Poonam Gaikwad. *et al.* / *International Journal of Biological & Pharmaceutical Research.* 2013; 4(3): 216-224.

e- ISSN 0976 - 3651
Print ISSN 2229 - 7480

International Journal of Biological & Pharmaceutical Research
Journal homepage: www.ijbpr.com

IJBPR

HPLC WITH UV AND MASS SPECTROMETRIC DETECTION FOR QUANTIFYING TOTAL EZETIMIBE IN HUMAN PLASMA: VALIDATION AND APPLICATION IN PHARMACOKINETIC STUDY

***[1]Poonam Gaikwad, [2]Ashok Bhagwat, [2]Suvarna Bhoir**

[1]School of Science, SVKM's NMIMS (deemed-to-be) University, Vile Parle (w), Mumbai-400056, India.
[2]C. B. Patel Research Centre, Vile Parle (w), Mumbai-400056, India.

ABSTRACT

In this article, two different methods were developed and compared for quantifying total Ezetimibe (EZM) in human plasma. The total EZM was quantified with liquid chromatography method, after liquid-liquid extraction; one with UV detection (LC-UV) and the other with mass spectrometric detection (LC-MS/MS). Chromatographic separation was achieved on Symmetry Shield C18 column (250 × 4.6 mm i.d., 5 µ) using an isocratic elution with mobile phase composition consisting of Acetonitrile and 1mM Ammonium acetate in the proportion of 60:40 (v/v). For the estimation of total EZM, the glucuronide form of EZM is hydrolyzed by using β-glucuronidase and extracted using t-butyl methyl ether from human plasma samples. 20 µL was loaded onto the system and eluent was monitored at 233 nm. Mass spectrometric data was acquired in multiple reaction monitoring mode. The calibration curve was linear in the concentration range of 30 – 500 ng/mL (LC-UV) and 1.02 – 303.07 ng/mL (LC-MS/MS) with correlation coefficients $r^2 \geq 0.997$ and $r^2 \geq 0.999$, respectively. The inter-day coefficients of variation (CVs) ranged from 6.51 to 9.56% (LC-UV) and 0.78 to 4.21% (LC-MS/MS) and that of intra-day ranged from 6.78 to 9.81% (LC-UV) and 0.86 to 4.76% (LC-MS/MS) at four different concentrations. The resulting method was successfully applied for the Pharmacokinetic study in healthy human subjects.

Key Words: Ezetimibe, β-glucuronidase, HPLC, Pharmacokinetics, Human plasma, LC-MS/MS.

INTRODUCTION

Ezetimibe [1-(4-fluorophenyl)-3(R)-[3-(4-fluoro phenyl)-3(S)-hydroxypropyl]-4 (S)- (4-hydroxy phenyl) -2-azetidinone] is the first in a new class of cholesterol lowering agents known as cholesterol absorption inhibitors. After oral administration ezetimibe (EZM) is rapidly absorbed and extensively conjugated to Ezetimibe Phenoxy glucuronide (EZMG), which is pharmacologically active in vivo. Thus, EZM and EZMG are the major drug derived compounds detected in plasma, constituting approximately 10-20% and 80-90% of the total drug in plasma, respectively (Rosenblum *et al*., 1998; Van Heek *et al*., 2000).

Although the direct detection of the glucuronide conjugate overcomes the critical limitations of approaches that involve enzymatic cleavage procedures and/or derivatization, indirect methods are still a choice for the routine pharmacokinetic analysis of EZM and its glucuronide in human biological matrices (Kaushik *et al*., 2006). In case of indirect measurement, the quantification methods for glucuronides involve an enzymatic (β-glucuronidase) or an acid/base hydrolysis of the glucuronide ether bond prior to HPLC analysis (Kadi *et al*., 2009) as described in Fig. 1. Enzymic hydrolysis is usually preferred due to the fact that although an acid/base hydrolytic procedure is time saving and cost effective; many drugs are destroyed by its extreme conditions (F.

