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Determination of Tadalafil in rat plasma by liquid chromatography tandam mass spectrometry: Application to a pharmacokinetic study

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ABSTRACT

An analytical method based on Liquid-liquid extraction has been developed and validated for analysis of Tadalafil in rat plasma. Tadalafil-D3 was used as an internal standard. Zorbax-SB C18, 75 x 4.6 mm, 3.5 µm column provided chromatographic separation of analyte followed by detection with mass spectrometry. The method involves simple isocratic chromatographic condition and mass spectrometric detection in the positive ionization mode using an API-4200 system. The total run time was 3.0 minutes. The proposed method has been validated with linear range of 0.50–1000.00 ng/mL for Tadalafil. The intra-run and inter-run precision values are within 1.37 - 2.25% and 2.23 - 5.31%. The overall recovery for Tadalafil and Tadalafil-D3 was 91.07% and 86.66%. This validated method was applied successfully in rat plasma samples for pharmacokinetic study.

Keywords: LC- MS/MS; Tadalafil; Rat plasma; Pharmacokinetic study;

INTRODUCTION

Tadalafil is used to treat male erectile dysfunction (impotence) and pulmonary arterial hypertension (PAH). Chemicallly it is (6R-trans)-6-(1,3-benzodioxol-5-yl)-2,3,6,7,12,12a-hexahydro-2-methyl- pyrazino [1',2':1,6]pyrido[3,4-b]indole-1,4-dione. The Chemical Formula of tadalafil is $C_{22}H_{19}N_3O_4$ with molecular weight of 389.41. After single oral-dose administration, the maximum observed plasma concentration (Cmax) of tadalafil is achieved between 30 minutes and 6 hours (median time of 2 hours). Absolute bioavailability of tadalafil following oral dosing has not been determined. The rate and extent of absorption of tadalafil are not influenced by food; thus, CIALIS may be taken with or without food. Tadalafil in plasma is bound to proteins is up to 94%. Tadalafil is predominantly metabolized by CYP3A4 to a catechol metabolite. The catechol metabolite undergoes extensive methylation and glucuronidation to form the methylcatechol and methylcatechol glucuronide conjugate, respectively. The major circulating metabolite is the methylcatechol glucuronide. Methylcatechol concentrations are less than 10% of glucuronide concentrations. In vitro, data suggests that metabolites are not expected to be pharmacologically active in observed metabolite concentrations. The mean oral clearance for tadalafil is 2.5 L/hr, and the mean terminal half-life is 17.5 hours in healthy subjects. Tadalafil is excreted predominantly as metabolites, mainly in the feces (approximately 61% of the dose) and to a lesser extent in the urine (approximately 36% of the dose) [1].

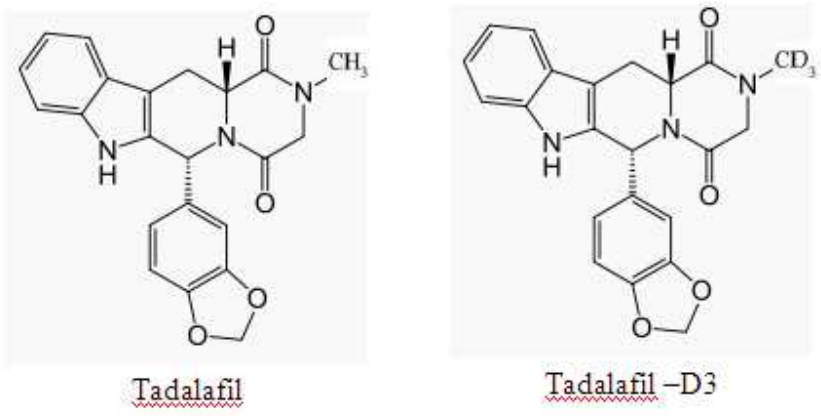

Fig. 1. Chemical structure of Tadalafil, Tadalafil –D3

As of now, several analytical methods were developed for quantification of Tadalafil in biological matrices [2-7], Herbal [8-26], pharmaceutical formulations [27-38]. These methods include different instruments like GCMS[2], HPLC[3,4,5,19,32,33,34], LCMS/MS[6, 8, 9, 10, 11, 12, 13, 14, 15, 27, 28, 29, 30], UPLC-MS/MS[16], mobility spectrometry[17], NMR[18,31]. Nicolaou .et.al., [2] developed the method in human whole blood by using GC/MS with the linearity range 2.00 to 500.00 µg/L using SPE extraction method. Cheng .et.al., [3] developed the method in rat blood by using HPLC-UV with the linearity range 10.00 to 2000.00 ng/ml using LLE extraction method. Farthing.et.al., [4] established the method in mouse plasma by using HPLC-fluorescence with the linearity range 100.00 to 2000.00 ng/ml using PPT extraction method. Shakya.et.al., [5] reported the method in mouse plasma by using HPLC-UV with the linearity range 5.00 to 600.00 ng/ml using LLE extraction method. Ramakrishna.et.al., [6] proved the method in human plasma by using LC-MS/MS with the linearity range 10.00 to 1000.00 ng/ml using LLE extraction method. Among all, Ramakrishna.et.al.,[6] developed rapid method by LC-MS/MS but the sensitivity wise and suitable internal standard selection wise this method has few draw backs. Based on the literature survey, it was observed that, there is a need to develop a simple, higly sensitive, rapid, rugged and reproducible method for quantification of tadalafil in biological matrices to extend clinical pharmacokinetic studies. The proposed method aims to develop a simple, highly sensitive, rapid, rugged and reproducible method in rat plasma. The developed method could be useful for human subjects for pharmacokinetics evaluation.

