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Highly sensitive LC-MS/MS-ESI method for simultaneous quantitation of albendazole and ricobendazole in rat plasma and its application to a rat pharmacokinetic study

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ABSTRACT: A highly sensitive and specific LC-MS/MS-ESI method was developed for simultaneous quantification of albenadazole (ABZ) and ricobendazole (RBZ) in rat plasma (50 µL) using phenacetin as an internal standard (IS). Simple protein precipitation was used to extract ABZ and RBZ from rat plasma. The chromatographic resolution of ABZ, RBZ and IS was achieved with a mobile phase consisting of 5 mM ammonium acetate (pH 6) and acetonitrile (20:80, v/v) at a flow rate of 1 mL/min on a Chromolith RP-18e column. The total chromatographic run time was 3.5 min and the elution of ABZ, RBZ and IS occurred at 1.66, 1.50 and 1.59 min, respectively. A linear response function was established for the ranges of concentrations 2.01–2007 and 6.02–6020 ng/mL for ABZ and RBZ, respectively. The intra- and inter-day precision values for ABZ and RBZ met the acceptance as per FDA guidelines. ABZ and RBZ were stable in battery of stability studies, viz. bench-top, auto-sampler and freeze–thaw cycles. The developed assay was applied to a pharmacokinetic study in rats. Copyright © 2011 John Wiley & Sons, Ltd.

Keywords: albendazole; ricobendazole; LC-MS/MS; method validation; rat plasma; pharmacokinetics

Introduction

Albendazole (Fig. 1, ABZ, CAS no. 54965-21-8), chemically methyl [6-(propylthio)-1*H*-benzoimidazol-2-yl]carbamate, is a benzimidazole carbamate broad spectrum oral anthelmetic used to treat a variety of worm infections caused by nematodes and cestodes. It was first approved for human use in 1982. ABZ is used in the treatment of intestinal and tissue nematode infections and in higher doses in the treatment of echinococcosis. ABZ exerts its vermicidal, ovicidal and larvicidal activity by binding to intracellular tubulin of parasites and inhibiting essential absorptive functions in the organism (Dollery, 1999). Because of its low aqueous solubility, it is poorly absorbed following oral administration. Following oral administration the percent absorbed in rats and humans was found to be 20-30 and 1.5%, respectively (Dollery, 1999). Following oral administration, ABZ undergoes extensive metabolism in the intestine and liver [by cytochrome P450 (CYP) 3A4 and/or flavin-containing monooxygenase (FMO)] into its major active metabolites, i.e. albendazole sulfoxide (ABZ-SOX) or ricobendazole (RBZ, Fig. 1, CAS no. 54029-12-8). RBZ further metabolizes by CYP2C into albendazole sulfone (ABZ-SON), which is pharmacologically inactive. Owing to extensive metabolism in all species, the plasma concentrations of ABZ are usually low when compared with the concentrations of its oxidized metabolites, viz. RBZ and ABZ-SON. RBZ has a chiral center and it was reported that formation of (–)-RBZ depends on CYP isozymes, whereas formation of (+)-RBZ depends on FMO in all species examined (Delatour *et al.*, 1991a, 1991b; Moroni *et al.*, 1995). In humans the (+)-RBZ plasma concentration was 80%, whereas that of (–)-RBZ was 20%; whereas in rat the concentrations of (+)-RBZ and (–)-RBZ were 59 and 41%, respectively (Delatour *et al.*, 1991a). Following oral administration of ^{14}C-ABZ, the peak level of radioactivity of ABZ and RBZ was reached within 2–3 h in animals and humans. Food increases the absorption of ABZ by 5-fold in animals and humans. The protein binding of ABZ and RBZ was found to be 89–92 and 62–67%, respectively (Jung *et al.*, 1992). Clearance of ABZ is very rapid, whereas clearance of the metabolites, viz. RBZ and ABZ-SON, is slower in all the species studied. It has also been reported that both the oxidative metabolites are excreted through bile.

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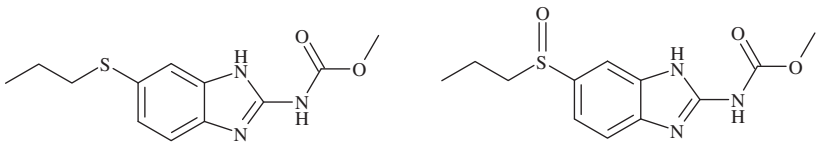
Abbreviations used: ABZ, albenadazole; ABZ-SON, albendazole sulfone; ABZ-SOX, albendazole sulfoxide; CYP, cytochrome P450; FMO, flavin-containing monooxygenase; MRM, multiple reaction monitoring; RBZ, ricobendazole.



Albendazole (ABZ) Ricobendazole (RBZ) or albendazole oxide

Figure 1. Structural representation of albendazole (ABZ) and ricobendazole (RBZ).

ABZ is not detected in plasma following its oral administration because of its low systemic bioavailability owing to its poor solubility, low gastric absorption and extensive metabolism. Hence the pharmacokinetics of ABZ has been studied by determining the plasma concentrations of RBZ and ABZ-SON (Jung *et al.*, 1992; Takayanagui *et al.*, 1997; Sotelo and Jung, 1998). Jung *et al.* (1992) have reported that, during steady state, the RBZ concentrations have higher inter-individual variability. Because of the rapid metabolism of ABZ and possible high variation in plasma concentrations of RBZ, the bioanalytical method for simultaneous determination of ABZ and RBZ should be efficient, rapid, accurate and precise.

