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Simultaneous estimation of atorvastatin and its two metabolites from human plasma by ESI-LC-MS/MS

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A selective, sensitive, and fast high performance liquid chromatography (HPLC) method with mass spectrometric (MS) detection mode has been developed and validated completely in human plasma. Atorvastatin (ATO), p-hydroxy atorvastatin (p-HATO), o-hydroxy atorvastatin (o-HATO) and internal standard (IS) are extracted from human plasma via solid phase extraction (SPE) technique. After elution, the solution is evaporated, then reconstituted with 250 μL of Mobile Phase and analyzed using HPLC/MS/MS system. An isocratic mode is used to separate interference peaks using a Symmetry C-18, 75 × 4.6 mm ID, 3.5 μ, column. The m/z of ATO, o-HATO and p-HATO are 559.2/440.2, 575.3/440.4 and 575.0/440.4 respectively. Linearity ranges are 0.05 to 252.92 ng/mL for ATO, p-HATO and o-HATO respectively. Calibration functions, lower limit of quantitation (LLOQ), stability, intra- and inter-day reproducibility, accuracy, and recovery are estimated. This method is free from matrix effects and any abnormal ionization. This method was successfully applied to a single dose 80 mg tablet bioequivalence (BE) study of Atorvastatin.

Keywords: atorvastatin; p-hydroxy atorvastatin; o-hydroxy atorvastatin; solid phase extraction; matrix effects; positional isomer

Introduction

Atorvastatin (ATO; Figure 1) a member of the drug class known as statins, is used for lowering blood cholesterol. ATO inhibits 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase, the rate-determining enzyme located in hepatic tissue that produces mevalonate, a small molecule used in the synthesis of cholesterol and other mevalonate derivatives. This lowers the amount of cholesterol produced which in turn lowers the total amount of low-density lipoprotein (LDL) cholesterol. The most important abnormalities in the lipid profile are an increase in triglyceride levels, the presence of small, dense LDL particles and low high-density lipoprotein (HDL) cholesterol levels. The increase in triglyceride levels is due to elevated levels of very-low-density lipoprotein (VLDL) remnants and intermediate-density lipoprotein (IDL). Each of these parameters has been associated with increased risk of cardiovascular disease.[1] ATO has developed a well-defined role in the primary and secondary prevention of cerebrovascular disease, and appears to have a particularly prominent place in preventing such disease in coronary heart disease (CHD) patients, and in the post-stroke and post-transient ischemic attack (TIA) setting in patients without CHD.[2] Statin therapy also decreases platelet activation and aggregation, and thereby may decrease the propensity toward thrombosis. Statin therapy inhibits tissue factor expression by macrophages, which plays an integral role in blood coagulation and is an important determinant of plaque thrombogenicity. There also may be a reduction in plasminogen activator inhibitor activity, which would facilitate fibrinolysis. All of these factors may contribute towards decreased risk for stroke seen in the statin studies. These statin effects may lead to decreased thrombus formation and thereby influence the development of clinical activity related to atherosclerotic plaque.

Several HPLC [3,4] and LC-MS/MS [5–18] methods have been reported for estimation of ATO alone or with its metabolites, i.e. ortho hydroxy atorvastatin (o-HATO) and para hydroxy atorvastatin (p-HATO). Among all these methods, few of them have LLOQ values as low as 0.10 ng/mL and others are much higher.[3–18] Those methods are either compromised by long analysis times or required the use of more sensitive instrumentation to achieve this sensitivity. However, there were no reported methods with LLOQ value as low as 0.05 ng/mL for ATO alone or in combination with its metabolite. This low LLOQ value will help to get better time-concentration profile of the analyte(s). This low LLOQ value also helps to apply the same analytical method for lower dosage of ATO. Moreover, some methods had used either one or two stable isotope labelled ATO, as an internal standard,[12,16] which is a very expensive material. But in this reported method only enalapril was used as IS, which is easily available. Moreover, some methods have long analysis time,[3,6,8,9,13] where as this reported method has very short analysis time of 3.2 min. So this method possesses the highest sensitivity with very short analysis time and good resolution between the positional isomers, though it is very difficult to achieve such a good resolution between the positional isomers with short analysis time.

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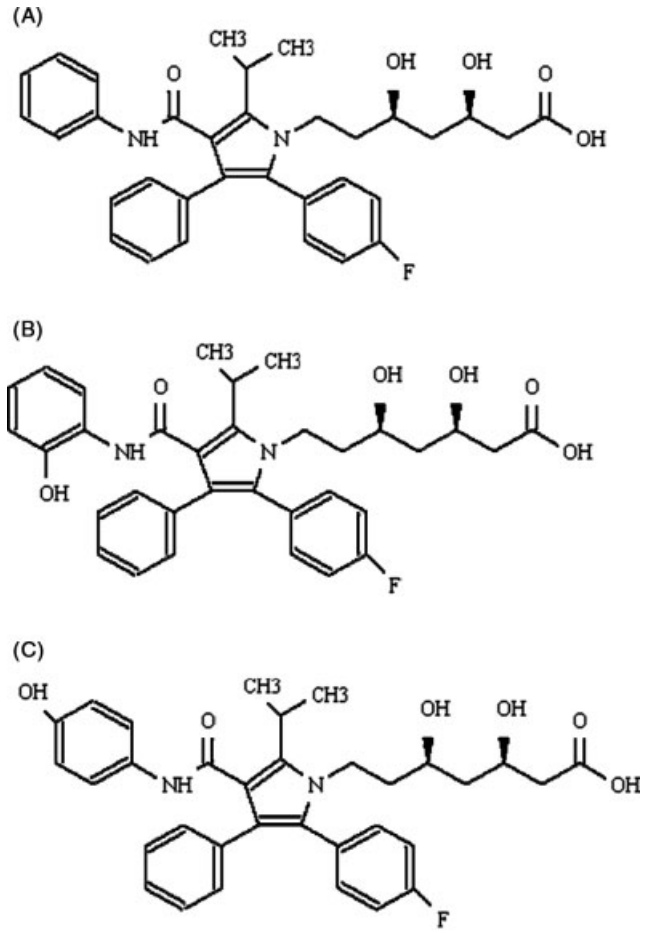


Figure 1. Chemical structure of A) Atorvastatin, B) o-Hydroxy Atorvastatin and C) p-Hydroxy Atorvastatin.

