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Determination of Chemical and in vitro Metabolic Stability Studies of New Triazole Antitubercular Compounds to Optimize Lead Compound Selection

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Abstract : Evaluation of new chemical entities for their drug like properties, early in the discovery phase will help to reduce late stage attrition due to poor stability or unsuitable pharmacokinetic properties. Four newly synthesized triazole compounds viz., 3,3,5-Trimethyl-1-(5-phenyl-4H-1,2,4-triazol-3-yl)cyclohexanol **(MSDRT 8),** 3,3,5-Trimethyl-1-(5-(pyridin-4-yl)-4H-1,2,4-triazol-3-yl)cyclohexanol **(MSDRT 10)**, Cyclohex-3-enyl(5-(pyridin-4-yl)-4H-1,2,4-triazol-3-yl)methanol **(MSDRT 11)** and Cyclohex-3-enyl(5-phenyl-4H-1,2,4-triazol-3-yl)methanol **(MSDRT 12)),** having in vitro antitubercular activity, were screened for chemical stability after acid and alkali hydrolysis. Metabolic stability was also studied after incubating each compound with rat liver microsomes. Based on the results of chemical and metabolic stability, MSDRT 12 was identified as a potential lead compound. A reverse phase high performance liquid chromatographic method with UV/Visible detector was developed for all four compounds and their degradation products were developed and separation were achieved on a Zorbax XDB C18 5 µm column with mobile phase containing a gradient mixture of 0.1 % formic acid and acetonitrile for MSDRT 8, MSDRT 10 and MSDRT 12 and a gradient mixture of 0.1 % formic acid and methanol for MSDRT 11. The method was validated as per International Conference of Harmonisation (ICH) guidelines.
Keywords: Antitubercular, stability, metabolism, rat liver microsomes, pharmacokinetic.

Introduction

Tuberculosis (TB) remains a major health problem throughout the world causing more number of deaths compared to any other single infectious disease [1]. Almost 50% of Multidrug-resistant Tuberculosis (MDR-TB) cases worldwide are estimated to occur in China and India [2]. No new antitubercular drugs have been approved in the last five decades except Bedaquiline which was approved by the United States Food and Drug Administration (USFDA) in January 2013 [3]. The emergence of drug resistant strains of *Mycobacterium Tuberculosis* (*Mtb)* , Human Immunodeficiency Virus –Tuberculosis (HIV-TB) coinfection and the problems

with the current antitubercular therapy like prolonged duration of treatment and drug toxicity highlights the urgency in the search for new antitubercular drugs and is a major social and medical need especially in the south east Asian region.

Triazoles are an important class of heterocyclic compounds which show various biological activities including antitubercular [4-6]. A series of triazoles have been synthesized at the Institute of Chemical Technology coordinated by Dr M S Degani (one of the coauthor of this research paper) and they were tested for antitubercular activities [7]. These compounds were identified and characterized using Proton Nuclear magnetic resonance (^{1}H- NMR) spectroscopy , Carbon Nuclear magnetic resonance spectroscopy (^{13}C –NMR), Infra Red Spectroscopy and Mass Spectroscopy.

Based on the antitubercular activity four triazole analogues, MSDRT 8 (A), MSDRT 10 (B), MSDRT 11 (C) and MSDRT 12 (D) were selected for chemical and metabolic stability evaluation. The structures are shown in Figure 1.

Regulatory requirements for Investigational New Drugs suggests performing stress studies in early development phase as these are essential for developing stability-indicating analytical procedures [8]. Since chemical stability helps to evaluate the inherent stability characteristic and metabolic stability helps in prediction of pharmacokinetic parameters these studies are used to verify the suitability of a molecule for further development as a lead compound [9].

The present study describes stability evaluation after forced degradation hydrolysis in acid and alkali and metabolic stability using rat liver microsomes. These four compounds and their degraded products were analyzed by using newly developed reverse phase High Performance Liquid Chromatography (HPLC) methods followed by their validation as per ICH guidelines [10, 11].

