Asian Journal of Chemistry; Vol. 25, No. 2 (2013), 981-985

ASIAN JOURNAL OF CHEMISTRY

http://dx.doi.org/10.14233/ajchem.2013.13310

Simultaneous Estimation of Atorvastatin Calcium and Fenofibrate in Rabbit Plasma by RP-HPLC

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(*Received*: 22 December 2011; *Accepted*: 29 August 2012) AJC-12026

An accurate, rapid and simple reversed-phase high performance liquid chromatography (RP-HPLC) method was developed and validated for simultaneous estimation of atorvastatin calcium and fenofibrate in rabbit plasma. Plasma samples were treated with acetonitrile to precipitate proteins. Chromatographic separation was accomplished using CAPCELL PAK C_8 (4.6 mm × 250 mm, 5 mm) analytical column with a mobile phase consisting of phosphate buffer and acetonitrile (28:72). Detection and quantification were performed by UV/VIS detection at 260 nm. The lower limits of detection and quantification were 0.05 μg mL^{-1} and 0.20 μg mL^{-1} for atorvastatin calcium and 0.07 μg mL^{-1} and 0.35 μg mL^{-1} for fenofibrate respectively. The calibration curves are linear over the concentration range 1 to 40 μg mL^{-1} for both atorvastatin and fenofibrate in rabbit plasma. The method was quantitatively evaluated in terms of linearity, precision, accuracy, recovery, selectivity and stability. The proposed method is simple, convenient and suitable for the analysis of atorvastatin calcium and fenofibrate in rabbit plasma.

Key Words: Atorvastatin calcium, Fenofibrate, Rabbit plasma, High performance liquid chromatography.

INTRODUCTION

Atorvastatin calcium, (β R, δ R)-2-(4-fluorophenyl)-β,δ-dihydroxy-5-(1-methyl ethyl)-3-phenyl-4-((phenyl amino)-carbonyl)-1*H*-pyrrole-1-hepatonoic acid is a selective competitive inhibitor of the enzyme HMG-CoA reductase that catalyze conversion of HMG-CoA to mevalonate, an important rate-limiting steps in cholesterol biosynthesis[1]. Statins, lipid lowering molecule independent to cholesterol reduction, also potentiate the antioxidant[2], antiinflammatory[3] and antimalerial[4]. 2-[4-(4-chlorobenzoyl) phenoxy]-2-methyl-propanoic acid, 1-methyl-ethyl ester, Fenofibrate drug reduces several types of fats in the blood[5]. Hence prescribed for the treatment of hypercholesterolemia and mixed dyslipidemia. Thus quantification methods of the drug in pharmacokinetics studies need to be sensitive and specific for simultaneous estimation. Several analytical methods for analysis of atorvastatin calcium and fenofibrate in biological fluid have been reported for single drug. However few methods are available for estimation of atorvastatin calcium and fenofibrate in biological samples. Amongst them HPLC-UV and Mass spectroscopy methods have been developed for the quantification of both atorvastatin calcium and fenofibrate either alone or in combination with other drugs in different matrices. LC/MS method has been reported for quantification of atorvastatin calcium in biological matrices[6-8]. HPLC methods have been reported for the determination of atorvastatin calcium alone in biological matrices[9,10], along with impurities in pharmaceutical preparation[11,12] and in various combinations[13-16]. Several LC/MS/MS and HPLC methods have also been developed for the determination of two or more than two statins simultaneously[17,18]. Similarly, HPLC-UV methods have been reported for the determination of fenofibrate alone in biological matrices[19], HPLC/MS/MS method[20,21], pharmaceutical preparation with combination[22] and its metabolite finofibric acid HPLC method was developed in biological matrices[23]. The present investigation made in this direction is to establish a simple and versatile isocratic reversed phase HPLC-UV method for simultaneous measurement of atorvastatin calcium and fenofibrate in rabbit plasma using a simple extraction procedure.

EXPERIMENTAL

Isocratic high pressure liquid chromatography (cyberlab-chrom-HPLC V 4.0) with LC-P-100 pump, variable wavelength programmable UV/VIS detector LC-UV 100 and operating software cyberstore version no 4-0512-039 was used. The chromatographic separation carried out by reverse phase capcell pak C_8 DDS5 column (4.6 mm LD × 250 mm i.d.

particle size 5 µm. The mobile phase consisted a mixture of acetonitrile: KH_2PO_4 (72:28 v/v) (pH 4.1) with a flow rate of 1.0 mL min^{-1}. The UV/VIS detector was set at a wavelength of 260 nm. An injection volume of 10 µL was used. Diclofinac was used as an internal standard.