Table 1. Tandem mass spectrometer main working parameters

Parameter	Value
Source temperature, °C	400
Dwell time per transition, msec	500
Ion source gas 1, psi	30
Ion source gas 2, psi	50
Curtain gas, psi	10
Collision gas, psi	11
Ion spray voltage, V	4600
Entrance potential, V	
	10(atorvastatin)
	11 (*ortho*-hydroxyatorvastatin)
	11 (*para*-hydroxyatorvastatin),
	11 (IS)
Declustering potential, V	
	60 (atorvastatin)
	120 (*ortho*-hydroxyatorvastatin)
	130 (*para*-hydroxyatorvastatin),
	100 (IS)
Collision energy, V	
	32 (atorvastatin)
	30 (*ortho*-hydroxyatorvastatin)
	30 (*para*-hydroxyatorvastatin)
	25 (IS)
Collision cell exit potential, V	
	12 (atorvastatin)
	12 (*ortho*-hydroxyatorvastatin)
	10 (*para*-hydroxyatorvastatin)
	12 (IS)
Resolution	Unit
Mode of analysis	Positive
Ion transition for atorvastatin, *m/z*	559.2/440.2
ortho-hydroxyatorvastatin, *m/z*	575.3/440.4
para-hydroxyatorvastatin, *m/z*	575.0/440.4
IS (enalapril), *m/z*	377.1/234.2

Experiment

Apparatus and software

The HPLC system with an auto sampler was a Shimadzu LC-20AD (Shimadzu, Japan) coupled with Applied Biosystem Sciex (MDS Sciex ON, Canada) API 4000 Tandem mass spectrometer. The auto sampler was SIL-HTC from Shimadzu, Japan. The solvent delivery module was LC-20AD from Shimadzu, Japan. The chromatographic integration was performed by Analyst software (version: 1.4.2; Applied Biosystems). Positive pressure unit used for SPE was from Orochem technologies Inc (Lombard, IL, USA). The Caliper turbovap LV concentration workstation used to evaporate the samples was purchased from Caliper Life Sciences (Hopkinton, MA, USA).

Chemicals and reagents

ATO calcium was procured from Cadila Pharmaceutical Ltd, Dholka, Ahmedabad, India; p-Hydroxy atorvastatin calcium salt and o-Hydroxy atorvastatin calcium salt were purchased from VARDA Biotech Ltd, Mumbai, India. Acetic acid and formic acid was procured from Merck Specialities Pvt. Ltd, Mumbai, India. Water used was collected from water purification systems (Billerica, Massachusetts Milli Q, Milli Pore, USA) installed in laboratory. Methanol and acetonitrile were of HPLC grade and were supplied by J. T. Baker (Billerica, Massachusetts, USA. Fresh frozen human plasma (K_2-EDTA as anticoagulant) was used during validation, and was supplied by Clinical department of Cadila Pharmaceuticals Limited, 1389-Trasad road, Dholka, Gujarat, India. Plasma was stored into -70 ± 5 °C.

Standards and working solutions

Individual stock standard solution of ATO, p-HATO and o-HATO containing 1 mg/mL was prepared by dissolving pure compound in methanol. Intermediate and working solutions of ATO, p-HATO & o-HATO were prepared from corresponding stock solutions by diluting with diluent (water: methanol 50 : 50 *v/v*). Calibration standards were established between 0.05 to 252.92 ng/mL of ATO, p-HATO and o-HATO, using eight concentration levels. Quality control (QC) standards of three different levels low (LQC) (0.15 ng/mL), medium (MQC) (26.55 ng/mL) and high (HQC) (140.35 ng/mL) for ATO, p-HATO and o-HATO were also prepared. All these stock solutions, calibration standards and QC samples were stored at 4 ± 2 °C. These solutions were found to be stable and used for the complete method validation.

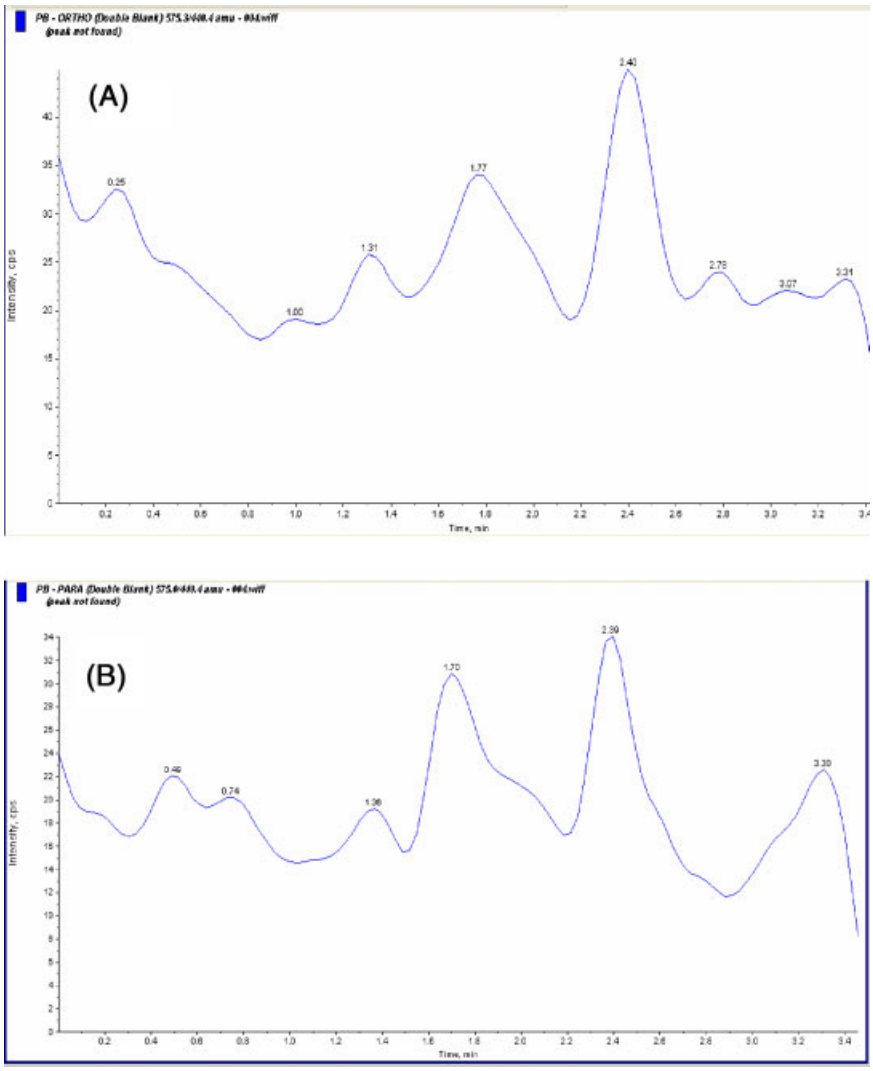


Figure 2. Representative chromatogram of Plasma Blank of A) o-HATO, B) p-HATO, C) ATO and D) IS.

