Egypt. J. Chem. **Vol. 65,** No. 2 pp. 209 - 221 (2022)

Egyptian Journal of Chemistry

http://ejchem.journals.ekb.eg/

Development and Validation of a Dual Column HPLC Method for Determination of Albendazole and Its Metabolites in Rat Plasma

Suddhasatya Dey[1*], Sudip Kumar Mandal[2], Mithun Bhowmick[1], Manik Ghosh[3], Pankaj Dagur[3], Shreya Shah[4], Biplab Kumar Dey[5], Ewies F. Ewies[6*]

[1]*Bengal College of Pharmaceutical Sciences & Research, Basu Sarani Burdwan, Bidhannagar, Durgapur, West Bengal 713212*

[2]*Dr B.C. Roy College of Pharmacy and Allied Health Sciences, Meghnad Saha Sarani, Bidhan Nagar, Durgapur 713206, West Bengal, India*

[3]*Department of Pharmaceutical Sciences and Technology, Birla Institute of Technology, Mesra-835215, Ranchi, Jharkhand, India*

[4]*Sigma Institute of Pharmacy, At-Bakrol, Waghodhai, Near Ajwa Nimata Road, Vadodara, Gujarat-390016, India*

[5]*Dean, Faculty of Pharmaceutical Science, Assam Down Town University, Panikhaiti, Guwahati, Assam, India*

[6]*Organometallic and Organometalloid Chemistry Department, National Research Centre, 33 El-Bohouth St., Dokki, PO-12622, Giza, Egypt.*

Abstract

To develop a quick, simple and reproducible dual column high performance liquid chromatography (HPLC) method to determine the albendazole and its metabolites in rat plasma. Albendazole (ABZ), albendazole sulfoxide (ABZSO) and albendazole sulfone ($ABZSO_2$) were analyzed in rat plasma by high performance liquid chromatography using UV-detector. Preparation of plasma samples was carried out by protein precipitation using 8.25% perchloric acid. This method involves two different mobile phases with two different columns and different wavelengths. Estimation of Albendazole was done using Enable C18 column (250 mm × 4.6 mm, 5µm: SpinCo Biotech Pvt. Ltd.), mobile phase acetonitrile: water in the ratio 60: 40, wavelength 225nm and Praziquantel as an internal standard (IS). The retention time for Albendazole and Praziquantel was 3.7 and 6.4 minutes respectively. But the estimation of Albendazole Sulfoxide, Albendazole Sulfone were done by using Phenomenex C18 Luna column (250 mm × 4.6 mm, 5µm: USA), mobile phase acetonitrile: methanol: phosphate buffer (20mM) in the ratio 20: 25: 55. The pH was adjusted to 6.9 using 0.1N NaOH solution, wavelength 290nm and oxfendazole an internal standard (IS). The retention time for Albendazole Sulfoxide, Albendazole Sulfone, and Oxfendazole was 5.5, 7.0 and 8.2 minutes respectively. Both the methods were validated over the range from 0.005-5µg/mL for Albendazole, 0.05-80µg/mL for Albendazole Sulfoxide and Albendazole Sulfone. Both the method showed % RSD and % DEV lower than 15% for all the analytes. The limit of quantitation was 0.005µg/mL for Albendazole whereas 0.05µg/mL for Albendazole Sulfoxide and Albendazole Sulfone. Metabolites of albendazole were analyzed in rat plasma samples using a single dose of Albendazole 50mg/kg was determined application of this method was also used to found the pharmacokinetic studies.

Keywords: Albendazole, Albendazole sulfoxide, Albendazole sulfone, HPLC–UV, Plasma.

Introduction

Consuming milk on a daily basis makes it one of the Albendazole (ABZ), chemically it is methyl-((5-propylthio)-1Hbenzimidazol-2-yl) carbamate, which is found to be a broad-spectrum anti-helminthic agent and hence it acts against the helminths parasites which are most commonly found in human and veterinary and that's why it is used in most of the cases for the treatment [1]. As ABZ is a prodrug, to afford its cytotoxic activity biotransformation is required via both cytochrome P450s (CYP) and flavin-containing monooxygenases (FMO) [2]. After administrating ABZ, due to extensive metabolism, it produces into albendazole sulphoxide (ABZSO) which is therapeutically active [3,4].Further, oxidation of ABZSO converts it to albendazole

*Corresponding author e-mail: : kuntal.kuni@gmail.com

Receive Date: 06 July 2021, Revise Date: 12 August 2021, Accept Date: 24 August 2021

DOI: 10.21608/EJCHEM.2021.84205.4130

©2022 National Information and Documentation Center (NIDOC)

sulfone (ABZSO2) [3-5] and finally, to albendazole 2-aminosulfone (ABZSO2NH2) which is the N-deacetylation product of albendazole sulfone [6]. The transformation is catalyzed by cytochrome P450. From the pharmacokinetics studies of the metabolites shows that ABZSO has anthelmintic activity [7] as well as toxic effects [8], whereas ABZSO2 and ABZSO2NH2 show no biological activity [8,9]. The Figure 1 displays the chemical structures of ABZ and its three metabolites. Two enantiomers, (+)-ABZSO and (−)-ABZSO of albendazole sulphoxide exist as there is a presence of stereogenic center at the sulfur atom. Clinical studies indicate that (+) - sulphoxide is found dominantly in the human and animal blood plasma [10]. Therefore an enantioselective clinical study is important to determine the disposition of (+) - and (−) - ABZSO, and to quantify the amount of $ABZSO_2$ and $ABZSO_2NH_2$, from which important information about the overall metabolism can be found out.

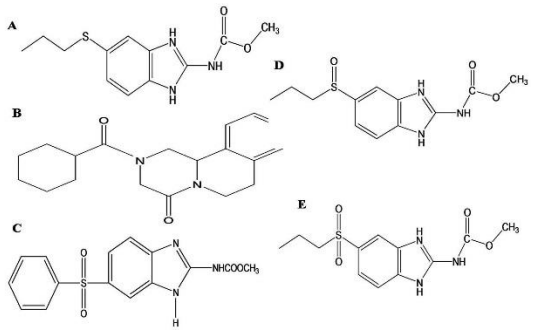

Figure 1. Chemical Structure of analytes. A. Albendazole B. Praziquantel (IS) C. Oxfendazole (IS); IS: Internal Standard D. Albendazole Sulfoxide E. Albendazole Sulfone

Likewise, in recent time it has become important to evaluate the existing anthelmintic drugs pharmacokinetic profiles of benzimidazoles in biological fluids for the maximum efficacy.

Racemic ABZSO marketed as ricobendazole (RBZ) is one example of this trend which is administered directly animals. (±) – ABZSO is slightly greater soluble in water than that of its parent molecule, which allows developing an injectable aqueous solution to administrate the animals. In Brazil two popular injectable products are used for cattle, these are Recover from Vet Brands Sau´ de Animal and Ricobendazole from Ouro Fino Sau´ de Animal. Several liquid chromatography methods have been developed for analyzing benzimidazoles in human [11,12] and sheep plasma, [13,14] human serum, [15] spermatozoa and seminal plasma, [16] animal tissue, [17] parasite animal, [18] and bovine milk [19] using chiral or achiral columns [20,22] and various compositions and different ratios of mobile phases. The liquid-liquid extraction method was used for sample clean-up prior to chromatographic analysis which is very common. Generally, aqueous extraction method is used in which high pH causes partitioning into an immiscible organic solvent [15,16]. Some of the samples are pre-treated using either matrix solid-phase dispersion method (MSPD), [23] or supercritical fluid extraction method [24] with multiple stages of liquid-liquid extraction and/or solid-phase extraction method [25]. Besides these tedious and time-consuming sample treatments, chiral analysis by an indirect method in the enantiomer selective assays of (±)-ABZSO was also performed, in which the fractions of different enantiomers (±) were collected. The enantiomers were again chromatographed through a chiral stationary phase after drying by evaporation, [26,29] except for studies assayed on Chiral Pak AD® column in normal elution mode and other developed methods [36-40]. Therefore to derogate between different steps throughout sample treatment and at the same time to study the major metabolites of albendazole in biological factors, high performance liquid chromatography (HPLC) method was developed and validated for albendazole (ABZ), albendazole sulphoxide (ABZSO) and albendazole sulfone ($ABZSO_2$) in rat plasma.

