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Chromatography–tandem mass spectrometry method for the simultaneous quantitation of metoprolol succinate and simvastatin in human plasma

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ABSTRACT

The liquid chromatographic–tandem mass spectrometry method was developed for the accurate quantitation of metoprolol succinate (MET) and simvastatin (SIM) in human plasma which were obtained from the pharmacokinetic (PK) study. The sample purification and pre-concentration was performed by protein precipitation technique using propranolol hydrochloride as working internal standard (WIS). The chromatographic separation was achieved using an isocratic mobile phase consisting of a mixture of acetonitrile and 0.5% formic acid (90:10 (v/v), pH 3.5) flowing through C18 column at a flow rate of 0.2 ml/min. Electro spray ionization (ESI) with multiple reaction monitoring (MRM) was used to acquire mass spectra. Ions were monitored in positive mode and the mass transitions measured were m/z 268.1 → m/z 103.2, m/z 441.3 → m/z 325.1 and m/z 260.0 → m/z 129.5 for MET, SIM and WIS, respectively. An extensive pre-study method validation was carried out in accordance with USFDA guidelines. The linearity for the calibration curve in the concentration range of 1.0–500.0 and 0.1–20 ng/ml for MET and SIM, respectively and the lower limits of quantitations (LLOQ) were 1.0 and 0.1 ng/ml for MET and SIM, respectively. The method was successfully applied to a PK study on fixed dose combination (FDC) tablet containing MET and SIM in healthy human subjects.

1. Introduction

Cardiovascular disorders (CVD) are still among the most common causes of death in the western industrialized countries. And they are using β-adrenergic blocking agents along with cholesterol lowering agents for the treatment of CVD. Widely, the β-adrenergic blocking agent of metoprolol succinate (MET) and HMG-CoA (3-hydroxy-3-methylglutaryl coenzyme-A) reductase inhibitor of simvastatin (SIM) are used in the treatment of CVD [1,2]. Also the combination of MET along with SIM has been proved as clinically more beneficial, because of the additive beneficial effects [3]. Here, our aspiration of this research is to develop a highly sensitive and validated bioanalytical method to evaluate the pharmacokinetics of fixed dose combination (FDC) tablet containing MET and SIM using healthy human subjects.

Basically, the quantitation of drugs in biological samples is a vital part of drug discovery and drug development. Furthermore, the liquid chromatography (LC) in combination with tandem mass spectrometry (MS/MS) is the state-of-the-art technique in quantitation of drugs in biological samples. Several bioanalytical methods were reported for the quantitation of MET and SIM individually. Such as, HPLC methods [1,4], LC–MS/MS with API source [5–8] and MET along with some other drugs using LC–MS/MS [9,10] were reported for the quantitation of MET. Same as, few methods like, UV/vis spectrometry [11] and LC–MS/MS methods [12,13] were reported for the quantitation of SIM. But the proposed method focuses on simultaneous quantitation of MET along with SIM in human plasma using LC–MS/MS. In addition, the efficacy of the formerly published methods is inadequate by lack of sensitivity, laborious in sample clean up procedures, need of derivatization in sensitivity, inability in quantitation of MET and SIM simultaneously and the use of less readily available working internal standard (WIS).

In this proposed method, a simple and single step precipitation technique was used for the isolation of drugs from the plasma and propranolol hydrochloride was used as WIS. The electro spray ionization (ESI) source was used for the ionization of molecules in LC–MS/MS. The validation report has shown good results in terms of precision, accuracy, ruggedness and reproducibility. In addition, this method was successfully applied to a pharmacokinetic (PK) study using healthy human subjects.