Chromatographic conditions

Chromatographic separation was performed on a Waters Symmetry C18, 75 × 4.6 mm ID, 3.5 µ analytical column. The mobile phase used was a mixture of acetonitrile and 0.60% (*v*/*v*) acetic acid in water at a ratio of 70 : 30 *v*/*v*. The flow rate was 0.500 mL/min. Total analysis time of single injection is 3.20 min. Column oven temperature was set to 40 °C.

Mass spectrometric conditions

The mass spectrometric parameters to determine the ATO, o-HATO, p-HATO and IS are presented in Table 1.

Sample treatment

Solid phase extraction technique was used to extract the ATO, p-HATO and o-HATO from human plasma samples. 2% *v*/*v* formic acid solution was used for providing acidic medium. 500 µL plasma samples were transferred to RIA vials for analysis. 50 µL of IS (250 ng/mL) sample was added and the samples were vortexed for 15 s, followed by the addition of 100 µL of 2% formic acid solution (*v*/*v*). The samples were again vortexed for 15 s. 500 µL of Milli-Q water was added to dilute the plasma sample and vortexed for 15 s. Oasis HLB 1 cc 30 mg SPE cartridge (Milford Waters Corporation, USA) was conditioned with 1 mL methanol, followed by equilibration with 1 mL of Milli-Q Water. Then the sample was loaded and washed with 1 mL of Milli-Q Water. Then the cartridge was dried under nitrogen for 1 min. and the sample was eluted with 1 mL of methanol, followed by evaporation under the nitrogen stream at 40 °C. The dry residue was reconstituted with 250 µL of mobile phase.

Results and discussions:

Optimization of chromatographic condition and sample clean-up:

The successful analysis of the analyte in biological fluids using LC-MS/MS relies on the optimization of chromatographic conditions, sample preparation techniques, chromatographic separation and post column detection, etc.[19,20] Thus, for better selectivity and sensitivity, different types of columns and mobile phases were used. The length of the column varied from 50 mm to 150 mm, and the particle size varied from 3.5 µ to 5 µ. Columns of

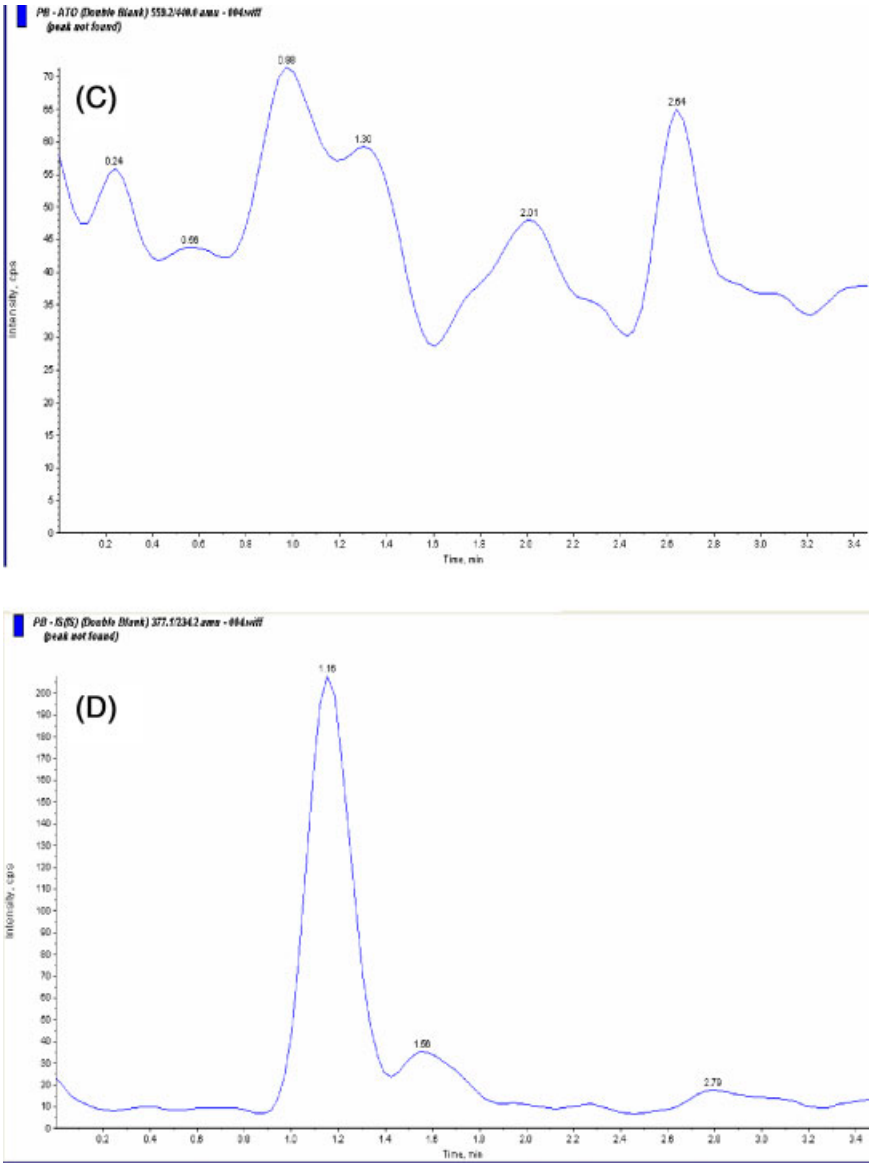


Figure 2. (*Continued*).

different stationary phases like C8, C18, cyano etc., were used which demonstrated significant effects on interference, resolution between positional isomers and peak shape. There was almost no resolution was obtained with a cyano column, whereas the use of C8 resulted in poor resolution. Finally, a Waters Symmetry C18, 75 × 4.6 mm ID, analytical column of 3.5 µ particle size was selected for analysis.

