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A simple, precise, and sensitive HPLC method for quantification of letrozole in rat plasma: development, validation, and preclinical pharmacokinetics

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Abstract

A simple bioanalytical liquid chromatographic method was developed and validated to quantify letrozole (LTZ) in rat plasma. Protein precipitation using acidified chilled acetonitrile (containing 0.1% orthophosphoric acid) was used to extract LTZ from the plasma. Chromatographic separation was carried out on Kinetex C18 reverse phase (RP) column (250 mm × 4.6 mm i.d., 5 μm) using a mixture of 20 mM acetate buffer (pH 5.5) and acetonitirile (60:40 %v/v) eluting at 1.0 mL/min flow rate with the method responses measured at 240 nm. The optimized method was selective and established good linearity with recovery ranging between 91.16 and 99.44%. The validation experiments revealed that the method showed acceptable precision (2.61–7.48%) and accuracy (97.44–102.70%) and was found to be stable. The sensitivity of the method was demonstrated by the lowest concentration (LLOQ) detected at 75 ng/mL. Using the developed method, single-dose oral pharmacokinetics in Sprague-Dawley rats was carried out to successfully confirm the applicability of the method for the quantification of LTZ in biological matrix.

Keywords: Letrozole, Bioanalytical, Validation, Rat plasma, Protein precipitation, Chromatographic

Introduction

A majority of the breast cancers are hormone dependent, of which estrogen plays an important role. Estrogen synthetase or aromatase is the catalytic enzyme involved in the local biosynthesis of estrogens from androgens. Exposure to circulating estrogen levels leads to the increased risk of developing breast cancer. Hence, inhibition of aromatase enzyme to reduce the peripheral conversion to estrogen is an attractive strategy in the therapy of hormone-dependent breast cancer (Chumsri et al. 2011). Conversion of androgens to estrogen can be effectively stopped by aromatase inhibitors (AIs), which block the aromatase enzyme thus impairing the growth and development of tumors (Osborne and Tripathy 2005; Scott and Keam 2006). Letrozole (LTZ) is a potent, non-steroidal, 3rd generation AI used to treat hormone-sensitive breast cancer. LTZ is approved for oral hormonal therapy by the USFDA and is sold under the brand name Femara® to treat local or metastatic breast cancer in post-menopausal women. LTZ undergoes complete absorption from the gastrointestinal tract and metabolizes to form the carbinol metabolite, which is inactive and further excreted in the urine (Acharjya et al. 2012)

Several analytical techniques such as UV spectrophotometry, liquid chromatography, gas chromatography, and mass spectrophotometry have been reported for the estimation of LTZ either alone or in combination (Berzas et al. 2003; Mareck et al. 2005; Mondal et al. 2007; Laha et al. 2008). In this regard, we have previously reported a sensitive, robust, and stable analytical liquid

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chromatographic (RP-HPLC) method to estimate LTZ in bulk and in nanoformulations (Hegde et al. 2018).

For the quantification of LTZ (either alone or in combinations) in biological fluids, several analytical methodologies have been reported. Previously, a few spectrophotometric methods have been developed to estimate LTZ; however, these methods are not very sensitive and accurate to quantify LTZ in low concentrations (Mondal et al. 2007; Ganesh et al. 2007; Ganesh et al. 2008). Fluorescence detection has been reported to quantify LTZ in human plasma and urine (Marfil et al. 1996; Zarghi et al. 2007). However, these solid-phase extraction methods resulted in longer retention times and time-consuming processing. Pfister et al. reported an enzyme immunoassay (EIA) and HPLC method for the quantification of LTZ in the plasma. While the EIA method was non-specific and resulted in cross-reactivity of metabolites, the HPLC method was based on liquid-liquid extraction, which was tedious and had low sensitivity (Pfister et al. 1994). The liquid chromatographic method utilizing MS-MS systems has been used for the analysis of LTZ letrozole in plasma, which are sensitive and selective with a low level of quantification. However, such methods require operation by trained personnel and sophisticated equipment (Joshi et al. 2011; Al-Shehri et al. 2019; Vanol et al. 2016). Acharjya et al. reported a fast and sensitive liquid chromatographic method using liquid-liquid extraction for the estimation of LTZ in the serum (Acharjya et al. 2012). However, the method reported a shorter retention time with observable interference corresponding to drug retention time. Recently, Dhakne et al. reported a simultaneous liquid chromatographic method for the simultaneous quantification of LTZ and abemaciclib in rat plasma using the liquid-liquid extraction method for extracting the drug from the plasma (Dhakne et al. 2020). However, the separation of analytes follows a gradient technique, which requires a dwell period for adjusting the column condition, in addition to significantly longer run times due to post-gradient re-equilibration of the column (Marina and Garcia 2000).