MATERIALS AND METHODS

1. Standards and chemicals

The reference standard of Tadalafil was provided by Dr.Reddy's Laboratories Ltd.(Hyderabad, India). The reference standard Tadalafil D3 was obtained from SynFine Research (Ontario, CANADA). Purity of both standards was higher than 99%. High purity water was prepared inhouse using a Milli-Q A10 gradient water purification system (Millipore, Bangalore, India). LC grade methanol, acetonitrile and methyl tertiary butyl ether (MTBE) were purchased from J.T. Baker Inc. (Phillipsburg, NJ, USA). AR grade acetic acid and ammonium acetate were procured from Merck (Mumbai, India). Blank rat plasma from healthy rats was obtained from Bioneeds, Bangalore.

2. Instrumentation

Tadalafil was analyzed using HPLC system (1200 Series Agilent Technologies, Germany). MS/MS (ABI-SCIEX,Toronto, Canada) using MRM. A turbo electrospray interface in positive ionization mode was used. Data processing was performed on Analyst 1.4.1 software package (SCIEX).

3. Detection

Mass spectra were obtained using an API-4200 from Applied Biosystems, Canada equipped with electrospray ionization source. The mass spectrometer was operated in the multiple reaction monitoring (MRM) mode. Sample introduction and ionization was electrospray ionization in the positive ion mode. Source dependent parameters optimized were as follows: nebulizer gas flow: 7 L/min; auxiliary gas flow: 8 L/min; ion spray voltage (ISV): 5500 V; temperature (TEM): 500 °C. The compound dependent parameters such as the declustering potential (DP), focusing potential (FP), entrance potential (EP), collision energy (CE), cell exit potential (CXP) were optimized

during tuning as 60, 40, 10, 45, 12 and 60, 50, 10, 40, 12 eV for Tadalafil and Tadalafil d3, respectively. The collision activated dissociation (CAD) gas was set at 5 psi, while the curtain gas flow was set at 25 L/min using nitrogen gas. Quadrupole 1 and quadrupole 3 were both maintained at unit resolution and dwell time was set at 300 ms for Tadalafil and Tadalafil d3. The mass transitions were selected as m/z 390.2 → 268.3 for Tadalafil and m/z 393.2 → 271.2 for Tadalafil d3. The parent and product ion spectra for Tadalafil and Tadalafil d3 are represented in Figs. 2a-2d respectively. The data acquisition was ascertained by Analyst 1.4.2 software. For quantification, the peak area ratios of the target ions of the analyte to those of the internal standard were compared with weighted ($1/x^2$) least squares calibration curves in which the peak area ratios of the calibration standards were plotted versus their concentrations.

4. Chromatographic conditions

Chromatographic separation was performed on an Agillent LC with a Zorbax-SB C18 column (75 x 3.5 mm, 3.5 µm) purchased from Agillent Technologies, Mumbai, India. A mobile phase consisting of 20 mM ammonium acetate (pH 4.5) and acetonitrile in the ratio of 10:90 v/v was delivered without splitter at a flow rate of 0.4 mL/min. The total run time for each sample analysis was 3.0 min.

5. Preparation of standards and quality control (QC) Samples

Two separate stock solutions of Tadalafil were prepared for bulk spiking of calibration curve and quality control samples for the method validation exercise as well as for sample analysis. The stock solutions of Tadalafil and Tadalafil d3 were prepared in methanol at free base concentration of 100 µg/mL. Primary dilutions and working standard solutions were prepared from stock solutions using water: methanol (50:50 v/v) solvent mixture. These working standard solutions were used to prepare the calibration curve and quality control samples. Blank rat plasma was screened prior to spiking to ensure it was free of endogenous interference at retention times of Tadalafil and internal standard Tadalafil d3. A Ten point standard curve and four quality control samples were prepared by spiking the blank plasma with an appropriate amount of Tadalafil. Calibration samples were made at concentrations of 0.50, 1.00, 5.00, 10.00, 50.00, 100.00, 200.00, 400.00, 600.00 and 1000.00 ng/mL and quality control samples were made at concentrations of 0.50, 1.50, 300.00 and 600.00 ng/mL for tadalafil.

6. Sample preparation

An internal standard working solution (150.00 ng/mL of Tadalafil-D3) of 50 µL was added to 100µL aliquot of rat plasma sample (respective concentration) into riavial. To this 3 mL of methyl t-butyl ether was added and vortexed for 5 minutes. Samples were then centrifuged for 5 minutes at 4000 rpm at 20°C. Supernatant from each sample was transferred into ria vial and evaporated to dryness. This was followed by reconstitute with 200 µL of reconstitution solution and vortex briefly. From this, 5 µL of sample was injected into the LC–MS/MS system through the auto sampler.

7. Precision and Accuracy

The intra-run (within a day) and inter-run (between days (*n=5*)) accuracy was determined by replicate analysis of quality control samples (*n* = 6) at LLOQ (lower limit of quantification), LQC (low quality control), MQC (medium quality control), HQC (high quality control) and ULOQ (upper limit of quantification) levels. The % CV should be less than 15% and accuracy should be within 15%, except LLOQ where it should be within 20%.

8. Recovery

The extraction efficiencies of Tadalafil and Tadalafil d3 were determined by analysis of six replicates at each quality control concentration level for Tadalafil and at one concentration for the internal standard Tadalafil d3. The percent recovery was evaluated by comparing the peak areas of extracted standards to the peak areas of nonextracted standards.