The influences of strength of the buffer, pH and types of organic modifier on the signal intensities were also studied. Based on the peak intensity of the ATO, p-HATO, o-HATO and IS, 0.6% acetic acid (*v*/*v*) and acetonitrile (30 : 70 *v*/*v*) as mobile phase at a flow rate of 0.500 mL/min were selected for further studies. Initially 90% acetonitrile: 10% of 0.6% acetic acid (*v*/*v*) at a flow rate of 0.500 mL/min was tried. However, a very high proportion of organic phase led to improper elution and peak deformation. Therefore, the 70 : 30 (*v*/*v*) organic phase to buffer were selected as optimum.

Different extraction techniques were tried to extract ATO and its two metabolites from plasma samples. In protein precipitation, almost no recovery was obtained even in different conditions like acidic, basic or neutral. Then liquid-liquid extraction was carried out, resulting in low recovery, which was not sufficient to detect the LLOQ value. Finally, solid phase extraction technique was adopted. In this method different cartridges were tested, but except HLB 1 mg 30 cc cartridge, other cartridges showed very poor and inconsistent recovery. So to get the optimum and consistent recovery for all three analytes, the above mentioned extraction technique was finalized for further study sample analysis.

Method validation

A full validation according to the FDA guidelines[21] was performed for the assay in human plasma.

Aqueous solution linearity

Aqueous solution linearity of calibration standards, i.e. spiking solution checking was assessed by subjecting the spiked concentrations and the respective peak areas using $1/X^2$ (X – concentration)

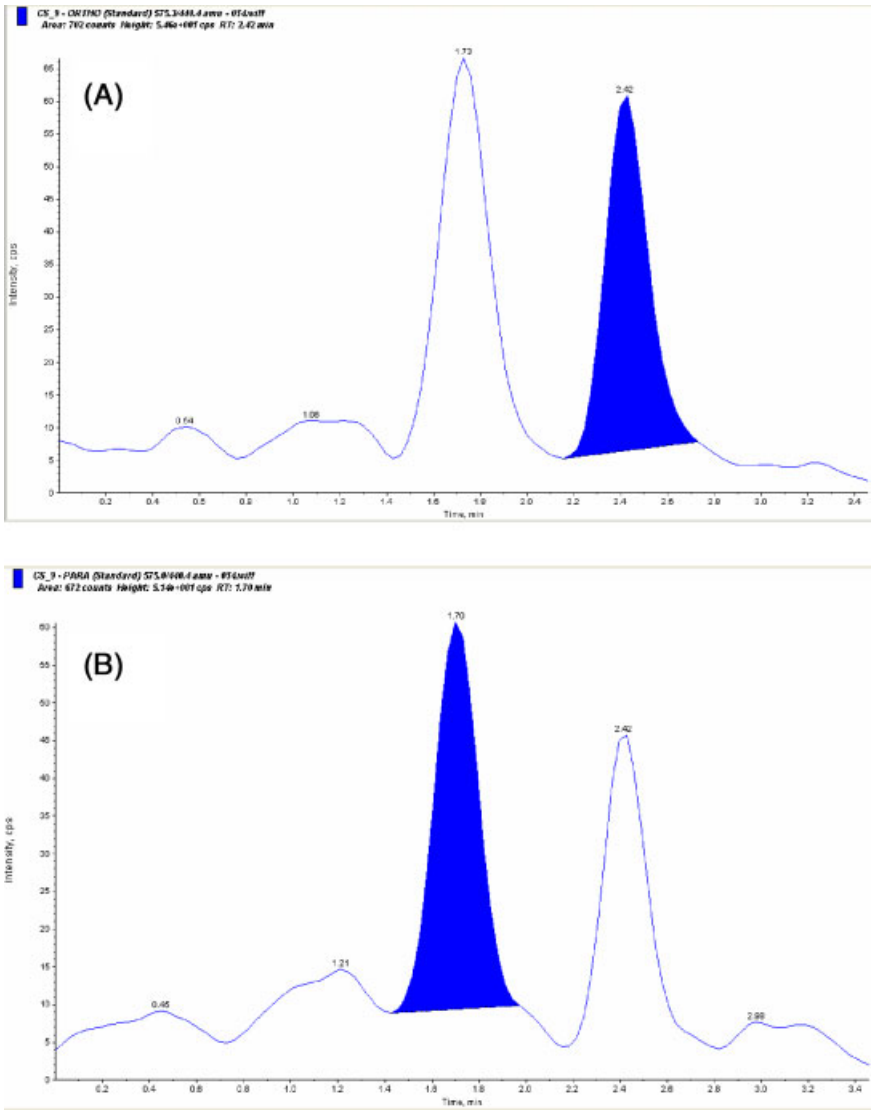


Figure 3. Representative chromatogram of LLOQ (0.05 ng/mL) of A) o-HATO, B) p-HATO, C) ATO and D) IS.

linear least-squares regression analysis. The calibration curves had a correlation coefficient (r) of 0.9900 or better. In aqueous solution linearity test all calibration standards accuracy were within 85–115%, except LLOQ where it was 80–120%.

Specificity and selectivity

Six different lots of plasma along with one lipemic plasma and one haemolyzed plasma were analyzed to ensure that no endogenous interferences were present at the retention time of ATO, p-HATO, o-HATO and IS. Six LLOQ level samples along with plasma blank from the respective plasma lots were prepared and analyzed. In all plasma blanks, the response at the retention time of ATO, p-HATO and o-HATO were less than 20% of LLOQ response and at the retention time of IS, the response was less than 5% of mean IS response in LLOQ. Figure 2 shows a typical chromatogram of plasma blank; Figure 3 represents the chromatogram of LLOQ.