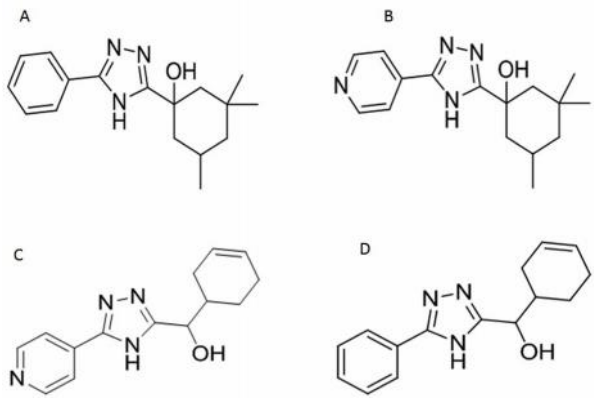

Figure 1:
A: Structure of 3,3,5-Trimethyl-1-(5-phenyl-4H-1,2,4-triazol-3-yl)cyclohexanol (MSDRT 8)
B: Structure of 3,3,5-Trimethyl-1-(5-(pyridin-4-yl)-4H-1,2,4-triazol-3-yl)cyclohexanol (MSDRT 10)
C: Structure ofCyclohex-3-enyl(5-(pyridin-4-yl)-4H-1,2,4-triazol-3-yl)methanol (MSDRT 11)
D: Structure ofCyclohex-3-enyl(5-phenyl-4H-1,2,4-triazol-3-yl) methanol (MSDRT 12)

Materials and Methods

Sample and Reagents

All four compounds (MSDRT 8, MSDRT 10, MSDRT 11 and MSDRT 12) were synthesized at Institute of Chemical Technology, Mumbai, India. HPLC grade methanol and acetonitrile were purchased from Rankem and Merck, respectively. Nicotinamide adenine dinucleotide phosphate reduced tetra sodium salt

(NADPH) (extra pure grade) was from Sisco Research Laboratories Pvt. Ltd. All other reagents of highest purity grade were locally purchased. Milli Q water (Millipore) was used throughout the experiments.

Rat liver microsomes

The rat livers (10 g) were homogenized in a Potter Elvejhem glass homogenizer equipped with Teflon pestle with 40 ml of 10 mM Tris (hydroxymethyl) aminomethane hydrochloride (Tris-HCl) buffer containing 0.25 M sucrose, pH 7.4. The homogenate was centrifuged at 13,000 x g for 10 min at 4°C in a refrigerated centrifuge and the precipitate was discarded. To the supernatant calcium chloride was added to the final concentration of 10 mM. The solution was stirred for 15-20 minutes with a glass rod and then centrifuged at 25,000 x g for 10 min at 4°C. Firmly packed pellets of microsomes were resuspended by homogenization in 100 mM Tris-HCl buffer containing 20 % w/v of glycerol and 10 mM Ethylene diamine tetraacetic acid (EDTA), pH 7.4 (1: 1 v/w of liver). The microsomes were stored at -70°C until further use.

Preparation of Standard Stock Solutions

All four compounds (MSDRT 8, MSDRT 10, MSDRT 11 and MSDRT 12) were dissolved individually in methanol to obtain a stock solution of 1000 µg/mL. Each stock solution was further diluted with methanol to obtain a standard solution of 250µg/mL.

Analytical conditions

All four compounds (MSDRT 8, MSDRT 10, MSDRT 11 and MSDRT 12) and their degraded products, obtained during stress studies and after incubation with rat liver microsomes, were separated on a Zorbax XDB C18, 4.6 X 150 mm column (particle size 5 µm) at a flow rate of 1.0 mL/min in Shimadzu HPLC (LC 2010) attached with a UV/Visible detector and the output signal was monitored and processed using Empower 2 software. The column oven temperature was maintained at 25°C and the injection volume was 20 µL. The analysis for compounds MSDRT 8, MSDRT 10 and MSDRT 12 was performed with the mobile phase composition of 0.1 % formic acid: acetonitrile with a linear gradient of 75: 25 to 25: 75 from 5 to 25 minutes which was maintained further for five minutes. The analysis for compound MSDRT 11 was performed with the same gradient profile with methanol as the organic solvent instead of acetonitrile. The absorbance was measured at 260 nm for MSDRT 8, MSDRT 10 and MSDRT 11 and at 245 nm for MSDRT 12.