9. Stability

Stock solution stability was performed by comparing area responses of analyte and internal standard to stability sample, with the area responses of sample prepared from fresh stock solution. Stability studies in plasma were performed at LQC and HQC concentration level using six replicates at each level. Analyte was considered stable if the % Change is less than 15% as per US FDA guidelines [39]. The stability of spiked rat plasma samples stored at room temperature (bench top stability) was evaluated for 24 h. The autosampler sample stability was evaluated by comparing the extracted plasma samples that were injected immediately (time 0 h), with the samples that were reinjected after storing in the autosampler at 10 °C for 55h. The reinjection reproducibility was evaluated by

comparing the extracted plasma samples that were injected immediately (time 0 h), with the samples that were re-injected after storing in the refrigerator at 2-8 °C for 26 h. The freeze–thaw stability was conducted by comparing the stability samples that had been frozen at –30 °C and thawed three times, with freshly spiked quality control samples. Six aliquots each of LQC and HQC concentration levels were used for the freeze–thaw stability evaluation. For long-term stability evaluation the concentrations obtained after 75 days intervals were compared with initial concentrations.

10. Dilution integrity

The dilution integrity experiment was performed with an aim to validate the dilution test to be carried out on higher analyte concentrations above the upper limit of quantification (ULOQ), which may be encountered during real subject samples analysis. Dilution integrity experiment was carried out at 1.5 times the ULOQ concentration. Six replicates each of 1/2 and 1/4 concentrations were prepared and their concentrations were calculated by applying the dilution factor 2 and 4 against the freshly prepared calibration curve. During study sample handling and analysis.

11. Application of method

The validated method has been successfully used to analyze Tadalafil concentrations in rat plasma. The study was conducted according to current GCP guidelines. Before conducting the study it was also approved by an authorized animal ethics committee. There were a total of 12 blood collection time points including the predose sample. The blood samples were collected in separate vacutainers containing K_2EDTA as anticoagulant. The plasma from these samples was separated by centrifugation at 3000 rpm within the range of 2–8 °C. The plasma samples thus obtained were stored at –30 °C till analysis. Post analysis the pharmacokinetic parameters were computed using WinNonlin software version 5.2 and 90% confidence interval was computed using SAS software version 9.2.

RESULTS AND DISCUSSION

3.1. Method Development

During method development different options were evaluated to optimize detection parameters, chromatography and sample extraction.

3.1.1. Mass spectra

Electro spray ionization (ESI) provided maximum response over atmospheric pressure chemical ionization (APCI) mode, and was chosen for this method. The instrument was optimized to obtain sensitivity and signal stability during infusion of the analyte in the continuous flow of mobile phase to electro spray ion source operated at both polarities at a flow rate of 20 µL/min. Tadalafil gave more response in positive ion mode as compare to the negative ion mode. The predominant peaks in the primary ESI spectra of Tadalafil and Tadalafil D3 corresponds to the MH+ ions at m/z 390.2 and 393.3 respectively. Product ions of Tadalafil and Tadalafil D3 scanned in quadrupole 3 after a collision with nitrogen in quadrupole 2 had an m/z of 268.3 and 271.0.

3.1.2. Chromatography

Initially, mobile phase consisting of ammonium formate and acetonitrile in varying combinations were tried, but low response was observed. The mobile phase containing 10 mM ammonium acetate: methanol (10:90 v/v) and 10 mM ammonium formate: methanol (10:90 v/v) gives better response, but poor peak shape was observed. A mobile phase of ammonium acetate in water in combination with methanol and acetonitrile with varying combinations were tried. Using a mobile phase containing 10 mM ammonium acetate in water in combination with acetonitrile (10:90, v/v), the best signal along with a marked improvement in the peak shape was observed for Tadalafil and Tadalafil d3. Short length columns, such as Symmetry Shield RP18 (50 x 2.1 mm, 3.5 µm), Inertsil ODS -2V (50 x 4.6 mm, 5µm), Hypurity C18 (50 x 4.6 mm, 5 µm), Hypurity advance (50 x 4.0 mm, 5 µm) and Zorbax-SB C18, 4.6 x 75 mm, 3.5 µm were tried during the method development. Symmetry Shield RP18 column gave a relatively good peak shape but the response was low. Using Hypurity C18 column poor chromatography was observed. The best signal was obtained using the Zorbax-SB C18 (75 x4.6 mm, 3.5µm) column. It gave satisfactory peak shapes for both Tadalafil and Tadalafil d3, and a flow rate of 0.4mL/min reduced the run time to 3.0 min. The column oven temperature was kept at a constant temperature of about 30 °C.

3.1.3. Extraction

Prior to LC injection, the co-extracted proteins should be removed from the prepared solution. Several organic solvents were employed to extract analytes from the plasma sample. Out of the tested solvents, (ethyl acetate,

chloroform, hexane, dichloromethane, methyl tertiary butyl ether, acetonitrile and mobile phase) methyl tertiary butyl ether is selected more effectively than other solvents. It was difficult to find a compound which could ideally mirror the analytes to serve as a good IS. Several compounds were investigated to find a suitable IS, and finally Tadalafil d3 belonging to a similar deuterated compound was found to be most appropriate for the present purpose. There was no significant effect of IS on analyte recovery, sensitivity or ion suppression. The results of method validation using Tadalafil d3 as the IS were acceptable in this study based on FDA guidelines. High recovery and selectivity was observed in the Liquid-Liquid extraction method. These optimized detection parameters, chromatographic conditions and extraction procedure resulted in reduced analysis time with accurate and precise detection of Tadalafil in rat plasma.