To date only three LC-MS/MS methods have been reported for simultaneous quantification of RBZ and ABZ-SON (Bonato *et al.*, 2003, 2007) or ABZ along with RBZ (Chen *et al.*, 2004) in human plasma. Bonato *et al.* (2003) reported an LC-MS/MS method for simultaneous quantification of RBZ and ABZ-SON in human plasma (1 mL) with a lower limit of quantification (LLOQ) of 0.5 and 5 ng/mL for ABZ-SON and RBZ, respectively. RBZ, ABZ-SON and internal standard (IS, phenacetin) were resolved on a cyano column using isocratic mobile phase. Both analytes and IS were monitored in multiple reaction monitoring (MRM) under positive-ion mode. The total run time was ~8 min (Bonato *et al.*, 2003). Chen *et al.* (2004) developed and validated an LC-MS/MS method (in selective reaction monitoring under positive-ion mode) for simultaneous quantification of ABZ and RBZ using 500 µL of human plasma with an LLOQ of 0.4 and 4 ng/mL for ABZ and RBZ, respectively. Estazolam was used as an internal standard (IS). Both analytes and IS were separated on a C_{18} column using an isocratic mobile phase. The total run time was 5 min (Chen *et al.*, 2004). Bonato *et al.* (2007) reported simultaneous quantification of RBZ and ABZ-SON along with praziquantel and its metabolite using 1 mL human plasma under similar conditions to those reported by them earlier (Bonato *et al.*, 2003). In the more recent report the LLOQ for all the analytes was 5 ng/mL. The total run time was 10 min (Bonato *et al.*, 2007). Following a review of all available LC-MS/MS methods, we found that Chen *et al.* (2004) used 500 µL of human plasma and achieved an LLOQ of 0.4 and 4 ng/mL for ABZ and RBZ, respectively, which corresponds to an on-column load of 40 and 400 pg of ABZ and RBZ, respectively. When compared with this method, we achieved an LLOQ of 2.01 and 6.02 ng/mL for ABZ and RBZ, respectively, by processing just 50 µL of plasma sample and on-column load of 2.23 and 6.68 pg of ABZ and RBZ, respectively. By comparing the plasma volume taken for analysis and the column loading reported by Chen *et al.* (2004) with our method, our LLOQ for ABZ and RBZ was 18- and 60-fold lower than the lowest reported LLOQ, respectively.

To the best of our knowledge no bioanalytical method has been reported for simultaneous quantification of ABZ and RBZ in rat plasma. In this manuscript, we are presenting method development and validation of an LC-MS/MS method for simultaneous quantification of ABZ and its active metabolite, i.e. RBZ in rat plasma. In preclinical setup, especially with rodents, only small volumes of blood (~100–200 µL) can be drawn at each time point during a pharmacokinetic/toxicokinetic study, in contrast to the clinical scenario, where large blood volumes (up to 3–5 mL) can be collected at each time point. Hence we felt that there is a need to develop and validate an LC-MS/MS method using small volumes of plasma for simultaneous quantification of ABZ and RBZ. The main advantages of our method are: small plasma volume (50 µL); higher sensitivity compared with earlier reported methods; simple sample processing; and shorter run time. The validated method was successfully applied to a rat pharmacokinetic study.

Experimental

Chemicals and reagents

Albendazole (purity 99.1%) was procured from Sigma-Aldrich (St Louis, MO, USA) and ricobendazole (purity 99.1%) was procured from Jai Radhe Sales (Ahemadabad, India), whereas phenacetin (purity 99.6%) was procured from Jubilant Organosys (Noida, New Delhi, India). HPLC-grade acetonitrile, methanol and analytical-grade tetrahydrofuran were purchased from Rankem, Ranbaxy Fine Chemicals Limited (New Delhi, India). Analytical-grade ammonium acetate was purchased from SD Fine Chemicals (Mumbai, India). The control rat plasma (Na_2EDTA) was procured from Animal House, Jubilant Biosys (Bangalore, India) and stored at −20 ± 5°C prior to use.

HPLC operating conditions

A Shimadzu HT (Shimadzu, Japan) LC system equipped with a degasser (DGU-20A5) and binary pump (LC-20AD) along with an autosampler (SIL-HTC) was used to inject 10 µL aliquots of the processed samples on a Chromolith Performance, RP-18e column (100 × 4.6 mm, Merck, Darmstadt, Germany), which was maintained at 40 ± 2°C in the column oven (CTO-10ASvp). The isocratic mobile phase, a mixture of 5 mM ammonium acetate (pH 6.0) and acetonitrile (20:80, v/v), was filtered through a 0.45 µm membrane filter (XI5522050; Millipore, USA) and then degassed ultrasonically for 5 min. It was delivered at a flow rate of 1 mL/min with splitter at 50% into the mass spectrometer electrospray ionization (ESI) chamber.

Mass spectrometry operating conditions

Quantitation was achieved by MS/MS detection in positive ion mode for analytes and IS using an MDS Sciex (Foster City, CA, USA) API-4000 mass spectrometer, equipped with a Turboionspray™ interface at 450°C. The common parameters, viz. curtain gas, GS1 and GS2, were set at 30, 35 and 40 psi. The compound parameters, viz. declustering potential, entrance potential, collision energy and collision exit potential for ABZ, RBZ and IS were 61, 10, 29 and 18 V; 36, 10, 19 and 12 V; and 46, 10, 31

and 12 V, respectively. Detection of the ions was performed in MRM mode, monitoring the transition of the *m/z* 266.1 precursor ion to the *m/z* 234.4 product ion for ABZ, *m/z* 282.2 precursor ion to the *m/z* 240.4 product ion for RBZ and *m/z* 180.1 precursor ion to the *m/z* 110.1 product ion for IS. Quadrupoles Q1 and Q3 where set on unit resolution. The dwell time was 200 ms. The analytical data were processed by Analyst software (version 1.5).