2. Materials and Methods

Albendazole (Figure 1A) was obtained from Mercury Labs Ltd, Vadodara, India, Praziquantel (Figure 1B) was obtained from Micro Labs Ltd., Goa, India, Oxfendazole (Figure 1C), were obtained from Hetero Pharma Ltd. Hyderabad India as a gift sample, Albendazole sulfoxide (Figure 1D) and Albendazole sulfone (Figure 1E) were synthesized in Pharmaceutical Chemistry lab of Sigma Institute of Pharmacy Vadodara Gujarat India. Acetonitrile and Water (obtained from S. D. Fine Chemicals Limited, Worli, Mumbai, India.) of HPLC grade were used. All the other reagents (Perchloric acid) used for the development of a liquid chromatographic method for determination of albendazole and its metabolites in rat plasma were of analytical grade obtained from Merck Specialties private limited, Worli, Mumbai, India.

3. Experimental Work

3.1 Synthesis of Albendazole Sulphoxide (ABZSO) and Albendazole Sulfone ($ABZSO_2$)

A racemic mixture of albendazole sulfoxide is produced due to the chemical oxidation of albendazole at the Thioether Bridge. The reaction was carried out by adding 0.47mL 30% H_2O_2 in glacial acetic acid (8mL) solution of 1gm albendazole at 0°C. The mixture was allowed to withstand until it

attains room temperature. The peracetic acid formed in situ act as an oxidizing reagent. After completion of the reaction, 10M NaOH solution is used to raise the pH up to 6 which is optimum for sulfoxide precipitation. The precipitate formed was filtered by using a vacuum and dried.TLC was used to evaluate the need for additional H_2O_2 and also to check product formation. The reaction time was 3 to 4 hours. Formation of albendazole sulfone is majorly influenced by temperature during the reaction, equivalents, and concentration of the reactant. The reaction is achieved at 40°C temperature using 2 equivalents of hydrogen peroxide as shown in Figure 2: (Scheme 1). Then TLC technique was used to find out whether the desired product is produced. It also helps to realize if additional H_2O_2 is required or not.

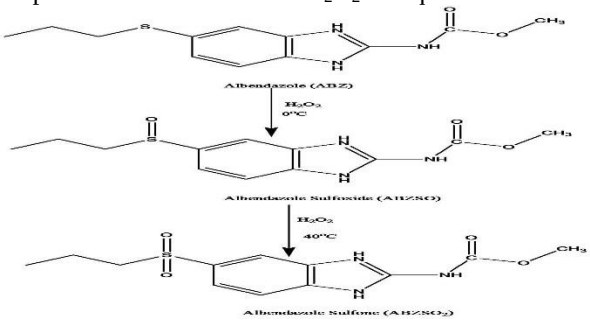

Figure 2. Reaction scheme used for the synthesis of Albendazole Sulphoxide (ABZSO) and Albendazole Sulfone ($ABZSO_2$) from Albendazole (ABZ)

3.2 Equipment and chromatographic conditions

A high-performance liquid chromatography (Shimadzu, Kyoto, Japan) containing an LC-20AT prominence solvent delivery system with a manual rheodyne injector having a 20-µl fixed loop and a prominence UV–visible detector (SPD-20A). Separation of albendazole and praziquantel (IS) was performed on Enable C18G column (Column Length: 250mm×4.6mm i.d.; particle size of 5µm; Enable) at an ambient temperature and as the mobile phase acetonitrile and water in the ratio of 60:40 was used for plasma samples at a flow rate of 1.0 mL/ min with U.V. detection set at 225nm. Separation of albendazole sulfoxide, albendazole sulfone and oxfendazole (IS) was achieved using Phenomenex C18 Luna column (Column Length: 250mm×4.6mm i.d.; particle size of 5µm; USA) at an ambient temperature and the mobile phase containing acetonitrile, methanol and phosphate buffer (20mM) in the ratio 20: 25: 55 pH was adjusted to 6.9 using 0.1N NaOH solution for plasma samples at a flow rate of 1.0 mL/ min with U.V. detection set at 290nm. The data acquisition was made by Spinchrom Chromatographic Station® CFR Version 2.4.195 (Spinchrom Pvt. Ltd., Chennai, India).

3.3 Preparation of stock and standard solutions

The standard stock solutions of 1mg/mL concentration of albendazole, albendazole sulfoxide, albendazole sulfone, praziquantel (internal standard) and oxfendazole (internal standard) were prepared in methanol. Standard solution of albendazole, albendazole sulfoxide, albendazole sulfone, praziquantel (internal standard) and oxfendazole (internal standard) were prepared by mixing and diluting the appropriate amounts from the individual stock solution by methanol. The final concentrations of the standard solution of albendazole were 50, 30, 20, 10, 1, 5, 0.5 and 0.05µg/mL and a rigid concentration of the praziquantel (internal standard) (50µg/mL). The final concentrations of the standard solutions of albendazole sulfoxide and albendazole sulfone were 800, 300, 100, 50, 10, 5, 1 and 0.5µg/mL and a rigid amount of the oxfendazole (internal standard) (5µg/mL). Precision and accuracy standards with concentrations of 50, 10, 5 and 0.05µg/mL for ABZ were also prepared in the same manner and a fixed concentration of the PRQ (50µg/mL). For precision and accuracy study different concentrations of 800, 50, 10 and 0.5µg/mL of standard ABZSO and $ABZSO_2$ were also prepared in the same manner and a fixed concentration of the oxfendazole (5µg/mL). Refrigeration was used for storing stock solutions when not they were not used and replaced two times in every week. New standard solutions of the drugs and metabolites were prepared for each day for analysis or validation.

3.4 Sample Collection

The procedure of animals study was permitted by GTU (Gujarat Technological University, Ahmadabad, Gujarat, India) and CPCSEA (Committee for the Purpose of Control and Supervision on Experimental Animals). Protocol no. - IAEC/SIP/05/2012-13. The rats were kept as one animal in every cage at Animal House of Sigma Institute of Pharmacy, Baroda. The environment was controlled with daily feeding and water.

Venous blood samples (2 mL) from healthy albino rats (weighing 150–250 gm) were collected into 2mL heparinized-coated micro-centrifuge tube from retro-orbital plexus of albino rats. The 2mL micro-centrifuge containing blood was centrifuged at 15000 rpm for 15 min and the plasma was collected carefully. The collected plasma was stored in -20°C till it was used. A blood sample was collected on a regular basis from different rats and plasma was separated until the study is been completed so that the analysis is unbiased in nature.

3.5 Calibration Curves

Blank plasma was collected from untreated anesthetized animals. Plasma calibration point was

prepared by spiking 200µl of plasma with 20µl of each ABZ, ABZSO and $ABZSO_2$ standard solutions (mention in section 2.4) and internal standards (PRQ for ABZ, oxfendazole for ABZSO and $ABZSO_2$) standard solutions (mention in section 2.4) were vortexed for few minutes. The calibration curves for ABZ was in the range of 0.005-5µg/mL using a fixed concentration of the internal standard (5µg/mL), for ABZSO and $ABZSO_2$were in the range 0.05-80µg/mL using a fixed concentration of internal standard (0.5µg/mL) in rat plasma. After plasma was spiked, it was treated to prepare the samples for analysis.