3.2. Method Validation

3.2.1. Specificity and Selectivity

Representative chromatograms obtained from blank plasma, plasma spiked with lower limit of quantification and real rat sample for Tadalafil and Tadalafil d3 is shown in Fig. 3. The mean % interference observed at the retention time of analyte between six different lots of rat plasma K2EDTA as an anti-coagulant calculated was not observed for Tadalafil and Tadalafil d3 which was within acceptance criteria. Six replicates of extracted samples at the LLOQ level in one of the plasma sample having least interference at the retention time of Tadalafil was prepared and analyzed. The % CV of the area ratios of these six replicates of samples was 3.38% for Tadalafil confirming that interference does not affect the quantification at LLOQ level. Utilization of selected product ions for each compound enhanced mass spectrometric selectivity. The product ions of m/z 268.3 and 271.2 were concluded to be specific for Tadalafil and Tadalafil d3.The LLOQ for Tadalafil was 0.50 ng/mL. The intra-run precision and intrarun accuracy of the LLOQ plasma samples containing Tadalafil was 2.25 and 99.97%.

3.2.2. Linearity and Range

The peak area ratios of calibration standards were proportional to the concentration of Tadalafil in each assay over the nominal concentration range of 0.50–1000.00 ng/mL. The calibration curves appeared linear and were well described by least-squares linear regression lines. As compared to the 1/x weighing factor, a weighing factor of 1/x2 properly achieved the homogeneity of variance and was chosen to achieve homogeneity of variance. The correlation coefficient was ≥0.9850 for Tadalafil. The observed mean back-calculated concentration with accuracy and precision (% CV) of four linearity's analyzed during method validation are given in table 1. The deviation of the back calculated values from the nominal standard concentrations were less than 15%. This validated linearity range justify the concentration observed during real sample analysis.

Table 1: Precision and accuracy data of back calculated concentrations of calibration samples of Tadalafil in Rat plasma

Spiked plasma concentration (ng/mL)	Concentration measured(mean) (ng/mL)	SD (Standard deviation)	(%) CV (*n* = 5) (Precision)	Accuracy %
0.50	0.50	0.01	1.02	99.36
1.00	1.01	0.02	1.91	100.76
5.00	5.13	0.07	1.45	102.68
10.00	9.90	0.25	2.53	99.04
50.00	49.80	0.60	1.20	99.60
100.00	100.00	1.22	1.22	100.00
200.00	195.20	2.59	1.33	97.60
400.00	401.60	6.62	1.65	100.40
600.00	602.80	7.01	1.16	100.47
1000.00	1001.25	12.05	1.20	100.13

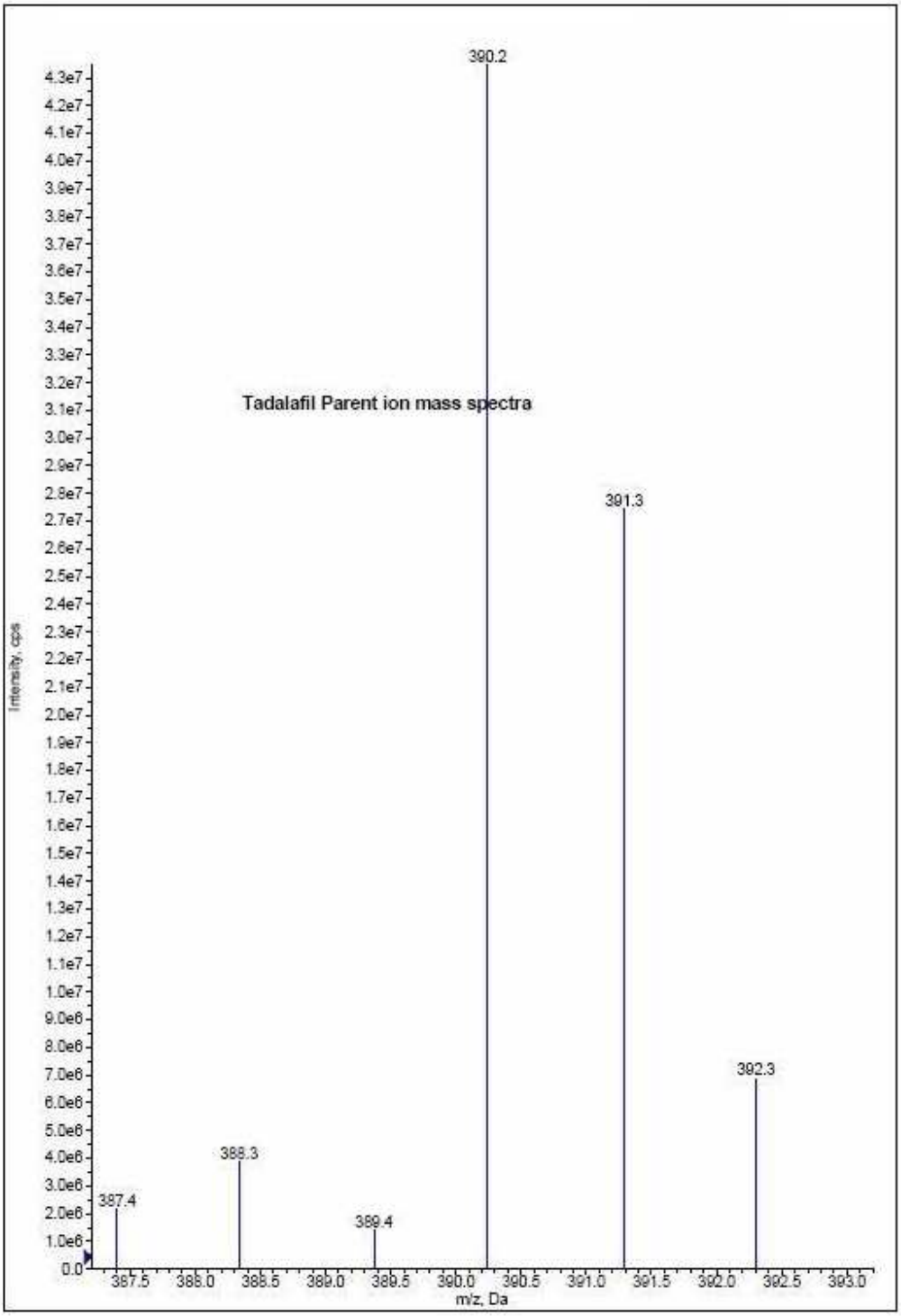

Fig.2a. Mass spectrum of Tadalafil Parent ion.