Corresponding Author

Poonam V. Gaikwad
Email: poonam.gaikwad@nmims.edu.in

Fish *et al*., 1974). β-glucuronidase is used for the enzymatic hydrolysis of glucuronides from urine (Xu *et al*., 2002; Staimer *et al*., 2001), plasma(Zhai *et al*., 2001; Andersen *et al*., 1999), and other fluids (Nobilis *et al*., 2004) prior to analysis by enzyme immunoassay, mass spectrometry, gas chromatography, high performance liquid chromatography, or other means.

To date there are few analytical methods reported for the estimation of EZM and EZMG. The reported bioanalytical methods were based either on HPLC coupled to UV detector (Sistla *et al*., 2005; Basha *et al*., 2007; Van Heek *et al*., 2000)/radio detector (Ghosal *et al*., 2004) or by the use of tandem mass spectrometers (Bahrami *et al*., 2010; Patrick *et al*., 2002; Li *et al*., 2006; Oliveira *et al*., 2006; Oswald *et al*., 2006). Tandem mass spectrometers, though sensitive, require skilled manpower and the initial setup of instrument is very expensive which may not be available in all the laboratories. On the other hand HPLC is less expensive and easier to operate. Although Van Heek *et al*. (2000) have reported the HPLC method for simultaneous estimation of EZM and EZMG; the method primarily served the need of assessing the formation of EZMG qualitatively. Also S. J. S. Basha *et al*. (2007) have reported HPLC method for the in vitro metabolism studies (phase I and II) and bile sample analysis for the simultaneous estimation of EZM and EZMG. To the best of our knowledge, there is no HPLC method for the estimation of pharmacokinetic parameters of total EZM from human volunteers. Thus, the aim of the present study was to develop an accurate and precise High Performance Liquid Chromatographic method for the determination of total EZM using Standard addition method. The application of the method was demonstrated by estimating the pharmacokinetic parameters for total EZM in healthy human subjects. Furthermore, the results obtained for the volunteer study from HPLC were compared with the results obtained from LC-MS/MS without standard addition.

MATERIALS AND METHODS

Chemicals and Reagents

Helix Pomatia β-glucuronidase [145700U (Type HP-2)] was obtained from Sigma-Aldrich. Ezetimibe (99.52%) and Ezetimibe Phenoxy Glucuronide (99.2%) were supplied by VARDA Biotech (P) Ltd., India. Tamsolusin (100.01%) was obtained from Corporate Analytical Development Lab, India. Ezetimibe D4 (97.14%) was procured from Vivan Life Sciences. Acetonitrile and Methanol of HPLC grade was purchased from E-Merck (India). Sodium acetate, Sodium Borate and Ammonium Acetate of analytical grade were procured from Qualigens. Glacial acetic acid (GAA) and Triethylamine (TEA) of analytical grade were procured from S. D. Fine Chem. Ltd. HPLC grade water, used for dilution, was prepared in-house using 'miniquartz distiller' of Qualigens.

Instruments and methods

Chromatographic separation was performed on HPLC system (JASCO 1500) equipped with JASCO PU-980 pump unit attached to a manual injector with a 20µL loop, and a JASCO UV-1575 detector. The data acquisition was carried on Borwin software version 1.50. The HPLC column used was Symmetry shield 100 5C18 (250 × 4.6 mm) 5 µm. The mobile phase consisted of Acetonitrile and 1mM NH_4COOCH_3 in the proportion 60:40 v/v. The flow rate was 1ml/min and the eluate was monitored with UV detection at 233nm. The injection volume was 20 µL and total run time was 12 min.

For the LC-MS/MS analysis, Schimadzu Prominence UFLC coupled with mass spectrometer API-3200 having ESI source was used. The Chromatography was carried out using Schimadzu Prominence UFLC system equipped with LC-20AD Pump, DGU-$20A_3$ Degasser, SIL-HTc Autosampler (6°C), CTO-10AS VP Column Oven. The mobile phase involved a mixture of Methanol and 5mM Ammonium Acetate in the proportion 90:10 (v/v), pumped at a flow rate of 0.9 mL/min through the analytical column (ACE, 5 C18, 50 × 4.6 mm, 5µ), at 40°C. The injection volume was 10 µL and the total run time was 3 min 30 s. The mass spectrometer (API-3200) equipped with an electrospray source was run in negative ion mode (ES-), and set up in multiple reaction monitoring (MRM), at a temperature of 500°C and a source voltage of -2000V, monitoring the transitions 408.100/271.100 and 412.100/275.100 for EZM and EZM D4, respectively. The values for the mass spectrometer variables are listed in Table 1. Data acquisition and analysis were performed using the Analyst Software version 1.5.1.