Accuracy and precision

For the validation of the assay, QC samples were prepared at three concentration levels (low, medium, and high). The respective concentrations were 0.15, 26.55, and 140.35 ng/mL for ATO, p-HATO and o-HATO. Six replicates of each QC sample were analyzed together with a set of calibration standards. The accuracy of each plasma sample preparation was determined by injection of calibration samples and LQC, MQC, and HQC samples in six replicates on each day for three days. The obtained accuracy and precision (inter- and intra-day) data are presented in Table 2 for ATO, p-HATO and o-HATO. The result showed that the analytical method was accurate, as the accuracy was within the acceptance limits of 100 $\pm$ 15% at their respective concentration levels. The precision around the mean value was never greater than 15% at any of the concentrations studied. Figure 4 represents the chromatogram of upper limit of quantization (ULOQ).

Recovery study

Recovery was evaluated by comparing extracted QC samples of three different levels in six replicate with aqueous samples of same level. The mean recovery of ATO was 66.18% and the %CV of mean recovery of all its three QC levels was10.28, where as the mean recovery of p-HATO was 54.01 and the %CV of mean recovery of

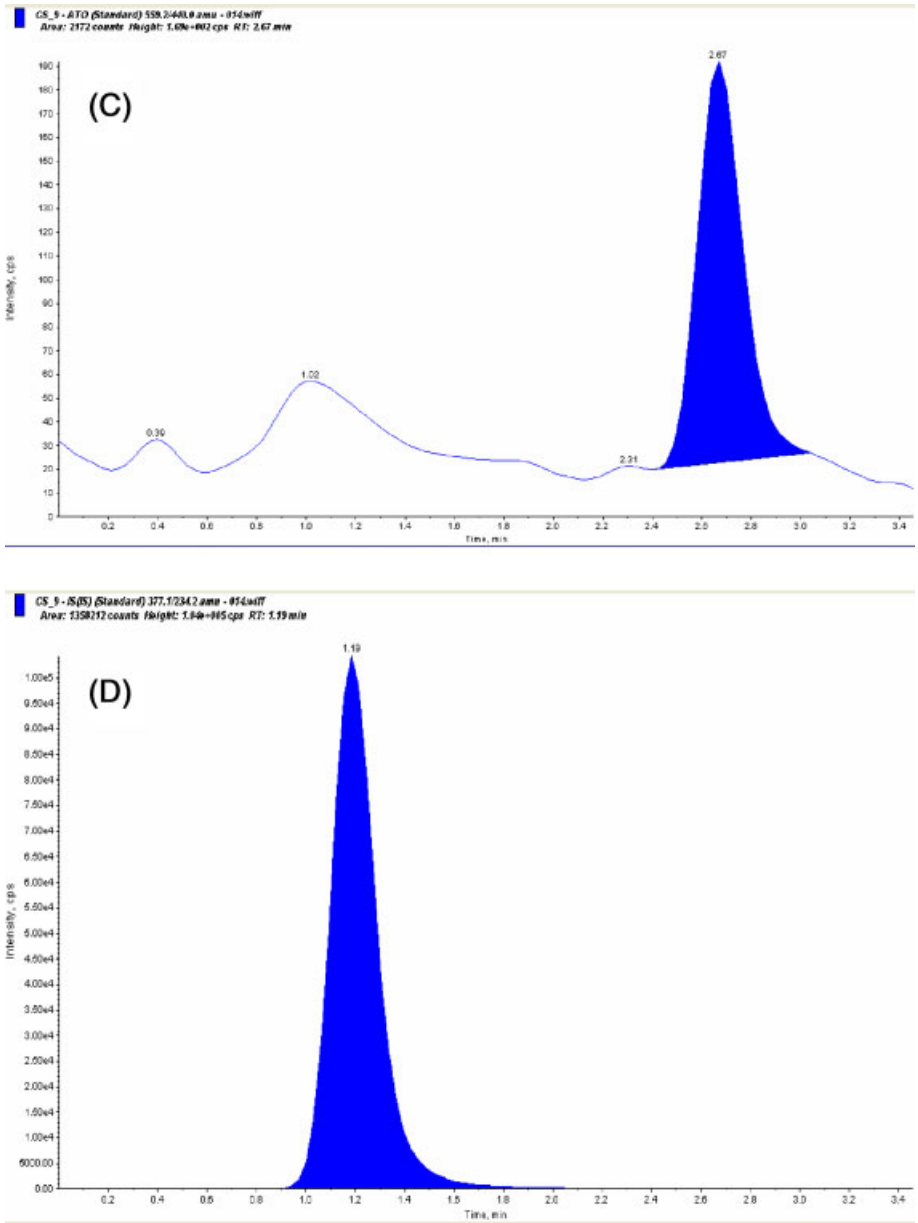


Figure 3. (*Continued*).

all its three QC levels was 7.87 and the mean recovery of o-HATO was 45.36% and the %CV of mean recovery of all its three QC levels was 10.36. Recovery of internal standard was 44.45%.

Haemolysis effects

To determine the haemolysis effects, six haemolysed plasma blanks and QC samples at two different concentration levels, i.e. LQC (0.15 ng/mL) and HQC (140.35 ng/mL) were prepared. Six replicates of each QC sample were analyzed together with a set of calibration standards prepared in normal plasma. The accuracy of each sample preparation was determined by injection of calibration samples and two QC samples in six replicates. The average % accuracy of LQC level was 99.83 and for HQC level was 98.67 for ATO; % accuracy for LQC level was 103.00 and for HQC level was 104.46 for p-HATO; and % accuracy for LQC level was 103.36 and for HQC level was 101.93 for o-HATO. The %CV of LQC was 4.29 and for HQC was 2.63 for ATO; %CV of LQC was 5.31and for HQC was 3.30 for p-HATO; and %CV of LQC was 4.09 and for HQC was 1.93 for o-HATO.

Matrix effects

The effect of human plasma constituents over the ionization of ATO, p-HATO, o-HATO and IS were determined by comparing the responses of the post-extracted plasma standard QC samples (n = 18) with the response of analytes from aqueous standard samples at low, medium, and high QC of equivalent concentrations. The % accuracy for ATO for LQC was 97.51 and for HQC was 98.70; % CV LQC and HQC was 4.22 and 0.96, respectively; for p-HATO % accuracy for LQC was 94.42 and for HQC was 100.19; % CV of LQC and HQC was 4.48 and 1.12, respectively; for o-HATO % accuracy for LQC was 98.90 and for HQC was 101.12; % CV of LQC and HQC was 4.10 and 1.32, respectively.