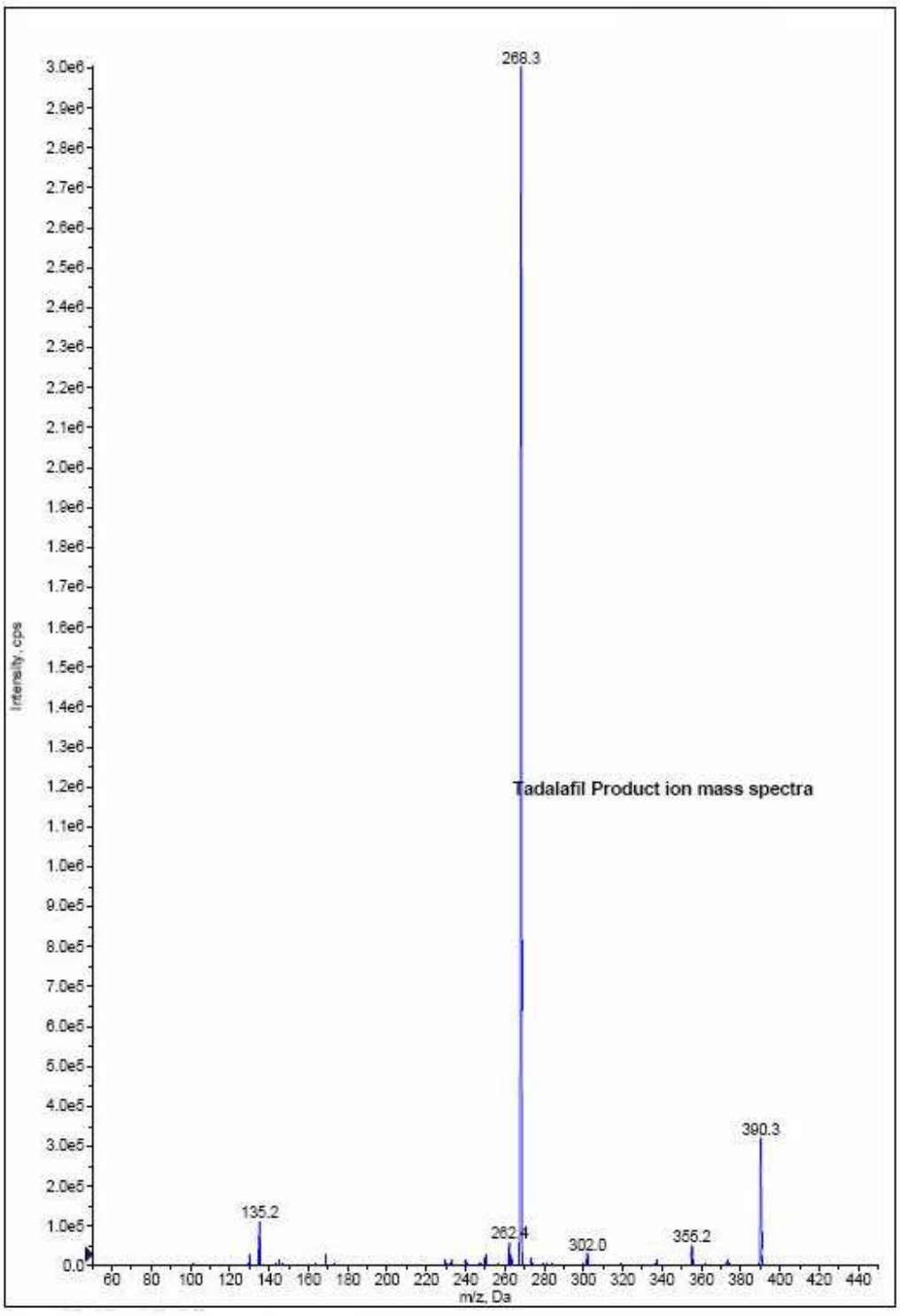

Fig.2b. Mass spectrum of Tadalafil Product ion.

