Study of Pharmacokinetics and Tissue Distribution of BITS-17 in Rat Plasma and Tissue Homogenate Using a Validated LC Method

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Abstract

Accurate, simple, sensitive, reproducible and specific liquid chromatographic method has been developed for estimation of BITS-17 in rat plasma and other biological tissues using β-naphthol as an internal standard (IS). The applicability of the validated method was tested to estimate the concentrations of BITS-17 (a novel anti-tubercular agent) in plasma and tissues that were harvested in a rat tissue distribution study. A liquid–liquid extraction technique was used to extract BITS-17 and internal standard from the plasma and various tissue homogenates. The main pharmacokinetic parameters obtained after intravenous administration were $t_{1/2} = 14 ± 0.20$ h, $K_e = 0.62 ± 0.10$ h$^{-1}$, mean plasma clearance $= 1.35 ± 0.16$ mg/h and mean volume of distribution $= 1.99 ± 0.49$L. The systemic absorption was tested with two formulations was slow after oral administration and maximum bioavailability was 33.02%. The peak plasma concentration of both the formulations was found to be $1.31 ± 0.06$ and $1.0 ± 0.12$ mg/mL, respectively, which was much above the levels of minimum inhibitory concentrations of BITS-17. The concentrations of BITS-17 were found in various tissues but the levels in lung and brain signifies a good therapeutic target.

Keywords: BITS 17; β-naphthol; Rat plasma; Method validation; HPLC; Pharmacokinetics; Tissue distribution

Introduction

Tuberculosis (TB) is the leading cause of mortality among all infectious diseases worldwide and is responsible for over two million deaths annually [1]. One of the most effective and widely used anti-tuberculosis (TB) drugs is isoniazid (INH); a pro-drug activated via oxidation that forms an adduct with NAD(+) to inhibit NADH dependent targets of Mycobacterium tuberculosis (MTB), such as the enoyl-acyl carrier protein reductase (InhA). In our earlier work we developed a novel, highly effective, and fast acting anti-TB drugs with low toxicity profiles and performing activity against both drug-sensitive and drug resistant MTB [2]. The INH moiety was incorporated in the 1,3-thiazinan scaffold and was evaluated for invitro MTB activity [3-7]. Compound N-(2-(4-(benzyloxy) phenyl)-4-oxo-1,3-thiazinan-3-yl) isonicotinamide (BITS 17) inhibited MTB with MIC of 0.12 µM and was three times more potent than INH was selected for further studies [2].

In this present study, a simple, robust and reproducible method has been developed and validated to estimate BITS-17 concentrations in rat plasma. This method meets the requirements and provides high degree of accuracy, sensitivity and specificity by simple extraction using high performance liquid chromatography and detection by ultraviolet -visible spectroscopy [8-11]. A tissue distribution study performed after oral and intravenous (i.v.) administration will provide the concentration of an NCE in various tissues relative to the circulatory levels of the NCE, and this aids in mapping tissue to plasma ratios during drug absorptive, distributive and elimination phases. The outcome of such key experiments will provide information regarding the propensity (or lack of it) to accumulate in tissue (s). By estimating the concentrations in plasma and other tissues, one can establish the tissue/plasma ratio, which will help the researcher to use plasma as a surrogate to assess the levels in other tissues during pharmacology, PK/ PD, toxicokinetic studies, etc. One of the protocols followed (following oral administration) is to collect both plasma and tissue samples, during the absorption phase, around $C_{max}$ (the time point at which maximum concentration of NCE is observed in plasma) and another time point during the elimination phase of the NCE. Nine tissues are harvested at the suggested time points and the concentration of NCE in each tissue will be analyzed to compute tissue/plasma ratios. It is tedious task for a bioanalyst to develop a method and validate it for the determination of an NCE in each tissue, especially when radio labeled NCE is not available. Therefore, we looked into the feasibility of applying a validated method (in plasma), without further modification, to measure NCE levels in the tissue samples. In the present paper, we are extending the applicability of a previously validated method in plasma to quantitate BITS-17 in various tissues. In addition, certain key validation experiments, such as accuracy, precision, matrix effect, recovery, stability studies (freeze–thaw, bench-top, in-injector and long-term), that were performed with each tissue matrix are reported.