Preparation of stock and standard solutions

Primary stock solutions of ABZ and RBZ for preparation of standard and quality control (QC) samples were prepared from separate weighings. The primary stock solutions were prepared in methanol (295 and 229 µg/mL for ABZ and RBZ, respectively). The IS stock solution of 1000 µg/mL was prepared in methanol. The stock solutions of ABZ, RBZ and IS were stored at 4°C, and were found to be stable for 2 months (data not shown). They were successively diluted with methanol–water (50:50, v/v) to prepare working solutions to prepare a calibration curve for ABZ and RBZ. Another set of working stock solutions of ABZ and RBZ were made in methanol–water (50:50, v/v; from primary stock) for preparation of QC samples. Working stock solutions were stored at approximately 4°C for a week (data not shown). Appropriate dilutions of ABZ and RBZ stock solutions were made in methanol–water (50:50, v/v) to produce working stock solutions. Working stocks were used to prepare plasma calibration standards. A working IS solution (100 ng/mL) was prepared in precipitating solution. Calibration samples were prepared by spiking 45 µL of control rat plasma with the appropriate working solution of the analytes (5 µL of pooled working solution) on the day of analysis. Samples for the determination of precision and accuracy were prepared by spiking control rat plasma in bulk with ABZ and RBZ at appropriate concentrations [for ABZ: 2.01 ng/mL (LLOQ, lower limit of quantitation), 4.01 ng/mL (LQC, low quality control), 903 ng/mL (MQC, medium quality control) and 1405 ng/mL (HQC, high quality control); for RBZ: 6.02 ng/mL (LLOQ), 12.0 ng/mL (LQC), 2709 ng/mL (MQC) and 4214 ng/mL (HQC)] and 50 µL plasma aliquots were distributed into different tubes. All the samples were stored at −80 ± 10°C.

Recovery

The efficiency of ABZ, RBZ and IS extraction from rat plasma was determined by comparing the responses of the analytes extracted from replicate QC samples ($n = 6$) with the response of analytes from post-extracted plasma standard samples at equivalent concentrations by protein precipitation. Recoveries of ABZ and RBZ were determined at LQC, MQC and HQC concentrations [for ABZ: 4.01 ng/mL (LQC), 903 ng/mL (MQC) and 1405 ng/mL (HQC); for RBZ: 12.0 ng/mL (LQC), 2709 ng/mL (MQC) and 4214 ng/mL (HQC)], whereas the recovery of the IS was determined at a single concentration of 100 ng/mL.

Sample preparation

A simple protein precipitation method was followed for extraction of ABZ and RBZ from rat plasma. An aliquot of 50 µL plasma was precipitated with 400 µL of 10% tetrahydrofuran in acetonitrile containing 100 ng/mL of IS and centrifuged at 14000 rpm on a Eppendorf 5430R centrifuge (Germany) at 10°C for 10 min. From the supernatant 10 µL was injected onto LC-MS/MS system for analysis.

Validation procedures

A full validation according to the FDA guidelines (US DHHS *et al.*, 2001) was performed for the assay in rat plasma.

Specificity and selectivity. The specificity of the method was evaluated by analyzing rat plasma samples from at least six different lots to investigate the potential interferences at the LC peak region for analytes and IS. The acceptance criterion for experiment was that at least four out of six lots should have response less than 5 times the LLOQ level response in the same matrix.

Matrix effect. The post-column infusion method, defined by Bonfiglio *et al.* (1999) was used to evaluate the matrix effect. Briefly, an infusion pump delivered a constant amount of analyte into the LC system outlet, entering the mass spectrometer inlet. The mass spectrometer was operated in MRM mode to follow the analyte signal. Rat plasma sample extract was injected onto the LC column under the same chromatographic conditions. Since the analyte was infused at a constant rate, a steady ion response was obtained as a function of time. Any endogenous compound that eluted from the column and caused a variation in ESI response of the infused analyte was seen as a suppression or enhancement in the response of the infused analyte. Separate experiments were conducted for ABZ and RBZ, i.e. each analyte was assessed separately for matrix effect. In addition to the post-column infusion method, to further evaluate the matrix effect, six different lots of rat plasma were spiked with analyte concentration levels at LLOQ, LQC, MQC and HQC levels. The acceptance criteria for each back-calculated concentration were ±15% deviation from the nominal value, except at the LLOQ, which was set at ±20%.

Calibration curve. The eight point calibration curves for ABZ (2.01, 3.61, 20.1, 251, 502, 1004, 1506 and 2007 ng/mL) and for RBZ (6.02, 10.2, 60.2, 753, 1505, 3010, 4515 and 6020 ng/mL) were constructed by plotting the peak area ratio of each analyte:IS against the nominal concentration of calibration standards in rat plasma. Following the evaluation of different weighting factors, the results were fitted to linear regression analysis with the use of a $1/x^2$ (x = concentration) weighting factor. The calibration curve had to have a correlation coefficient (r) of 0.99 or better. The acceptance criteria for each back-calculated standard concentration were ±15% deviation from the nominal value except at LLOQ, which was set at ±20% (US DHHS *et al.*, 2001).

Precision and accuracy. The intra-assay precision and accuracy were estimated by analyzing six replicates containing ABZ and RBZ at four different QC levels [for ABZ: 2.01 (LLOQ), 4.01 (LQC), 903 (MQC) and 1405 ng/mL (HQC); for RBZ: 6.02, 12.0 (LQC), 2709 (MQC) and 4214 ng/mL (HQC)] in plasma. The inter-assay precision was determined by analyzing the four levels of QC samples on five different runs. The acceptance criteria for each back-calculated standard concentration were 85–115% accuracy from the nominal value except at LLOQ, which was set at 80–120% (US DHHS *et al.*, 2001).

