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SIMULTANEOUS QUANTIFICATION OF EPIGALLOCATECHIN-3-GALLATE AND ATORVASTATIN BY USING HPLC-UV METHOD AND ITS APPLICATION TO PHARMACOKINETIC STUDIES IN RATS

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ABSTRACT

A sensitive, precise and simple LC method for the simultaneous quantification of epigallocatechin-3-gallate and atorvastatin in rat plasma has been developed and validated. The chromatographic separation was achieved on a C_{18} column (250mm×4.6 mm, 5 µm) maintained at room temperature, using gradient elution with 0.1% formic acid (Solvent A) and acetonitrile (Solvent B), and detected using UV-visible detector. Protein precipitation followed by liquid-liquid extraction of epigallocatechin-3-gallate and atorvastatin from rat plasma resulted in their high recoveries. LC calibration curves based on the extracts from rat plasma were linear in the range of 30–1000 ng/ml for both the analytes. The limits of quantification were 30ng/ml for both epigallocatechin-3-gallate as well as atorvastatin. The precision and accuracy of the method were well within the generally accepted criteria for biomedical analysis. The described method was successfully applied to study the effect of epigallocatechin-3-gallate, which is reported as a P-glycoprotein inhibitor on the pharmacokinetics of atorvastatin (P-glycoprotein substrate) in *Wistar* rats. The results of the study inferred that epigallocatechin-3-gallate significantly improved the oral bioavailability of atorvastatin.

Keywords: Column liquid chromatography,P-glycoprotein, Epigallocatechin-3-gallate, Atorvastatin, Pharmacokinetics.

INTRODUCTION

Atorvastatin (Fig. 1a) is the most preferred molecule among statins for treatment of moderate as well as severe hypercholesterolemia [1]. It is a selective and competitive inhibitor of hydroxyl methyl glutaryl-coenzyme A (HMG-CoA) reductase, which is responsible for converting HMG-CoA to mevalonate. Mevalonate is the precursor for cholesterol biosynthesis. Atorvastatin by inhibiting this enzyme decreases the mevalonate level, and subsequently reduces hepatic cholesterol levels and increases uptake of low density lipoprotein cholesterol (LDL-CH) [2]. However, the oral bioavailability of atorvastatin is very low (only 14%) due to poor water solubility, rapid metabolism in the gut and liver and incomplete intestinal absorption and/or extensive gut wall extraction [1, 3]. One of the reasons for incomplete intestinal absorption may be its efflux by P-glycoprotein, as atorvastatin is known to be a substrate for P-glycoprotein [4]. Various researchers have tried to improve its oral bioavailability by developing different formulations, which resolved the solubility issue [2, 3]. However, the problem associated with P-glycoprotein mediated efflux still remained unaddressed. P-glycoprotein inhibition approach which has come to forefront in few years may be opted for improving the oral bioavailability of atorvastatin. Researchers have found that P-glycoprotein inhibitors may have a great impact on altering pharmacokinetics of a drug and may be preferred for improving the oral bioavailability of its substrates [5]. These inhibitors act either by blocking the drug binding site or interferingATP hydrolysis [6]. Based on these

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findings, we tried to evaluate the effect P-glycoprotein inhibition on oral bioavailability of atorvastatin.

Epigallocatechin-3-gallate (EGCG) (Fig. 1b), is the major flavanoid found in green tea [7]. It inhibits P-glycoprotein in human Caco-2 cells [8]. It is also found that EGCG improves the bioavailability of its substrates *viz.* diltiazem [9], tamoxifen [10], doxorubicin [11], irinotecan [12]. Thus, we tried to determine the effect of EGCG on oral bioavailability of atorvastatin. In order, to perform bioavailability studies, we need some analytical method for quantifying EGCG and atorvastatin in plasma. However, no method is reported for quantifying these two compounds simultaneously in biological matrix.