Hence, in the present study, we present an accurate, sensitive, and reproducible RP-HPLC method to quantify LTZ in rat plasma. The proposed method is simple and economic over the previously reported methods as we employed an isocratic mode of elution that is highly efficient, utilizing protein precipitation technique for the extraction of drug from the plasma. The method was developed and validated in accordance with the USFDA guidelines. In addition, the optimized method was applied to determine the pharmacokinetics of LTZ in healthy female Sprague-Dawley (SD) rats.

Methods

Materials and reagents

Letrozole (LTZ) was a kind gift sample from Gland Pharma Ltd., Malur, Karnataka, India. Carbamazepine (CBZ), ketoconazole (KCZ), and paracetamol (PA), which were used as internal standard (IS), were obtained from Sun Pharmaceutical Industries Ltd., Vadodara, India, and Kumar Organic Products Ltd., Bengaluru, India. Ammonium acetate, glacial acetic acid, and methyl tertbutyl ether (MTBE) were procured from Spectrochem Pvt. Ltd., Mumbai, India. Ortho-phosphoric acid (OPA) and acetonitrile (ACN) were procured from Merck Ltd. (Mumbai, India). Methanol of HPLC grade was acquired from Finar Ltd. (Ahmedabad, India). Potassium dihydrogen orthophosphate was purchased from Sisco Research Laboratories Pvt. Ltd (Mumbai, India). Ultrapure water used in the preparation of sample and mobile phase was obtained from a Millipore Direct-Q® 3 water purification system (Millipore Corporation, MA, USA).

Animals

The experiments were carried out in SD rats (weighing 200–250 g). Before commencing the study, approval was sought from the Institutional Animal Ethical Committee, Manipal, India (Approval No.: IAEC/KMC/76/2020). The animals were handled according to the institutional and national guidelines for the use and care of animals.

Instrumentation and chromatographic specifications

The chromatographic separation of LTZ was effected using a HPLC system (Shimadzu, Kyoto, Japan) equipped with LC-10 AD pump, CBM-20A Prominence system controller (Shimadzu, Kyoto, Japan), DGU-20A5 degasser unit, and SIL-10AXL auto-injector. The responses were recorded using SPD-10A UV-Vis detector and analyzed using LC Solution 5.57 system control (Hegde et al. 2018). A mixture of 20 mM acetate buffer (pH 5.5) and ACN (60:40 %v/v) was used to separate LTZ and IS on a Kinetex C18 column maintained 25 °C column temperature and 10 °C autosampler temperature. Samples was eluted at a flow rate of 1.0 mL/min, and the run time for each sample was set to 12 min. Twenty microliters of the sample was injected into the HPLC and analyzed at a detector wavelength of 240 nm.

Extraction procedure

Extraction of drug and IS from rat plasma was performed by protein precipitation technique. Ninety microliters of clear plasma, 10 μL of LTZ (10 μg/mL), and 10 μL of CBZ (50 μg/mL) were mixed using a vortex mixer (CM101, Remi Equipment, Mumbai, India). This mixture was acidified using OPA (0.1 %v/v) and further vortexed for 2 min. Later, the volume was made up using

chilled ACN as a precipitating agent and centrifuged at 15,000 rpm and 10 °C for 10 min in a cold centrifuge (Hegde et al. 2017). The clear supernatant was removed, and 20 μL was injected into the HPLC.