Validation of the methods

Developed analytical methods were validated using the following parameters,

Linearity range

The linearity of the method was demonstrated by injecting the prepared solutions in triplicate. A stock solution of about 1000 µg/mL of MSDRT 8, MSDRT 10, MSDRT 11 and MSDRT 12 was prepared for each compound which was serially diluted to obtain concentrations in the range of 0.200 - 498.750, 0.286 - 500.500, 2.562 - 512.424 and 0.199 - 498.155 µg/mL for MSDRT 8, MSDRT 10, MSDRT 11 and MSDRT 12 respectively.

Precision

Repeatability of the method was evaluated by injecting six replicates of the standard solution (250 µg/mL) for each compound. Intermediate precision was also determined by injecting six replicates of the standard solution for each compound using a different analyst, a different column and on a different day.

Limit of detection and limit of quantitation

Limits of detection (LOD) and quantification (LOQ) represent the concentration of the analyte that would yield signal-to-noise ratios of about 3 for LOD and 10 for LOQ. To determine the LOD and LOQ dilutions of stock solution of MSDRT 8, MSDRT 10, MSDRT 11 and MSDRT 12 were prepared. The diluted samples were injected in LC system and measured signal from the samples was compared with those of blank samples.

Specificity

Specificity is the ability of the method to measure the analyte response in the presence of its potential impurities or degradation products.

Accuracy

The accuracy of the assay method was evaluated in triplicate using three concentration levels 80, 100 and 120 %. The percentage recovery and percentage Relative Standard Deviation (RSD) was calculated.

Solution Stability

The stability of all four compounds (MSDRT 8, MSDRT 10, MSDRT 11 and MSDRT 12) in solution was determined by leaving test solutions of the sample in tightly capped polypropylene vials in a cooling cabinet maintained at 2 – 8°C. The stored solution was assayed at least after seven days storage and the results were compared with those obtained from freshly prepared solution.

Range used for metabolic stability experiments

For metabolic stability experiments, each compound was dissolved individually in methanol to obtain a stock solution of 5 mM. Each stock solution was serially diluted with methanol to obtain concentrations in the range of 5 to 50 µM and the prepared solutions were injected in duplicate for confirming the linearity.

Repeatability of the method for the range used in metabolic stability was evaluated by injecting six replicates of the standard solution (25µM) for each compound and calculating the % Relative Standard Deviation (RSD).

Forced degradation studies in alkaline and acidic condition:

Stability in alkaline and acidic condition was evaluated by mixing about 2 mg each of these compounds in 2 mL of 0.02 N sodium hydroxide (NaOH) and 0.1 N hydrochloric acid (HCl) respectively and then incubating it in a water bath at 60°C for a specific time period of 0, 15, 30 and 60 minutes. After cooling the sample to room temperature alkali and acid treated compounds were neutralized by adding 2 mL of 0.02 N HCl and 0.1 N NaOH respectively. 4 mL of methanol was added to each tube, mixed well and transferred to auto sampler vials. All chemical stability samples were analysed at an initial concentration of 250 µg/mL.

Metabolic Stability Studies

Susceptibility to biotransformation was studied by mixing 60 µl of 5 mM stock solution of each compound in dimethyl sulfoxide, with 300 µl of rat liver microsomes (RLM) (20 mg/mL protein). The total volume was then adjusted with mixed phosphate buffer having pH 7.4, to 6 mL to obtain final concentration of 50 µM and 1 mg/mL of test compound and RLM protein respectively. The reaction was initiated by addition of 300 µl of 12 mM NADPH in mixed phosphate buffer and incubation samples were maintained at 37°C. The samples were withdrawn at 0, 15, 30, 45, 60 and 120 minutes and the reaction was terminated by addition of a volume of acetonitrile to the sample aliquot. The samples were centrifuged and the supernatant was transferred to autosampler vials and analysed by HPLC.

Results and discussion

Method Development and Optimization

The aim of method development was to develop methods with similar chromatographic conditions since all four compounds are structurally similar. Trials were initiated to simultaneously evaluate the suitability of columns, mobile phase, gradient and programme for all four compounds.