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2. Experimental

2.1. Chemicals and supplies

The reference substances were purchased from the following manufactures: metoprolol succinate from Aurobindo Pharma (Hyderabad, India), simvastatin from ZydusCadila (Ahmedabad, India) and propranolol hydrochloride from Psyco Remedies (Ludhiana, India). The reference products were purchased from the following manufactures: metoprolol succinate (Seloken® XL-100 mg tablet) from AstraZeneca, Sweden and simvastatin (BIOSIM-20 mg tablet) Biochem Pharmaceutical Industries Ltd., India. The test product (FDC tablet containing 100 mg of MET as sustained release and 20 mg of SIM as immediate release) was manufactured as in-house product. Formic acid and ammonium acetate were procured from Sigma–Aldrich (Bangalore, India) and acetonitrile (LiChrosolv®) was purchased from Merck (Mumbai, India).

2.2. Instrumentation

A SHIMADZU HPLC system consisting of LC-20AD pump; SIL-20AC auto-sampler and CTO-10ASvp column oven (Shimadzu, Kyoto, Japan) was used for the separation of analytes. A triple quadrupole MS/MS system consisting of API-2000® source (AB Sciex Instruments, Foster, CA) was coupled with the HPLC for the detection of analytes. The data integration was performed with Analyst 1.4.1 software version (AB Sciex Instruments, Foster, CA).

2.3. Liquid chromatography

The LC separation was achieved using Gemini-C18 column (50 mm × 2.0 mm i.d., 3 μm, Phenomenex, Torrance, CA, USA) with a Security Guard C18 guard column. The mobile phase consisting of a mixture of acetonitrile and 0.5% formic acid (90:10 (v/v), pH 3.5) was delivered at a flow rate of 0.2 ml/min. The column temperature was maintained at 20 ± 2 °C and the injection volume was maintained at 10.0 μl for each sample.

2.4. Mass spectrometry

The ESI source was operated on the positive ionization mode for all the molecules and channel electron multiplier (CEM) was used as detector. Zero air was used as an ion source gas and heater gas. Ultra high purity nitrogen was served as curtain gas and collisional gas. The simultaneous determination of precursor ions and fragment ions of both analyte and WIS were done by using multiple reaction monitoring (MRM) mode.

2.5. Calibration and control samples

Stock solutions of MET, SIM and WIS were prepared individually at the concentration of 10 μg/ml and stored in a cold storage (2–8 °C). Methanol and water were used as solvent in the ratio of 1:1 throughout the analysis. The stock solutions of MET and SIM were further diluted to give a series of combined standard solutions with the concentrations of 10.0, 25.0, 50.0, 100.0, 250.0, 500.0, 1000.0, 2500.0, 5000.0 ng/ml and 1.0, 2.5, 5.0, 10.0, 25.0, 50.0, 100.0, 150.0, 200.0 ng/ml, respectively. A solution containing 300.0 ng/ml of WIS was prepared only with methanol (HPLC grade). The plasma calibration samples were prepared at the concentrations of 1.0, 2.5, 5.0, 10.0, 25.0, 50.0, 100.0, 250.0, 500.0 ng/ml and 0.1, 0.25, 0.5, 1.0, 2.5, 5.0, 10.0, 15.0, 20.0 ng/ml for MET and SIM, respectively, by spiking 0.1 ml of the series standard solutions with 0.9 ml of blank plasma which were obtained from healthy human volunteers. The quality control (QC) samples were prepared in triplicate using the pooled blank plasma at low, medium and high concentrations of 2.5, 25.0, 250.0 ng/ml and 0.25, 2.5, 15.0 ng/ml for MET and SIM, respectively. The spiked samples were then treated as following as the sample preparation procedure as indicated in Section 2.6.

2.6. Sample processing

The protein precipitation technique was used as an extraction technique on sample preparation. WIS (400 μl) was added to 200 μl of plasma sample containing the analytes. The sample was mixed briefly by vortex-mixing up to 2 min and centrifuged at 10,000 × *g* for 10 min to separate the supernatant liquid. The eluate was filtered through 0.2 μm membrane and 10 μl aliquot was injected onto the HPLC system with MS/MS detection.