The present study was focussed on developing a simple, economical, sensitive and specific RP-LC method for simultaneous determination of EGCG and atorvastatin in rat plasma. The developed method was then applied for the pharmacokinetic study in rats. In this method, tinidazole (Fig. 1c) was selected as the internal standard, and chromatographic separation of EGCG and atorvastatin was completed within 15 min.

MATERIALS AND METHODS

Chemicals and reagents

Atorvastatin (purity 99.3%) and tinidazole (purity 99.4%) were obtained as gift samples from Astron Research Limited (Ahmedabad, India) and Troikaa Pharmaceuticals Limited (Ahmedabad, India), respectively. Epigallocatechin-3-gallate (purity 99.1%) was provided as a gift sample by Dr. D. W. Han, Pusan National University, Korea. All the solvents and chemicals used for the study were of chromatographic grade and purchased from Qualigen Fine Chemicals, Mumbai, India. Heparin was purchased from Biological E. Ltd, Hyderabad, India. Deionised water for LC was prepared in-house using a Milli-Q water purifier system (Millipore Elix, Germany).

Chromatographic conditions

The chromatographic system consisted of Agilent 1200 Infinity Series (Agilent, USA) pump, auto-sampler and UV-visible detector, set at 240 nm. The data were analysed by EZ Chrome Elite Software. Chromatographic separation was achieved by using a Kromasil C_{18} (250 mm×4.6 mm, 5 µm) column maintained at room temperature. Gradient elution technique was followed for separating the analytes due to difference in their polarity. Elution of EGCG and atorvastatin was carried out with 0.1% formic acid as solvent A and acetonitrile as solvent B using gradient elution in 0.01-14 min with 20-80% B, and 14-14.5 min with 80-20% B. The flow rate and equilibration period were 1ml/min and 5 min, respectively. Samples were quantified by determining the peak area ratio (Peak area$_{Drug}$/Peak area$_{IS}$).

Extraction procedure

Protein precipitation followed by liquid-liquid extraction method was followed for efficient recovery of EGCG, atorvastatin and tinidazole (internal standard) from rat plasma to reduce the interference of plasma impurities. Twenty five microlitre of internal standard (20 µg/ml) was added to 100 µL of rat plasma, and vortex for 1 min. Four hundred microlitre of acetonitrile was added to the above sample and vortex for 1 min. The resulting sample was centrifuged at 3000×*g* for 15 min at 4°C. The supernatant was collected in another microcentrifuge tube to which 1.5 ml of ethyl acetate: *t*-butyl methyl ether (1:1) was added. The resulting sample was vortex for 90 sec and then centrifuged at 1000×*g* for 10 min at 4°C. The organic layer (supernatant) was transferred into a 10 ml conical glass tube and evaporated under the gentle stream of nitrogen. The samples were reconstituted in 100 µl mobile phase and 50 µl of it was injected onto LC system.

METHOD VALIDATION

Calibration curve

Ten milligram each of EGCG and atorvastatin was dissolved in methanol (final adjusted volume 100 ml) to obtain a stock solution of concentration 100 µg/ml. The working solutions were prepared from the stock solution by dilution with methanol. Spiked calibration curve samples containing EGCG and atorvastatin at concentrations of 30, 100, 200, 400, 600, 800 and 1000 ng/ml was prepared using the stock and working solutions in order to plot seven point calibration curve. The lower limit of quantification was determined as the lowest concentration of the analytes in plasma that could be quantified with acceptable precision and accuracy under the experimental conditions (less than 20% variation in precision). The analyte response at the lower limit of quantitation (LLOQ) was found to be 5 times the response of the blank response. The limit of detection was determined by injecting the serial diluted standard solutions to obtain a signal-to noise ratio of 3. The working solution concentration of internal standard was 20 µg/ml, from which 25 µl was added to each sample. The extraction of the plasma samples were carried out as per the procedure described above. Standard curves were constructed by plotting ratio of the peak areas of EGCG and atorvastatin to internal standard, individually *versus* concentration. The calibration curves were obtained by least square linear regression analysis using weight scheme as 1/c (c = concentration) using EZ Chrome Elite Software.