HPLC method development trials were initiated using a Hypersil BDS, 150 X4.6 mm, 5 µ C18 column and different mobile phases in both isocratic and gradient mode were evaluated but the trials for compound

MSDRT11 were not satisfactory with respect to resolution of the degradants. Trials with Zorbax Eclipse XDB C18 column and replacing methanol in the mobile phase with acetonitrile helped in increasing the response , reducing the retention time along with complete separation of the degradants for MSDRT 8, MSDRT 10 and MSDRT 12. For MSDRT 11 the same gradient method was finalized but methanol was used in the mobile phase.

The retention times for MSDRT 8, MSDRT 10, MSDRT 11 and MSDRT 12 were found to be 17.74, 7.21, 16.78 and 16.01 minutes respectively (Figure 2: A, B, C and D respectively).

Method validation

The validation results for the HPLC methods have been summarized in **Table I**.

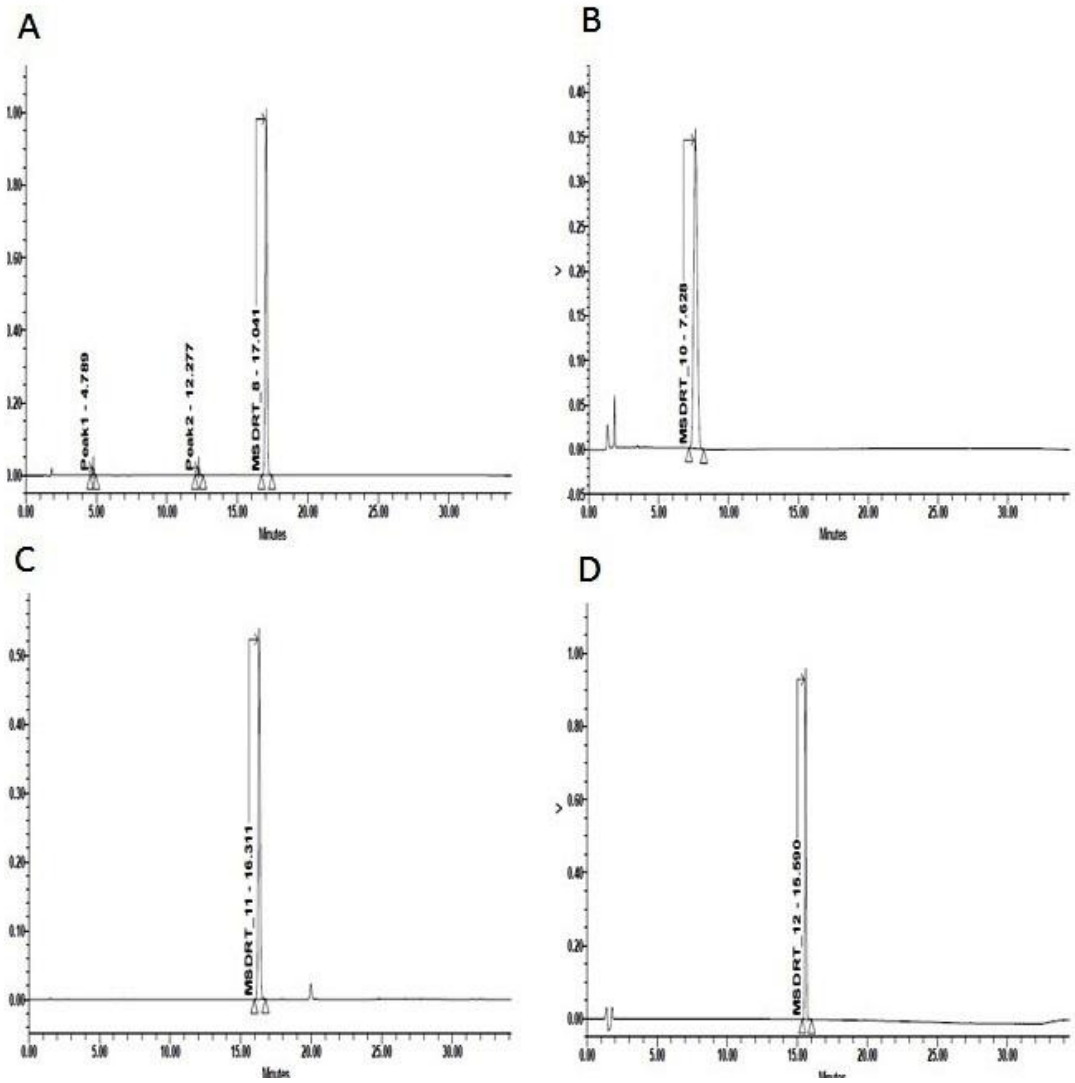

Figure 2:

A: HPLC chromatogram of standard MSDRT 8 (250 µg/mL)

B: HPLC chromatogram of standard MSDRT 10 (250 µg/mL)

C: HPLC chromatogram of standard MSDRT 11 (250 µg/mL)

D: HPLC chromatogram of standard MSDRT 12 (250 µg/mL)

Table I: Summary of method validation

Parameter	MSDRT 8	MSDRT 10	MSDRT 11	MSDRT 12
Linearity range µg/mL	0.200- 498.750	0.286-500.500	2.562-512.424	0.199-498.155
Precision % RSD	0.89	0.15	0.17	0.09
Intermediate Precision (%)	0.78	0.33	0.15	0.47
LOD µg/mL	0.057	0.086	0.788	0.051
Accuracy (%)	99.10 to 101.24	98.40 to 99.39	100.28 to 100.45	99.19 to 99.37
LOQ µg/mL	0.200	0.286	2.562	0.199
Correlation Coefficient	0.996	0.998	0.999	0.999

Linearity

The analyte response was linear over the concentration range of 0.200 – 498.750, 0.286 – 500.50, 2.562 – 512.424 and 0.199 – 498.155 µg/mL with the correlation coefficient values of 0.996, 0.998, 0.999, 0.999 for MSDRT 8, MSDRT 10, MSDRT 11 and MSDRT 12 respectively.

The analyte response was linear in the range used in metabolic stability evaluation and correlation coefficient values were 0.996 for MSDRT 8, 0.993 for MSDRT 10, 0.999 for MSDRT 11 and 0.999 for MSDRT 12 over the concentration range of 5 to 50 µM.

Precision

The RSD (%) of the method at a concentration of 250 µg/mL was 0.89, 0.15, 0.17 and 0.09 for MSDRT 8, MSDRT 10, MSDRT 11 and MSDRT 12 respectively. For intermediate precision the RSD (%) of the method at a concentration of 250 µg/mL was 0.78, 0.33, 0.15 and 0.47 for MSDRT 8, MSDRT 10, MSDRT 11 and MSDRT 12 respectively. The RSD (%) of the method at a concentration of 25 µM was 0.49, 0.31, 1.75 and 0.21 for MSDRT 8, MSDRT 10, MSDRT 11 and MSDRT 12 respectively.

Limit of detection and limit of quantitation

The LOD was 0.057, 0.086, 0.788 and 0.051 µg/mL for MSDRT 8, MSDRT 10, MSDRT 11 and MSDRT 12 respectively. LOQ was 0.200, 0.286, 2.562 and 0.199 µg/mL for MSDRT 8, MSDRT 10, MSDRT 11 and MSDRT 12 respectively.

Specificity

No interfering peak was seen at the retention time of the analytes for all four compounds indicating the specificity of the method.

Accuracy

The percent recovery was well within limits for all the four compounds and was in the range from 98.40 to 101.24 % w/w at various added concentrations..

Solution Stability

The results from solution stability experiments confirmed that standard solutions were stable up to 7 days when stored at a temperature of 2 - 8°C.

Stability of compounds in hydrolytic stress conditions

The stability results of the triazole compounds after incubation with acid and alkali has been summarized in Table II.

Table II: Acid and Alkali Degradation

0.1 N HCl				
Compound	**MSDRT 8**	**MSDRT 10**	**MSDRT 11**	**MSDRT 12**
0 mins	49.47	15.03	94.14	99.57
15 mins	23.17	5.54	95.04	99.65
30 mins	32.72	5.16	95.06	99.54
60 mins	34.06	2.28	95.43	99.35
0.02 N NaOH				
Compound	**MSDRT 8**	**MSDRT 10**	**MSDRT 11**	**MSDRT 12**
0 mins	94.51	81.72	95.61	98.73
15 mins	93.55	9.38	95.96	97.26
30 mins	91.38	2.70	94.65	88.81
60 mins	87.45	0.39	94.80	84.29