3.6 Preparation of sample

The plasma samples were treated with various protein precipitating agents for the analysis of ABZ, ABZSO, and $ABZSO_2$. To each 200µl of plasma spiked with ABZ, ABZSO, and $ABZSO_2$ (different concentration) praziquantel and oxfendazole (fixed concentration) were taken in 1.5mL microcentrifuge tubes and simultaneously a blank (200µl of plasma without analytes and internal standard) was also taken in a 1.5mL microcentrifuge tube. Different protein precipitating agents were used such as ammonia sulfate, 15% trichloroacetic acid, 10% sodium tungstate in water, 5-sulphosalicylic acid, zinc sulfate in methanol, different ratios of organic solvents (Acetone, Acetonitrile, Methanol) and different percentages of perchloric acid in water. Lastly, it was found that 8.25% of perchloric acid gave a clear chromatogram without any interference with ABZ, ABZSO and $ABZSO_2$ and internal standards (Praziquantel and Oxfendazole) so 8.25% of perchloric acid was chosen to be used as a protein precipitating agent to precipitate proteins throughout the whole Bioanalytical study. To each 200µl of plasma spiked sample was mixed with 45µl of 8.25% perchloric acid for 30 seconds. The samples were centrifuged at 15000 rpm for 15min this gives a clear supernatant liquid at the top in comparison to the above mention protein precipitating agents. 20µl of the clear supernatant liquid was transferred in Hamilton Syringe and injected through the rheodyne injector to HPLC column for analysis.

4. Validation of the Developed Method

Validation of the above method was done by studying different validation parameter like recovery study, linearity, precision, accuracy, quantitation limit and stability of the developed method. The coefficients of variation and relative errors should be within acceptable limit which is less than 15%, but in case of quantification limit, coefficients of variation and relative errors should not be more than 20%, as per the literature survey [30].

4.1 Linearity

Linearity study was performed using the calibration curve prepared as it is described in preparation of calibration curve. By taking the proper standard solution of albendazole and its other two derivatives in plasma, calibration standards of the analytes were prepared in triplicate with the plasma in which no drug was present. The following concentrations: 5; 3; 2; 1; 0.5; 0.1; 0.05 and 0.005µg/mL for ABZ using a fixed concentration of IS (praziquantel) 5µg/mL; 80; 30; 10; 5; 1; 0.5; 0.1 and 0.05µg/mL for ABZSO and $ABSO_2$ using a fixed concentration of IS (oxfendazole) 0.5µg/mL were prepared. Then three calibration curves were constructed and evaluated using its coefficient of determination (r^2). The calibration curve of ABZ (peak area quotient of ABZ to IS vs. the concentration of ABZ) and for ABZSO and $ABZSO_2$ (peak area quotient of ABZSO and $ABZSO_2$ to IS vs. the concentrations ABZSO and $ABZSO_2$) were plotted by an identical study (n = 8) of all concentrations. The method having least square was used to evaluate the linear relationship using within Microsoft Excel® of Microsoft Corporation. For minimum error with precise, concise and accurate data 8 different concentration were been taken which gave a wide range for linearity. The coefficient of determination (r^2) for ABZ is 0.998, ABZSO is 0.999 and $ABZSO_2$ is 0.999 in rat plasma.

4.2 Accuracy

Accuracy study of the two above methods was accomplished by analyzing five sets of samples multiple times which were spiked with four different concentration levels of ABZ (0.005, 0.5, 1 and 5µg/mL) with a fixed amount of IS (praziquantel) (5µg/mL). The other four different concentration levels of ABZSO and $ABZSO_2$ (0.05, 1, 5 and 80µg/mL) with a fixed amount of IS (oxfendazole) (0.5µg/mL). Then the variation between the amount spiked (theoretical value) and the amount found actually was compared.

4.3 Precision

The study of precision of the above-developed methods based on intraday repeatability was calculated by analyzing five sets of samples multiple times, spiked with four different concentrations of ABZ (0.005, 0.5, 1 and 5µg/mL) with a fixed amount of IS (praziquantel) (5µg/mL). The other four different concentration of ABZSO and $ABZSO_2$ (0.05, 1, 5 and 80µg/mL) with a fixed concentration of IS (oxfendazole) (0.5µg/mL). The reproducibility or inter-day variations of the methods were validated by taking the same concentration range of plasma as it is described in the previous section (Accuracy) though only one single determination was made on

three different days for every concentration. Percentage relative standard deviation (R.S.D.) and standard deviation (S.D.) were calculated.
Where, %R.S.D = (SD/mean)*100

4.4 Limit of quantitation
The limit of quantitation value (LOQ) of the above-developed methods were determined from the minimum concentration of ABZ, ABZSO and $ABZSO_2$ (in spiked plasma sample) which formed a three times more higher peak height than that of the baseline noise having a sensitivity of 0.005 aufs (absorbance unit full scale) for ABZ and 0.05aufs (absorbance unit full scale) for ABZSO and $ABZSO_2$ in a 200µl of plasma sample.

4.5 Stability
The stability was performed for all the three analytes (ABZ, ABZSO, and $ABZSO_2$). The stability study for ABZ was performed by keeping spiked plasma samples at the concentrations of 0.005, 1 and 5µg/mL with a fixed concentration of IS (praziquantel) (5µg/mL). For ABZSO and $ABZSO_2$ stability study was also done by preserving spiked plasma of the samples at the concentrations of 0.05, 5, and 80µg/mL with a fixed amount of IS (oxfendazole) (0.5µg/mL). Samples were analyzed at least three times for each concentration in a −20°C freezer (Sanyo, Japan) for a period of 6 months. The measurement was done periodically that was for 1, 2, 4, 6 months. Freeze and thaw stability study was achieved by freezing samples at −20°C for at least 24 h and defrosted by keeping it at room temperature (25°C). After completion of thaw process, the samples were again kept in freeze and refrozen for at least 24 h. The process is repeated at least three times according to the CDER guideline [35].

4.6 Quality control
Samples for quality control (QC) of ABZ was prepared in plasma by taking a stock solution discretely from the stock solution that was prepared for the calibration curve plotting. The concentrations of ABZ were 0.005, 0.5, 1 and 5µg/mL along with a fixed concentration of IS (5µg/mL of praziquantel). Another QC samples for ABZSO and $ABZSO_2$ were made up in plasma using a stock solution separated from that used to prepare the calibration curve, at the concentrations of 0.05, 1, 5 and 80µg/mL along with a fixed concentration of IS (oxfendazole 0.5µg/mL). These samples were kept in cryovials at −20°C for freezing which was later run for analysis. The base of accepting or rejecting the test run depends on the results obtained from the run of the quality control samples. Nonetheless, two out of four samples should have been within ±20% of their individual minimum value. Two out of four samples might be outside the ±20% of their individual minimum value, however, it must not be for the same concentration.