Preparation of quality control (QC) samples and spiked samples

Initially, a primary stock solution of LTZ standard (500 μg/mL) was prepared by weighing LTZ (5 mg) and dissolved in methanol (5 mL) in a volumetric flask (10 mL). The final volume was made up using methanol. Aliquots of primary stock solution were further diluted to make a series of dilutions. Standard solution of IS (500 μg/mL) was prepared by adding CBZ (5 mg) in methanol (5 mL) in a volumetric flask (10 mL), and the volume was made up using methanol. Further dilutions were made to get a working standard solution of 50 μg/mL concentration.

For plasma linearity, LTZ standard solutions having concentrations viz. 0.075, 0.1, 0.25, 0.5, 0.75, 1, 2.5, 5, and 10 μg/mL were prepared. The least detectable quantity in the plasma, also called lower limit of quantification (LLOQ), was observed at 75 ng/mL. Known concentrations of LTZ standard solutions were spiked with plasma and extracted following protein precipitation to obtain low quality control (LQC; 0.225 μg/mL), middle quality control (MQC; 1 μg/mL), and high quality control (HQC; 8 μg/mL) samples.

Method optimization

Several buffers such as acetate buffer pH 4.5, acetate buffer pH 6.0, and phosphate buffer pH 3.0 were trialed. Varying mobile phase compositions comprising buffer and acetonitrile were evaluated to achieve optimum separation of drug and IS. Active pharmaceutical ingredients such as PA, KCZ, and CBZ were screened for the selection of IS. To achieve maximum drug extraction, protein precipitation as well as liquid-liquid extraction was trialed. Chilled ACN containing 0.1 %v/v OPA was used as precipitating solvent to precipitate proteins from the plasma whereas liquid-liquid extraction was carried out using solvents such as diethyl ether, ethyl acetate, and MTBE (Acharjya et al. 2012; Dhakne et al. 2020).

Method validation

After optimizing the chromatographic method based on optimum separation, satisfactory peak shape, and good resolution between the analytes, validation of the developed method was performed as per the USFDA guidelines for the following recommended parameters (Managuli et al. 2017).

System suitability

The method suitability was assessed by injecting six replicates of LTZ solution (1 μg/mL) and evaluating the system suitability parameters.

Selectivity

Three replicates each of diluent (mobile phase), blank plasma, and spiked plasma were injected separately to assess the specificity of the method. The absence of interference at the R_t of LTZ and IS due to diluent or blank plasma indicates that the method passes the selectivity test.

Linearity

Linearity was established by spiking known concentrations of LTZ solution with plasma to obtain a series of concentrations ranging from 0.075 to 10 μg/mL. The peak area ratio of drug/IS versus drug concentrations was plotted to establish the regression equation. Accuracy and precision of the calibration standards were determined by back calculating the drug concentrations from the regression equation.

Precision and accuracy

Inter- and intra-day precision and accuracy runs were carried out at LQC (n = 6), MQC (n = 6), and HQC (n = 6) by injecting the spiked samples into HPLC.

Recovery

For recovery studies, the percent drug recovery was calculated at LQC, MQC, and HQC by comparing the area ratios of extracted analytes with those of unextracted analytes (indicating 100% recovery).

Stability studies

Short-term stability studies were determined by storing LQC and HQC samples (three replicates each) at normal room temperature (RT) for 7 days followed by analysis using HPLC. Freeze-thaw stability studies were determined by freezing the LQC and HQC samples at −20 °C for 24 h and further thawing at RT. Upon complete thawing, the samples were again kept at −20 °C for 12 h. This freeze-thaw cycle continued thrice after which a sample analysis was carried out at the end of the third cycle. For benchtop stability studies, spiked plasma QC levels (LQC and HQC) were kept on benchtop for 8 h, following which the samples were injected. Autosampler stability was assessed by storing the processed LQC and HQC samples in the autosampler for 10 h at 10 °C. Standard stock solution stability of LTZ and IS was assessed by keeping the samples on benchtop for 24 h at RT followed by analysis using HPLC.