Materials and Methods

Chemicals and reagents

Test substance BITS 17 (Figure 1) was synthesized at Drug research laboratory, BITS-PILANI Hyderabad Campus. β-naphthol, Phosphat buffer saline and Ammonium acetate were supplied by Sigma Chemicals. Acetonitrile (HPLC grade) and Methanol (HPLC grade) were supplied by Sigma-Aldrich. The reagents Glacial acetic acid and ethyl acetate were of HPLC grade.

Analytical method

Analysis of the anti tubercular drug was performed using a validated
HPLC technique as described below. Analyses were performed on a reverse phase HPLC (Perkin Elmer LC system equipped with Flexar quaternary pump along with peltier controlled Flexar auto-sampler and Flexar PDA) on Brownlee Analytical C18 column (4.6 × 150 mm, 3 µm, Perkin Elmer Corporation, U.S.A) column. The mobile phase used is a mixture of 0.01 M ammonium acetate (pH=4.5) and acetonitrile mixture (62:38,v/v). The detection wavelength was set at 315 nm. Chromatography separation was performed at room temperature and flow rate was maintained at 1 mL/min.

Preparation of stock solutions and calibration standards

Primary stock solutions of BITS-17 and IS for preparation of standard and quality control (QC) samples were prepared by weighing separately. The primary stock solutions (1.0 mg/mL) of the analyte and IS were prepared in methanol and stored at -20°C, which were found to be stable for one month (data not shown).

Preparation of calibration and quality control samples

Appropriate dilutions were made in methanol for BITS-17 to produce working stock solutions of 2.5, 4.0, 6.0, 8.0, 10, 20, 40, 60 µg/ml on the day of analysis and these stocks were used to obtain a calibration curve (CC). Another set of working stock solutions was made in methanol (from primary stock) at 50, 9, 3 and 2.5 µg/mL of which 50 and 9 µg/mL were used for QC high and QC medium respectively. Whereas working stock solutions at 3 and 2.5 µg/mL were used for QC low and lower limit of quantitation (LLOQ) of BITS 17, respectively. A working solution of IS (100 µg/mL) was also prepared in methanol. Calibration samples were prepared by spiking 100 µL of control rat plasma with the appropriate amount of analyte (10 µL) and IS (20 µL) on the day of analysis. Samples for the determination of recovery, precision and accuracy were prepared by spiking control rat plasma in bulk at appropriate concentrations (0.25, 0.4, 0.6, 0.8, 1, 2, 4 and 6 µg/ml) prepared by spiking 100 µL of blank plasma with 10 µL of standard stock solution and 10 µL of IS 200 µg/mL. Standard tissue samples were prepared in similar manner with appropriate blank tissues. The acceptance criterion for each back-calculated standard concentration was ± 15% deviation from the nominal value except at LLOQ, which was set at ± 20%.

Inter/intra assay precision and accuracy was determined by analyzing six replicates at four different QC levels as described above on four different days. The criteria for acceptability of the data included accuracy within ± 15% deviation (S.D) from the nominal values and a precision of within ± 15% relative standard deviation (R.S.D), except for LLOQ, where it should not exceed ± 20% of S.D.

The stability of BITS-17 and IS in the injection solvent was determined periodically by injecting replicate preparations of processed samples up to 24 h (in auto-sampler at 4°C) after the initial injection. The ratio of peak-areas of the BITS-17 and IS obtained at initial cycle were used as the reference to determine the relative stability of the BITS-17 at subsequent points. Stability of BITS-17 in the biomatix after 10 h (bench top) exposure at ambient temperature (25 ± 2°C) was determined at two concentrations in six replicates. Freezer stability of the BITS 17 in biomatix was assessed by analyzing the low and high QC samples stored at -80 ± 10°C for at least 30 days. The stability of BITS 17 in biomatix following repeated three freeze-thaw cycles (stored at -80 ± 10°C between cycles) was assessed using QC samples spiked with analyte. Samples were processed as described above. Samples were considered to be stable if assay values were within the acceptable limits of accuracy (i.e., ± 15% S.D) and precision (i.e., 15% R.S.D).