Stability experiments. The stability of ABZ, RBZ and IS in the injection solvent was determined periodically by injecting replicate preparations of processed plasma samples for up to 48 h (in the autosampler at 10°C) after the initial injection. The peak areas of the analyte and IS obtained at the initial cycle were used as the references to determine the stability at subsequent points. Stability of ABZ and RBZ in plasma during 6 h (bench-top) was determined at ambient temperature (24 ± 2°C) at two concentrations (4.01 and 1405 ng/mL for ABZ and 12.0 and 4214 ng/mL for RBZ) in six replicates. Freezer stability of ABZ and RBZ in rat plasma was assessed by analyzing the LQC and HQC samples stored at −80 ± 10°C for at least 28 days. The stability of ABZ and RBZ in rat plasma following three freeze–thaw cycles was assessed using QC samples spiked with ABZ and RBZ. The samples were stored at −80 ± 10°C between freeze–thaw cycles for at least 12 h for each cycle. The samples were thawed by allowing them to stand (unassisted) at room temperature for approximately 2 h. The samples were then returned to the freezer. The samples were processed using the same procedure as described in the Sample Preparation section. Samples were considered stable if assay values were within the acceptable limits of accuracy (i.e. 85–115% from fresh samples) and precision (i.e. ±15% RSD).

Dilution effect. Dilution effect was investigated to ensure that samples could be diluted with blank matrix without affecting the final concentration. Rat plasma samples spiked with ABZ and RBZ were prepared at 5- and 10-fold MQC level, i.e. 4517 and 9034 ng/mL for ABZ and 13545 and 27091 ng/mL for RBZ, and diluted with pooled rat plasma

at dilution factors of 5 and 10 in six replicates, then analyzed. As part of validation, the replicates had to comply with a precision of ≤15% and an accuracy of 100 ± 15%, similar to other QC samples.

Pharmacokinetic study

All the experiments were approved by institutional animal ethical committee. Male Sprague–Dawley rats were procured from Bioneeds, Bangalore, India. The animals were housed in a Jubilant Biosys animal care facility in a temperature- and humidity-controlled room with a 12:12 h light:dark cycle. They had free access to food (Lipton India) and water. Blood samples were obtained following oral administration of ABZ (using a self micro-emulsfying drug delivery system formulation) at a dose of 30 mg/kg to overnight fasted rats (~12 h, during fasting animals had free access to water; $n = 4$, 189–193 g). The blood samples were placed in polypropylene tubes containing Na_2EDTA solution as anti-coagulant pre-dose and at 0.25, 0.5, 1, 2, 4, 8, 10, 18, 20 and 24 h. Plasma was harvested by centrifuging the blood using an Eppendorf 5430R centrifuge (Germany) at 5000 rpm for 5 min and stored frozen at −80 ± 10°C until analysis. Animals were allowed to feed 2 h post-dose of ABZ.

An aliquot of 50 µL of thawed plasma samples was processed as described in the Sample Preparation section. Along with study samples, QC samples at LQC MQC and HQC were assayed in duplicate and were distributed among unknown samples in the analytical run. The criteria for acceptance of the analytical runs encompassed the following: (i) not more than 33% of the QC samples deviated by more than ±15% from the nominal concentration; (ii) not less than 50% at each QC concentration level met the acceptance criteria. Plasma concentration–time data of ABZ and RBZ were analyzed by noncompartmental method using WinNonlin Version 5.1 (Pharsight Corporation, Mountain View, CA, USA).

Results and discussion

Liquid chromatography

The feasibility of various mixture(s) of solvents such as acetonitrile and methanol using different buffers such as ammonium acetate, ammonium formate and formic acid along with altered flow rates (in the range of 0.7–1.5 mL/min) was tested for complete chromatographic resolution of ABZ, RBZ and IS (data not shown). The resolution of peaks was achieved with 5 mM ammonium acetate (pH 6.0)–acetonitrile (20:80, v/v) with a flow rate of 1 mL/min, on a Chromolith Performance RP-18e column and was found to be suitable for the determination of electro-spray response for ABZ, RBZ and IS.

Mass spectroscopy

In order to optimize ESI conditions for ABZ, RBZ and IS, quadrupole full scans were carried out in positive-ion detection mode. During a direct infusion experiment, the mass spectra for ABZ, RBZ and IS revealed peaks at *m/z* 266.1, 282.2 and 180.1, respectively, as protonated molecular ions, $[M + H]^+$. Following detailed optimization of mass spectrometry conditions (provided in the Mass Spectrometry Operating Conditions section), the *m/z* 266.1 precursor ion to the *m/z* 234.4 was used for quantification of ABZ and the *m/z* 282.2 precursor ion to the *m/z* 240.4 was used for quantification of RBZ. Similarly, for IS the *m/z* 180.1 precursor ion to the *m/z* 110.1 was used for quantification purposes. As the earlier publication (Chen *et al.*, 2004) has discussed extensively the fragmentation patterns of ABZ and RBZ, we are not presenting the data pertaining to this.

Recovery

A simple protein precipitation method proved to be robust and provided the cleanest samples. The results of the comparison of neat standards vs plasma-extracted standards were estimated for ABZ and RBZ. The recoveries at LQC, MQC and HQC for ABZ were 87.8 ± 6.89, 83.3 ± 1.10 and 84.9 ± 1.41%, respectively, and for RBZ were 86.8 ± 4.91, 84.1 ± 0.77 and 85.4 ± 0.85%, respectively. The recovery for IS at 100 ng/mL was 102 ± 2.79%.