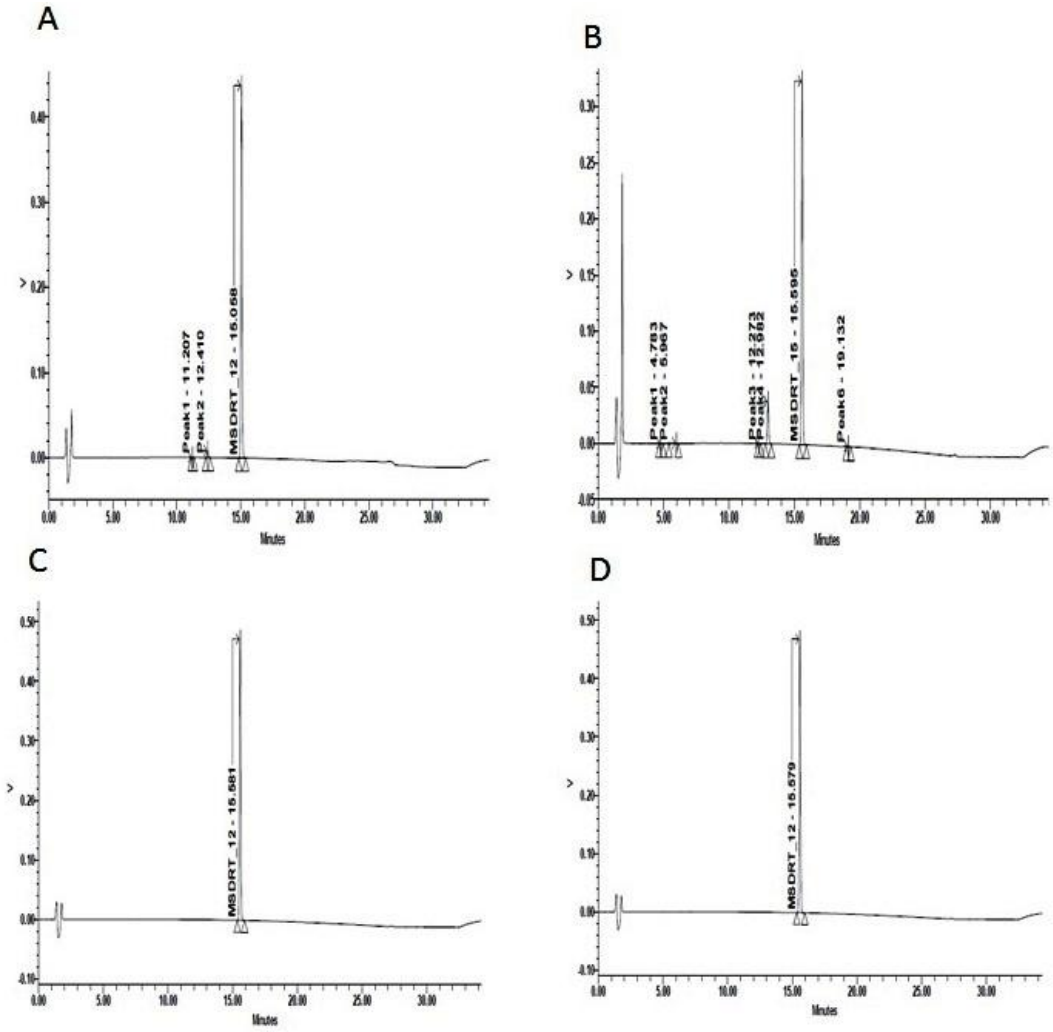

Figure 3:
A: Degradation of MSRDT 12 in 0.1 N HCl (0 minutes)
B: Degradation of MSRDT 12 in 0.1 N HCl (after 60 minutes)
C: Degradation of MSRDT 12 in 0.02 N NaOH (0 minutes)
D: Degradation of MSRDT 12 in 0.02 N NaOH after (60 minutes)