Atorvastatin calcium (Emcure Pharmaceutical, Pune), Fenofibrate (Smruthi Organics, Solapur), Diclofinac (Emcure Pharmaceutical, Pune), Acetonitrile (Merk chemicals) and all other chemicals used were of analytical grade. Doubly distilled water was used for preparing mobile phase solutions.

Preparation of standard solution: Stock solution containing 1 mg mL^{-1} each of atorvastatin calcium, fenofibrate and diclofinac (IS) were prepared in methanol. Internal standard was further diluted with methanol to get final concentration of 200 µg mL^{-1}. Further the stock solutions of atorvastatin calcium and fenofibrate were diluted in methanol to obtain various concentrations of 1, 2, 4, 8, 16, 32 and 40 µg mL^{-1}. Phosphate buffer (50 mM) was prepared in water and adjusted to pH 4.1 with *ortho*-phosporic acid. All solutions were stored at 4 ºC before use.

Chromatographic condition: The mobile phase consisting of (50 mM) phosphate buffer -acetonitrile (28:72), (v/v) at pH 4.1 was degassed and filtered by using Millipore vacuum filter system equipped with 0.45 µm membrane filter. Chromatography was performed at an ambient temperature by pumping the mobile phase with a flow rate of 1 mL min^{-1}. The column effluent was monitored at 260 nm.

Extraction procedure: Calibration standards were prepared by adding 50 µL of the appropriate atorvastatin calcium and fenofibrate working solutions (1-40 µg mL^{-1}) to 500 µL of blank plasma. Calibration standards, samples and controls were processed by adding 1.0 mL of acetonitrile to 0.50 mL of above processed plasma to labeled 15 mm × 75 mm glass culture tubes. Then, this solution was vortex-mixed for 30s and centrifuged at 5000 rpm for 15 min.

The supernatant was transferred to a clean, similarly labeled 10 mL glass conical centrifuge tube. The acetonitrile was evaporated under a nitrogen steam at 15 psi in a water bath set to temperature of 40 ºC. The dried extracts were reconstituted in 400 µL mobile phase. All tubes were vortex-mixed and 10 µL aliquots of the extracted solutions were injected onto the HPLC system[24,25].

To develop a precise, accurate and reproducible HPLC method for the estimation of atorvastatin calcium and fenofibrate in rabbit plasma, various mobile phases, stationary phases and sample preparation methods were employed and the proposed chromatographic condition was found to be appropriate for the quantitative determination. After optimization of the analytical conditions, the evaluation of the parameters, such as accuracy, precision, linearity, selectivity, recovery and stability were performed for the validation of method[26,27].

System suitability test: The system suitability test was performed before analysis of every batch of sample to ensure the reproducibility of the chromatographic system. The HPLC system suitability test was performed by running six injections of diluted drug and IS in the linear region of the calibration curve and measuring the coefficient of variation in percentage (% CV). Number of theoretical plates, tailing factor and resolution were also determined as a means of validation parameter. The values obtained are listed in Table-1.

TABLE-1
SYSTEM SUITABILITY TEST FOR ATR AND FB

Ob. No.	Concen. (µg mL^{-1})	Resolution		Theoretical plate		Tailing factor	
		ATR	FB	ATR	FB	ATR	FB
1	16	3.18	17.51	3196.01	11381.12	1.05	1.09
2	16	3.17	17.45	3131.95	11095.33	0.94	1.08
3	16	3.17	17.51	3121.96	10811.27	0.95	1.09
4	16	3.17	17.41	3121.75	10730.64	0.95	1.04
5	16	3.18	17.50	3112.57	10879.45	1.02	1.06
6	16	3.17	17.21	3112.60	10748.38	1.02	0.98
	Mean	3.17	17.43	3131.80	10941.03	0.98	1.05
	S.D (±)	0.004	0.115	29.409	252.78	0.047	0.042
	C.V (%)	0.12	0.65	0.93	2.310	4.75	4.00

Linearity: The linearity was studied using six concentrations as 1, 2, 4, 8, 16, 32 and 40 µg mL^{-1} of atorvastatin calcium and fenofibrate. Linearity experiment was performed six times to check the detector response to the drug to be linear in function with various concentrations (1 to 40 µg mL^{-1}). The working standards were prepared by adding different concentrations of atorvastatin calcium, fenofibrate and fixed concentrations of diclofinac (IS) (200 µg mL^{-1}) solution spiked in plasma to obtain the required concentration range. These samples were extracted using standard procedure and then injected into the HPLC system[24,25]. The drug/IS peak area was plotted against the concentration of the drug and measured in terms of correlation coefficient.