Specificity and selectivity

Figure 2(a–e) shows chromatograms for the blank rat plasma (free of analytes and IS), blank rat plasma spiked with IS, blank rat plasma spiked with ABZ at LLOQ, blank rat plasma spiked with ABZ at the upper limit of quantitation (ULOQ) and IS, and an *in vivo* plasma sample obtained at 1.00 h after oral administration of ABZ, respectively. Similarly, Fig. 3(a–e) shows chromatograms for the blank rat plasma (free of analytes and IS), blank rat plasma spiked with IS, blank rat plasma spiked with RBZ at LLOQ, blank rat plasma spiked with RBZ at ULOQ and IS, and an *in vivo* plasma sample showing the peak of RBZ at 1.00 h following oral administration of ABZ, respectively. No interfering peaks from endogenous compounds were observed at the retention times of analytes and IS in the matrix. The retention times of ABZ, RBZ and IS were ~1.66, 1.50 and 1.59 min, respectively. The total chromatographic run time was 3.5 min. The specificity of the method was evaluated by analyzing rat plasma samples from six different animals to investigate the potential interferences at the LC peak region for analytes and IS. No significant response was observed in the LC region for any of the blank samples analyzed, as compared with the corresponding LLOQ-level response in the same matrix lot (data not shown).

Matrix effect

Figure 4(a, b) represents a matrix effect chromatogram overlaid by an aqueous standard chromatogram to indicate the elution profile for the analyte over the analyte infusion matrix effect baseline for ABZ and RBZ, respectively. No significant signal suppression was observed in the region of elution of the two analytes of interest, viz. ABZ and RBZ. Six different lots of rat plasma, spiked with analyte concentration levels at LLOQ, LQC, MQC and HQC levels, were analyzed. The results showed that the precision and accuracy for analyzed samples were within the acceptable range (data not shown).

Calibration curve

The plasma calibration curve was constructed using eight calibration standards (2.01–2007 ng/mL for ABZ and 6.02–6020 ng/mL for RBZ). The calibration standard curve had a reliable reproducibility over the standard concentrations across the calibration range. The calibration curve was prepared by determining the best fit of peak-area ratios (peak area analyte/peak area IS) vs concentration, and fitted to $y = mx + c$ using a weighting factor ($1/x^2$). The average regression ($n = 5$) was found to be ≥0.997 for both ABZ and RBZ. The lowest concentration with the RSD <20% was taken as the LLOQ and was found to be 2.01 and 6.02 ng/mL for ABZ and RBZ, respectively. The percentage accuracies observed for the mean of back-calculated concentrations for five calibration curves for ABZ and RBZ were