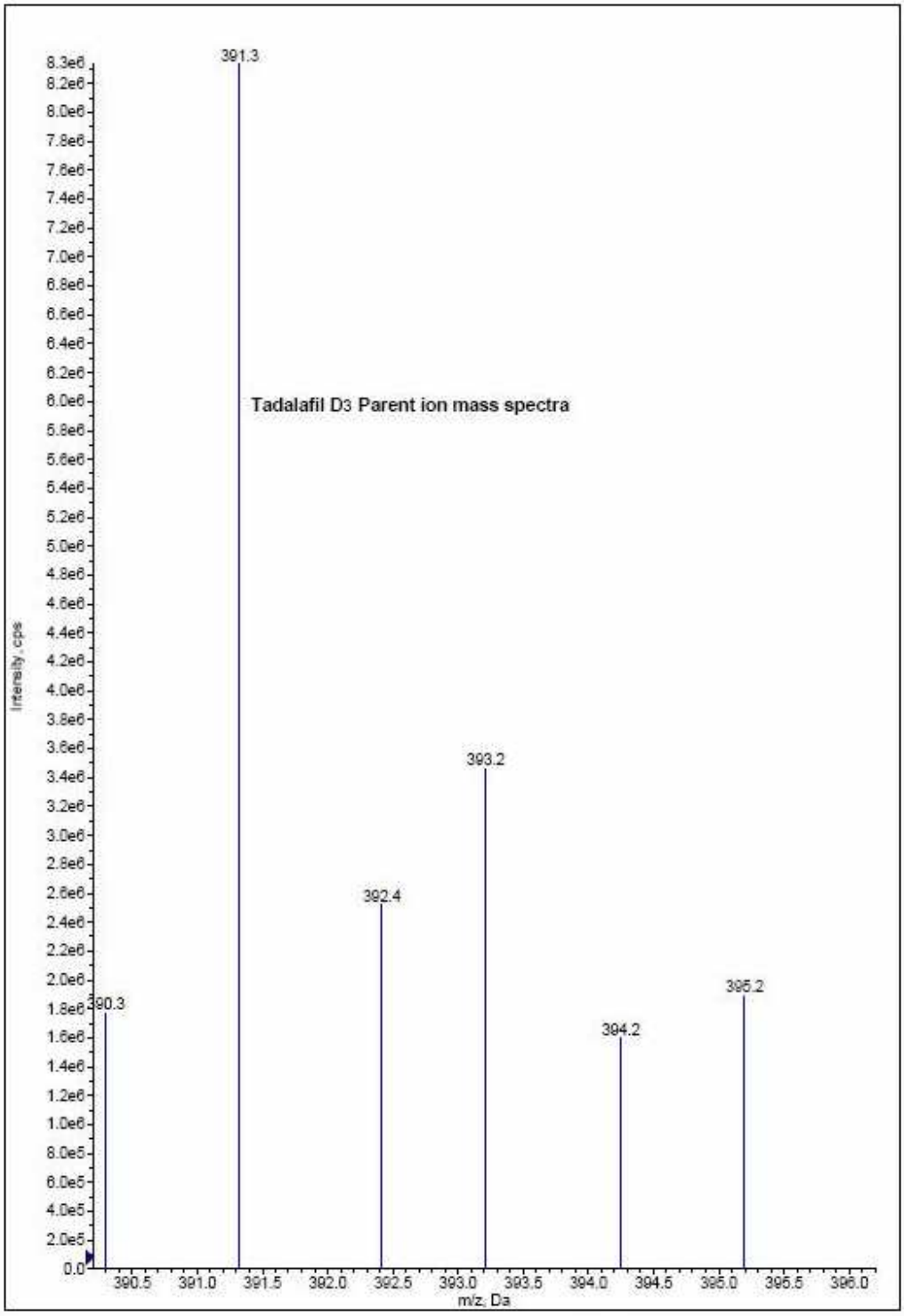

Fig.2c. Mass spectrum of Tadalafil D3 Parent ion.