Preparation of quality control samples

The quality control (QC) samples used during the validation and pharmacokinetic studies were prepared by spiking appropriate volumes of EGCG and atorvastatin from the dilution solution into blank rat plasma and stored at -80ºC till further use. The QC sample concentrations of both the drugs in plasma were 900, 500 and 150 ng/ml to represent high, medium and low QC samples, respectively.

Precision and accuracy

Intra-day precision and accuracy were calculated by taking six replicates of QC samples of EGCG and atorvastatin which were then extracted and analyzed using LC method as described above. Inter-day accuracy and precision were calculated by taking six replicates of all the three QC sample concentrations for EGCG and atorvastatin for three consecutive days along with the standard calibration curve. Concentrations of the analytes were calculated from the calibration curve.

Recovery

Recovery of the extraction procedure was calculated by analyzing six extracted samples at concentrations of 900, 500 and 150 ng/ml for EGCG and atorvastatin. The peak areas of the extracted samples were then compared with those of unextracted (non-plasma samples of identical standards prepared in the mobile phase which have not undergone extraction procedure) EGCG and atorvastatin samples with same concentration. The recovery value of the IS was determined in the same way at a single concentration of 500 ng/ml.

Stability studies

Stability of EGCG and atorvastatin in rat plasma during storage and processing were checked using QC samples. Bench-top stability was determined for the aliquots of each of the low and high QC samples, which were spiked into rat plasma and kept at room temperature for 6 h prior to their analysis. Six replicates of high and low control samples were frozen at -80°C and analysed for three freeze–thaw cycles to determine their freeze-thaw stability. A dry extract stability study was performed for six replicates of high and low controls after extracting the analytes from plasma and storing the dried samples at -80°C for 24 h. The samples were processed and kept at room temperature for 6 h and then analysed after reconstitution with the mobile phase. For checking the auto sampler stability, six replicates of high and low QC samples were reconstituted and kept in auto sampler and analyzed at 0 and 12 h. Long-term stability of EGCG and atorvastatin were checked for six replicates of the high and low control samples after storing them for 30 days at -80ºC. The samples were then processed and analysed as per the method described above.

Animals

Male *Wistar* rats weighing 280–300 g were obtained from the animal house of B. V. Patel PERD Centre, Ahmedabad. Animal housing and handling was performed in accordance with Good Laboratory Practice (GLP) mentioned in CPCSEA guidelines. Animal house is registered with the Committee for the Purpose of Control and Supervision of Experiments on Animals, Ministry of Social Justice and Empowerment, Government of India, vide registration no. 1661/PO/a/12/CPCSEA, dated 21/11/2012. All experimental protocols were reviewed and accepted by the Institutional Animal Ethics Committee prior to initiation of the experiment. The animals were housed in polypropylene cages (three animals per cage) and placed in the experimental room where they were allowed to acclimatize for a week before experiment. A 10% air exhaust conditioning unit was maintained along with a relative humidity of 60±5% and a temperature of 25±3ºC in the animal house facility. A 12:12 h light: dark cycle was also regulated for the experimental animals. Amrut certified rodent diet (Maharashtra Chakan Oil Mill Ltd) and tap water (boiling hot water cooled to room temperature) were provided *ad libitum* to the experimental animals.