Application to pharmacokinetic study

Male Wistar rats, ~3 months of age and weighing between 200-250 g were used in this study following the approval from ethics committee.
for animal use. The two oral formulations (50 mg/kg) consisted of a suspension of drug firstly, in sodium CMC and tween 80. Second formulation constituted of cremophore ELP, sodium CMC and SLS. The animals were fasted overnight (~14 h) and had free access to water throughout the experimental period. Animals were provided with standard diet 3 h post-dosing. The formulation for intravenous administration (10 mg/kg) was prepared with propylene glycol, cremophore ELP, N-methyl-pyrrolidone in normal saline. The rats were anaesthetized in ether and blood samples (~0.50 mL) were collected from retro-orbital plexus into microfuge tube (containing 20 L of saturated EDTA) at 0.5, 1, 1.5, 2, 3, 5, 8, 10 and 24 h post-dosing. Plasma was harvested by centrifuging the blood using micro centrifuge at 6000 rpm for 5 min. Rat plasma (100 µL) samples were spiked with IS and processed as described above.

Pharmacokinetic parameters were calculated by employing a non-compartmental analysis (Gibaldi and Perrier, 1982). The peak plasma concentrations (C_{max}) and the corresponding time (T_{max}) were directly obtained from the raw data. The area under the plasma concentration vs. time curve up to the last quantifiable time point, AUC (0-t) was obtained by a linear and log-linear trapezoidal summation. The AUC (0-t) extrapolated to infinity (i.e. AUC (0-∞)) by adding the quotient of Clast/Kel, where Clast represents the last measurable time concentration and Kel represents the apparent terminal rate constant. Kel was calculated by the linear regression of the log-transformed concentrations of the drug in the terminal phase [12-15]. The half-life (t½) of the terminal elimination phase was obtained using the relationship t½=0.693/Kel by non-compartmental method were evaluated by using Win Nonlin™ Enterprise, Version: 5.3 (Pharsight Corporation, Mountain View, CA).

**Application to tissue distribution study**

The tissue distribution study was performed in overnight (~12 h) fasted healthy male Wistar rats (n=6, weight range 200–240 g) following the approval from the ethics committee for animal use [16,17]. During the fasting time animals had free access to water. BITS-17 was administered to rats by oral administration at a dose of 50 mg/kg (in the form of a suspension, prepared using cremophore ELP, sodium CMC and SLS). At each time point, viz. 1 h (during the initial absorption phase), 2 h (around C_{max}) and 5 and 8 h (elimination phase), two rats were sacrificed and various tissues (heart, lung, liver, stomach, small intestine, large intestine and brain along with blood and plasma) were collected separately from each animal. Tissue homogenate samples (50 mL) were spiked with IS solution and processed as described in the ‘extraction procedure for biosamples’ section. The standard curve was generated using individual tissue homogenates. Along with respective samples, QC samples at low, medium and high concentrations spiked in respective blank tissue homogenates were assayed in duplicate and were randomly distributed among standard calibrators and unknown samples in the analytical run; not more than 33% of the QC samples were greater than ± 15% of the nominal concentration.
Results
Optimization of chromatographic detection

Chromatographic conditions were optimized to achieve the good separation, high reproducibility of BITS-17 and IS from the endogenous interferences of the biological matrix. Wavelength of 315 nm was selected at the λ_{max} of BITS-17 as an optimum response of IS was also observed. Different buffer like di-potassium hydrogen phosphate, potassium di-hydrogen phosphate, ammonium acetate of different pH also observed. Different buffer like di-potassium hydrogen phosphate, ammonium acetate of different pH and acetonitrile at a ratio of 68:32 with a Brownlee Analytical C18 column (4.6×150 mm, 3 µm, using 10 mM of ammonium acetate (pH 4.5) and acetonitrile at a ratio 100∶30 and 100∶50 respectively. The optimum resolution, proper run time of the analyte, with good peak shape was achieved by using 10 mM of ammonium acetate (pH 4.5) and acetonitrile at a ratio of 68:32 with a Brownlee Analytical C18 column (4.6×150 mm, 3 µm, Perkin Elmer Corporation, U.S.A). Robustness studies showed that a small variation in pH of the buffer in the mobile phase, C18 columns from different vendor like Thermo, Merck and Phenomenex did not affect the analysis much.

Validation parameters

Specificity and selectivity: A typical chromatogram for the biomatrix (free of analyte and IS) and spiked with analyte and extracted standards were observed at the retention times of analyte and IS. The retention time of BITS-17 and IS were 10.5 and 8.5 min, respectively. The total chromatographic run time was only 13 mins.