5. Assessment of the biological samples using the developed method
The pharmacokinetic study of albendazole and praziquantel along with their metabolites was performed using the developed method. Twelve healthy albino rats of three groups (weighing 150–250 gm.) were taken and a single dose through oral route as 50mg/kg body weight for ABZ was given. Approval for the procedure of the pharmacokinetic study was granted by the CPCSEA (Committee for the Purpose of Control and Supervision on Experimental Animals). Using heparinized-coated micro centrifuge plastic tubes (2mL) samples of venous blood (1.5 mL) were withdrawn from retro-orbital plexus of albino rats at the following time interval: 0, 0.5, 1, 1.5, 2, 4, 6, 7.5, 24, 28, 32, and 48 hrs of dosing.
Blood was collected from the retro-orbital plexus and immediately centrifuged at 15000 rpm for 15 min and the plasma was collected carefully. After that, the supernatant liquid from the plasma was taken and kept at −20 °C until it was investigated. For ABZSO (active metabolite) and $ABZSO_2$ the plasma samples were analyzed as described above after sample preparation. When ABZ is given orally, ABZ is readily converted to ABZSO as an active metabolite so ABZ cannot be measured. The pharmacokinetic parameters for ABZSO and $ABZSO_2$ such as T_{max}, C_{max}, $T_{1/2}$, AUC, AUMC, and MRT were calculated by using the software Phoenix(R) WinNolin. The maximum concentrations (C_{max}) and the time (T_{max}) of the maximum concentrations observed for ABZSO and $ABZSO_2$ were reported.

6. Results
Albendazole sulfoxide: $C_{12}H_{15}N_3O_3S$
IR (KBr, cm^{-1}): 3331.44 (N-H), 2957.91 (Ar. C-H), 2917 (C-H), 1709.64 (C=O), 1625.23 (Ar. C-C), 1583.93 (C-C), 1323.21 (S=O), 1269.08 (C-O), 1094.58 (C-N) (See Figure 2).
1H NMR (400MHz, d_6-DMSO), δ: 11.496 (s, 3H, H-3); 7.759 (s, 1H, H-4); 7.300 (d, 1H, H-6); 7.506 (d, 1H, H-7); 2.998 (t, 2H, H-9); 1.653 (m, 2H, H-10); 1.258 (t, 3H, H-11); 10.188 (s, 1H, H-12); 3.862 (s, 3H, H-14). For IR and NMR peaks see Supplementary Figures S1 and S2.
(d_6-DMSO- Dimethyl sulfoxide-d_6- 2.466)

Albendazole sulfone: $C_{12}H_{15}N_3O_4S$
IR (KBr, cm^{-1}): 3331.35 (N-H), 2957.99 (Ar. C-H), 2917.54 (C-H), 1709.97 (C=O), 1623.47 (Ar. C-C), 1584.07 (C-C), 1323.17 (S=O), 1269.64 (C-O), 1095.88 (C-N) (See Figure 2).

1H NMR (400MHz, d_6-DMSO) δ: 11.496 (s, 3H, H-3); 7.759 (s, 1H, H-4); 7.300 (d, 1H, H-6); 7.506 (d, 1H, H-7); 3.205 (t, 2H, H-9); 1.792 (m, 2H, H-10); 1.190 (t, 3H, H-11); 10.188 (s, 1H, H-12); 3.862 (s, 3H, H-14). For IR and NMR peaks see Supplementary Figures S3 and S4. (d_6-DMSO-Dimethyl sulfoxide-d_6- 2.466)

6.1 Chromatographic separation

Different HPLC chromatographic systems were tested and tried for the optimization of complete separation of ABZ, ABZSO, and $ABZSO_2$ from their internal standards. A C18 reverse phase column as a stationary phase and acetonitrile and distilled water (60:40) (v/v) as an appropriate mobile phase were chosen for optimal separation of albendazole along with praziquantel at a wavelength of 225nm. For ABZSO and $ABZSO_2$ along with oxfendazole Phenomenex C18 Luna reverse phase column (stationary phase) and the mobile phase containing acetonitrile, methanol: and phosphate buffer (20mM) in the ratio 20: 25: 55 pH was adjusted to 6.9 using 0.1N NaOH solution was chosen as an appropriate elution solvent at a wavelength of 290nm. The retention times (RT) of ABZ and Praziquantel (IS) were found to be 3.7 and 6.4 minutes respectively using enable C18 column. For ABZSO, $ABZSO_2$ and Oxfendazole (IS) retention times were 5.5, 7.0 and 8.2 minutes respectively using Phenomenex C18 Luna column. Good baseline separation was found in the chromatograms for both the mobile phases. Chromatogram of ABZ (5µg/mL) and Praziquantel (5µg/mL) (IS) and the chromatogram of ABZSO, $ABZSO_2$ (80µg/mL) and Oxfendazole (0.5µg/mL) (IS) shown in Figures 3 and 4.

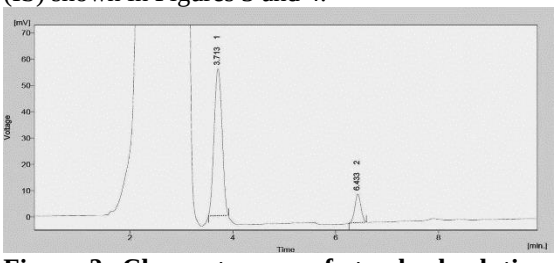

Figure 3: Chromatogram of standard solution of ABZ (5 µg/ml) and PRQ (5µg/ml) with retention times 3.7 and 6.4 minutes

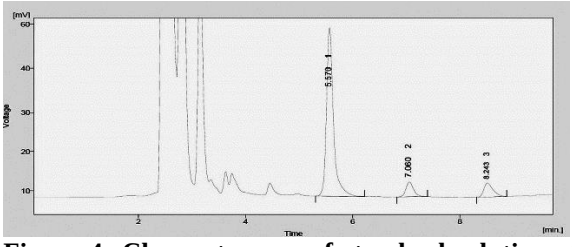

Figure 4: Chromatogram of standard solution of ABZSO, $ABZSO_2$ (80µg/ml) and Oxfendazole (5µg/ml) with retention times 5.5, 7.0 and 8.2 minutes

6.2 Sample preparation

For the analysis of the samples were prepared by protein precipitation. Perchloric acid (8.25% of perchloric acid) of 45µl was used to precipitate the proteins from plasma. The above method was found to be the most suitable method to prepare the samples which in turn produced uncontaminated chromatogram.

6.3 Calibration curves

Plasma analysis for ABZ concentration range of 0.005–5µg/mL using IS (Praziquantel) (5µg/mL) and plasma analysis for ABZSO and $ABSO_2$ concentration range of 0.05-80µg/mL using IS (Oxfendazole) (0.5µg/mL). A linear relationship was found from the calibration plot. The correlation coefficients(r) were found to be 0.9989, 0.99949 and 0.99949 for ABZ, ABZSO and $ABZSO_2$ with equations Y=115.2x+12.03, Y=3.996x+1.564 and Y=1.913x+0.575 are summarized in table no. 1.

6.4 Method validation

6.4.1 Linearity

The linearity was tested for the concentration range of 5, 3, 2, 1, 0.1, 0.05 and 0.005µg/mL for ABZ with a fixed concentration of Praziquantel (IS) (5µg/mL) and 80; 30; 10; 5; 1; 0.5; 0.1 and 0.05µg/mL for ABZSO and $ABSO_2$ using a constant amount of oxfendazole (IS) (0.5µg/mL). The calibration curves were plotted and assessed by its correlation coefficient. The least squares method was used to calculate the linear regression equations using Microsoft Excel® program. The coefficient of determination (r^2) for plasma was 0.998, 0.999 and 0.999 for ABZ, ABZSO and $ABZSO_2$ representing a strong proportional relationship with the variable are summarized in table no. 1.