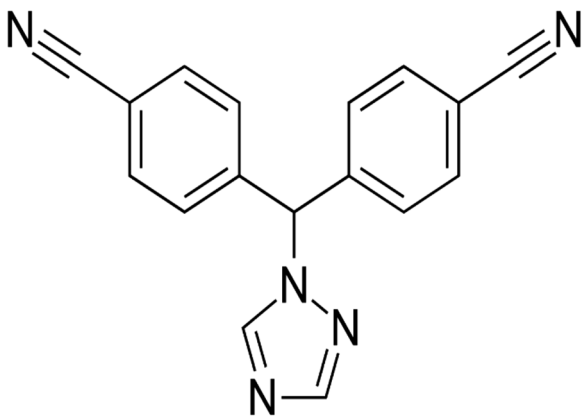

Fig. 1 Molecular structure of letrozole (LTZ)

Pharmacokinetics study

The applicability of the developed bioanalytical method was assessed by carrying out the pharmacokinetic studies in female SD rats. Before commencing the study, rats were housed in polypropylene cages and quarantined for a week to acclimatize with the environmental conditions. An air-conditioned room (25 ± 2 °C) was used to keep the cages, and the animals were exposed to light and dark cycles (12 h each).

For the pharmacokinetic study, rats (n = 6) were dosed with LTZ at 2 mg/kg body weight. Following oral administration, blood (0.4 mL) was withdrawn by retro-orbital puncture at intervals of 0.5, 1, 2, 4, 6, 12, 24, 36, and 72 h into 2-mL Eppendorf tubes containing 20 μL disodium EDTA solution. Clear plasma was separated by centrifuging the samples at 10,000 rpm for 8 min at 4 °C. Until analysis, all plasma samples were stored at −80 °C until analysis. The amount of LTZ present in the plasma was quantified using HPLC, and the pharmacokinetic parameters were determined using the Winonlin software.

Results and discussion

Method optimization

Considering the pKa (2.17) of LTZ (Fig. 1), buffer having a pH > 3 was used as the mobile phase. Based on previous literature, drugs such as paracetamol (PA), ketoconazole (KCZ), and carbamazepine (CBZ) were trialed to select a suitable internal standard (IS) with optimum retention time, favorable resolution, and good peak shape (Al-Shehri et al. 2019; Dhakne et al. 2020). The stationary phase comprised Kinetex C18 column (250 × 4.6 mm, 5 μ). The results of the optimization trials with PA and KCZ as IS are given in Fig. S1 and Fig. S2 (supplementary information).

Being an extremely basic drug with a pKa value of 2.3, CBZ was used as the IS, to assess the elution of LTZ by liquid-liquid extraction using MTBE. Initially, trials were carried out using acetate buffer pH 4.5 and ACN in the ratio (50:50 %v/v) eluting at a flow rate of 1 mL/min. Drug and IS were extracted from the plasma using liquid-liquid extraction with MTBE. Following injection, it was observed that the drug peak was distorted in addition to significant interference with plasma (Fig. 2). Hence, additional trials were carried out by changing the pH of the mobile phase to 5.5 based on the results observed with other IS (supplementary information). The mobile phase comprised a combination of acetate buffer pH 5.5 and ACN (60:40 %v/v) flowing at 1.0 mL/min. The peaks of LTZ and CBZ were well resolved with an R_t of 7.02 min and 5.23 min, respectively. No interference was observed at R_t of drug or IS when compared to blank plasma. An additional trial was carried out using the protein precipitation technique (using chilled ACN