Standard preparations and quality control samples

The stock solutions of Ezetimibe phenoxy glucuronide (EZMG), Ezetimibe (EZM), Ezetimibe D4 (EZM D4) and Tamsolusin (TAM) were prepared by dissolving accurately weighed standard compound in methanol to give final concentration of 2.5 mg/mL, 1 mg/mL, 1mg/mL and 1 mg/mL respectively. The stock solution was diluted to suitable concentrations using methanol and HPLC grade water (50:50, v/v) for spiking into plasma to obtain calibration curve (CC) standards, with concentrations of 30, 36, 50, 120, 200, 300, 400 and 500 ng/mL for LC-UV. TAM (300 ng/mL) was used as an internal standard. For LC-MS/MS analysis, the calibration standards were prepared by spiking human plasma with standard solutions containing EZM to give standards of 1.02, 2.04, 14.57, 29.14, 58.28, 116.57, 233.013 and 303.07 ng/mL. A fixed concentration of EZM D4 (400 ng/mL) was added to all assay tubes as an internal standard. Zero concentration plasma samples used in each run were prepared containing internal standard only. In each run, a blank plasma sample (no IS) was also analyzed. The linear regression of the peak area ratio of analyte/IS vs. concentration was obtained. A weighted

(1/concentration^2) equation was used to obtain calibration curve. The regression equation of the calibration curve was then used to calculate the plasma concentration. The back calculated values of the concentrations were statistically evaluated.

Quality control samples were prepared using the fresh stock solution. Four levels of QC samples in plasma were 30 ng/mL and 1.03 ng/mL (lower limit of quantitation), 90 ng/mL and 2.94 ng/mL (low), 250 ng/mL and 134.23ng/mL (medium), 380 ng/mL and 247.26 ng/mL (high) for the LC-UV and LC-MS/MS, respectively. All stock solutions and working standard solutions were stored in refrigerator below -20°C.

Sample Collection and preparation

The developed method was applied to study the pharmacokinetic parameters for the total EZM in 6 healthy subjects under fasting conditions. The human plasma samples were obtained as gift samples from C. B. Patel Research Centre (India).

The blood samples were drawn at 0.33, 0.67, 1.0, 1.33, 1.67, 2.0, 2.50, 3.0, 3.5, 4.0, 4.5, 5.0, 5.5, 6.0, 7.0, 8.0, 9.0, 10.0, 12.0, 14.0, 16.0, 18.0, 24.0, 48.0, and 72.0 hours interval after administration of EZM 10 mg tablets by 6 healthy subjects.

Frozen human plasma samples were thawed at ambient temperature and 1mL of samples were placed in glass tubes. 50 µL of internal standard working solution and a fixed concentration of 50 ng/mL of EZM were added and vortexed for 30 s. 2 mL of 0.5M sodium acetate buffer (pH 4.5 with GAA) and 100 µL of β-glucuronidase (1457 Fishman Units) was added. After a thorough vortex mixing for 30 s, samples were incubated at 50-55°C for 6 hours in a water bath. Post incubation, 2 mL of 0.1M sodium borate buffer (pH 9.80 with TEA) was added and Ezetimibe was extracted with 5 mL of *tert*-butyl methyl ether (MTBE). The organic layer is separated and evaporated to dryness using N_2 gas. The reconstitution was done with 100 µL of mobile phase (LC-UV) or 500 µL of mobile phase (LC-MS/MS) and transferred to an injection vial for analysis.

Validation

The methods were validated in compliance with US Food and Drug Administration guidelines (Bressole *et al*., 1996; Shah *et al*., 1991). To evaluate the performance of the LC-UV and LC-MS/MS methods, a comparison was done between spiked and calculated QC concentrations by calculating precision values and the mean differences between spiked and calculated concentrations.

