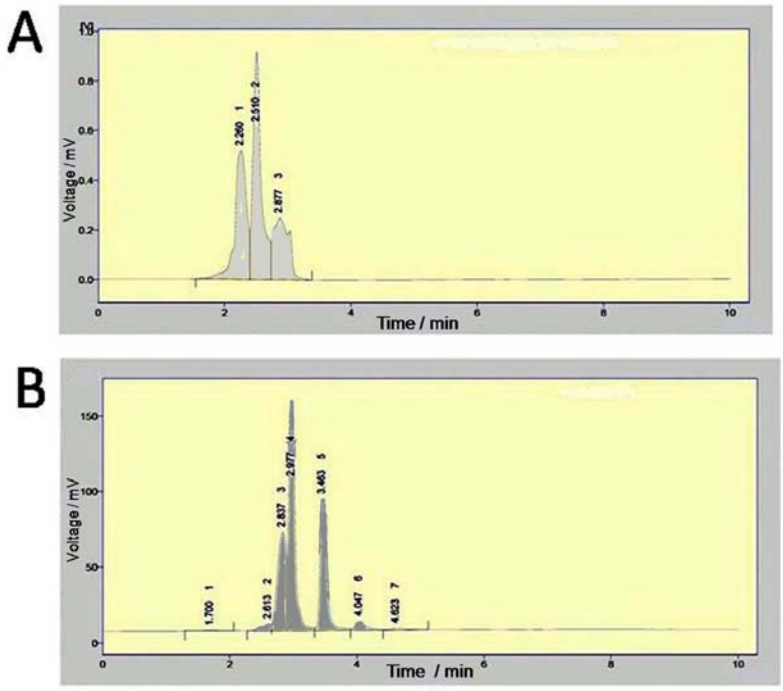

Fig. (6). Chromatogram of blank rat plasma in two columns **A.**Without ABZ, PZQ and Diazepam **B.**Without ABZSO, $ABZSO_2$ and Oxfendazole.

3.6.5. Specificity

Interference (overlapping peaks) was obtained only due to the junk plasma endogenous components being within 2–4 min (Fig. **6A** and **6B**). Later on, there was no significant interference from blank plasma that affected the response of PZQ, ABZ, ABZSO, $ABZSO_2$, Diazepam and Oxfendazole.

3.6.6. Limit of Quantification

The limit of quantification (LOQ) in rat plasma for ABZ, PZQ, ABZSO and $ABZSO_2$ was accepted as 0.005, 0.005, 0.05and 0.05 μg mL^{-1}in rat plasma, respectively.

3.6.7. Stability

The stability of plasma samples was found unaffected in storage under cold condition (-20ºC) for the duration of six months, as no significant degradation product was found after the above mentioned period. Mean deviation (%) of measured concentrations after storage at the observed periods (1, 2, 4 and 6 months) varied between -12.00-9.16%, -8.00-6.00%, -10.00-1.6% and -10.00-1.6% for ABZ, PZQ, ABZSO and $ABZSO_2$, respectively. Similarly, freeze thawing stress did not incur into degradation of the active ingredients. Standard deviation from the mean value ranged within -2.00-4.02%, 0.2-2.8%, 0.6-0.9% and -1.3-2.5% for ABZ, PZQ, ABZSO and $ABZSO_2$, respectively. The detailed storage-stability data have been summarized in Tables **3** and **4**.

3.6.8. Quality Control

0.005, 0.5, 1 and 5μg mL^{-1} of ABZ and PZQ and 0.05, 1, 5 and 80μg mL^{-1} of ABZSO and $ABZSO_2$in spiked plasma were analyzed before and after the analytical run. Results were all within the acceptable limit (±20% of their respective nominal values).