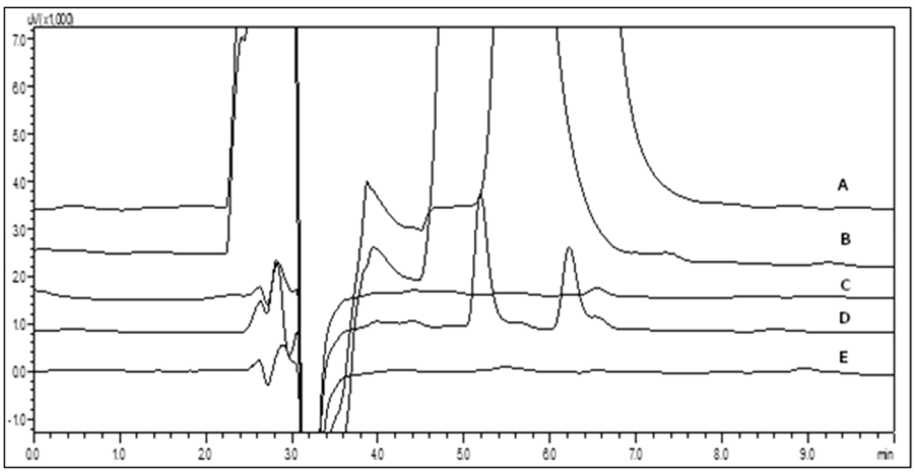

Fig. 2 Effect of mobile phase composition and CBZ (IS). Chromatograms of (A) LTZ, (B) CBZ, (C) blank plasma, (D) spiked plasma, and (E) diluent using ACN and acetate buffer pH 4.5 (50:50 %v/v) at 1 mL/min

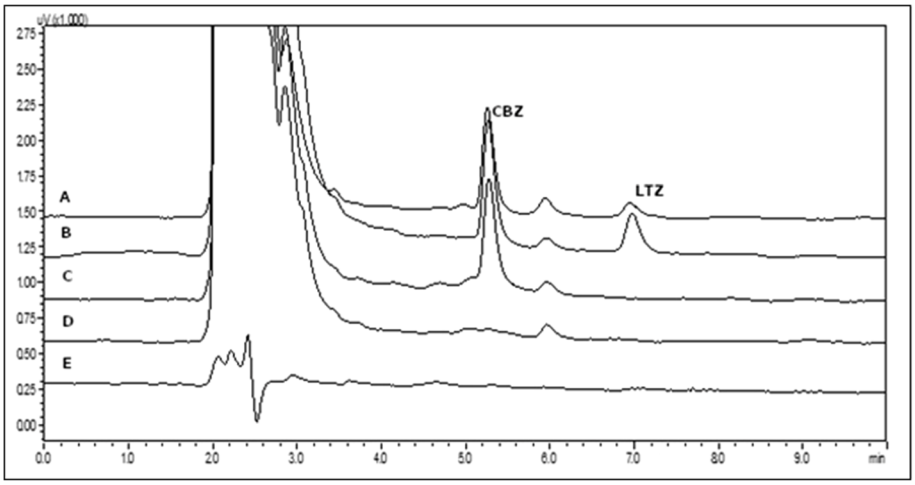

Fig. 3 Chromatograms of plasma samples and diluent eluted using the optimized chromatographic method. (A) Plasma sample from pharmacokinetic study, (B) plasma spiked with LTZ and CBZ, (C) plasma spiked with CBZ, (D) blank plasma, and (E) diluent

and 0.1 %v/v OPA) to extract the analytes in the plasma. Protein precipitation technique is very suitable for biological matrices such as the whole blood, serum, or plasma. Since the recovery of the analytes was comparable in both the techniques of extraction, it was decided to use protein precipitation considering that it is simple, economical with minimal loss of sample, and can be adapted for a wide range of chemistries (Bylda et al. 2014; Stone 2017). In addition, the inclusion of OPA as the acidic agent further aided the process of precipitation due to the interaction with proteins in the plasma leading to the formation of an insoluble salt. Compared to the methods reported previously, the novelty of the present method is the use of a simple drug extraction method with rapid cleanup of the sample.