The strength of alkali and acid, for evaluating the chemical stability, was selected in such a way that it was able to distinguish and identify the most stable compound. Since these are preliminary screening for ranking of compounds based on stability experiments with a strong alkali like sodium hydroxide and a strong acid like hydrochloric acid was performed. Stability in the acidic environment of the stomach is especially important for compounds which have to be developed as solid oral dosage formulations like in the case of antitubecular drugs. The chemical stability of these antitubercular agents showed that MSDRT 8, MSDRT 11 and MSDRT 12 were resistant to hydrolytic degradation in alkaline condition. However, MSDRT 8 and 10, the cyclohexanol derivatives of 1, 2, 4 triazole are highly unstable in acidic conditions. MSDRT 11 and MSDRT 12, the cyclohex-3-enyl derivatives of 1, 2, 4 triazole are totally resistant to acid hydrolysis even after 60 minutes. Chromatograms showing the degradation, immediately and after 60 minutes incubation in alkaline and acidic condition for MSDRT 12 are given in Figure 3: (A, B) and Figure 3: (C, D) respectively.

Incubation conditions for metabolic stability studies were optimized after evaluation of different protein concentrations and different drug concentrations so as to obtain optimum detector response and also to avoid complete metabolism in less than 30 minutes. Metabolic stability studies with rat liver microsomes show that MSDRT 11 followed by MSDRT 12 are the most stable compounds. The results of in vitro metabolism studies have been summarized in Table III. Standard pharmacokinetic calculations were performed and the results are described in Table IV. MSDRT 11 shows high metabolic stability, with very low intrinsic clearance. The half life of MSDRT 11 is greater than 20 hours which may lead to poor acceptance as a probable lead compound since it is almost completely resistant to metabolism. MSDRT 12 seems a promising candidate as it has reasonable metabolic stability with a half life of about 2.6 hours (Figure 4).

Table III: Summary results for *in vitro* metabolic stability

Time	**% PARENT REMAINING**			
Min	**MSDRT8**	**MSDRT 10**	**MSDRT 11**	**MSDRT 12**
0	100.0	100.0	100.0	100.0
15	89.4	92.4	87.8	87.2
30	63.3	81.3	97.4	78.0
45	51.6	76.5	96.5	74.8
60	44.5	69.0	94.3	63.1
120	15.7	44.9	89.2	57.9
Time	**LOG % PARENT REMAINING**			
Min	**MSDRT8**	**MSDRT 10**	**MSDRT 11**	**MSDRT 12**
0	2.000	2.000	2.000	2.000
15	1.951	1.966	1.944	1.941
30	1.802	1.910	1.988	1.892
45	1.712	1.884	1.985	1.874
60	1.648	1.839	1.974	1.800
120	1.195	1.653	1.950	1.763

Table IV: *In vitro* pharmacokinetic study

	Formulae	Unit	MSDRT8	MSDRT10	MSDRT11	MSDRT12
Slope			0.0068	0.0029	0.00025	0.0019
Elimination rate constant (ke)	2.303*slope	**min-1**	0.016	0.007	0.001	0.004
Half life (t1/2)	0.693/ke	**min**	44.3	103.8	1203.6	158.4
Half life (t1/2)	**t1/2 in mins/60**	**hour**	0.7	1.7	20.0	2.6
Intrinsic clearance (CL)	Ke*V	**µl/min**	15.66	6.68	0.58	4.38
Intrinsic clearance (CL) normalized to protein content	Cl/protein per incubation (mg)	**µl/min/ mg of protein**	15.66	6.68	0.58	4.38
Intrinsic clearance (CL) scaled to microsomal protein per gm of liver	microsomal protein 45mg/g of liver	**µl/min/g of liver**	704.72	300.54	25.91	196.91
Intrinsic clearance (CL) scaled to liver per Kg body weight		**ml/min/ kg body wt**	31.71	13.52	1.17	8.86