Pharmacokinetic study

Twelve animals (six animals per group) were used in the study. A single dose of 10 mg/kg bodyweight was decided for EGCG and atorvastatin, based on the previous reports on pharmacokinetic studies in rats [13, 14]. A pilot study was done with an objective to finalise the dosing time of EGCG, where EGCG was dosed concomitantly with atorvastatin in one group whereas in two other groups EGCG was dosed 30 and 45 min prior to the dosing of atorvastatin. In this study, it was observed that the animals pre-dosed with EGCG showed significantly greater AUC and C_{max} ($p \leq 0.05$) as compared to other group. However, the results between 30 and 45 min pre-dose of EGCG were not significantly different. Thus, EGCG was dosed 30 min prior to atorvastatin in final studies. The first group was dosed with atorvastatin, second with EGCG and atorvastatin and third with EGCG only. All the compounds were orally administered as a suspension in 0.2 % agar. The jugular veins of all the animals were cannulated for collection of the blood at different sampling time points. Blood samples of 0.3 ml were withdrawn from each rat at 15, 30, 45 min, 1, 1.5, 2, 4, 6, 8 and 12 h, post dose of atorvastatin. Zero hour blood sample was collected prior to dosing of EGCG. The samples were collected into heparinised microcentrifuge tubes and centrifuged at 1,500×*g* for 7 min at 4°C. The resulting plasma samples were kept frozen at -80ºC prior to LC analysis.

The maximum plasma concentration (C_{max}) and the time to reach the maximum concentration (T_{max}) was directly determined from the plasma concentration *versus* time curves. The area under the curve from 0 to t (AUC_{0-t}) was calculated following linear trapezoidal rule by summing the area from 0 to t h. Elimination rate constant (K_{el}) was determined by taking the absolute value of the slope of any three points lying on a straight line of the curve after the C_{max}, i.e. during the elimination phase. Elimination half life ($t_{1/2}$) was determined using the relationship $t_{1/2} = 0.693/K_{el}$.

RESULTS AND DISCUSSION

A new analytical method for simultaneous

estimation of EGCG and atorvastatin in rat plasma was developed and validated. The method was found to be reproducible and specific. The chromatograms of blank rat plasma, unextracted standards of the drugs, plasma spiked with standards of EGCG and atorvastatin at a concentration of 600 ng/ml and plasma samples from the animals after administration of EGCG and atorvastatin in combination are shown in Fig. 2 (a–d), respectively. Retention times of EGCG, tinidazole (internal standard) and atorvastatin were 5.74, 6.74 and 14.01 min, respectively. Total LC run time was 15 min. EGCG and atorvastatin were unambiguously identified in plasma upon comparison of the retention times with those of their respective standards.

Linearity and lower limit of quantification

Standard curves were constructed by plotting ratio of peak area ratio of drugs to internal standard *versus* their respective concentration. The standard curves were linear in the range of 30–1000 ng/mL EGCG and atorvastatin. The calibration curves for EGCG and atorvastatin from spiked plasma samples could be described by the equation $y = 0.031 (\pm 0.006)x + 0.007(\pm 0.003)$ ($r^2 = 0.9998$) and $y = 0.039(\pm 0.005)x + 0.006(\pm 0.002)$ ($r^2 = 0.9996$), respectively. The lower limits of quantification were found to be 30 ng/ml for both the analytes. The lower limits of detection were 5 ng/ml. No carryover was observed upon the injection of standard sample of highest concentration.

Precision and accuracy

Table 1 shows intra-day and inter-day precision and accuracy. The intra-day precisions i.e., coefficient of variation (CV) of high, medium and low QC samples of EGCG were 3.6, 1.9 and 4.7%, respectively and of atorvastatin were 4.2, 2.9 and 5.1%, respectively. Inter-day precisions (CV) of high, medium and low QC samples of EGCG were 2.2, 3.6 and 5.5% respectively and of atorvastatin were 3.5, 2.8 and 4.8%, respectively. Intra-day and inter-day accuracies ranged between 92.3 and 96.8% for EGCG and 91.8 to 96.4% for atorvastatin. These results inferred that the developed method was accurate and precise.

Recovery

Recovery of EGCG and atorvastatin was in the range of 84-87% and 82-85%, respectively. The recovery of internal standard was 88%.

Stability study of plasma samples

Table 2 shows the results for bench-top stability, freeze–thaw stability, dry extract stability, auto sampler and long-term stability. The results illustrates that EGCG and atorvastatin were stable during processing and storage for up to one month.

Pharmacokinetic parameters

Pharmacokinetic parameters of the different animal groups are shown in Table 3 and mean plasma concentration curves are shown in Fig. 3(a-b).