2.7. Bioanalytical method validation

The method was validated to meet the acceptance criteria industrial guidance for the bioanalytical method validation [14]. Batches, consisting of triplicate calibration standards at each concentration, were analysed on three different days to complete the method validation. In each batch, the QC samples of low, medium and high concentrations were analysed in sets of six replicates to evaluate the intra- and inter-day precision and accuracy. For testing the specificity, six randomly selected control drug free human plasma samples were processed as per the sample preparation procedure (Section 2.6) and injected into the LC–MS/MS system in order to determine the extent to which endogenous plasma components may interference at retention times (Rt)s of MET, SIM and WIS.

To establish the linearity, a series of calibration standards (Section 2.5) were prepared by adding known concentration of working standards of analytes and WIS in drug free human plasmas and analysed ($n = 6$). The lowest standard on the calibration curve was to be accepted as lower limit of quantitations (LLOQ) if the detector response of analytes was five times that of drug free (blank) processed human plasma [14]. The concentrations of analytes in plasma samples were determined by back-calculation of the observed peak-area ratios of analytes and WIS from the best-fit calibration curve using a weighted ($1/x$) linear regression. During routine analysis, each analytical run included a set of standard sample, a set of QC sample in duplicate and plasma samples to be determined.

The extraction recovery of MET, SIM and WIS was determined at low, medium and high QC concentrations by comparing the responses from plasma samples spiked before extraction with those from plasma samples extracted and spiked after extraction. The matrix effect (ME) was investigated by preparing two sets of samples with low and high QC concentrations of MET, SIM. Set-1 was prepared to evaluate the MS/MS response of reference standard solutions injected in mobile phase. Reference standard solutions of MET and SIM were diluted with mobile phase to reach the concentration expected in plasma spiked samples. Set-2 consisted of six blank plasma samples spiked with reference standard solutions after extraction. The WIS was not added to standards. The mean peak area and its R.S.D. values were calculated for set-1 and set-2. The ME% values of 100% indicates absences of any matrix effects, where less than 100% indicates ion suppression and value more than 100% indicates ion enhancement.

The stability tests were performed in low and high QC samples in terms of short-term, long-term storage, freeze/thaw stabilities, auto-sampler stability and stock solution stability. The short-term (room temperature) stability was assessed at room temperature for 24 h and long-term stability was assessed at −20 °C for 45 days. Freeze–thaw stability was performed by freeze–thawing for 4 times specifically. Freezing was performed at −20 °C for 24 h and thawed