Precision and accuracy: Precision and accuracy of the developed method was determined by analysis of quality control samples at three different concentrations covering the low, medium and higher ranges of the calibrations curves. Injecting six samples at each concentration on the same day assessed intraday variation of the assay. Inter day variation was assessed by injecting nine sample of each concentration (on 15 days) over a period of two weeks. The precision of the method was expressed in terms of percent coefficient of variation (% CV) and accuracy was expressed as a percentage of the theoretical concentration (observed concentration × 100/ theoretical concentration)[28].

Recovery: The absolute recoveries for the atorvastatin calcium, fenofibrate and diclofenac were determined by spiking known amount of atorvastatin calcium, fenofibrate and diclofenac into drug-free rabbit plasma to obtain three different concentration covering the low, medium and higher ranges of the calibration curves. Absolute recoveries were determined by comparing the peak area of extracted quality control samples with the peak area of recovery standards at the same nominal concentrations.

Selectivity: The selectivity was verified by checking the interference of endogenous compound in rabbit plasma at the retention time of the drug and IS by evaluating six lots of plasma.

Stability: Stability was evaluated by Bench top stability; freeze thaw stability and auto sampler stability methods by using quality control samples. Bench top stability was determined at lower and higher quality control samples and by evaluating six replicate samples at each level. The samples

were processed after keeping them at bench top for 2 h at room temperature and then analyzed against freshly spiked standard calibration curve. Freeze thaw stability was performed for three cycles with freezing and thawing. Spiked plasma was kept in the deep freeze at -20 ºC for 12 h (1st cycle) followed by unassisted thawing at room temperature. The samples were extracted and analyzed with freshly extracted quality control samples. The remaining samples were kept for 2nd and 3rd cycles and then analyzed. Autosampler stability was assessed by extracting six replicate of quality control samples at low and high concentration and putting the processed samples in atmosphere. Finally the samples were injected after 120 h along with freshly spiked calibration curve to validate the stability.

RESULTS AND DISCUSSION

A typical chromatogram of a working solution containing atorvastatin calcium, fenofibrate and IS is given in Fig. 1. The representative chromatogram of drug free (blank) rabbit plasma is shown in Fig. 2. Drug free rabbit plasma was screened and no endogenous interference was observed at retention time of atorvastatin calcium, fenofibrate and IS. Fig. 3 illustrates the representative chromatogram of blank plasma spiked with 4 µg mL^{-1} atorvastatin calcium, fenofibrate and diclofenac as IS.

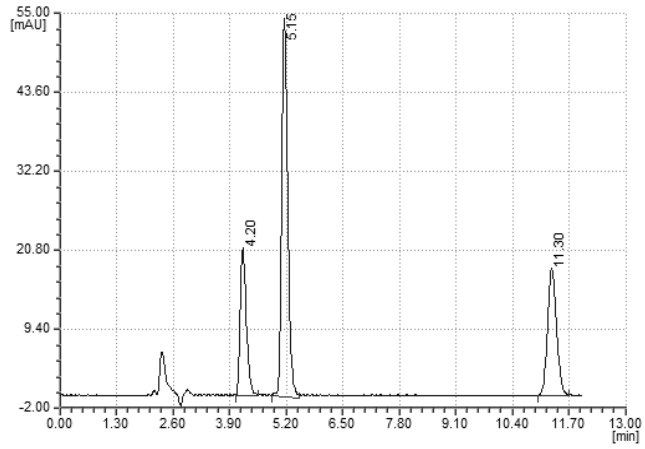

Fig. 1. Typical chromatogram standard solution of ATR and FB and diclofinac As IS