3.7. Application of Assay and Pharmacokinetics Study

The developed method was applied to quantify PZQ, ABZSO and $ABZSO_2$ concentration in pharmacokinetics study carried out on three groups each containing twelve albino rats. Using the same mobile phase, PZQ was eluted at 6.4 min in the first column (Fig. **5C**), whereas ABZSO and $ABZSO_2$ were eluted at 5.5 and 7.0 min in the second column (Fig. **5D**). The elimination curves of ABZSO, $ABZSO_2$ and PZQ have also been provided (Fig. **7A**, **7B**, and **7C**). Various other pharmacokinetic parameters of PZQ, ABZSO and $ABZSO_2$ have been summarized (Table **5**). Amongst majors, it is noteworthy to mention that T_{max} of PZQ is 2 h, whereas the same for ABZSO and $ABZSO_2$ is 6 and 8 h, respectively. Also, the retention of ABZSO has been almost 4 days within body which could lead the subject exposed to the active metabolite for sufficiently high amount of time. This in turn, leads to improved therapeutic efficacy of Albendazole when administered with Praziquantel.

In a control study, we evaluated pharmacokinetics parameters after the administration of ABZ alone to same weight of albino rats under same condition. All the pharmacokinetic parameters of ABZSO showed remarkable improvement in combined administration compared to the control (Table **6**). The elimination rate constant (K_{el}) and total renal clearance were lowered to three times for ABZSO in combination therapy leading to three times improvement of plasma half-life ($t_{1/2}$) of the same. The increase in C_{max} for ABZSO in combination could be explained by PZQ induced enhanced activity of CYP3A4 [39]. The improved CYP3A4 activity increases metabolism of ABZ into ABZSO, thereby increasing plasma concentration of ABZSO. Furthermore, the increased residence of ABZSO inside body for combination could be explained by two proposed mechanisms. First,

Table 3. Storage stability data of ABZ and PZQ in plasma at concentrations 0.005, 1 and 5 μg mL^{-1} ([a] %DEV = deviation of single mean value from theoretical value (%)T.P. = Time Period, mts = months, C.A. = Concentration Added).

T.P(mts)	C.A (μg/mL)	Concentration measured (μg mL^{-1})											
		Assay 1 ABZ	Assay 1 PZQ	Assay 2 ABZ	Assay 2 PZQ	Assay 3 ABZ	Assay 3 PZQ	Mean ABZ	Mean PZQ	S.D. ABZ	S.D. PZQ	%DEV[a]ABZ	%DEV[a]PZQ
a. Long term stability													
1	0.005	0.0051	0.0054	0.0050	0.0052	0.0051	0.0056	0.0050	0.0053	0.0001	0.0001	0	6
	1	1.1470	1.0142	1.0140	1.0286	1.1140	1.0404	1.0916	1.0277	0.0692	0.0131	9.16	2.7
	5	5.1160	5.0024	5.1173	5.0374	5.1473	5.0224	5.1268	5.0207	0.0177	0.0175	2.53	0.4
2	0.005	0.0050	0.0056	0.0048	0.0050	0.0050	0.0050	0.0049	0.0051	0.0001	0.0003	-2	2
	1	1.0550	1.0196	0.9965	1.0354	1.0258	1.0554	1.0257	1.0368	0.0292	0.0179	2.57	3.6
	5	5.0020	5.1107	4.8879	5.0206	5.0020	5.0722	4.9639	5.0678	0.0658	0.0452	-0.7	1.3
4	0.005	0.0049	0.0049	0.0047	0.0047	0.0049	0.0048	0.0048	0.0047	0.0001	0.0001	-4	-6
	1	0.9953	0.9875	0.9765	0.9745	0.9255	0.9966	0.9657	0.9862	0.0361	0.0111	-3.4	-1.3
	5	4.8657	4.9956	4.8814	4.9657	4.9111	4.9888	4.8860	4.9833	0.0230	0.0156	-2.2	-0.3
6	0.005	0.0046	0.0047	0.0045	0.0046	0.0046	0.0045	0.0045	0.0046	0.0001	0.0001	-10	-8
	1	0.8876	0.9625	0.8654	0.9145	0.8857	0.9689	0.8795	0.9486	0.0123	0.0297	-12	-5.1
	5	4.7641	4.8899	4.8743	4.9711	4.7889	4.9803	4.8091	4.9471	0.0578	0.0497	-3.8	-1.0
b. Freeze and thaw stability													
	0.005	0.0051	0.0054	0.0049	0.0052	0.0049	0.0049	0.0049	0.0051	0.0001	0.0002	-2	2
	1	0.9945	1.0254	1.0255	1.0453	1.1007	1.0142	1.0402	1.0283	0.0546	0.0157	4.02	2.8
	5	4.9988	5.0143	5.1041	5.0244	5.0027	5.0044	5.0352	5.0143	0.0597	0.0100	0.70	0.2