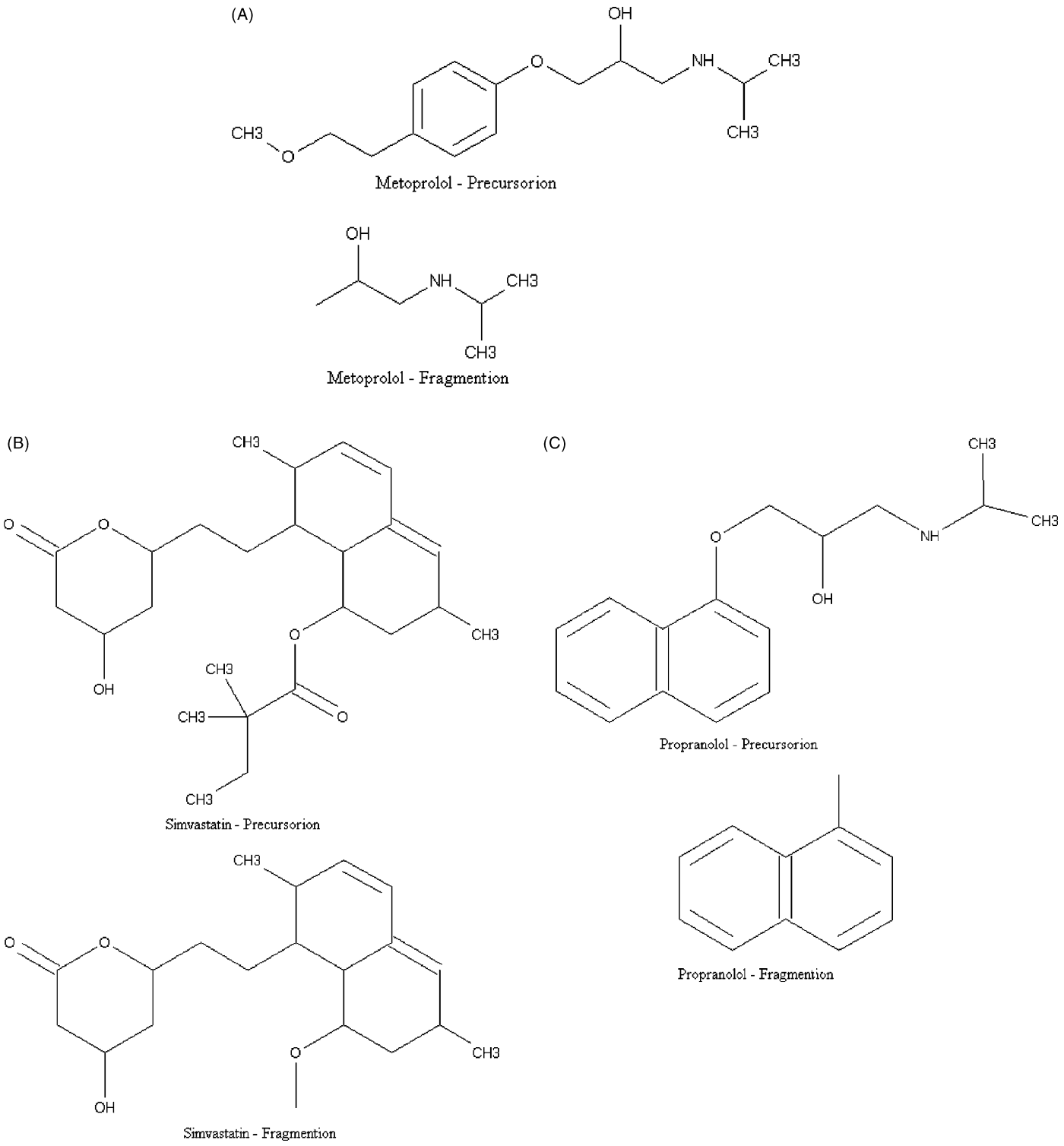

Fig. 1. Chemical structure of (A) metoprolol, (B) simvastatin and (C) propranolol (WIS).

at room temperature. Auto-sampler stability (post-preparative stability) was tested by comparing after-day analysis with the first intra-day analysis results. It has estimated by storing the extracted samples at 10 °C in an auto-sampler for 30 h. The stock solution stability samples were prepared in methanol with water (1:1 ratio) and stored at 2–8 °C for 35 days. The samples were further diluted before injection. The obtained results were compared with the results which were obtained from the freshly prepared solutions of the same concentration.

2.8. Application to pharmacokinetic study

The proposed LC–MS/MS method was successfully applied to a PK study on FDC tablet using healthy human subjects. Six Indian healthy human volunteers, aged 22–32 years were admitted in Bioequivalence Study Center, Jadavpur University. The in-house product was used as "test" product and the "reference" product was purchased from the market (Section 2.1). About 5.0 ml of blood samples were collected from the forehand vein into heparinized tubes

Table 1
Accuracy and precision of intra- and inter-run analysis for the quantitation of MET and SIM in human plasma. (n = 3 days, six replicates per day).