Hence, the optimized method comprised a mobile phase mixture containing ACN and 20 mM ammonium acetate buffer pH 5.5 (60:40 %v/v) eluted at 1.0 mL/min, maintained at 25 °C column temperature and 10 °C autosampler temperature. Drug and IS (CBZ) were extracted by protein precipitation from plasma matrix using acidified (0.1 %v/v OPA) chilled ACN. Samples were injected at 20 μL, and the total run time was set to 12 min with method responses measured at 240 nm. This resulted in the optimum separation of LTZ at R_t of 7.02 min and CBZ at 5.23 min with a $Tf_{10\%}$ of 1.4 (LTZ) and 1.3 (CBZ) with a plate count (N) of 5639 (LTZ) and 6145 (CBZ) (Fig. 3).

Method validation

The optimized method was validated in accordance with the USFDA guidelines (US Department of Health and Human Services, Food and Drug Administration 2018) for various validation parameters.

System suitability

The method was found to be suitable for the intended application, which was demonstrated by the compliance of the system suitability parameters with the acceptance criteria. The relative standard deviation (RSD) of the peak area for LTZ was observed to be 1.4% (< 2.0%) with a $Tf_{10\%}$ of 1.2 (< 2.0) and a plate count (N) of 5639 (> 2000).

Selectivity

No interference of the diluent or blank plasma was observed at the R_t of drug or IS which indicates the specificity of the proposed method (Fig. 3). In addition, the peak area in the blank plasma was < 20% compared to the peak area of the LLOQ sample at the R_t of the drug. The LLOQ was observed at 0.075 μg/mL, which indicates the sensitivity of the chromatographic method.

Table 1 Precision and accuracy data for the estimation of LTZ in rat plasma

QC level	Inter-day				Intra-day			
	Mean conc. detected (μg/mL)	SD	CV (%)	Accuracy (%)	Mean conc. detected (μg/mL)	SD	CV (%)	Accuracy (%)
LQC	0.219	0.014	6.34	94.02	0.217	0.016	7.48	95.52
MQC	1.037	0.056	5.44	102.13	1.030	0.063	6.14	103.62
HQC	7.653	0.200	2.61	95.00	7.657	0.234	3.05	94.91

HQC high quality control, *MQC* middle quality control, *LQC* low quality control, *SD* standard deviation, *CV* coefficient of variance

Though a few previous reports have shown better sensitivity, these methods demonstrated very short retention times for LTZ which is not desirable (Al-Shehri et al. 2019; Zarghi et al. 2007). The sensitivity of the present method is considerably more compared to previous reports where LLOQ was observed at 0.15 μg/mL and 0.1 μg/mL (Acharjya et al. 2012; Dhakne et al. 2020).

Linearity

The developed method demonstrated good linearity between the concentration range 0.075–10 μg/mL with an R^2 value of 0.9997. Accuracy and precision (%CV) of the calibration standards determined by back calculation ranged from 99.08 to 108.23% and 1.02 to 7.89%, respectively.

Accuracy

Method accuracy was determined by back calculating the concentrations for six replicates of the QC samples (Table 1). The percentage accuracy ranged from 94.02 to 103.62%. The accuracy levels were well within the acceptance limit of ±15% for LQC, MQC, and HQC.

Precision

The precision of the chromatographic method was determined at intra-batch and inter-batch levels. For intra-batch precision determined by injecting various sets of QC samples within the same day, the values for the coefficient of variance (CV) ranged from 3.05 to 7.48%. The inter-batch precision of the QC samples was assessed on different days, and the values for CV ranged from 2.61 to 6.34%. The results of the precision studies, shown in Table 1, complied with the acceptance limits (%CV < 15) which demonstrates the repeatability of the method.

Extraction recovery

The efficiency of the method of drug extraction was determined, and the results are shown in Table 2. The mean drug recovery from the plasma was observed to range from 98.39 to 99.74% at the three QC levels, which indicates the efficiency of the sample extraction technique. The drug recovery is comparable with the methods reported previously (Acharjya et al. 2012; Al-Shehri et al. 2019).