Selectivity and Sensitivity

To ascertain selectivity of the method, 6 different sources of plain human plasma along with 6 LLOQ (30 ng/mL for LC-UV and 1.02 ng/mL for LC-MS/MS) samples were analyzed. The degree of interference was assessed at the retention time or *m/z* of analyte and I.S. to verify that each observed peak eluted free of any potential interference. Sensitivity test was performed by injecting six LLOQ (30 ng/mL for LC-UV and 1.02 ng/mL for LC-MS/MS) samples along with one set of calibration curve standard of concentration equivalent to that used for the calculation of precision and accuracy.

Matrix Effect

Undetected matrix components co-eluting with analytes may adversely affect the reproducibility of analyte ionization in the electrospray source of the mass spectrometer (Matuszewski *et al*., 1998). To verify the absence of ion suppression or ion-enhancement effects attributable to the matrix, two sets of each blank sample i.e. six sources of blank plasma were extracted. Then 500 µL of recovery dilution of each level LQC (1.21 ng/mL) and HQC (98.37 ng/mL) containing internal standard (40.12 ng/mL) was added in each plasma lot and termed as post extracted spiked samples. Recovery comparison samples at LQC and HQC containing internal standard, with their respective concentrations, representing 100% extraction, was prepared. The area response of drug and internal standard of post extracted spiked samples was compared against the recovery comparison solution to evaluate matrix effect.

Precision and Accuracy

Precision and Accuracy of the method were assessed by analyzing QC samples at the concentrations mentioned above along with a calibration curve. To evaluate intra-day precision and accuracy, 6 samples of each QC concentration were extracted and injected them on the same day. To evaluate inter-day precision and accuracy, 6 samples of each QC concentration were analyzed per day on different days. Percent accuracy was evaluated as: [(mean found concentration)/ (nominal concentration)] × 100. Precision was expressed as the CV.

Recovery

Recovery experiments were performed by comparing the analytical results for extracted samples at three concentrations (low, medium, and high) with un-extracted standards that represent 100% recovery.

Pharmacokinetics and statistical analysis

Pharmacokinetic analysis was performed by use of Winolin version 5.2 computer program. Various pharmacokinetic parameters such as area under curve (AUC), peak plasma concentration (C_{max}), time to reach the peak concentration (T_{max}), elimination rate constant (K_{el}), elimination half-life ($T_{1/2}$) and absorption efficiency was determined for each volunteer. The elimination rate constant (K_{el}) was obtained as the slope of the linear regression of the log-transformed concentration versus time data in the terminal portion of the curve. AUC_{0-t} was determined by linear trapezoidal rule. $AUC_{0-\infty}$ was

calculated as $AUC_{0-t}+C_{last}/K_{el}$, where C_{last} is the last measured concentration. Pharmacokinetic parameters such as C_{max} and T_{max} were determined by the inspection of the individual plasma concentration-time profiles.

Table 1. Tandem mass spectrometer working parameters

Analyte	*MRM Transition (m/z)*	*DP (V)*	*EP (V)*	*CEP (V)*	*CE (%)*	*CXP (V)*	*Dwell Time (ms)*
Total EZM	408.100/271.100	-40	-4	-15	-20	-2.50	300
EZM D4	412.100/275.100	-40	-4	-15	-28	-2.50	300

DP, Declustering Potential; EP, Entrance Potential; CEP, Collision Entrance Potential; CE, Collision Energy; CXP, Collision Cell Exit Potential.