Table 4. Storage stability data of ABZSO and$ABZSO_2$ in plasma at concentrations 0.05, 5 and 80 μg mL^{-1} ([a] %DEV = deviation of single mean value from theoretical value (%)T.P. = Time Period, mts = months, C.A. = Concentration Added).

T. P. (mts)	C.A. (μg/m)	Concentration Measured (μg mL^{-1})											
		Assay 1 ABZSO	Assay 1 AB-ZSO_2	Assay 2 ABZSO	Assay 2 AB-ZSO_2	Assay 3 ABZSO	Assay 3 AB-ZSO_2	Mean ABZSO	Mean AB-ZSO_2	S.D. AB-ZSO	S.D. AB-ZSO2	[a]%DEVABZSO	[a]%DEVABZSO$_2$
a. Long term stability													
1	0.05	0.051	0.051	0.049	0.051	0.050	0.049	0.050	0.050	0.001	0.001	0.0	0.6
	5	5.011	5.114	5.014	5.112	5.217	5.021	5.081	5.082	0.118	0.053	1.6	1.6
	80	80.167	81.357	82.364	81.254	80.257	81.254	80.929	81.288	1.243	0.059	1.1	1.6
2	0.05	0.051	0.049	0.048	0.049	0.048	0.048	0.049	0.049	0.002	0.001	-2.0	-2.6
	5	5.112	4.987	5.047	5.002	5.007	4.998	5.055	4.996	0.053	0.008	1.1	-.08
	80	80.156	81.257	81.254	81.579	79.998	79.335	80.469	80.724	0.684	1.213	0.5	0.9
4	0.05	0.048	0.049	0.048	0.047	0.047	0.046	0.048	0.047	0.001	0.002	-4.6	-5.3
	5	4.857	4.887	4.768	4.822	4.865	4.875	4.830	4.861	0.054	0.035	-3.4	-2.7
	80	79.888	78.254	79.996	79.667	79.667	78.223	79.850	78.715	0.168	0.825	-0.1	-1.6
6	0.05	0.046	0.045	0.044	0.045	0.045	0.044	0.045	0.045	0.001	0.001	-10	-10
	5	4.754	4.789	4.699	4.687	4.699	4.678	4.717	4.718	0.032	0.062	-5.6	-5.6
	80	78.225	78.024	77.899	78.221	78.257	77.886	78.127	78.044	0.198	0.168	-2.3	-2.4
b. Freeze and thaw stability													
	0.05	0.051	0.049	0.049	0.051	0.051	0.048	0.050	0.049	0.001	0.002	0.6	-1.3
	5	5.011	5.144	5.112	5.124	5.024	5.112	5.049	5.127	0.055	0.016	0.9	2.5
	80	80.561	80.247	81.254	80.267	80.001	79.147	80.605	79.887	0.628	0.641	0.7	-0.1