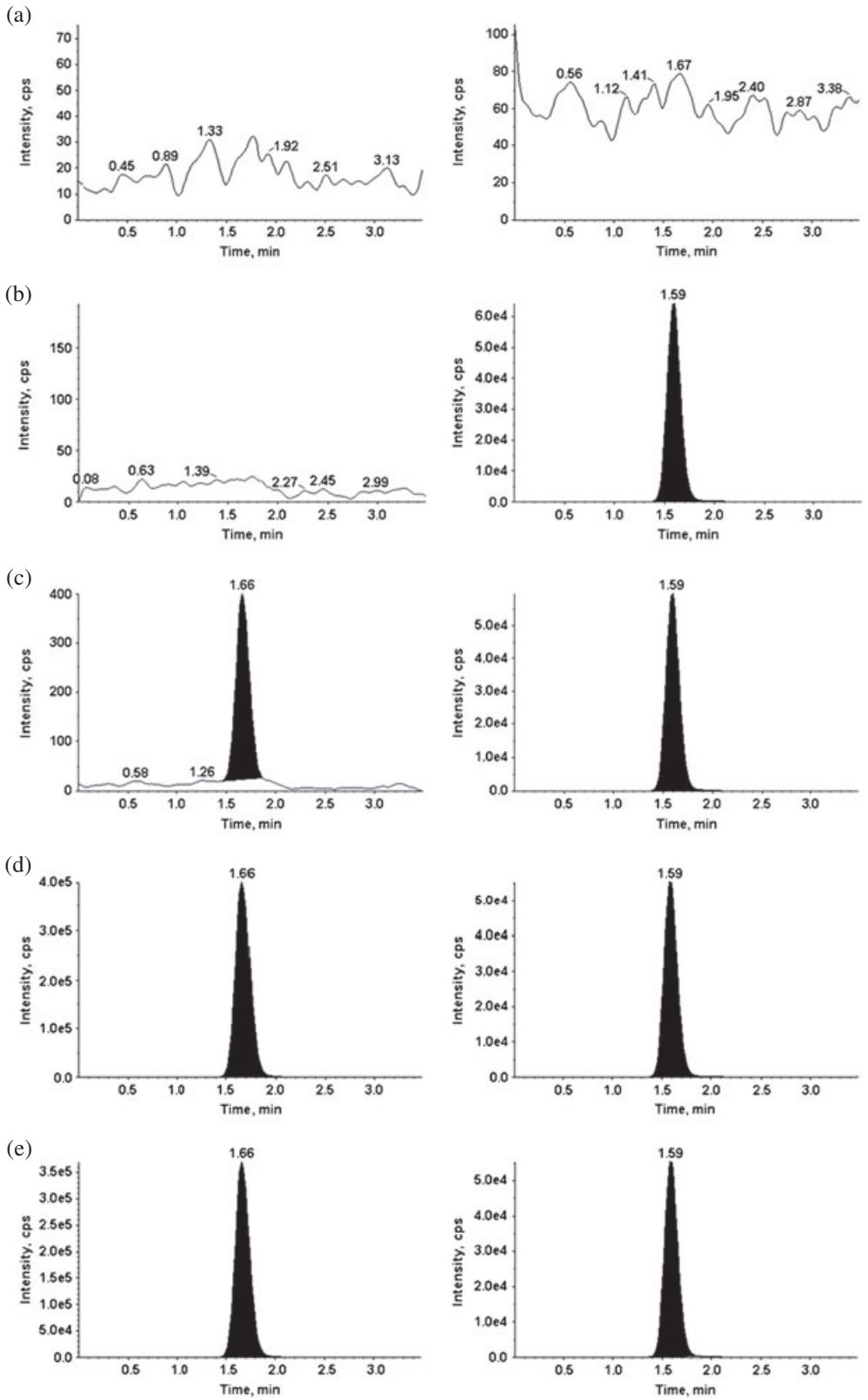


Figure 2. Typical multiple reaction monitoring chromatograms of albenadazole (ABZ; left panel) and IS (right panel) in (a) rat blank plasma, (b) rat blank plasma spiked with IS, (c) rat blank plasma spiked with ABZ at LLOQ (2.01 ng/mL) and IS, (d) rat blank plasma spiked with ABZ at ULOQ (2007 ng/mL) and IS, and (e) an 1 h *in vivo* plasma sample showing ABZ peak obtained following oral dose of ABZ to rats along with IS.

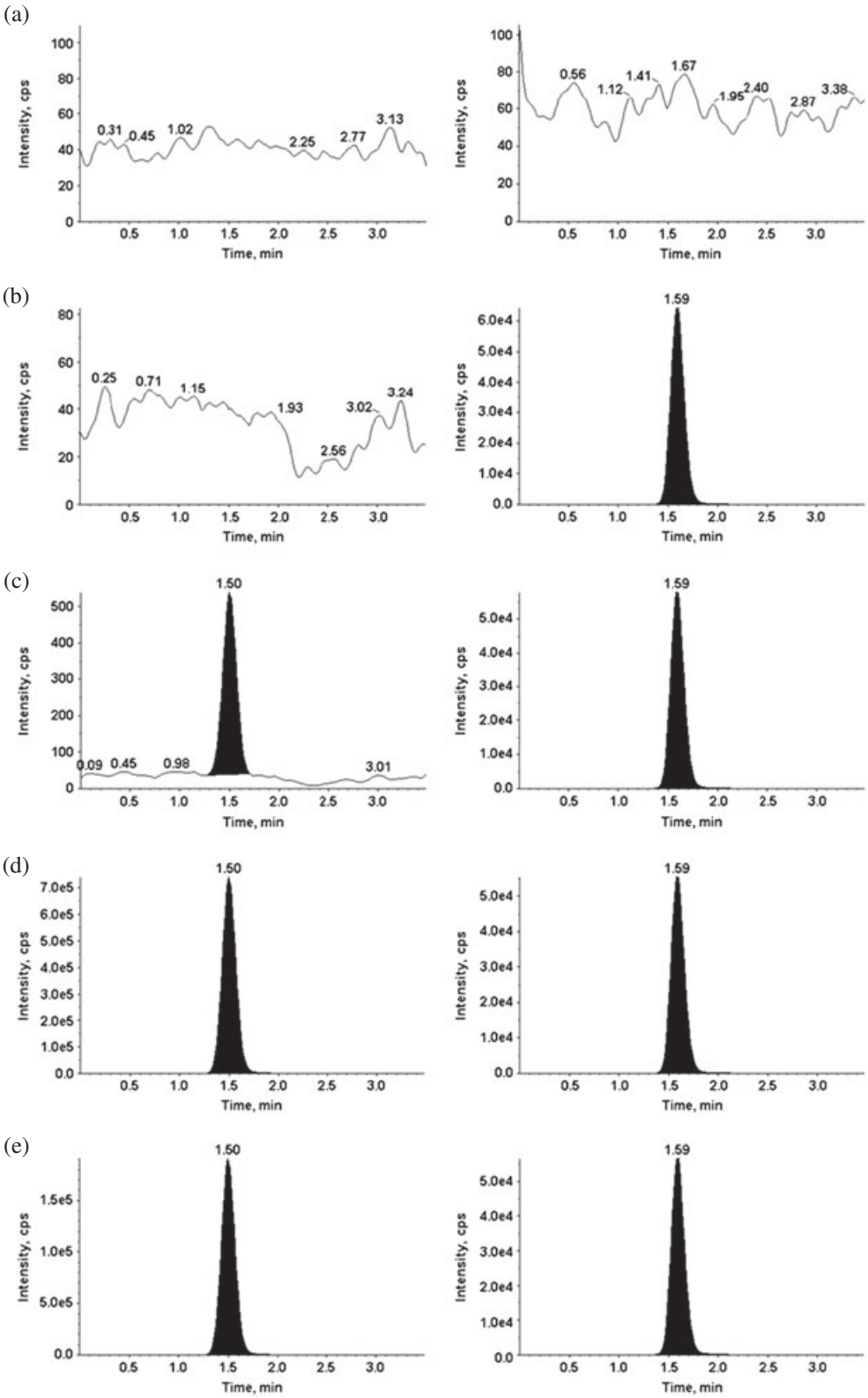


Figure 3. Typical multiple reaction monitoring chromatograms of ricobendazole (RBZ; left panel) and IS (right panel) in (a) rat blank plasma, (b) rat blank plasma spiked with IS, (c) rat blank plasma spiked with RBZ at LLOQ (6.02 ng/mL) and IS, (d) rat blank plasma spiked with RBZ at ULOQ (6020 ng/mL) and IS, and (e) an 1 h *in vivo* plasma sample showing RBZ peak obtained following oral dose of albendazole to rats along with IS.

within 97.3–105 and 92.2–102, respectively, while the precision (%CV) values were 0.95–10.1 and 1.28–12.1 for ABZ and RBZ, respectively.