Table 2 Recovery data for LTZ in rat plasma

QC level	Mean conc. detected (μg/mL)	Recovery (%)	RSD (%)
LQC	0.212	98.39	2.59
MQC	0.994	99.74	3.46
HQC	7.651	98.86	2.56

HQC high quality control, *MQC* middle quality control, *LQC* low quality control, *RSD* relative standard deviation

Table 3 Stability studies of LTZ in rat plasma

QC level	%RE			
	Freeze-thaw stability	Bench-top stability (6 h)	Autosampler stability (10 h)	Short-term stability
LQC	−10.73	−6.32	−3.08	−2.17
HQC	2.34	3.30	2.98	4.71

HQC high quality control, *LQC* low quality control, *RE* relative error

Stability studies

The stability of LTZ in the plasma was assessed, and the data is shown in Table 3. The percentage relative error (%RE) for the QC stability samples was determined by evaluating the mean accuracy of the back calculated concentrations. LTZ was observed to be stable following three cycles of freeze-thaw of the QC samples. The results of the benchtop stability (for 6 h) and autosampler stability (for 10 h) also indicated that LTZ was stable under these conditions. Short-term stability studies carried out for 7 days with the QC samples demonstrated that the results were well within the acceptance limits of ±15%. In addition, standard stock solutions of LTZ and CBZ were observed to be stable when kept on benchtop with %RE < 1.5%.

Pharmacokinetic studies

The optimized method was applied to determine the pharmacokinetic parameters for LTZ following oral administration. The Winonlin software was used to calculate the parameters, and data is shown in Table 4. The mean plasma concentration of LTZ versus time profile in rat plasma is shown in Fig. 4. Oral gavage of LTZ in rats resulted in a peak plasma concentration of (C_{max}) of 0.415 ± 0.036 μg/mL and exhibited T_{max} at 44.000 ± 9.798 h. The $AUC_{(0\text{-}72)}$ was observed to be 25.007 ± 1.425 μg h/mL while $AUC_{(0\text{-}\infty)}$ was observed to be 52.541 ± 2.46 μg h/mL. The elimination rate constant (K_{el}) was observed to be 0.011 ± 0.001/h. LTZ exhibited a mean residence time (MRT) of 101.900 ± 7.316 h and

Table 4 Pharmacokinetic parameters of LTZ in SD rats following oral administration

Parameters	Observed values*
K_{el} (h^{-1})	0.011 ± 0.001
$t_{1/2}$ (h)	60.573 ± 3.018
C_{max} (μg/mL)	0.415 ± 0.036
T_{max} (h)	44.000 ± 9.798
$AUC_{(0\text{-}t)}$ (μg h mL^{-1})	25.007 ± 1.425
$AUC_{(0\text{-}\infty)}$ (μg h mL^{-1})	52.541 ± 2.460
MRT (h)	101.900 ± 7.316

$AUC_{(0\text{-}t)}$ area under the curve from zero to the last measurable time t, *$AUC_{(0\text{-}\infty)}$* area under the curve from zero to the last detectable time point, *C_{max}* peak plasma concentration, *K_{el}* elimination rate constant, *MRT* mean residence time, *$t_{1/2}$* half-life, *T_{max}* time to reach peak plasma concentration
*All values are expressed as mean ± S.D.; n = 6