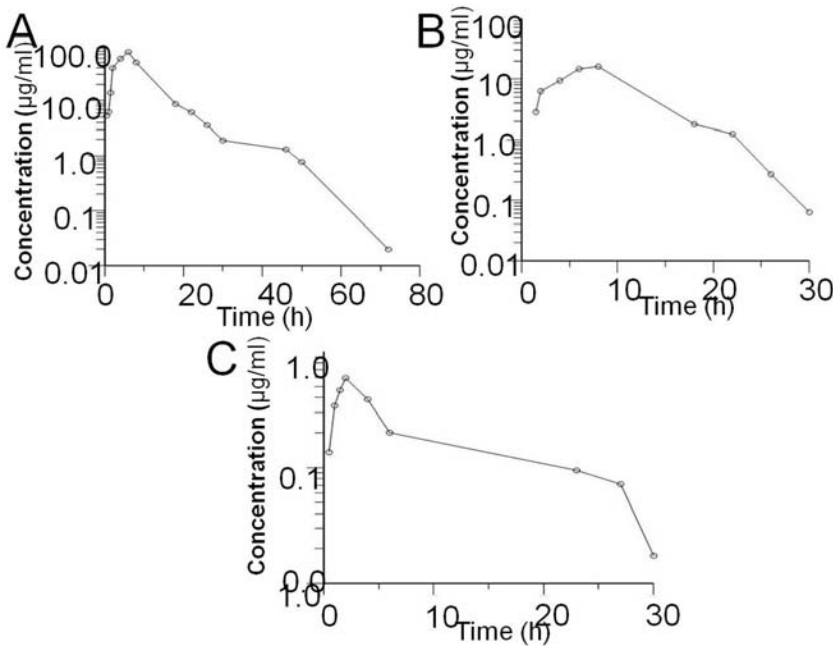

Fig. (7). Plasma log concentration–time profile of drugs in healthy rats following a single oral dose of (50+40) mg kg^{-1} body weight of ABZ and PZQ **A.** ABZSO **B.** $ABZSO_2$**C.** PQZ.

Table 5. Pharmacokinetic parameters of analytes after oral dose of ABZ and PZQ to wistar rats.

SL. No.	Pharmacokinetic Parameters	Observed Value for PZQ	Observed Value for ABZSO	Observed Value for $ABZSO_2$
1	Elimination rate constant, k_{el} (h^{-1})	0.0874	0.1629	0.3732
2	Time required for maximum plasma concentration, T_{max} (h)	2.0000	6.0000	8.0000
3	Maximum plasma concentration, C_{max} (µg mL^{-1})	0.5918	77.8078	16.1641
4	Plasma half life, $t_{1/2}$ (h)	7.9311	4.2553	1.8575
5	Area under curve at 48 h, $AUC_{(0\rightarrow48)}$, (µg h mL^{-1})	5.1627	777.0053	173.3521
6	Area under curve at infinite time, $AUC_{(0\rightarrow\infty)}$ (µg h mL^{-1})	5.3615	777.1266	173.5202
7	Area under momentum curve at 48 h, $AUMC_{(0\rightarrow48)}$ (µg h^2 mL^{-1})	46.9873	7613.5738	1413.0000
8	Mean residence time, MRT (h)	9.1014	9.7986	8.1510

Table 6. Comparative pharmacokinetic parameters after oral dose of ABZ (combination) and ABZ (Single).

SL. No.	Pharmacokinetic Parameters	Observed Value for ABZSO (Combination)	Observed Value for ABZSO (Single)
1	Elimination rate constant, k_{el} (h^{-1})	0.1629	0.5020
2	Time required for maximum plasma concentration, T_{max} (h)	6.0000	6.0000
3	Maximum plasma concentration, C_{max} (µg mL^{-1})	77.8078	69.685
4	Plasma half life, $t_{1/2}$ (h)	4.2553	1.3803
5	Area under curve at 48 h, $AUC_{(0\rightarrow48)}$, (µg h mL^{-1})	777.0053	631.03
6	Area under curve at infinite time, $AUC_{(0\rightarrow\infty)}$ (µg h mL^{-1})	777.1266	631.37
7	Area under momentum curve at 48 h, $AUMC_{(0\rightarrow48)}$ (µg h^2 mL^{-1})	7613.5738	5681.7
8	Mean residence time, MRT (h)	9.7986	9.0164
9.	Volume of Distribution (V_d) (mL)	311.11	311.11
10.	Total Renal Clearance (h^{-1})	46.66	156.18