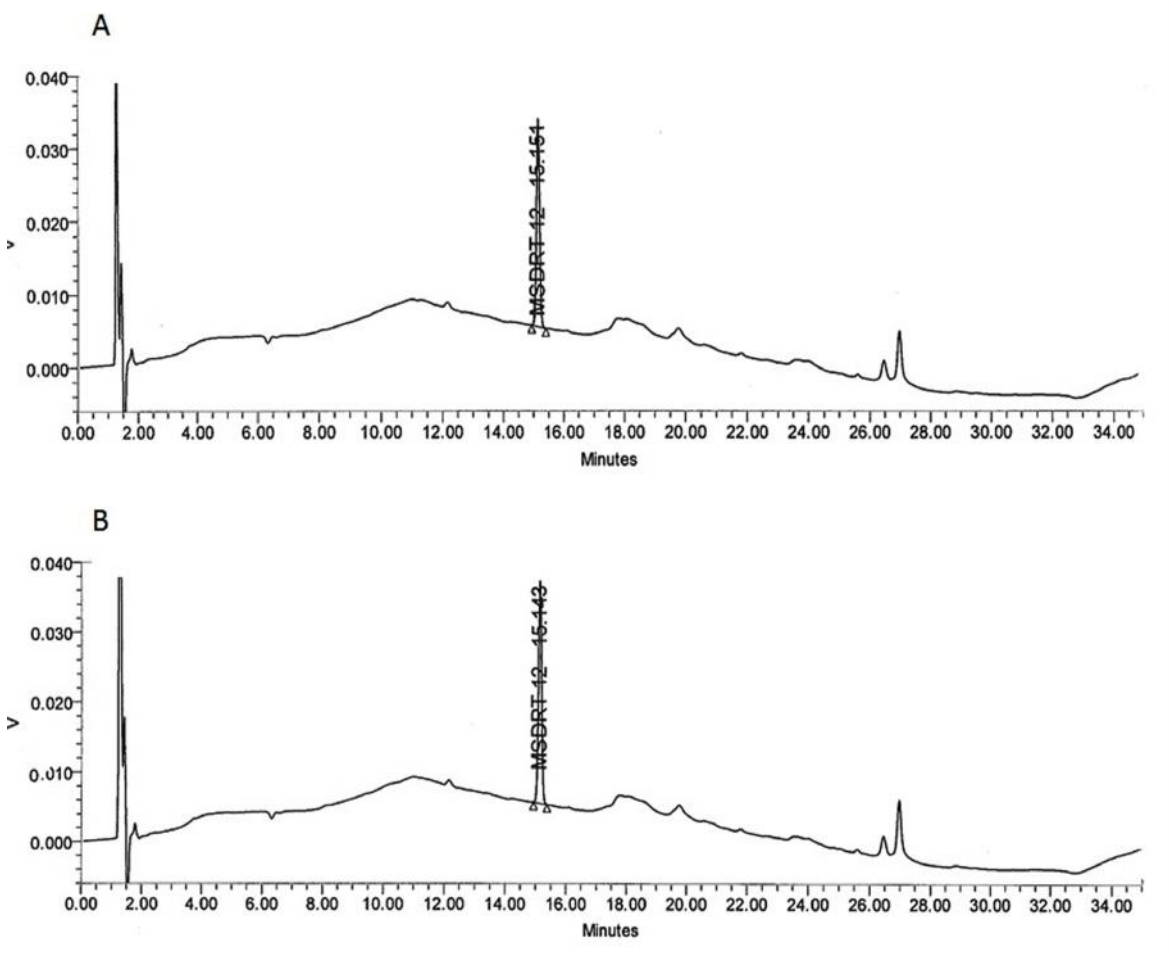

Figure 4:
A: Metabolic stability studies of MDSRT 12 with rat liver microsomes (0 minute)
B: Metabolic stability studies of MDSRT 12 with rat liver microsomes (after 120 minutes)

Conclusion

The newly developed HPLC methods were simple, linear, accurate, precise and selective for these compounds and can be used conveniently and reliably for the estimation of these compounds as they progress through the drug discovery cycle. The methods are similar and with slight changes in mobile phase or detection wavelength they may be applied for other novel triazole compounds. The method has a low LOQ and is sensitive enough to be used for testing the impurities/degradants and the wide linearity range allows the methods to be used both for assays, stability studies and for metabolic stability studies.

This study confirmed that compound MSDRT 12 was very stable and resistant to degradation under both acidic and alkaline conditions. Moreover, data from the in vitro metabolic study with rat liver microsomes suggests that MSDRT 12 has an acceptable half- life and sufficient metabolic stability to avoid immediate metabolism. Therefore MSDRT 12 can be selected as a potential lead candidate for further development as an antitubercular agent. Stress studies used early in the drug discovery phase can thus be used to rank a series of compounds based on their stability characteristics and help in selection of the potential lead compound.

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