Statistical analysis

Student's *t*-test was applied to determine the difference in oral bioavailability of atorvastatin when administered alone and in combination with EGCG. The results showed that EGCG significantly ($p \leq 0.05$) increased the oral bioavailability of atorvastatin. However, no significant change in the pharmacokinetic profile of EGCG was observed amongst the animal group treated with EGCG alone and in combination with atorvastatin. The results are expressed as mean±SD.

Previous findings suggest regarding the potential of EGCG as a P-glycoprotein inhibitor [11]. Researchers have also suggested P-glycoprotein inhibition as an approach to overcome P-glycoprotein mediated drug efflux [5]. Based on these findings, we tried to evaluate the potential of EGCG for improving the oral bioavailability of atorvastatin. The results showed that EGCG significantly improved the oral bioavailability of atorvastatin. Thus, it may be inferred that EGCG would have either inhibited P-glycoprotein or modulated its activity, which subsequently increased the bioavailability of atorvastatin. However, further mechanistic studies are required to understand this observed altered bioavailability.

Table 1.Intra-day and inter-day precision and accuracy for EGCG and atorvastatin in rat plasma.

Nominal concentration (ng/mL)	Calculated concentration (ng/mL)		Precision (% CV)		Accuracy (%)	
	EGCG	Atorvastatin	EGCG	Atorvastatin	EGCG	Atorvastatin
[a]Intra-day (n = 6)						
900	854.5	867.5	3.6	4.2	94.9	96.4
500	468.1	459.2	1.9	2.9	93.6	91.8
150	138.4	141.5	4.7	5.1	92.3	92.3
[b]Inter-day (n = 18)						
900	871.5	859.7	2.2	3.5	96.8	95.5
500	471.2	451.1	3.6	2.8	94.1	94.4
150	141.3	138.5	5.5	4.8	94.2	92.3

[a]Intra-day precision: Data expressed as mean (n = 6)

[b]Inter-day precision: Data is expressed as mean (n = 18)

Table 2.Stability data of EGCG and atorvastatin in rat plasma.

QC samples	Mean concentration observed at 0 h		Mean concentration observed at last h		% Deviation	
	EGCG	Atorvastatin	EGCG	Atorvastatin	EGCG	Atorvastatin
Bench top stability (n = 6) (after 6 h)						
High	872.4	863.1	870.2	858.7	3.1	3.9
Low	141.4	139.5	139.7	137.2	4.7	4.2
Freeze thaw stability (n = 6) (three cycles)						
High	871.5	865.4	862.7	852.6	3.9	4.1
Low	143.4	140.1	138.2	138.7	5.6	2.9
Dry extract stability (n = 6) (24 h)						
High	878.3	862.4	869.2	855.8	3.9	3.1
Low	142.2	138.1	140.6	136.4	2.5	4.3
Long- term stability (n = 6) (30 days)						
High	862.4	870.3	856.2	866.1	3.5	2.7
Low	140.2	142.1	137.2	139.5	1.3	3.6
Auto-sampler stability (n = 6) (12 h)						
High	864.7	860.2	858.3	851.7	2.9	1.7
Low	143.1	140.2	139.6	138.1	5.4	4.2

Data is expressed as mean and %CV

Table 3. Pharmacokinetic parameters of EGCG and atorvastatin in rats upon oral administration in different animal groups

Pharmacokinetic parameters	Group 1 (Atorvastatin)	Group 2 (EGCG+Atorvastatin) Atorvastatin profile	Group 2 (EGCG+Atorvastatin) EGCG profile	Group 3 (EGCG)
C_{max}(ng/ml)	256.59 ± 7.98	479.17 ± 13.71	191 ± 8.76	202.25 ± 9.34
T_{max}(h)	0.45 ± 0.00	0.45 ± 0.00	0.75 ± 0.00	0.75 ± 0.00
$AUC_{(0-t)}$ (ng h/ml)	55245.64 ± 821.57	82852.51 ± 1023.43	47827.51 ± 791.87	48157.69 ± 695.88
K_{el}(1/h)	1.25 ± 0.11	1.98 ± 0.14	0.92 ± 0.13	0.89 ± 0.15
$t_{1/2}$(h)	4.17 ± 0.25	4.86 ± 0.79	1.2 ± 0.05	1.09 ± 0.03

Data are expressed as Mean±SD, (n=6)

Figure 1. Chemical structures of (a) atorvastatin, (b) epigallocatechin-3-gallate and (c) tinidazole.