6.4.2 Precision

Inter-day and intra-day precision study of ABZ ABZSO and $ABZSO_2$ gave a %R.S.D. within 5.27, 7.75 and 11.35 [as per CDER guidance for Bio-analytical Method Validation (38) must be less than 15]. That shows the recommended methods are highly precise. Little variation of ABZ, ABZSO and $ABZSO_2$ assay was detected. The Relative standard deviation for all different concentrations of ABZ, ABZSO, and $ABZSO_2$ were less than 15%. The intraday precision and interday precision study for ABZ, ABZSO and $ABZSO_2$ were performed using the concentration between 0.005–5µg/mL (0.005, 0.5, 1 and 5µg/mL) and 0.05-80µg/mL (0.05, 1, 5 and80µg/mL) are summarized in table 2a, 2b, 3a and 3b.

Table 1: Spectral and statistical data for determination of ABZ, ABZSO and $ABZSO_2$ by proposed HPLC method

Parameters	Value for ABZ	Value for ABZSO	Value for $ABZSO_2$
Absorption maxima, λmax (nm)	290nm	290nm	290nm
Linearity range (µg/mL)	0.005-5	0.05-80	0.05-80
Coefficient of determination (r^2)	0.998	0.999	0.999
Correlation coefficient (r)	0.9989	0.99949	0.99949
Regression equation (Y^a)	Y=115.2x+12.03	Y=3.996x+1.564	Y=1.913x+0.575
Slope (b)	115.2	3.996	1.913
Intercept (a)	12.03	1.564	0.575
Limit of detection, LOD (µg/mL)	0.0707	0.2236	0.2236
Limit of quantitation, LOQ (µg/mL)	0.005	0.05	0.05

[a] Y = mx + c, where x is the concentration (µg/mL)

Table 2a: Inter-day (n=5) precision (%R.S.D.) measured for QC points for ABZ in plasma

	T.C.	Day 1		Day 2		Day 3	
Plasma	µg/mL	E.C. ABZ	%R.S.D. ABZ	E.C. ABZ	%R.S.D. ABZ	E.C. ABZ	%R.S.D. ABZ
1	5.000	5.136	0.860	5.195	0.490	5.222	0.270
2	1.000	0.917	1.960	0.937	1.450	0.920	0.690
3	0.500	0.468	1.460	0.492	1.230	0.476	1.620
4	0.005	0.005	4.700	0.005	5.270	0.005	1.420

T.C. denotes theoretical concentration and E.C. denotes experimental concentration.

Table 2b: Intra-day (n=5) precision (%R.S.D.) measured for QC points for ABZ in plasma

	T.C.	Intra-day	
Plasma	µg/mL	E.C. ABZ	%R.S.D. ABZ
1	5.000	5.197	0.100
2	1.000	0.921	0.840
3	0.500	0.495	0.350
4	0.005	0.005	1.370

T.C. denotes theoretical concentration and E.C. denotes experimental concentration.

Table 3a: Inter-day (n=5) precision (%R.S.D.) measured for QC points for ABZSO and $ABZSO_2$ in plasma

	TC	Day 1				Day 2				Day 3			
Plasma	µg/mL	E.C. ABZSO	E.C. $ABZSO_2$	%R.S.D. ABZSO	%R.S.D. $ABZSO_2$	E.C. ABZSO	E.C. $ABZSO_2$	%R.S.D. ABZSO	%R.S.D. $ABZSO_2$	E.C. ABZSO	E.C. $ABZSO_2$	%R.S.D. ABZSO	%R.S.D. $ABZSO_2$
1	80	80.34	80.78	0.28	0.91	80.4	81.34	0.35	0.80	80.45	82.33	0.58	0.74
2	5	5.01	4.88	2.34	1.82	5.04	4.87	2.65	1.13	5.048	4.97	3.57	1.00
3	1	1.05	1.21	6.28	3.10	1.11	1.12	7.75	1.05	1.130	1.13	3.80	2.30
4	0.05	0.05	0.05	1.66	7.52	0.04	0.04	0.44	4.29	0.050	0.05	0.36	11.3

T.C. denotes theoretical concentration and E.C. denotes experimental concentration

Table 3b: Intra-day (n=5) precision (%R.S.D.) measured for QC points for ABZSO and $ABZSO_2$ in plasma

	T.C.	Intra-day			
Plasma	µg/mL	E.C. ABZSO	E.C. $ABZSO_2$	%R.S.D. ABZSO	%R.S.D. $ABZSO_2$
1	80	80.214	81.611	0.334	0.355
2	5	5.055	4.956	2.415	0.594
3	1	1.121	1.139	4.395	1.308
4	0.05	0.049	0.050	1.238	8.567

T.C. denotes theoretical concentration and E.C. denotes experimental concentration.

6.4.3 Accuracy
The inter-day and intra-day accuracy study show the results were ranged from 92.0552-103.9391%, 98.9789-112.1121% and 98.2498-115.5543% for ABZ, ABZSO, and $ABZSO_2$ (Table 4a and 4b). There was no meddling by the endogenous components that were present in the plasma is confirmed by the above accuracy data. Inter-day as well as intra-day range was 0.005–5µg/mL for ABZ (0.005, 0.5, 1 and 5µg/mL) and 0.05-80µg/mL (0.05, 1, 5 and 80µg/mL) for ABZSO and $ABZSO_2$ along with each fixed concentration of Praziquantel (5µg/mL) and Oxfendazole (0.5µg/mL) are abridged in the table 4a and 4b.

6.4.4 Recovery
In recovery study the average recoveries in plasma were found to be 0.005–5µg/mL for ABZ and 0.05-80µg/mL for ABZSO and ABZSO2 which was more than 90%. There is nearly 100% recovery which specifies there is no meddling due to sample treatment of the spiked plasma.

6.4.5 Selectivity
Nonappearance of meddling peaks from endogenous material of plasma establishes the selectivity of the developed method. Figures 5, 6, 3, 4 demonstrates archetypal chromatograms of absolute plasma in both mobile phases, spiked plasma with ABZ (5µg/mL) and Praziquantel(IS) 5µg/mL with retention times of 3.7 and 6.4 minutes and spiked plasma with ABZSO, $ABZSO_2$ (80µg/mL) and Oxfendazole (IS) 0.5µg/mL with retention times 5.5, 7.0 and 8.2.

6.4.6 Specificity
Coinciding peaks of any possible meddling of plasma endogenous components were in 2 to 4 min for blank. Later there was no noteworthy interference from the spiked plasma in both the mobile phases and in different columns (Figures 3 and 4) that affected the response of ABZ ABZSO, ABZSO2, Praziquantel (IS) and Oxfendazole (IS).

6.4.7 Limit of quantitation
The limit of quantitation (LOQ) for ABZ, ABZSO, and $ABZSO_2$ was established as 0.005µg, 0.05µg and 0.05µg respectively using 200µl of rat plasma.

6.4.8 Stability
Plasma samples containing ABZ at concentrations of 0.005, 1 and 5µg/mL and 0.05, 5 and 80µg/mL for ABZSO and $ABZSO_2$ were found to be stable after storing in a freezer at −20 °C for at least 6 months without major decay of the drug. There was no effect in the quantification value of the analytes in long-term storage. Concentrations were measured in the interval of 1, 2, 4 and 6 months after storing in freezing conditions. The mean deviancies (%) was found to fluctuate between -12.00 to 9.16%, -10.00 to 1.6% and -10.00 to 1.6% for ABZ, ABZSO, and $ABZSO_2$ (table 5a and 5b). After repeating the three consecutive cycles of freezing and defrosting did not alter the estimated concentrations. The mean percentage variation of the actual values from the theoretical values lies within -2.00 to 4.02%, 0.6 to 0.9% and -1.3 to 2.5% for ABZ, ABZSO, and $ABZSO_2$ (table 6a and 6b).