Added C[a] (ng/ml)	Found C (ng/ml)[a] mean ± S.D.[b]	Intra-run R.S.D. (%)[c]	Inter-run R.S.D. (%)[c]	Mean recovery (%)	(%) R.E.[d]
MET					
2.5	2.53 ± 0.12	2.04	1.62	100.87	1.37
25.0	25.73 ± 1.17	2.82	3.49	102.43	2.99
250.0	252.69 ± 3.54	3.81	4.14	103.55	2.68
SIM					
0.25	0.25 ± 0.01	1.24	2.35	98.93	2.69
2.5	2.55 ± 0.14	3.16	3.18	100.42	3.51
15.0	15.72 ± 1.39	3.29	4.51	99.65	4.30

[a] Concentration.
[b] Standard deviation.
[c] Relative standard deviation.
[d] Relative error.

before (0 h) and at 0.5, 1.0, 1.5, 2.0, 2.5, 3.0, 4.0, 6.0, 8.0, 10.0, 12.0, 18.0 and 24.0 h after dosing. Plasma was separated by centrifugation at 3000 × g for 10 min and kept frozen at −20 °C until analysis. The PK parameters of T_{max} and C_{max} were estimated directly from the experimental observations of plasma concentrations. The AUC_{0-t} was estimated by a combination of linear and logarithmic trapezoidal methods and the $AUC_{0-\infty}$ was estimated by the following equation: $AUC_{0-\infty} = AUC_{0-t} + C_{last}/K_{el}$. The K_{el} was estimated by performing log linear regression on the concentration versus time data points. The $t_{1/2}$ was calculated by using the equation of $0.693/K_{el}$.

3. Results and discussion

3.1. Separation and quantitation

The chromatographic separation was achieved using C18 column with mobile phase consisting of mixture of acetonitrile and 0.5% formic acid (90:10 (v/v), pH 3.5). The compounds of MET, SIM and WIS were exhibited with the total run time of 5.0 min at the Rt(s) of 1.34, 2.23 and 1.56 min, respectively. Methanol was used as a solvent for the precipitation of protein in the sample processing technique and propranolol hydrochloride was used as WIS. Clean chromatograms were obtained with the yield of highest recovery and no significant matrix effect was found.

In mass spectrometry, the parameters involving turbo spray needle temperature, heater temperature, flow rate of nebulizing gas and curtain gas were optimized to obtain the protonated molecules of MET, SIM and WIS. The collision energy was optimized to achieve maximum response of the fragment ion peak. The transitions selected were m/z 268.1 → m/z 103.2; m/z 441.3 → m/z 325.1 and m/z 260.0 → m/z 129.5 for MET, SIM and WIS, respectively. The MET and WIS were given the most intense signal (precursor ion) of $[M+H]^+$. The SIM was given the most intense signal (precursor ion) of $[M+Na+H]^+$, due to formation of sodium adduct by neutral loss of acetonitrile to gain $[M+Na]^+$ [15]. The precursor-ion and product-ion structures of MET, SIM and WIS were shown in Fig. 1 and the representative extracted-ion MRM chromatograms of MET, SIM and WIS were shown in Fig. 2.

3.2. Validation

3.2.1. Accuracy and precision

The accuracy and precision results were summarized in Table 1. Accuracy was determined by calculating the percentage deviation observed in the analysis of QC samples and expressed as relative error (R.E.). The intra- and inter-run precision was expressed as RSD. In each level of QC samples, both intra- and inter-run precisions of MET and SIM were found to be between 1.24 and 4.51%. The accuracy of MET and SIM was found to be between 1.37 and 4.30%, it indicating the acceptable accuracy and precision of the proposed method.

3.2.2. Specificity

The specificity of the method was investigated by comparing the chromatograms of blank plasma samples with known concentration spiked blank plasma sample chromatograms. The chromatograms were indicating that no significant interferences from endogenous substances in plasma. The precursor ions were observed at m/z 268.1; m/z 441.3; m/z 260.0 for MET, SIM and WIS, respectively. Precursor-to-product-ion transitions were m/z 268.1 → m/z 103.2; m/z 441.3 → m/z 325.1 and m/z 260.0 → m/z 129.5 for MET, SIM and WIS, respectively.