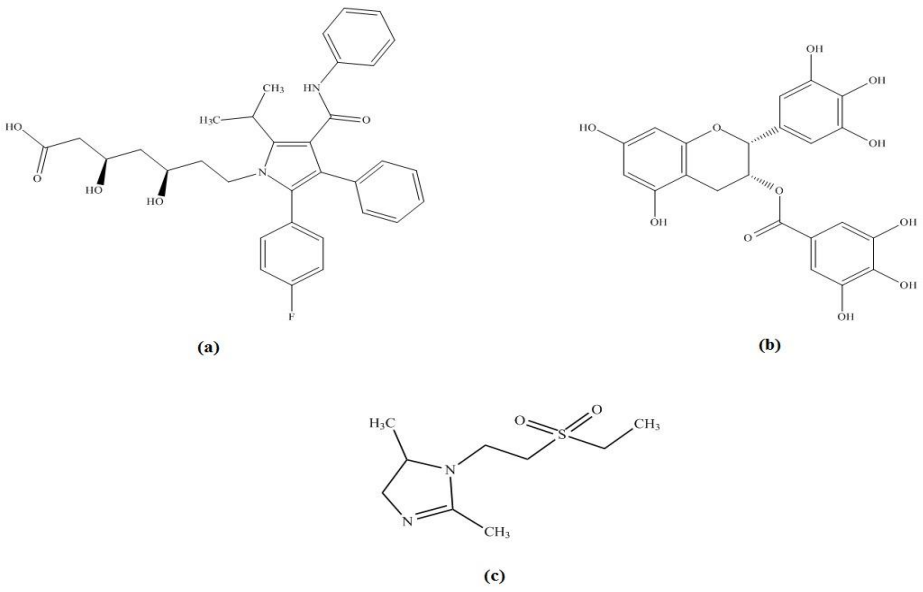


Figure 2. LC chromatograms

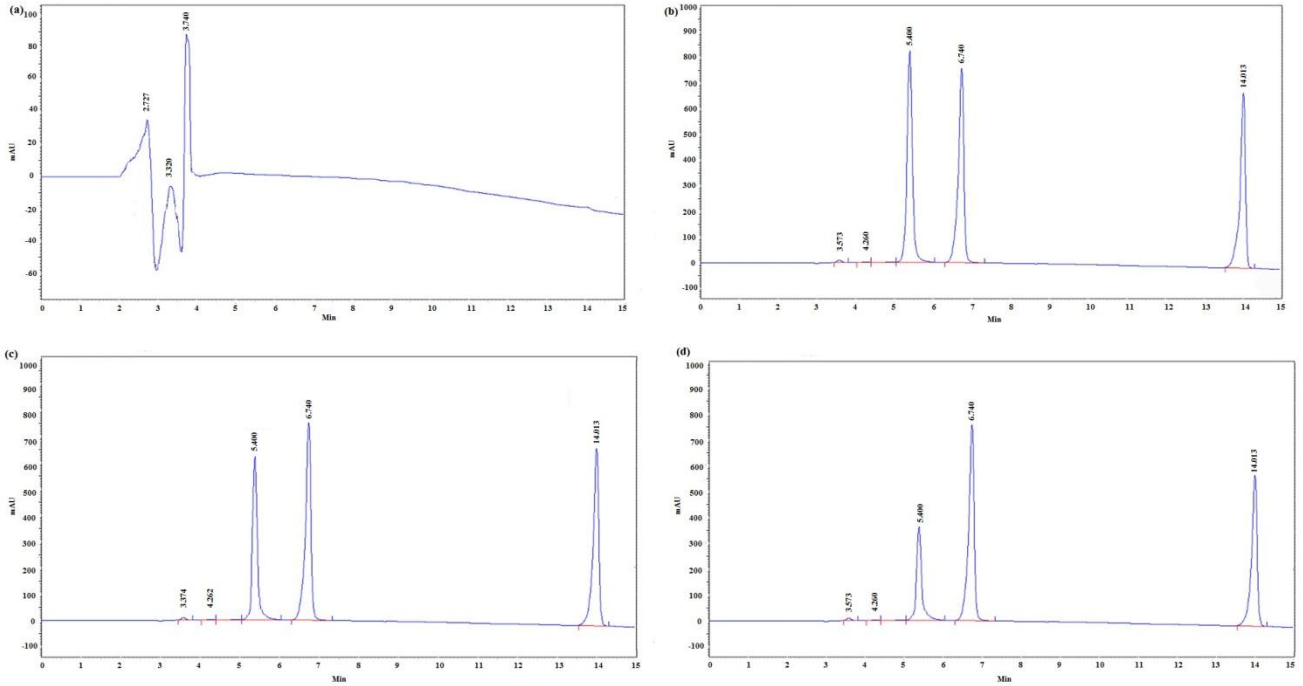
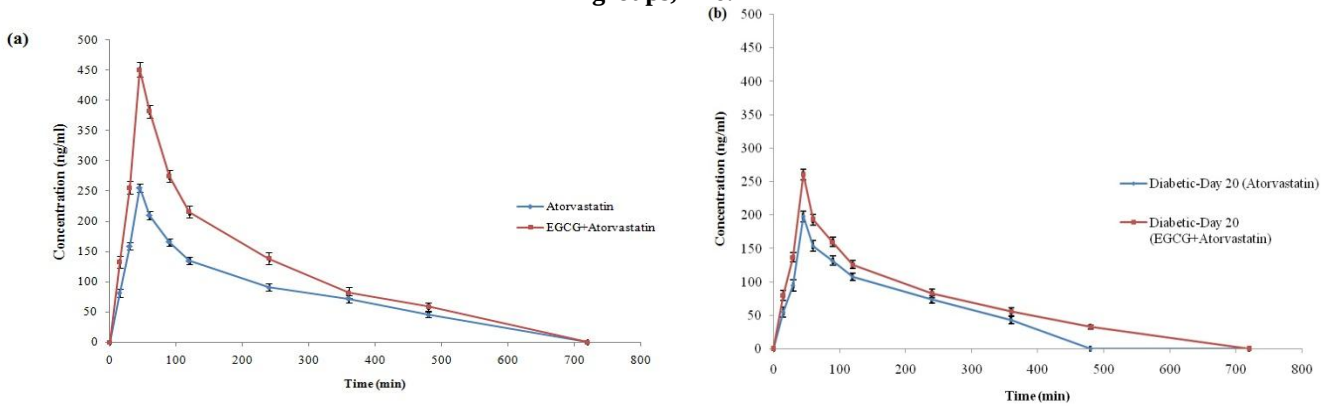


Figure 3. Mean (± SD) plasma concentration of (a) atorvastatin and (b) epigallocatechin-3-gallate in different animal groups, n=6.



CONCLUSION

A simple, precise, specific and sensitive method for simultaneous quantification of EGCG and atorvastatin in rat plasma was developed and validated. This is the first reported LC-UV method for simultaneous quantification of these two analytes in biological matrix. Stability studies showed that EGCG and atorvastatin were stable for short and long-term periods (30 days). This analytical procedure was successfully applied to the pharmacokinetic study in male *Wistar* rats. The results showed that EGCG significantly improved the oral bioavailability of atorvastatin. This method may be applied for preclinical pharmacokinetic studies of any new formulation containing EGCG and atorvastatinin combination and can be also tried for human pharmacokinetic studies.

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