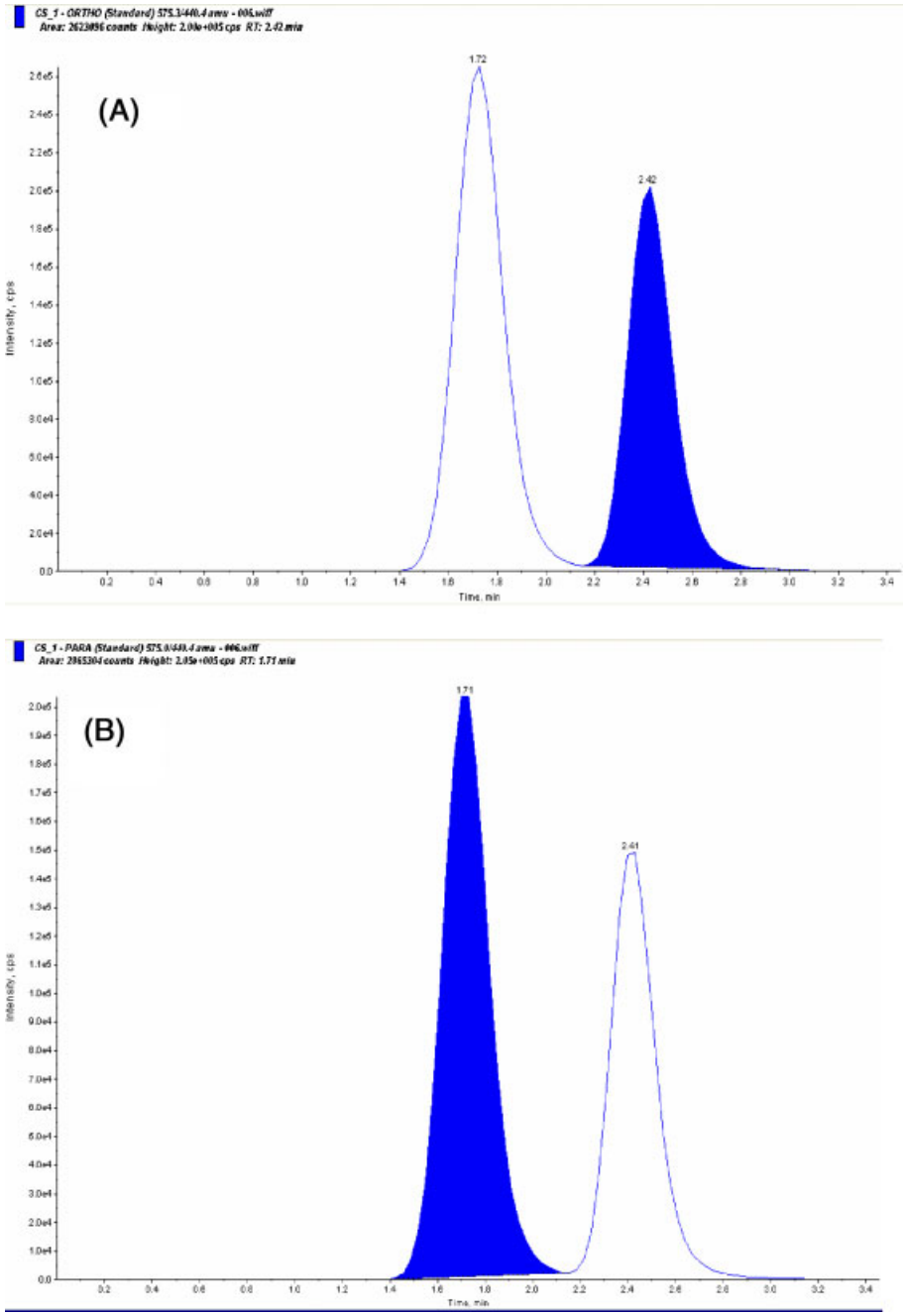


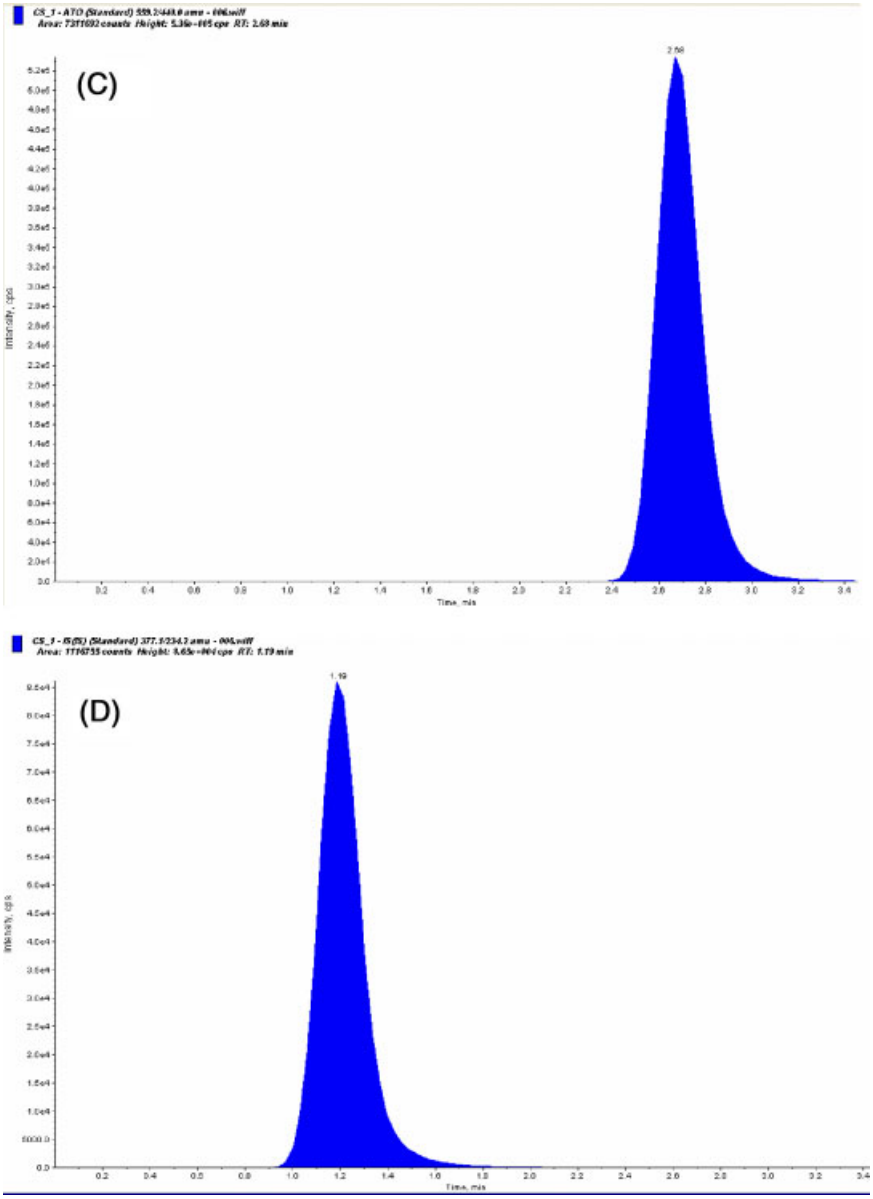
Figure 4. Representative chromatogram of ULOQ (252.92 ng/mL) of A) o-OATO, B) p-HATO, C) ATO and D) IS.