Accuracy and precision

Accuracy and precision data for intra- and inter-day plasma samples for ABZ and RBZ are presented in Table 1. The assay values on both the occasions (intra- and inter-day) were found to be within the accepted variable limits.

Stability

The predicted concentrations for ABZ at 4.02 and 1405 ng/mL and for RBZ at 12.0 and 4214 ng/mL samples deviated within ±15% of the concentrations of the fresh samples in a battery of

Table 1. Intra and inter-day precision determination of ABZ and RBZ quality controls in rat plasma

	ABZ				RBZ			
Theoretical concentration (ng/mL)	Measured concentration (ng/mL) Mean	SD	RSD	Accuracy (%)	Mean	SD	RSD	Accuracy (%)
Intra-day variation (six replicates at each concentration)								
ABZ, 2.01 RBZ, 6.02	2.12	0.15	6.90	106	5.96	0.10	1.74	99.0
ABZ, 4.01 RBZ, 12.0	3.74	0.45	12.1	93.2	11.6	0.91	7.88	96.2
ABZ, 903 RBZ, 2709	905	22.3	2.46	100	2800	67.5	2.41	103
ABZ, 1405 RBZ, 4214	1405	64.0	4.56	100	4317	185	4.29	102
Inter-day variation (30 replicates at each concentration)								
ABZ, 2.01 RBZ, 6.02	2.20	0.11	5.14	110	6.07	0.18	2.88	101
ABZ, 4.01 RBZ, 12.0	3.97	0.34	8.62	98.9	12.2	0.85	6.99	101
ABZ, 903 RBZ, 2709	888	29.2	3.28	98.3	2713	81.2	2.99	100
ABZ, 1405 RBZ, 4214	1399	63.9	4.57	100	4183	145	3.46	99.3

RSD, Relative standard deviation (SD × 100/mean).

Table 2. Stability data of ABZ and RBZ quality controls in rat plasma

Nominal concentration (ng/mL)	Stability	ABZ Mean ± SD,[a] $n = 6$	ABZ Accuracy (%)[b]	ABZ Precision (%CV)	RBZ Mean ± SD,[a] $n = 6$	RBZ Accuracy (%)[b]	RBZ Precision (% CV)
ABZ, 4.01	0 h (batch 1)	3.74 ± 0.45	NA	12.1	11.6 ± 0.91	NA	7.88
	6 h (bench-top)	3.74 ± 0.56	100	14.9	11.5 ± 1.35	99.2	11.8
	48 h (in-injector)	4.05 ± 0.42	108	10.4	11.9 ± 1.20	101	10.1
RBZ, 12.0	0 h (batch 2)	3.87 ± 0.30	NA	7.79	12.3 ± 0.24	NA	1.94
	Third freeze–thaw	3.71 ± 0.24	95.7	6.52	12.3 ± 0.18	99.9	1.49
	28 days at –80°C	3.58 ± 0.27	92.3	7.62	12.2 ± 0.33	99.1	2.68
ABZ, 1405	0 h (batch 1)	1405 ± 64.0	NA	4.56	4317 ± 185	NA	4.29
	6 h (bench-top)	1388 ± 39.7	98.8	2.86	4268 ± 116	98.9	2.73
	48 h (in-injector)	1362 ± 54.7	97.0	4.01	4077 ± 130	94.4	3.19
RBZ, 4214	0 h (batch 2)	1329 ± 27.3	NA	2.05	4143 ± 45.4	NA	1.10
	Third freeze–thaw	1310 ± 25.6	98.6	1.95	4172 ± 91.5	101	2.19
	28 days at –80°C	1317 ± 32.4	99.1	2.46	4240 ± 96.0	102	2.26

[a]Back-calculated plasma concentrations.
[b](mean assayed concentration/mean assayed concentration at 0 h i.e fresh samples) × 100.