6.4.9 Quality control
The plasma was analyzed by three different validated analysts. Each time the analysis involved a standard curve and samples of QC. The samples containing a minimum amount of 0.005, 0.5, 1 and 5µg/mL of ABZ and 0.05, 1, 5 and 80µg/mL of ABZSO and $ABZSO_2$ were investigated at the starting and at the completion of the analytical test. Results were found to be in the suitable perimeter that is ±20% of the minimum amount.

6.4.10 System suitability
In chromatographic analysis system suitability study is an essential stage that is intended to validate that the reproducibility and resolution of the developed method and it is suitable for the study. [34] As per USP, a system suitability study was executed for standard and test solutions by obtaining their chromatograms to find out the different parameters which were previously mentioned. The outcomes of the six duplicate injections of the standard are abridged in table 7.

6.4.11 Assay of specimens
The above analytical method was used to quantify ABZSO and $ABZSO_2$ concentration in a pharmacokinetic study carried out on three groups each containing twelve albino rats. HPLC chromatograms of rat plasma after 2hrs and 6hrs of oral drug administration (suspension) of ABZ 50mg/kg body weight with retention times of 5.5 for ABZSO and 7.0 for $ABZSO_2$ given in Figures 5 and 6. Plasma concentration Vs time profiles of ABZSO and $ABZSO_2$ given in Figure 7. Various other parameters of a pharmacokinetic study of ABZSO and $ABZSO_2$ have been summarized in table 8 and 9. The Cmax and Tmax of ABZO and $ABZSO_2$ in the present study to establish the clinical applicability of the method, plasma concentrations of ABZSO and $ABZSO_2$ were carried out on three groups each containing twelve albino rats following a single oral dose of 50mg/kg body weight of ABZ.

Table 4a: Summary of inter-day (n = 5) and intra-day (n = 5) precision and accuracy of the method in rat plasma for ABZ

Nominal Conc. (µg/mL) ABZ	Mean Conc. Found[a] (µg/mL) ABZ	S.D. ABZ	Precision (%R.S.D.) ABZ	Mean accuracy[b] (%) ABZ
Inter-day (n=5)				
5	5.1841	0.0282	0.5453	103.6828
1	0.9243	0.0127	1.3730	92.4268
0.5	0.4784	0.0069	1.4408	95.6727
0.005	0.0050	0.0002	3.8004	99.1686
Intra-day (n=5)				
5	5.1970	0.0054	0.1039	103.9391
1	0.9206	0.0078	0.8428	92.0552
0.5	0.4952	0.0018	0.3549	99.0322
0.005	0.0050	0.0001	1.3762	100.2778

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.

Table 4b: Summary of inter-day (n = 5) and intra-day (n = 5) precision and accuracy of the method in rat plasma for ABZSO and $ABZSO_2$

Nominal Conc. (µg/mL)	Mean Conc. Found[a] (µg/mL) ABZSO	Mean Conc. Found[a] (µg/mL) $ABZSO_2$	S.D. ABZSO	S.D. ABZSO2	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	98.24987
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	99.04931
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	99.11134
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	99.71772

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.

Table 5: Storage stability data of ABZ in plasma at concentrations 0.005, 1 and 5µg/mL

Months	Conc. (µg/mL)	Assay 1 ABZ	Assay 2 ABZ	Assay 3 ABZ	Mean ABZ	S.D. ABZ	%DEV[a] ABZ
a. Long term stability							
1	0.005	0.0051	0.0050	0.0051	0.0050	0.0001	0
	1	1.1470	1.0140	1.1140	1.0916	0.0692	9.16
	5	5.1160	5.1173	5.1473	5.1268	0.0177	2.53
2	0.005	0.0050	0.0048	0.0050	0.0049	0.0001	-2
	1	1.0550	0.9965	1.0258	1.0257	0.0292	2.57
	5	5.0020	4.8879	5.0020	4.9639	0.0658	-0.7
4	0.005	0.0049	0.0047	0.0049	0.0048	0.0001	-4
	1	0.9953	0.9765	0.9255	0.9657	0.0361	-3.4
	5	4.8657	4.8814	4.9111	4.8860	0.0230	-2.2
6	0.005	0.0046	0.0045	0.0046	0.0045	0.0001	-10
	1	0.8876	0.8654	0.8857	0.8795	0.0123	-12
	5	4.7641	4.8743	4.7889	4.8091	0.0578	-3.8
b. Freeze and thaw stability							
	0.005	0.0051	0.0049	0.0049	0.0049	0.0001	-2
	1	0.9945	1.0255	1.1007	1.0402	0.0546	4.02
	5	4.9988	5.1041	5.0027	5.0352	0.0597	0.70

a %DEV = deviation of single mean value from theoretical value (%)

Table 6: Storage stability data of ABZSO and $ABZSO_2$ in plasma at concentrations 0.05, 5 and 80µg/mL

Months	Conc. (µg/mL)	Assay 1 ABZSO	Assay 1 $ABZSO_2$	Assay 2 ABZSO	Assay 2 $ABZSO_2$	Assay 3 ABZSO	Assay 3 $ABZSO_2$	Mean ABZSO	Mean $ABZSO_2$	S.D. ABZSO	S.D. $ABZSO_2$	%DEV[a] ABZSO	%DEV[a] $ABZSO_2$
a. Long term stability: Concentration measured (µg/mL)													
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: Concentration measured (µg/mL)													
	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

a %DEV = deviation of single mean value from theoretical value (%)

Table 7: System suitability parameters of ABZ, PRQ, ABZSO, $ABZSO_2$ and Oxfendazole

Sl. No.	Parameters	ABZ	PRQ (IS)	ABZSO	$ABZSO_2$	Oxfendazole (IS)
1	Retention time, Rt (min)	3.7	6.4	5.5	7.0	8.2
2	Capacity factor (k)	1.341	3.07	2.235	3.11	3.85
3	Separation factor (α)	2.289	-----	1.39	1.237	------
4	Theoretical plates (USP)	2433	7281	3025	4900	6724
5	HETP (mm)	0.1027	0.0343	0.082	0.051	0.037
6	Resolution (Rs)	2.98	------	2.664	2.535	------

Table 8: Pharmacokinetic parameters of ABZSO after a single oral dose of 50mg/kg ABZ to three groups each containing 12 albino rats

S. No.	Pharmacokinetic parameters	Observed value for ABZSO
1	Elimination rate constant, kel (h^{-1})	0.5020
2	Time required for maximum plasma concentration, T_{max} (h)	6.0000
3	Maximum plasma concentration, C_{max} (µg/mL)	69.6852
4	Plasma half life, $T_{1/2}$ (h)	1.3808
5	Area under curve $AUC_{(0\rightarrow48)}$, (µg h/mL)	646.1751
6	Area under curve at infinite time, $AUC_{(0\rightarrow\infty)}$ (µg h/mL)	646.4679
7	Area under momentum curve $AUMC_{(0\rightarrow48)}$ (µg h^2/mL)	5863.7853
8	Mean residence time, MRT (h)	9.0746

Table 9: Pharmacokinetic parameters of $ABZSO_2$ after a single oral dose of 50mg/kg ABZ to three groups each containing 12 albino rats

S. No.	Pharmacokinetic parameters	Observed value for ABZSO2
1	Elimination rate constant, kel (h^{-1})	0.3752
2	Time required for maximum plasma concentration, T_{max} (h)	8.0000
3	Maximum plasma concentration, C_{max} (µg/mL)	16.1641
4	Plasma half life, $T_{1/2}$ (h)	1.8475
5	Area under curve $AUC_{(0\rightarrow48)}$, (µg h/mL)	216.4622
6	Area under curve at infinite time, $AUC_{(0\rightarrow\infty)}$ (µg h/mL)	216.6241
7	Area under momentum curve $AUMC_{(0\rightarrow48)}$ (µg h^2/mL)	1759.0291
8	Mean residence time, MRT (h)	8.1263