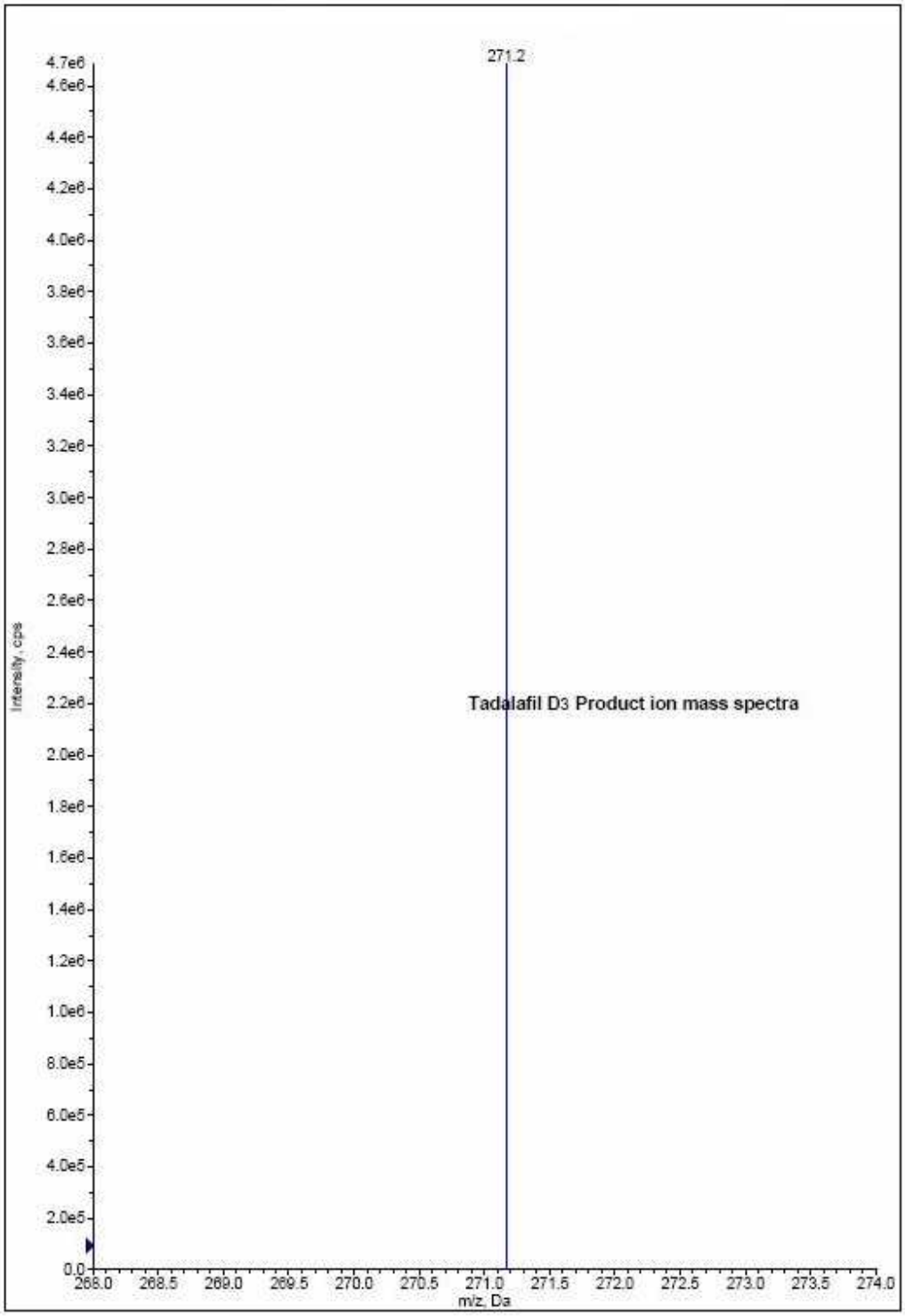

Fig.2d. Mass spectrum of Tadalafil D3 Product ion.

3.2.3. Precision and Accuracy

The inter-run precision and accuracy were determined by pooling all individual assay results of replicate (n = 6) quality control over three separate batch runs analyzed on four different days. The inter-run precision (% CV) and inter-run accuracy was ≤4.32% and ≤-96.00% for Tadalafil. The intra-run precision and accuracy were determined by pooling all individual assay results of replicate (n = 6) quality control of two separate batch runs analyzed on the same day. The intra-run precision (% CV) and intra-run accuracy was 1.37-2.25% and 97.56 - 99.97% for Tadalafil. All these data presented in Table 2 indicate that the method is precise and accurate.

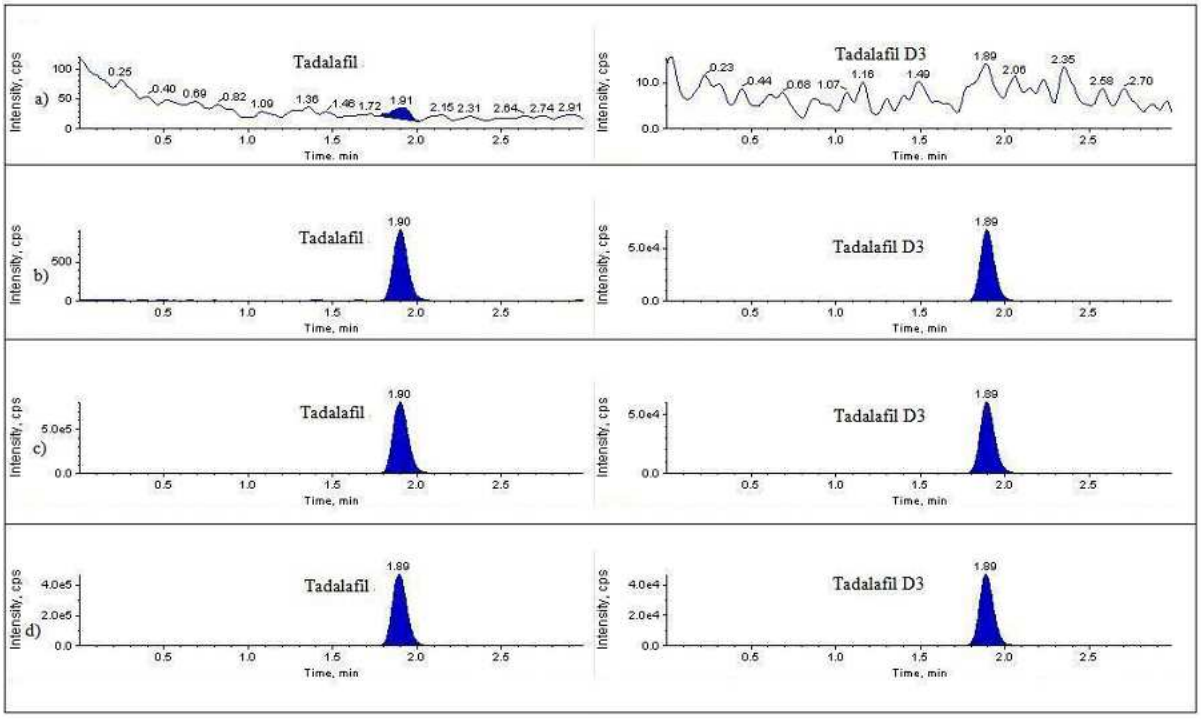

Fig.3. Chromatograms of a) Blank Plasma b) LOQ c) HQC d) Post dose 7 hr

Table 2: Precision and accuracy (analysis with spiked plasma samples at four different concentrations)

Spiked plasma concen-Tration (ng/mL)	Within-run			Between-run		
	Concentration measured (n=6) (ng/mL) (mean ± S.D)	(%)CV Precision	% Accuracy	Concentration measured (n=30) (ng/mL) (Mean ±S.D.)	(%)CV Precision	% Accuracy
0.50	0.50±0.01	2.25	99.97	0.50±0.03	5.31	100.12
1.50	1.46±0.02	1.60	97.56	1.46±0.04	2.64	97.42
300.0	297.29±4.06	1.37	99.10	294.25±7.71	2.62	98.08
600.0	588.04±11.04	1.88	98.01	587.36±13.07	2.23	97.89

3.2.4. Recovery

Six aqueous replicates at low, medium and high quality control concentration levels for Tadalafil were prepared for recovery determination and the areas obtained were compared versus the areas obtained for extracted samples of the same concentration levels from a precision and accuracy batch run on the same day. The mean recovery for Tadalafil was 91.07% with a precision of 3.60% and the mean recovery for Tadalafil D3 was 86.66% with a precision of 4.41%. This indicates that the extraction efficiency for the Tadalafil as well as Tadalafil D3 was consistent and reproducible.