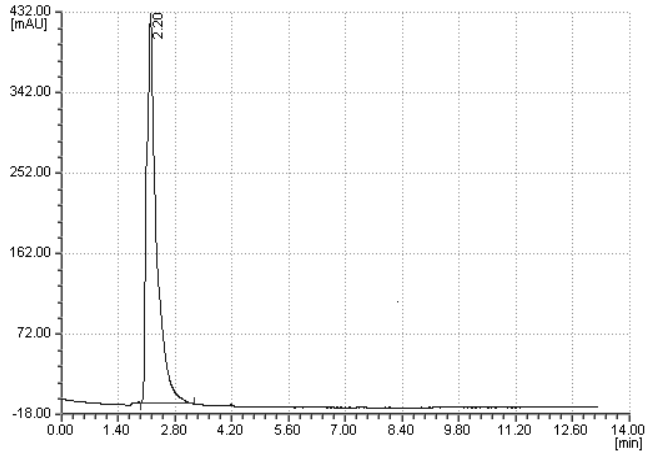

Fig. 2. Typical Chromatogram of rabbit blank plasma

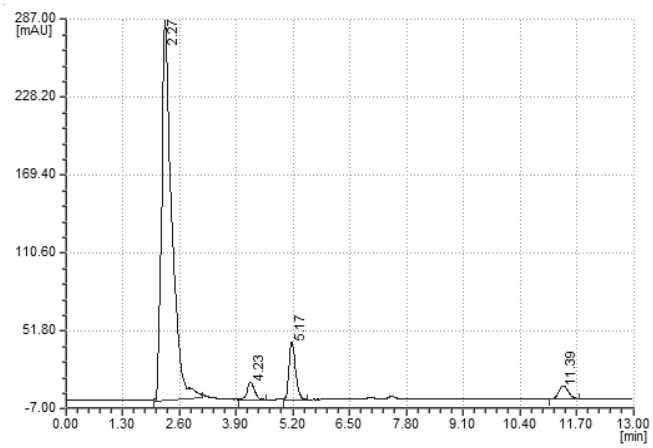

Fig. 3. Typical chromatogram obtained from blank plasma spiked with 4 µg mL^{-1} atorvastatin cadmium and fenofibrate and diclofinac as IS

During method development, different solvents like methanol, 10 % perchloric acid, 10 % trichloroacetic acid were investigated for extraction of atorvastatin calcium and fenofibrate that showed very low recovery (< 25 %) and also interference from plasma peak. However, extraction with acetonitrile was optimized as the sample treatment procedure[24,25]. The mobile phase was optimized to provide sufficient selectivity. Phosphate buffer contributed high sensitivity and selectivity when compared with other buffers. Acetonitrile as an organic component resulted in better sensitivity as compared to methanol but variation in the amount of acetonitrile in the mobile phase affected resolution and runtime. Variation of mobile phase pH resulted in bad peak shape and increased interference from the plasma. Therefore buffer pH was adjusted at 4.1. The optimized mobile phase was phosphate buffer:acetonitrile (28:72) (v/v) at pH 4.1. Injection volume was optimized to 10 mL because use of greater volume resulted in loss of peak of symmetry, the column temperature maintained at 25 ºC (ambient). The atorvastatin calcium, fenofibrate and IS were eluted at 4.23, 11.39 and 5.17 min respectively. The standard calibration curves were linear over the concentration range of 1-40 µg mL^{-1}. The correlation coefficients for calibration curves were 0.9993 for atorvastatin calcium and 0.9991 for fenofibrate. LOD and LOQ were found to be approximately 0.05 and 0.20 µg mL^{-1} for atorvastatin calcium and 0.07 and 0.35 µg mL^{-1} for fenofibrate respectively, indicative of high sensitivity of the method. The results of the linearity experiment are given in Table-2.

The precision of the method was measured by the percentage coefficient of variation (% CV) over the concentration range of high, middle and low quality control samples respectively of drug during course of validation. Intraday precision of the method ranged from 0.471 to 3.241 % CV. Inter-day precision of the method was found to be 0.548 to 2.917 % CV. Nominal value (%) for recovery of atorvastatin calcium and fenofibrate from quality control samples were tested of intra-day and inter-day. Intra-day accuracy ranged from 99.73 to 101.24 %. Interday accuracy ranged from 100.04 to 101.93 %. Result from determination of intraday and interday, accuracy and precision are listed in Table-3.

The data indicate that the assay method is reproducible within the same day and at different days, coefficient of variation

TABLE-2
LINEAR REGRESSION ANALYSIS OF CALIBRATION CURVES (n = 6)

Drug	Linearity range (μg mL^{-1})	Intercept (Mean ± SD)	Slope (Mean ± SD)	Correlation coefficient (r^2) (Mean ± SD)	LOD (μg mL^{-1})	LOQ (μg mL^{-1})
ATR	1-40	3859.333 ± 33.55394	2410.833 ± 433.3167	0.999374 ± 0.000381	0.05	0.20
FB	1-40	2967.167 ± 30.14244	3011.167 ± 668.2178	0.999164 ± 0.000345	0.07	0.35