3.2.3. Linearity and LLOQ

The linearity of each calibration curves were determined by plotting the peak-area ratio (y) of analytes to WIS versus the nominal concentration (x) of MET and SIM. The calibration curves were obtained by weighted ($1/x$) linear regression analysis. To evaluate the linearity of the LC–MS/MS method, plasma calibration curves were determined in triplicate on three separate days. Each calibrations, good linearity was observed over the concentration range of 1.0–500.0 and 0.1–20 ng/ml for MET and SIM, respectively. And the correlation co-efficient (r^2) values were 0.9972 and 0.9936 for MET and SIM, respectively. No significant changes in the values of slope, intercept and correlation co-efficient on both inter- and intra-day calibrations. The % R.E. and % co-efficient of variation (CV) were found to be less than 5.0%, which are sufficient for PK study of MET and SIM, respectively in human subjects. The lower limit of detections (LLOD) were found to be 0.3 and 0.03 ng/ml for MET and SIM, respectively and the LLOQ were found to be 1.0 and 0.1 ng/ml for MET and SIM respectively.

3.2.4. Recovery (extraction efficiency)

The extraction efficiency of MET and SIM from human plasma was determined by comparing peak areas from plasma samples spiked before extraction with those from plasma samples extracted and spiked after extraction. The results showed that the extraction recoveries were 87.32 ± 3.21, 89.67 ± 4.01, 90.11 ± 3.64% and 85.19 ± 2.92, 84.63 ± 4.41, 83.91 ± 3.75% from human plasma at the QC concentrations of 2.5, 25.0, 250.0 ng/ml and 0.25, 2.5, 15.0 ng/ml for MET and SIM, respectively and the WIS was found to be 81.69 ± 4.15% at concentration of 20.0 ng/ml.

3.2.5. Matrix effect

The matrix effect of the method was considerably reduced and suppressed by utilizing the ESI source and by eliminating a