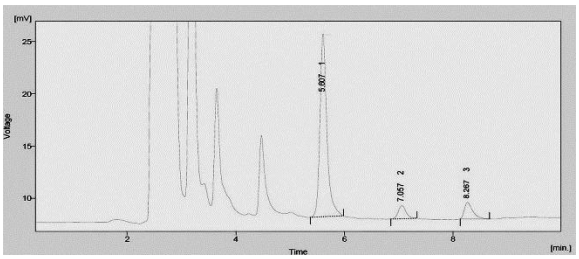

Figure 5: Chromatogram of rat plasma after 2hrs of oral administration of ABZ

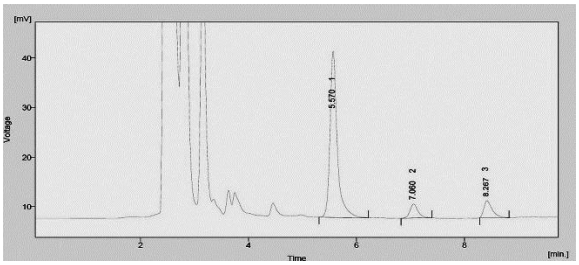

Figure 6: Chromatogram of rat plasma after 6hrs of oral administration of ABZ

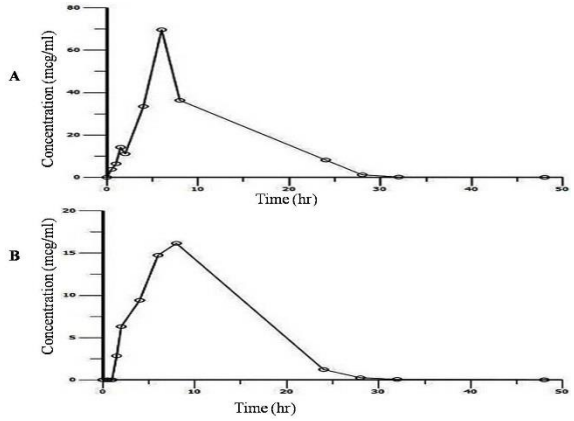

Figure 7: Plasma concentration–time profile of drug and its metabolite in healthy rats following a single oral dose of 50 mg/kg body weight of Albendazole. A. Albendazole Sulfoxide B. Albendazole Sulfone

7. Discussion and Conclusions

Simultaneous analysis of ABZ, ABZSO, and $ABZSO_2$ in plasma by HPLC is being reported in this paper. The above-developed method is fund to be quick, simple and reproducible. The LOQ achieved by taking 200µl plasma was comparable the previously reported methods. A small amount of the analyte is adequate for effective pharmacokinetic studies using the above-developed method. The method is the highly selective (as no interference from endogenous peaks) than the early reported methods in the literature [12-22] which gives it an important advantage in calculating the pharmacokinetic parameters (table no. 8 and 9) for the analytes. In this method, the sample preparation step is single step uses 8.25% perchloric acid which is effective in giving clear chromatograms and does not need evaporation of solvents which will rather increase the cost of analysis and increases the time of analysis. Total run time was within 10 min. Inter-day and intra-day precision study of ABZ, ABZSO, and $ABZSO_2$ show %R.S.D. less than 11.00% which must remain less than 15 according to CDER guidance for Bioanalytical Method Validation [35]. Another advantage of this method is the use of dual columns for determination of the parent drug and its metabolites which renders the analyte peaks to non-interfering/ non-overlapping with each other thus enabling the method to be more accurate, sensitive and selective.

8. Acknowledgments

We are indebted to the Principal Dr. U.M. Upadhyay and to the Assistant Professors of the Quality Assurance Department of the Sigma Institute of Pharmacy, Vadodara, Gujarat. We also thank the management team for their immense support and for making available of required chemicals and equipment for this work. We also like to thank Mercury Pharmaceutical Ltd, Vadodara, Gujarat, from where the Albendazole was provided and also thankful to Micro Labs Ltd, Goa, for providing Praziquantel, both free of charge as a gift samples.

9. Conflict of Interest

There is no conflict of interest among all authors.

10.Abbreviations

DPPH: 2, 2-diphenyl-1-picrylhydrazyl; **LC-MS/MS:** Liquid Chromatography-Mass Spectroscopy/ Mass Spectroscopy; E. coli**:** Escherichia coli; S. aureus**:** Staphylococcus aureus; **ILRI:** Indian Lac Research Institute; **EC 50:** Half maximal effective concentration; **PHBP:** Plasma Hyaluronan-Binding Protein; **IC50:** Half maximal inhibitory concentration; **ESI:** Electrospray Ionization; **HPLC:** High-Performance Liquid Chromatography; **PDA:** Photodiode Array; **IR:** Infrared; **UV-Vis:** Ultraviolet–Visible; **DAD:** Diode Array Detector; **IINRG:** Indian Institute of Natural Resins and Gums; **K3 [Fe(CN)6]:** Potassium Ferricyanide; **KH2PO4:** Potassium Dihydrogen Phosphate Buffer or Phosphate Buffer; **FeCl3:** Ferric Chloride; **rpm:** Revolution per minute; **C:** Chloroform; **EA:** Ethyl acetate; **M:** Methanol; **W:** Water; **EtOAc:** Ethyl acetate; **h:** Hour; **H2O2:** Hydrogen Peroxide; **CFU:** Colony Forming Units; **MHA:** Muller-Hinton Agar; **mm:** Millimeter; **nm:** Nanometer; **H2O:** Water; **CO2:** Carbon dioxide; **min:** Minutes; **amu:** Atomic Mass Unit; **ROS:** Reactive Oxygen Species.