3.2.5. LOD and LOQ

The LOD and LOQ of the method for Tadalafil were 0.01 pg/mL and 0.50 ng/mL respectively. These results confirm the significant sensitivity of the method for drug analysis.

3.2.6. Stability

Reinjection reproducibility exercise was performed to check whether the instrument performance remains unchanged after hardware deactivation due to any instrument failure during real sample analysis. % Change was less than 4.25% for LQC and HQC level concentration; hence batch can be reinjected in case of instrument failure during real sample analysis. Stock solution stability was performed to check stability of Tadalafil and Tadalafil D3 in stock solutions prepared in methanol and stored at 2-8 °C in refrigerator. The freshly prepared stock solutions were compared with stock solutions prepared before 9 days. The % Change for Tadalafil and Tadalafil D3 were 0.51% and 0.45% respectively indicates that stock solutions were stable at least for 10 days. Bench top, and autosampler stability for Tadalafil was investigated at LQC and HQC levels. The results revealed that Tadalafil was stable in plasma for at least 24 h at room temperature, and 55h in an autosampler at 10 °C. It was confirmed that repeated freezing and thawing (three cycles) of plasma samples spiked with Tadalafil at LQC and HQC levels did not affect their stability. The long term stability results also indicated that Tadalafil was stable in matrix up to 75 days at a storage temperature of –30 °C. The results obtained from all these stability studies are tabulated in Tables 3.

Table 3: Stability of the samples

Spiked plasma concentration (ng/ mL)	Room Temperature stability 24.0 h		Autosampler stability 55 h		Long term stability 75 days		Freeze and thaw stability Cycle 3 (48 h)	
	Concentration measured (n=6) (ng/ mL) (mean ±S.D)	%CV (*n*=6)	Concentration measured (n=6) (ng/ mL) (mean ± S.D)	%CV (*n*=6)	Concentration measured (n=6) (ng/ mL) (mean ± S.D)	%CV (*n*=6)	Concentration measured (n=6) (ng/ mL) (mean ± S.D)	%CV (*n*=6)
1.50	1.47±0.03	1.86	1.43±0.03	2.13	1.46±0.06	3.89	1.46±0.03	1.99
600.00	585.90±10.51	1.79	555.84±14.85	2.67	552.08±4.21	0.76	554.93±7.48	1.35

3.3. Application

The validated method has been successfully applied to quantify Tadalafil concentrations in to a single dose(0.2mg/200g) in rats. Male Sprague-Dawley rats were obtained from Bioneeds,Bangalore. After i.v administration of drug via left femoral vein 0.3 ml of blood samples for analytical determinations were collected via the right femoral vein at specific time intervals for 16 h. Plasma samples were stored at −30 ◦C until analysis. The study was carried out after approval from an independent animal ethics committee.The pharmacokinetic parameters evaluated were Cmax (maximum observed drug concentration during the study), AUC0-16 (area under the plasma concentration–time curve measured 16 hours, using the trapezoidal rule), *T*max (time to observe maximum drug concentration), Kel (apparent first order terminal rate constant calculated from a semi-log plot of the plasma concentration versus time curve, using the method of least square regression) and *T*1/2 (terminal half-life as determined by quotient 0.693/Kel). Pharmacokinetic details were shown in Table 4. The mean concentration versus time profile of Tadalafil in rat plasma is shown in Fig.4.

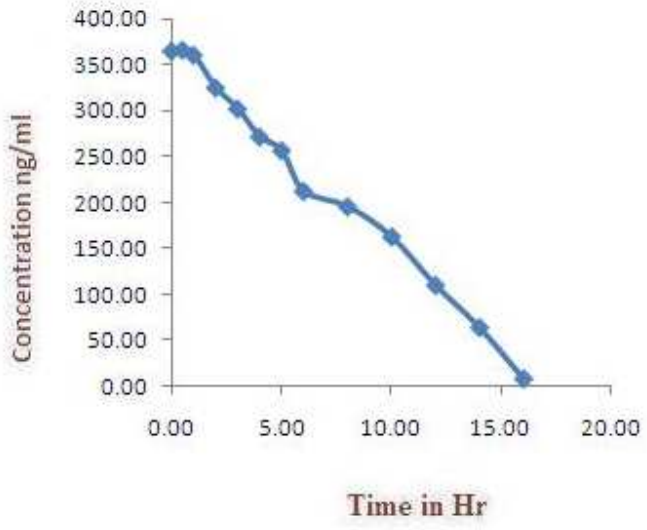

Fig.4. Mean Pharmacokinetic graph of Tadalafil in Rat plasma

Table 4: Mean Pharmacokinetic Parameters of Tadalafil in Rat Plasma

Pharmacokinetic Parameter	values
AUC_{0-t} (ng · h/mL)	3091.82
Cmax (ng/ mL)	365.88
$AUC_{0-\infty}$ (ng · h/mL)	3115.57
Kel	0.11759
Tmax (h)	0.5 hr
$t_{1/2}$	1.93
ct	8.54

$AUC_{0-\infty}$: area under the curve extrapolated to infinity;
AUC_{0-t}: area under the curve up to the last sampling time;
Cmax: the maximum plasma concentration;
Tmax: the time to reach peak concentration;
Kel: the apparent elimination rate constant.

CONCLUSION

The developed LC-MS/MS assay for tadalafil is simple, highly sensitive, selective, rugged and reproducible method. This simple high throughput Liquid-Liquid extraction method for an extraction of tadalafil in rat plasma using LC-MS/MS has been successfully applied to the pharmacokinetic study in healthy male rats. The pharmacokinetic study could be useful for estimation of tadalafil in human subjects to conduct bioequivalence study.

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