Dilution integrity

First, a dilution quality control sample (758.78 ng/mL), which was three times of ULOQ, was prepared in plasma. Then six samples each of 1/5th (151.75 ng/mL) and 1/10th (75.87 ng/mL) dilution from the above prepared sample were processed in plasma and analyzed with freshly processed calibration standards as per the extraction method. For ATO, % CV were found 1.06 and 1.41 for 1/5th and 1/10th diluted samples, respectively, and % nominal were found 90.97 and 89.63 for 1/5th and 1/10th diluted samples respectively. For p-HATO, % CV were found 1.51 and 1.76 for 1/5th and 1/10th diluted samples, respectively, and % nominal were found 91.84 and 90.00 for 1/5th and 1/10th diluted samples, respectively. For o-HATO, % CV were found 0.93 and 1.20 for 1/5th and 1/10th diluted samples, respectively, and % nominal were found 92.47 and 90.79 for 1/5th and 1/10th diluted samples, respectively.

Stability studies

The stability of ATO, p-HATO, o-HATO, and IS were investigated in the stock and working solutions, in plasma during storage, during processing, after four freeze-thaw cycles, at dry extract stage and in the final extract. Stability samples were compared with freshly processed calibration standards and QC samples. Analyte, metabolites, and IS were considered stable when the percent change of concentration was ± 10 with respect to initial



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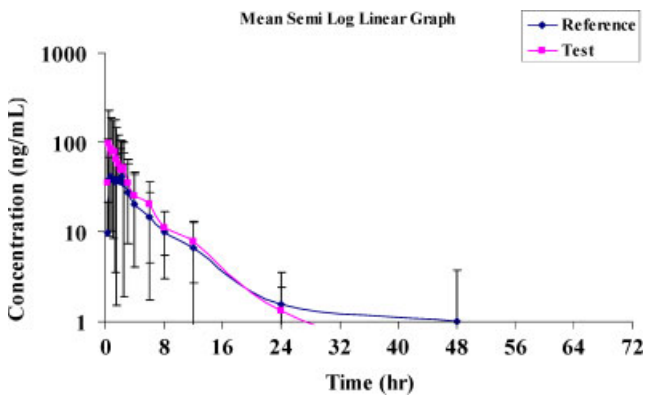

Figure 5. Concentration versus time profile of ATO in human plasma from 12 subjects receiving a single oral dose of 80 mg atorvastatin tablet as test and reference.

concentration. Summary of stability data is presented in Table 3A for ATO, Table 3B for p-HATO, and Table 3C for o-HATO.

Calibration curve

The plasma calibration curve was constructed using eight calibration standards (viz. 0.05 to 252.92 ng/mL for ATO, p-HATO and o-HATO respectively). The calibration curve had a reliable reproducibility over the standard concentrations across the calibration range. The calibration curve was prepared by determining the best fit of peak-area ratios (peak area analyte/peak area IS) *vs* concentration, and fitted to the $y = mx + c$ using weighing factor ($1/X^2$). The average regression ($n = 3$) was found to be >0.9980. The percentage accuracy observed for the mean of back-calculated concentrations for three calibration curves for ATO was within 94.33–106.84, while the precision (% CV) values ranged from1.68–6.57; for p-HATO was within 93.20–110.00, while the precision (% CV) values ranged from 0.01–10.00 and for o-HATO

Table 2. Inter- and intra-day accuracy and precision

Days	Conc. (ng/mL)	ATO Mean Accuracy (n = 6)	ATO Mean Precision [% CV] (n = 6)	p-HATO Mean Accuracy (n = 6)	p-HATO Mean Precision [% CV] (n = 6)	o-HATO Mean Accuracy (n = 6)	o-HATO Mean Precision [% CV] (n = 6)
Day 1	0.15	109.33	8.54	105.33	6.33	106.00	5.03
	26.55	95.27	2.35	92.97	2.70	92.11	2.10
	140.35	94.00	2.90	97.68	3.29	96.30	3.11
Day 2	0.15	109.33	3.05	110.00	4.24	104.00	8.97
	26.55	94.60	1.05	93.32	1.48	95.29	2.22
	140.35	92.32	4.36	96.20	3.40	94.50	4.99
Day 3	0.15	104.67	8.28	108.00	9.88	104.67	8.28
	26.55	93.56	2.88	93.11	3.30	93.56	2.88
	140.35	93.45	4.04	95.76	2.68	93.45	4.04
Inter Day	0.15	101.74	8.20	94.18	8.73	103.82	7.53
	26.55	104.50	5.92	102.37	3.93	107.44	6.57
	140.35	99.05	6.15	98.97	6.44	107.26	7.76

Table 3A Summary of stability data of atorvastatin

Stability	QC level	Mean Precision (%CV)	Mean Accuracy	Percent Change	Stability Duration
Bench top	LQC	3.44	100.28	1.12	6 : 30 Hrs
	HQC	1.79	96.86	−0.44	
Freeze thaw	LQC	0.94	104.17	1.01	4 Cycles
	HQC	1.82	96.62	−0.68	
Dry extract	LQC	2.08	103.24	−1.71	35 : 30 Hrs
	HQC	3.99	91.92	−0.75	
Auto sampler	LQC	3.44	96.06	−2.24	26 Hrs
	HQC	7.43	101.23	8.08	