11. References

1. Dayan A. D., Albendazole, mebendazole and praziquantel. Review of non-clinical toxicity and pharmacokinetics. Acta Trop, **86**(2-3), 141-59 (2003).
2. Rawden H. C., Kokwaro G. O., Ward S. A. and Edwards G., Relative contribution of cytochromes P-450 and flavin-containing monoxygenases to the metabolism of albendazole by human liver microsomes. Br. J. Clin. Pharmacol, **49**, 313-322 (2000).
3. Lacey E., Mode of action of benzimidazoles. Parasitol. Today, **6**, 112-115 (1990).
4. Gotschall D. W., Theorides V. J. and Wang R., The metabolism of benzimidazole anthelmintics. Parasitol. Today, **6**, 115-124 (1990).
5. Lanusse C. E., Nare B., Gason L. H. and Prichard R. K., Metabolism of albendazole and albendazole sulphoxide by ruminal and intestinal fluids of sheep and cattle. Xenobiotica, **22**, 419-424 (1992).
6. Souhaili-El-Amri H., Mothe O., Totis M., Masson C., Batt A. M., Delatour P. and Siest G., Albendazole sulfonation by rat liver cytochrome P-450c. J. Pharmacol. Exp. Ther, **246**, 758-764 (1988).
7. Bol ´ as-Fern´ andez F., Rama-Iˇniguez S. and Torrado J. J., Ex Vivo Anthelmintic Activity of Albendazole-Sulphoxide Enantiomers. J. Parasitol, **90**, 407-409 (2004).
8. Navvaro M., Canut L., Carretero A., Cristo` fol C., Pe´ rez-Aparicio F. J., Arboix M. and Ruberte J., Developmental toxicity in rat fetuses exposed to the benzimidazole netobimin. Reprod. Toxicol, **13**, 295-302 (1999).
9. Villaverde C., Alvarez A. I., Redondo P., Voces J., Del Estal J. L. and Pietro J. G., Small intestinal sulphoxidation of Albendazole. Xenobiotica, **25**, 433-441 (1995).
10. Delatour P., Benoit E., Besse S. and Boukraa A., Comparative enantioselectivity in the sulphoxidation of albendazole in man, dogs and rats. Xenobiotica, **21**, 217-221 (1991).
11. Sarin R., Dash A. P. and Dua V. K., Albendazole sulphoxide concentrations in plasma of endemic normals from a lymphatic filariasisendemic region using liquid chromatography. J. Chromatogr. B, **799**, 233–238 (2004).
12. Chen X., Zhao L., Xu H. and Zhong D., Simultaneous determination of albendazole and its major active metabolite in human plasma using a sensitive and specific liquid chromatographic-tandem mass spectrometric method. J. Pharmaceut. Biomed. Anal, **35**, 829–836 (2004).
13. Wu Z., Medlicott N. J., Razzak M. and Tucker I. G., Development and optimization of a rapid HPLC method for analysis of ricobendazole and albendazole sulfone in sheep plasma. J. Pharmaceut. Biomed. Anal, **39**, 225–232 (2005).
14. Capece B. P. S., Castells G., Pe´ rez F., Arboix M. and Cristo` fol C., Pharmacokinetic Behaviour of Albendazole Sulphoxide Enantiomers in Male and Female Sheep. Vet. Res. Commun, **24**, 339-348 (2000).
15. Mirfazaelian A., Dadashzadeh S. and Rouini M. R., A high per-formance liquid chromatography method for simultaneous determination of albendazole metabolites in human serum. J. Pharmaceut. Biomed. Anal, **30**, 1249–1254 (2002).
16. Batzias G. C., Theodosiadou E. and Delis G. A., Quantitative determination of albendazole metabolites in sheep spermatozoa and seminal plasma by liquid chromatographic analysis with fluorescence detection. J. Pharm. Biom. Anal, **35**, 1191-1202 (2004).
17. Shaikh B., Rummel N. and Reimschuessel R., Determination of Albendazole and Its Major Metabolites in the Muscle Tissues of Atlantic Salmon, Tilapia, and Rainbow Trout by High Performance Liquid Chromatography with Fluorometric Detection. J. Agri. Food Chem, **51**, 3254-3259 (2003).
18. Mottier L., Alvarez L. and Lanusse C., Quantitative chromatographic determination of

several benzimidazole anthelmintic molecules in parasite material. J. Chromatogr. B, **798**, 117-125 (2003).
19. Fletouris D. J., Botsoglou N. A. , Psomas I. E. and Mantis A. I., Determination of the marker residue of albendazole in milk using ion-pair liquid chromatography and fluorescence detection. Anal. Chem. Acta, **345**, 111-119 (1997).
20. Solana H. D, Rodriguez J. A. and Lanusse C., Comparative metabolism of albendazole and albendazole sulphoxide by different helminth parasites. Parasitol. Res, **87**, 275-280 (2001).
21. Goudah A., Aspects of the Pharmacokinetics of Albendazole Sulphoxide in Sheep. Vet. Res. Commun, **27**, 555-556 (2003).
22. Delatour P., Garnier F., Benoit E. and Caude I., Chiral behaviour of the metabolite albendazole sulphoxide in sheep, goats and cattle. Res. Vet. Sci, **50**, 134-138 (1991).
23. Su S. C., Chou H. H., Chang P. C., Lui C. H. and Chou S. S., Simultaneous determination of febantel, fenbendazole, oxfendazole and oxfendazole sulfone in livestock by matrix solid dispersion extraction technique and HPLC. J. Food Drug Anal, **12**, 244-253 (2004).
24. Danaher M., Keeffe M. O. and Glennon J. D., Development and optimisation of a method for the extraction of benzimidazoles from animal liver using supercritical carbon dioxide. Anal. Chim. Acta, **483**, 313-324 (2003).
25. Moreno L., Echevarria F., Mu˜noz F., Alvarez L., Sanchez Bruni S. and Lanusse C., Dose-dependent activity of albendazole against benzimidazole-resistant nematodes in sheep: relationship between pharmacokinetics and efficacy. Exp. Parasitol, **106**, 150-157 (2004).
26. Lanchote V. L., Takayanagui O. M. and Mateus F. H., Enantioselective renal excretion of albendazole metabolites in patients with neurocysticercosis. Chirality, **16**, 520–525 (2004).
27. Takayanagui O. M., Bonato P. S., Dreossi S. A. C. and Lanchote V. L., Enantioselective distribution of albendazole metabolites in cere-brospinal fluid of patients with neurocysticercosis. Br. J. Clin. Pharmacol, **54**, 125–130 (2002).
28. Lanchote V. L., Garcia F. S., Dreossi S. A. C. and Takayanagui O. M., Pharmacokinetic Interaction Between Albendazole Sulfoxide Enantiomers and Antiepileptic Drugs in Patients With Neurocysticercosis. Ther. Drug Monitor, **24**, 338-345 (2002).
29. Marques M. P., Takayanagui O. M. and Lanchote V. L.,Albendazole metabolism in patients with neurocysticercosis: antipyrine as a multifunctional marker drug of cytochrome P450. Br. J. Med. Biol. Res, **35**, 261-269 (2002)
30. ANVISA. Agˆencia Nacional de Vigilˆancia Sanit´aria. RE 899 de 29 de maio de, http://www.anvisa.gov.br/legis/resol/2003/re/89903.htm, (2003).
31. Guidance for the Industry: Analytical Method Validation, US Food and Drug Administration, Center for Drug Evaluation and Research (CDER), Rockville, MD, (2000).
32. ICH Guidelines: Validation of Analytical Procedures: Q2B, (1996).
33. ICH Guidelines: Validation of Analytical Procedures: Q2A, (1994).
34. Meyyanathan S. N., Ramasarma G.V.S. and Suresh B., Analysis of simvastatin in pharmaceutical preparations by thin layer chromatography, high-performance. ARS Pharma, **45**, 121–129 (2004).
35. Guidance for the Industry: Bioanalytical Method Validation, US Food and Drug Administration, Center for Drug Evaluation and Research (CDER), (2001)
36. Dey S., De A., Mandal S. K., Pradhan P. K., Patel C., Shah S. and Lad B., Development and Validation of RP-HPLC Method for the Estimation of Simvastatin in Bulk and Pharmaceutical Dosage Form. Indo American Journal of Pharmaceutical Research, **3**, 7376-7384 (2013).
37. Sony A., Mandal S. K. and Sen D. J., Development and validation of RP–HPLC method for simultaneous determination of a combined formulation of olmesartan medoxomil & hydrochlorothiazide, World Journal of Pharmacy and Pharmaceutical Sciences,**9**, 1468-1488 (2020).
38. Khawas S., Parui S., Dey S., Mandal S. K and Sarkar S., Simultaneous Spectrophotometric Estimation of Rifampicin, Isoniazid and Pyrazinamide in their Pharmaceutical Dosage Form. Asian Journal of Research in Chemistry, **13**, 117-122 (2020).
39. Dastider D., Mandal S. K. and Sen D. J., Chromatographic development & validation of 2-chloromethyl-4-methyl quinazoline for quantification of quality. European Journal of Pharmaceutical and Medical Research, **7**, 787-813(2020).
40. Ghosh S., Mandal S. K., Roy T. and Ravikumar B. V. V., A Novel RP – HPLC Analytical Method Development and Validation of Fulvestrant Injection as per ICH Guidelines. Egypt J Chem, **64**, 2047-2055 (2021).