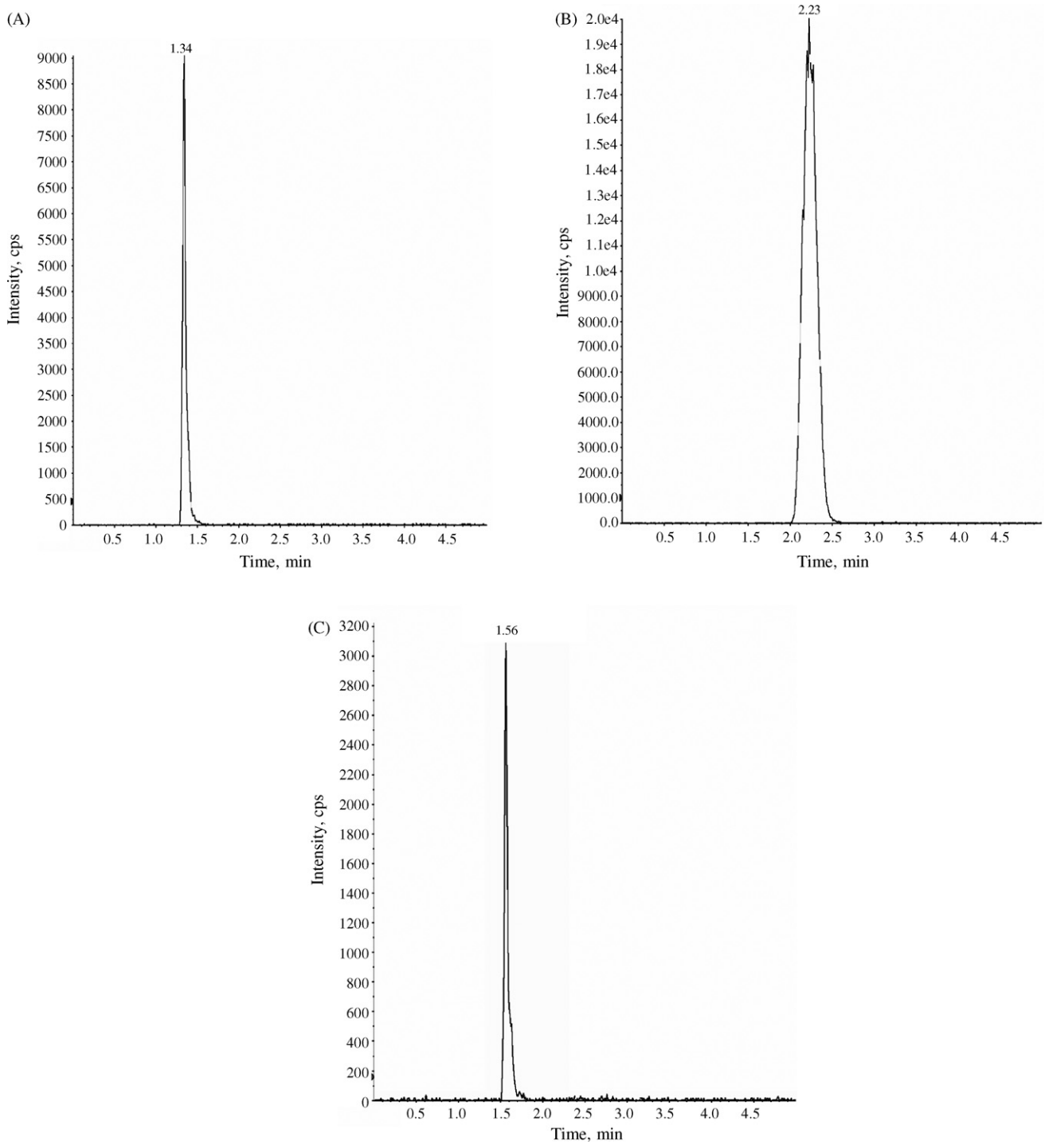

Fig. 2. The representative extracted-ion MRM chromatograms of (A) metoprolol, (B) simvastatin and (C) propranolol from plasma sample. The chromatographic separation was achieved using C18 column with mobile phase consisting of acetonitrile and 0.5% formic acid (90:10 (v/v), pH 3.5). The Rt(s) of MET, SIM and WIS were 1.34, 2.23 and 1.56 min, respectively.

number of endogenous components from plasma extracts during sample preparation. ME—the possibility of ionization suppression or enhancement was evaluated by comparing the results of analysis of two sets of samples as follows:

$$\text{ME}(\%) = \frac{B}{A} \times 100$$

[where A is the mean peak area of set-1 and B is the mean peak area of set-2].

3.2.6. Stability

The stability tests were assessed by the low and high QC samples as described in Section 2.7. The short-term stability samples were assessed in triplicate in three different days and the concentrations obtained were compared with the actual values of QC samples. The mean% deviation was found to less than 2.0% for both MET and SIM. The long-term stability was assessed in triplicate and the mean% deviation was found to be less than 3.0%. The freeze–thaw stability was performed and the deviation between the freeze values found to be less than 3.0% of the nominal values for both MET and SIM.