TABLE-3
INTRADAY AND INTERDAY PRECISION OF ATR AND FB IN RABBIT PLASMA

Concentration added (μg mL^{-1})	Intraday[a]			Interday[b]		
	Measured concentration (mean ± SD)	CV (%)	Accuracy (%)	Measured concentration (mean ± SD)	CV (%)	Accuracy (%)
ATR						
4	4.049 ± 0.131	3.241	101.24	4.037 ± 0.117	2.917	100.92
16	15.957 ± 0.160	1.004	99.73	16.006 ± 0.169	1.056	100.43
32	32.017 ± 0.150	0.471	100.05	32.063 ± 0.176	0.548	100.19
FB						
4	4.039 ± 0.092	2.295	100.99	4.077 ± 0.109	2.692	101.93
16	16.010 ± 0.182	1.138	100.06	16.006 ± 0.177	1.107	100.04
32	32.066 ± 0.203	0.636	100.20	32.066 ± 0.191	0.597	100.20

[a]Mean values represent six different plasma samples for each concentration; [b]Interday was determined from nine different runs over two-week period. The concentration of each run was determined from a single calibration curve run on the first day of the study

TABLE-5
RESULT FROM STABILITY STUDY FOR ATR AND FB

Statistical properties	Bench top stability				Freeze thaw stability				Auto sampler stability			
	ATR		FB		ATR		FB		ATR		FB	
	LQC	HQC	LQC	HQC	LQC	HQC	LQC	HQC	LQC	HQC	LQC	HQC
Mean	3.94	31.90	4.05	32.14	3.89	31.87	4.19	32.03	3.75	32.28	4.19	32.22
SD (±)	0.22	0.70	0.10	0.49	0.15	0.71	0.20	0.39	0.23	0.79	0.19	0.49
CV (%)	5.70	2.20	2.62	1.54	3.97	2.24	4.96	1.23	6.27	2.46	4.74	1.53
Nominal (%)	98.70	99.70	101.25	100.44	97.29	99.59	104.83	100.09	93.75	100.87	104.75	100.68

(% CV) are less than 15 % for both sample types over the concentration ranges assayed. The absence of peak at migration time of atorvastatin calcium, IS and fenofibrate indicates specifying of the method.

The absolute recovery for the atorvastatin calcium, fenofibrate and IS were determined by spiking known quantitative of atorvastatin calcium, fenofibrate and IS into drug free rabbit plasma to obtain three different concentration covering the low, medium and higher ranges of the calibration curve. The samples were then extracted and analyzed as described earlier. The absolute recovery was calculated by comparing the peak areas of the drugs with those obtained from pure standards in methanol and IS at the same concentration[28]. The absolute recovery of atorvastatin calcium and fenofibrate ranges from 93.21 ± 3.16 to 99.94 ± 0.79 %, while the absolute recovery for IS was 94.29 ± 2.25 % (Table-4). The relative recovery of the atorvastatin calcium and fenofibrate were calculated by comparing the concentration of the drug-spiked plasma with the actual added concentration. Relative recovery of the atorvastatin calcium and fenofibrate ranged from 99.73 ± 1.00 and 101.24 ± 3.28 % (Table-4).

Bench top stability, freeze thaw stability and autosampler stability were determined at lower and higher quality control samples by evaluating six replicate samples at each level. Low value of percentage difference (< 15 %) between area ratio for stability test samples and fresh QC samples confirm the stability of drug on the bench top for 2 h, in an auto sampler for 12 h and inside the freezer for 120 h[19,24,29]. Results of stability are given in Table-5.

TABLE-4
ABSOLUTE AND RELATIVE RECOVERY OF ATR, FB AND IS

Concentration added (μg mL^{-1})	Absolute recovery[a] (% mean ± SD)	Relative recovery[a] (% mean ± SD)
ATR		
4	93.21 ± 3.16	101.24 ± 3.28
16	97.98 ± 1.00	99.73 ± 1.00
32	99.94 ± 0.79	100.05 ± 0.47
FB		
4	94.01 ± 2.06	100.99 ± 2.31
16	98.32 ± 1.17	100.06 ± 1.13
32	99.53 ± 0.81	100.20 ± 0.63
IS	94.29 ± 2.25	

[a]Mean values represent six different plasma samples for each concentration

Conclusion

The present work described an accurate, rapid and simple assay procedure for simultaneous determination of atorvastatin calcium and fenofibrate in rabbit plasma sample. The method involves a simple extraction procedure, separation on a reversed phase column with an IS and UV detector. The validation data demonstrated good precision and accuracy, which proves the reliability of proposed developed method. Thus the method suits for routine TDM and pharmacokinetic study of atorvastatin calcium and fenofibrate.

ACKNOWLEDGEMENTS

The authors thank Prof. Kishore Singh Chatripati, President and Prof. R.C. Hariprasanna, Principal, RMES's College of Pharmacy for providing the necessary research facilities.

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