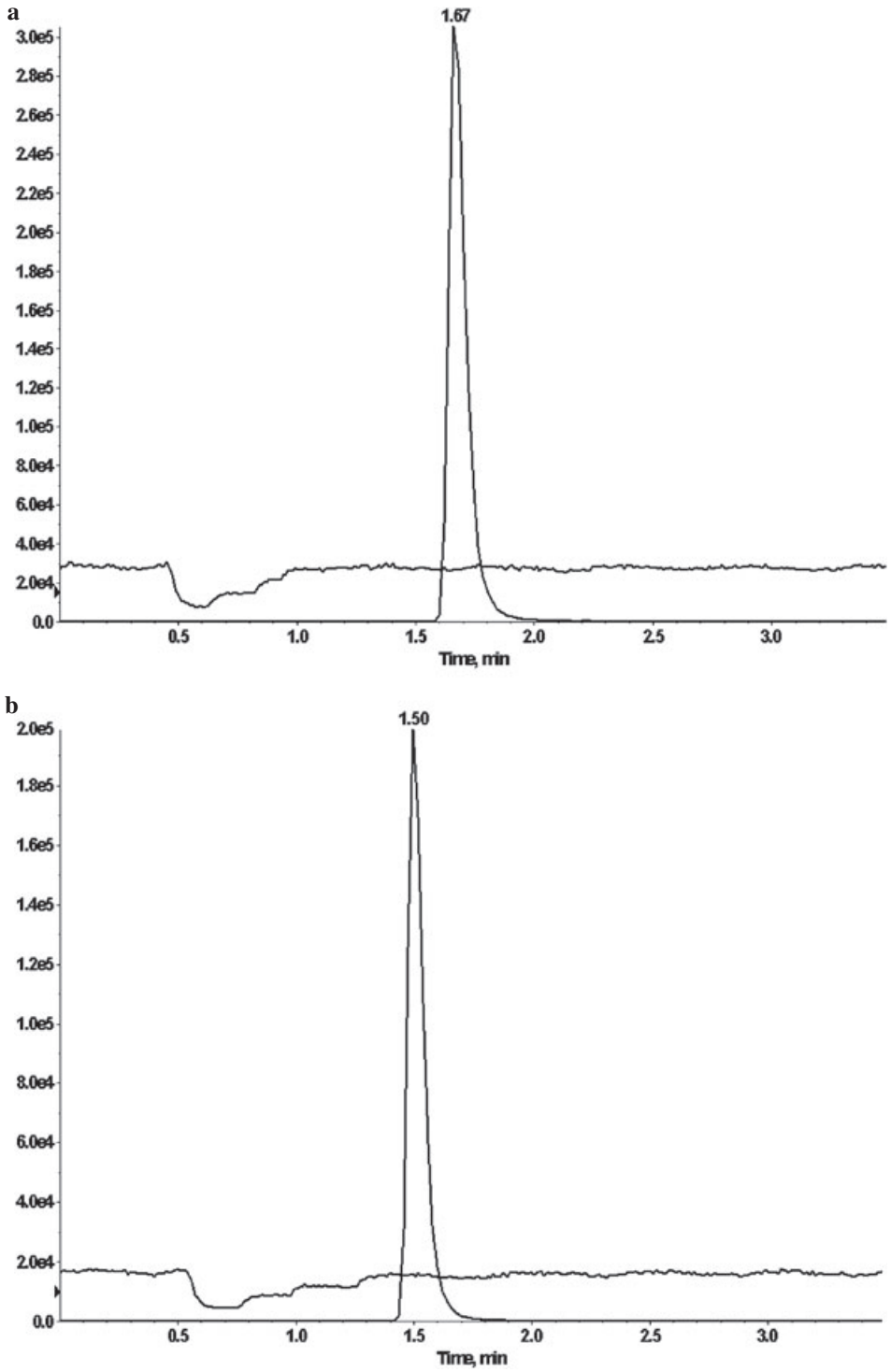


Figure 4. Overlay chromatograms showing the matrix effect for (a) albendazole (ABZ) and (b) ricobendazole (RBZ).

stability tests, viz. in-injector (48 h), bench-top (6 h), three repeated freeze–thaw cycles and freezer stability at −80 ± 10°C for at least for 28 days (Table 2). The results were found to be within the assay variability limits during the entire process.

Dilution effect

The dilution integrity was confirmed for QC samples that exceeded the upper limit of the standard calibration curve. The results showed that the precision and accuracy for two sets of six replicates of diluted samples were within the acceptance range (data not shown).

Pharmacokinetic study

The present method was applied to the analysis of plasma samples obtained from four rats following oral administration of 30 mg/kg of ABZ customized formulation. The sensitivity and

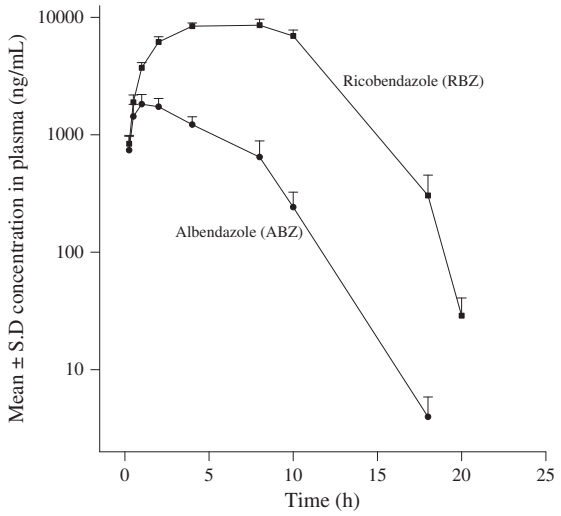


Figure 5. Mean plasma concentration–time profile of albendazole (ABZ) and ricobendazole (RBZ) (formed from ABZ) in rat plasma following oral dosing of ABZ.

specificity of the assay were found to be sufficient for accurately characterizing the plasma pharmacokinetics of ABZ and RBZ in rats. Figure 5 depicts the plasma concentration vs time profile of ABZ and RBZ (formed from ABZ) in rats. ABZ and RBZ concentrations were quantifiable up to 18 and 20 h post-dosing, respectively. Following oral administration of ABZ tablets to rats, ABZ attained mean peak plasma concentrations of 1876 ± 281 ng/mL at 1.75 h, whereas RBZ (formed from ABZ) attained mean peak plasma concentrations of 8976 ± 752 ng/mL at 6.00 h. The AUC_{0-t} values for ABZ and RBZ were found to be 11167 ± 1666 and 99962 ± 6334 ng*h/mL, respectively. The terminal half-life ($t_{½,\beta}$) was found to be 1.78 ± 0.57 and 1.63 ± 0.61 h for ABZ and RBZ, respectively.

Conclusions

In summary, we have developed and validated a highly sensitive, specific, reproducible and high-throughput LC-MS/MS assay to quantify ABZ and RBZ simultaneously in rat plasma. This method enabled us to characterize the disposition of ABZ and RBZ in rats following oral administration of ABZ. From the results of all the validation parameters, we can conclude that the present method can be useful for pre-clinical studies with the desired precision and accuracy along with high-throughput.

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