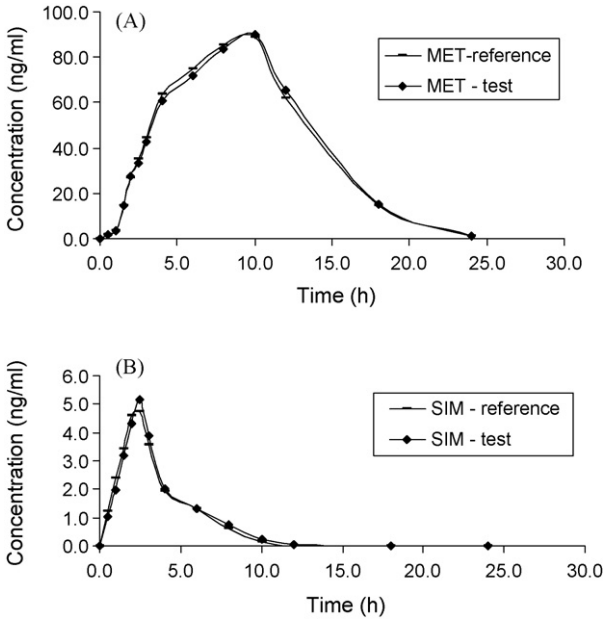

Fig. 3. The mean plasma concentration–time profiles of (A) metoprolol succinate 100 mg as SR and (B) simvastatin 20 mg as IR after oral administration of reference and test preparation to six healthy human subjects.

Table 2
Pharmacokinetic parameters of single dose of FDC tablet containing 100 mg of metoprolol succinate as SR and 20 mg of simvastatin as IR after oral administration to six healthy human volunteers.

Pharmacokinetic parameters	Reference		Test	
	Mean	±S.D.[a]	Mean	±S.D.[a]
Metoprolol				
C_{max} (ng/ml)	95.62	2.11	94.85	2.34
T_{max} (h)	10.00	1.27	11.00	1.10
AUC_{0-t} (ng/ml h)	968.36	108.28	1013.83	50.00
$AUC_{0-\alpha}$ (ng/ml h)	1296.25	216.84	1392.65	106.07
$t_{1/2}$ (h)	2.99	0.38	3.11	0.32
K_e (1/h)	0.23	0.03	0.24	0.04
Simvastatin				
C_{max} (ng/ml)	5.37	0.49	5.39	0.60
T_{max} (h)	2.25	0.27	2.33	0.26
AUC_{0-t} (ng/ml h)	17.93	2.96	18.56	1.56
$AUC_{0-\alpha}$ (ng/ml h)	18.06	3.00	18.66	1.53
$t_{1/2}$ (h)	2.42	0.64	2.22	0.10
K_e (1/h)	0.50	0.08	0.45	0.02

[a] Standard deviation.

Auto-sampler stability was performed and the mean% deviation of the stability results was found to be less than 4.0% of the nominal results for both MET and SIM. Finally the stock solution stability was performed on MET, SIM and WIS samples and the mean% deviations results were found to be less than 2.0%.

3.3. Application to pharmacokinetic study

The proposed LC–MS/MS method was successfully applied to a PK study on FDC tablet in healthy human subjects. The basic PK parameters (T_{max}, C_{max}, AUC_{0-t}, $AUC_{0-\infty}$, $t_{1/2}$ and K_{el}) obtained for test and reference in healthy human subjects used in this study were consistent with the previously reported PK data [5,12,15]. And the FDC tablet exhibited no significant difference with respect to the PK parameters of individual tablets of MET and SIM. With no serious clinical adverse events, it was concluded that the test and reference were bioequivalent. The PK parameters of both MET and SIM were summarized in Table 2. The mean (±S.D.) plasma concentration–time profile of healthy human volunteers, after single oral dose administration of reference and test products of MET and SIM tablets were shown in Fig. 3.

4. Conclusions

The CVD(s) like chronic diseases, the simultaneous quantitation methods are very much essential, because of the need of routine therapeutic drug monitoring. The proposed method is the first method for the simultaneous determination of MET and SIM in human plasma using LC–MS/MS in electro spray ionization source with MRM mode. It is a simple, rapid, sensitive, specific and highly validated one as per the USFDA guidelines [14]. It was showing very good precision and accuracy in the quantitation of MET and SIM in human plasma obtained from pharmacokinetic studies. The simplicity, speed of sample processing, shorter run time and cost-effectiveness were the other advantages of this method. Finally, the wide range of linearity and LLOQ of this method facilitates its application in the bioavailability, bioequivalence and clinical studies.

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