Table 3B Summary of stability data of para hydroxy atorvastatin

Stability	QC level	Mean Precision (%CV)	Mean Accuracy	Percent Change	Stability Duration
Bench top	LQC	2.97	108.93	1.81	6 : 30 Hrs
	HQC	2.27	98.76	0.73	
Freeze thaw	LQC	4.66	105.88	−1.04	4 Cycles
	HQC	1.94	97.90	−0.14	
Dry extract	LQC	3.14	102.76	0.58	35 : 30 Hrs
	HQC	4.62	94.04	0.63	
Auto sampler	LQC	2.94	91.95	−4.40	26 Hrs
	HQC	14.84	91.75	1.20	

Table 3C Summary of stability data of ortho hydroxy atorvastatin

Stability	QC level	Mean Precision (%CV)	Mean Accuracy	Percent Change	Stability Duration
Bench top	LQC	3.55	106.12	−0.76	6 : 30 Hrs
	HQC	2.79	99.36	0.14	
Freeze thaw	LQC	1.98	108.16	1.25	4 Cycles
	HQC	3.35	98.59	−0.64	
Dry extract	LQC	5.19	100.29	−4.41	35 : 30 Hrs
	HQC	3.12	91.42	−1.05	
Auto sampler	LQC	5.15	9.42	5.56	26 Hrs
	HQC	97.80	97.51	8.71	

was within 95.51 – 111.31, while the precision (% CV) values ranged from 0.23 to 5.69.

Application

The above-described fully validated method was applied to determine the concentration time profile following single dose administration of atorvastatin in healthy human volunteers. After LC-MS/MS analysis the plasma concentration of ATO, p-HATO, and o-HATO for all volunteers at times (0.0) and at 0.25, 0.50, 0.75, 1.00, 1.25, 1.50, 1.75, 2.00, 2.25, 2.50, 3.00, 4.00, 6.00, 8.00, 12.00, 24.00, 48.00, and 72.00 h for the test (new formulation) and reference (marketed formulation) products were measured. The concentration–time profile of ATO, o-HATO, and p-HATO is presented in Figures 5, 6 and 7. Table 4 represents the detailed pharmacokinetic parameters of ATO, o-HATO and p-HATO.

Conclusion

A simple, sensitive, selective, precise, and accurate LC-MS/MS method for the determination of ATO, p-HATO and o-HATO in human plasma was developed and validated. Unlike the already published methods, the present method features high sensitivity, throughput, reproducibility, and precision. Moreover, this method does not have any matrix effect, such as Ionization abnormalities. We believe that this method is a useful tool for the determination of ATO, o-HATO, and p-HATO in human plasma. This method can be successfully applied for bio-equivalence study of atorvastatin.

Acknowledgement

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Table 4. Pharmacokinetic parameters

		ATO		o-HATO		p-HATO	
		Test	Reference	Test	Reference	Test	Reference
Parameter	N	Mean (±SD)					
C_{max} (ng/mL)	12	119.92 (±131.91)	64.44 (±58.94)	65.90 (±59.86)	42.89 (±51.99)	14.64 (±17.36)	4.62 (±5.67)
AUC_{0-t} (hr X ng/mL)	12	383.213 (±324.048)	287.337 (±209.243)	376.915 (±202.205)	288.473 (±182.452)	92.238 (±72.500)	60.910 (±51.683)
$AUC_{0-\alpha}$ (hr X ng/mL)	12	399.041 (±321.585)	330.549 (±229.001)	389.684 (±201.974)	300.867 (±180.339)	127.915 (±71.405)	115.314 (±48.504)
T_{max} (hr)	12	1.458 (±0.916)	1.250 (±1.055)	2.437 (±1.940)	1.854 (±0.956)	6.250 (±4.640)	12.979 (±12.529)
K_{el} (1/hr)	12	0.127 (±0.044)	0.113 (±0.056)	0.115 (±0.040)	0.100 (±0.037)	0.054 (±0.030)	0.049 (±0.033)
$T_{1/2}$ (hr)	12	5.998 (±1.833)	8.599 (±6.993)	6.834 (±2.748)	7.976 (±3.4800)	16.608 (±8.605)	24.641 (±22.027)

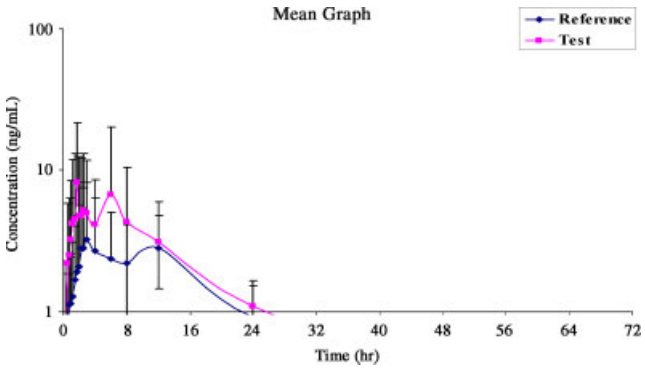

Figure 6. Concentration versus time profile of o-HATO in human plasma from 12 subjects receiving a single oral dose of 80 mg atorvastatin tablet as test and reference.

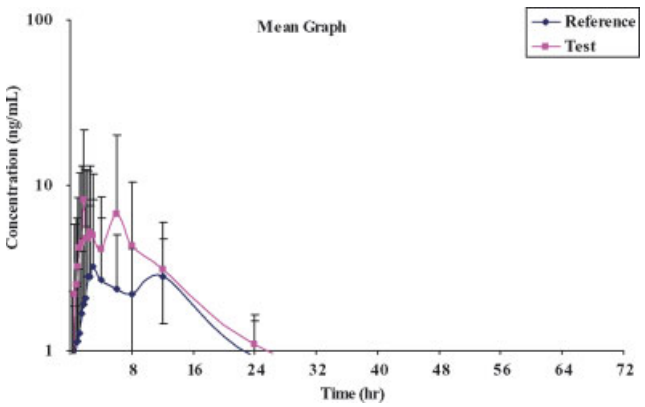

Figure 7. Concentration versus time profile of p-HATO in human plasma from 12 subjects receiving a single oral dose of 80 mg atorvastatin tablet as test and reference.

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