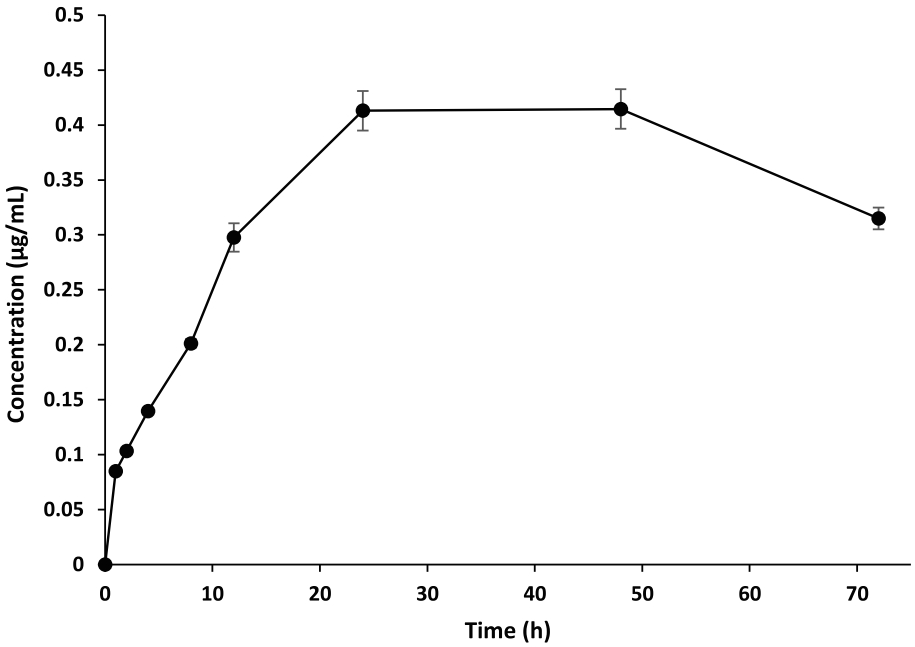

Fig. 4 Mean plasma concentration—time profile of LTZ in SD rats following oral administration (n = 6; dose = 2 mg/kg). Values are expressed as mean ± S.D.

$t_{1/2}$ equivalent to 60.573 ± 3.018 h. These results are in close agreement with the results reported previously for oral administration of LTZ (Wempe et al. 2007; Dhakne et al. 2020). This clearly demonstrates the appropriateness of the chromatographic method to estimate LTZ in rat plasma.

Conclusion

A precise and sensitive reverse phase liquid chromatographic method was developed to quantify LTZ in rat plasma. Drug extraction from the plasma was carried out using a simple protein precipitation technique without the need of additional processing steps. The optimized method was validated according to the USFDA guidelines for the recommended validation parameters. The results of the validation study demonstrated that the developed method was precise, accurate, and sensitive over a wide linear range of LTZ concentrations in rat plasma. The applicability of the method was corroborated following oral administration of LTZ to determine the pharmacokinetic parameters

Abbreviations

RP: Reverse phase; HPLC: High-performance liquid chromatography; ACN: Acetonitrile; AI: Aromatase inhibitor; LTZ: Letrozole; USFDA: United States Food and Drug Administration; EIA: Enzyme immunoassay; UV: Ultraviolet; MS: Mass spectrometry; SD: Sprague-Dawley; S.D.: Standard deviation; OPA: Orthophosphoric acid; IS: Internal standard; CBZ: Carbamazepine; PA: Paracetamol; KCZ: Ketoconazole; LQC: Low quality control; MQC: Middle quality control; HQC: High quality control; MTBE: Methyl tertbutyl ether; LLOQ: Lower limit of quantification; QC: Quality control; RT: Room temperature

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s40543-021-00276-4.

Additional file 1. Method optimization – effect of mobile phase compositions and internal standard (IS). Fig. S1. Effect of mobile phase composition and PA (IS). Chromatograms of a) diluent, b) LTZ, c) PA, d) blank plasma and e) spiked plasma using ACN and acetate buffer pH 4.5 (50:50 %v/v) at 0.8 mL/min. Fig. S2. Effect of mobile phase composition and KCZ (IS). Chromatograms of a) diluent, b) LTZ, c) KCZ, d) blank plasma and e) spiked plasma using ACN and phosphate buffer pH 5.5 (65:35 %v/v) at 0.8 mL/min.

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Authors' contributions

All authors have equal contribution to this research work. All authors read and approved the final manuscript.

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Availability of data and materials

Not applicable

Declarations

Competing interests

The authors declare that they have no competing interests.

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