improved metabolism of ABZ into ABZSO produces large quantity of metabolites that prompt reversible tissue binding of the compound. This later slowly releases ABZSO when plasma concentration of the same is reduced due to renal clearance. Second, basic nature of PZQ shifts the renal pH towards alkaline side, where most of the ABZSO remain unionized and thus, undergoes tubular reabsorption. The increased residence inside plasma also suggests lower frequency of administration of ABZ in clinical practice.

In subsequent calculations, extrapolation of rat administered dose to human equivalent dose (HED) elicited that HED for ABZ and PZQ reveals to be 8.11 mg kg^{-1} and 6.488 mg kg^{-1}, respectively where

$$\text{Human Equivalent Dose (HED in mg kg}^{-1}\text{)} = \text{Animal Dose (mg kg}^{-1}\text{)} \times \frac{\text{(Animal Km)}}{\text{(Human Km)}}$$

Where, K_m= Correlation Factor reflecting the relationship between body weight and body surface area of an adult individual (K_m=37). Assuming the body weight of a healthy adult to be 60 kg, it can be applied on human subjects in a fixed dose combination of ABZ: PZQ = 486.6mg: 389.28mg. While the usual clinical practice recommends 400mg ~ 800 mg of ABZ and 2.0~4.0 g of PZQ for neurocysticercosis treatment to an adult individual, our pharmacokinetics profiling revealed that the combination therapy of the two could significantly reduce the individual dosage regimen. Actually, a study in 2004 and 2005 on African school children reported that several school children suffered from toxicity like headache, stomach ache, nausea, vomiting and dizziness when administered with such high doses of ABZ and PZQ in combination [43]. Thus, this dose reduction evolved through this study, is practically remarkable for PZQ where 8~ 10 times diminution of dose is calculated suggesting high cost effectiveness for both the industries and the patients together with significant enhancement of safety parameter for the drug administration.

CONCLUSION

This manuscript demonstrates the first attempt of simultaneous estimation of PZQ, ABZSO and $ABZSO_2$ in plasma by column switching HPLC using UV detector. The method tried herein is rapid, sensitive, accurate and reproducible. The method supersedes the previously reported methods due to its high selectivity for exploiting dual columns for analyte separation. In this method, the sample preparation involves perchloric acid which has been effective in giving clear chromatograms and did not need evaporation of solvents which is both cost and time intensive. In addition, new mobile phases have been developed for better resolution of the compounds. Most interestingly, the study revealed a newer dosage regimen of the drug combination where both the drugs suggested improved therapeutic profile at a lower dose than that established clinically in the current days. This output, if optimized by subsequent studies could open a new horizon in antihelminthic drug research, where combined dosage form could be used clinically in order to lower toxicity and improve therapeutic efficacy.

LIST OF ABBREVIATIONS

ABZ	=	Albendazole
PZQ	=	Praziquantel
ABZSO	=	AlbendazoleSulphoxide
$ABZSO_2$	=	AlbendazoleSulphone
C_{max}	=	Maximum plasma concentration
T_{max}	=	Time to attain maximum plasma concentration
AUC	=	Area under curve
AUMC	=	Area under momentum curve
MRT	=	Mean residence time
K_{el}	=	Elimination rate constant
HETP	=	Height equivalent to theoretical plate
LOD	=	Limit of detection
LOQ	=	Limit of quantification
SD	=	Standard deviation
IS	=	Internal standard

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

Not applicable.

HUMAN AND ANIMAL RIGHTS

No Animals/Humans were used for studies that are base of this research.

CONSENT FOR PUBLICATION

Not applicable.

CONFLICT OF INTEREST

The authors declare no conflict of interest, financial or otherwise.

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