Recovery: The extraction recoveries of the BITS-17 from biomatrix were calculated by comparing the response of biological samples spiked with the analyte and extracted with a suitable solvent to that of standard solutions of equivalent concentration. The results of the comparison of neat standards vs biological samples extracted standards were estimated for BITS-17 at low, mid, high QC concentrations and peak area ratios (analyte/IS) versus concentration, and fitted to the equation \( y=mx+c \). The results demonstrated the reproducibility of the devised method.

Precision and accuracy: The method provides accuracy and precision for the determination of BITS-17. The accuracy, intra- and inter-assay precision was determined by analyzing six replicates of QC samples at four concentrations. Table 2 gives a summary of the recoveries for rat biological samples spiked with different concentrations of BITS-17. The intra-assay precision ranged from 0.86 to 6.22 and inter day precision ranges from 0.71 to 4.83 for all the biological samples. The results demonstrated the reproducibility of the devised method.

Stability: The stability was predicted at LQC and HQC concentrations in a battery of stability tests viz., in-injector, bench-top, repeated three freeze/thaw cycles and at -80 ± 10°C for 20 days (Tables 3 and 4). These results indicate that the analyte was stable in-injector (24 h), bench top (room temperature for 10 h), after freeze thaw condition (3 cycles). The results were also proved that the analyte are stable for 20 days in biological samples. The stability was predicted at LQC and HQC concentrations in a battery of stability tests viz., in-injector, bench-top, repeated three freeze/thaw cycles and at -80 ± 10°C for 20 days (Tables 3 and 4). These results indicate that the analyte was stable in-injector (24 h), bench top (room temperature for 10 h), after freeze thaw condition (3 cycles). The results were also proved that the analyte are stable for 20 days in biological samples.

Pharmacokinetic analysis: The concentration of BITS-17 in plasma and tissue homogenates. The recovery of IS at 20.0 µg/mL was 96.5%. The mean recovery of >91% was found in plasma and >75% in other tissues. The recovery of IS at 20.0 µg/mL was 96.5%. The mean recovery of >91% was found in plasma and >75% in other tissues. The recovery of IS at 20.0 µg/mL was 96.5%. The mean recovery of >91% was found in plasma and >75% in other tissues. The recovery of IS at 20.0 µg/mL was 96.5%. The mean recovery of >91% was found in plasma and >75% in other tissues.
a plasma sample was determined by the same method. Plasma concentration time data of the analyte were analyzed by non-compartmental method. The basic pharmacokinetic parameters assessed were half life (t½), elimination rate constant (Kel), mean plasma clearance (CL) and mean volume of distribution (Vd) as reported in Table 5. After a single i.v. bolus dose of 10 mg/kg body weight, BITS-17 had a t½ of 1.14 h with moderate clearance (22.48 mL/min/kg) and volume of distribution (1.99 L). Oral administration of a single dose of 50 mg/kg body weight the peak concentration was achieved in 1.31 ± 0.06 and 1.0 ± 0.12 μg/mL with formulation one and two, respectively. The plasma levels then declined rapidly with formulation one and the levels sustained with the second one (Figure 3). The oral bioavailability also increased from 16.7% to 33.0%.

The low systemic bioavailability of the compound can be attributed to its very poor aqueous solubility in the gastrointestinal fluids, therefore low and delayed absorption. The levels of BITS-17 are much above the MIC values.
Tissue distribution analysis: There is a wide tissue distribution of BITS-17 in rats after oral administration, shown in figure 4. Drug concentrations in highly perfused tissues were highest after 2 hours post dosing except for stomach. Table 6 comprises of the tissue to plasma ratios, assessing the tissue–plasma ratio, the plasma could be used as a surrogate to determine the concentrations of BITS-17 in other tissues in a pharmacodynamic, efficacy and toxicokinetic study. Comparing the distribution profiles, the maximum concentration was in intestine as the drug has low absorption due to its poor solubility. Presence of drug concentrations significantly above MIC levels in brain till 5 hours and in lungs at 2 hours post administration signifies that brain and lungs could be good therapeutic targets for the drug.

References

Table 6: Biological sample concentrations (µg/ml) of BITS-17 at various time points along with ratios (tissue to plasma).