Table 2. Precision and Accuracy of Calibration samples in human plasma by LC-UV and LC-MS/MS

***LC-UV* (n=4)**			***LC-MS/MS* (n=4)**		
Nominal Concentration (ng/mL)	**Accuracy %**	**CV %**	**Nominal Concentration (ng/mL)**	**Accuracy %**	**CV %**
30	102.19	7.21	1.02	98.82	0.95
36	100.45	10.98	2.04	102.60	2.08
50	102.89	8.30	14.57	102.23	1.49
120	91.17	10.74	29.14	100.33	0.91
200	99.98	5.31	58.28	99.44	1.32
300	103.16	6.44	116.57	99.77	0.21
400	97.93	5.77	233.13	98.88	0.47
500	101.41	3.94	303.07	98.22	0.48

Table 3. Intra-day and inter-day coefficient of variation and accuracy for the determination of Ezetimibe in human plasma

Analytical method	*Theoretical concentration (ng/ml)*	*Intra-day*			*Inter-day*		
		Mean concentration (ng/ml)	**CV (%)**	**Accuracy (%)**	**Mean concentration (ng/ml)**	**CV (%)**	**Accuracy (%)**
LC-UV		**(n=6)**			**(n=18)**		
	30	29.20	9.81	97.34	29.60	9.56	98.68
	90	84.89	7.15	94.32	87.70	7.01	97.44
	250	244.23	8.91	97.69	243.82	7.96	97.53
	380	377.34	6.78	99.30	382.96	6.51	100.78
LCMS/MS		**(n=18)**			**(n=24)**		
	1.03	1.086	4.76	105.44	1.087	4.21	105.53
	2.94	2.881	2.22	97.99	2.880	2.32	97.96
	134.23	133.561	0.86	99.50	133.386	0.78	99.37
	247.26	247.643	0.90	100.15	247.965	0.96	100.29

Table 4. Mean Pharmacokinetic parameters obtained for total EZM by LC-UV and LC-MS/MS.

Pharmacokinetic parameters	***HPLC Mean ± S. D.***	***LC-MS/MS Mean ± S. D.***
C_{max}(ng/ml)	52.58 ± 15.00	67.59 ± 43.59
T_{max}(h)	1.83 ± 1.11	1.39 ± 0.77
$T_{1/2}$(h)	15.66 ± 12.61	9.38 ± 5.79
AUC_{0-t}(ng.h/ml)	786.14± 821.20	867.88 ± 631.21
$AUC_{0-\infty}$(ng.h/ml)	826.56 ± 813.64	887.59 ± 676.43
K-elimination	0.0714 ± 0.0436	0.0879 ± 0.0274

Fig. 1. Hydrolysis of Ezetimibe Phenoxy Glucuronide (EZMG) to Ezetimibe (EZM)

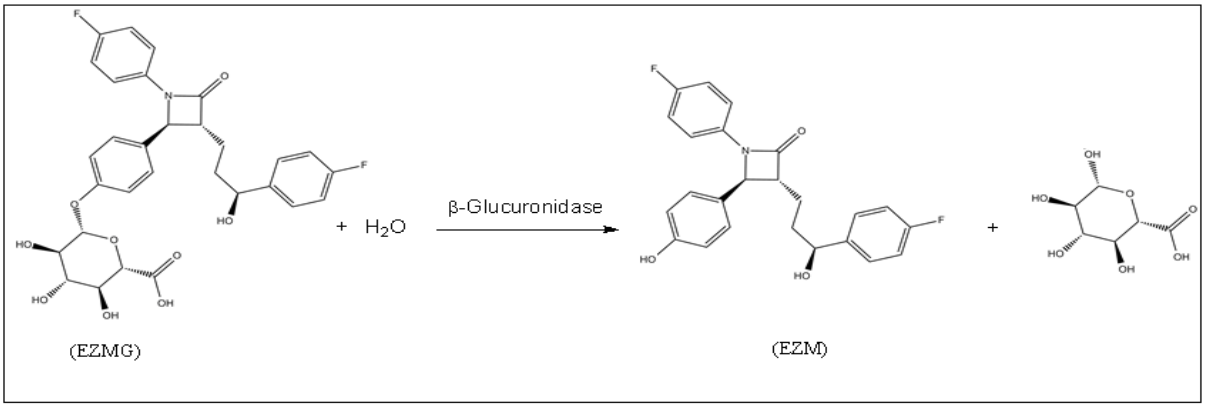


Fig. 2. Representative chromatograms of plasma extracts from LC-UV (A) Plasma extract from a subject at pre-dose; (B) Plasma extract from a subject 1.67 hrs post dose (10 mg). Ezetimibe – 5.492 min, Tamsolusin (IS) –10.667 min

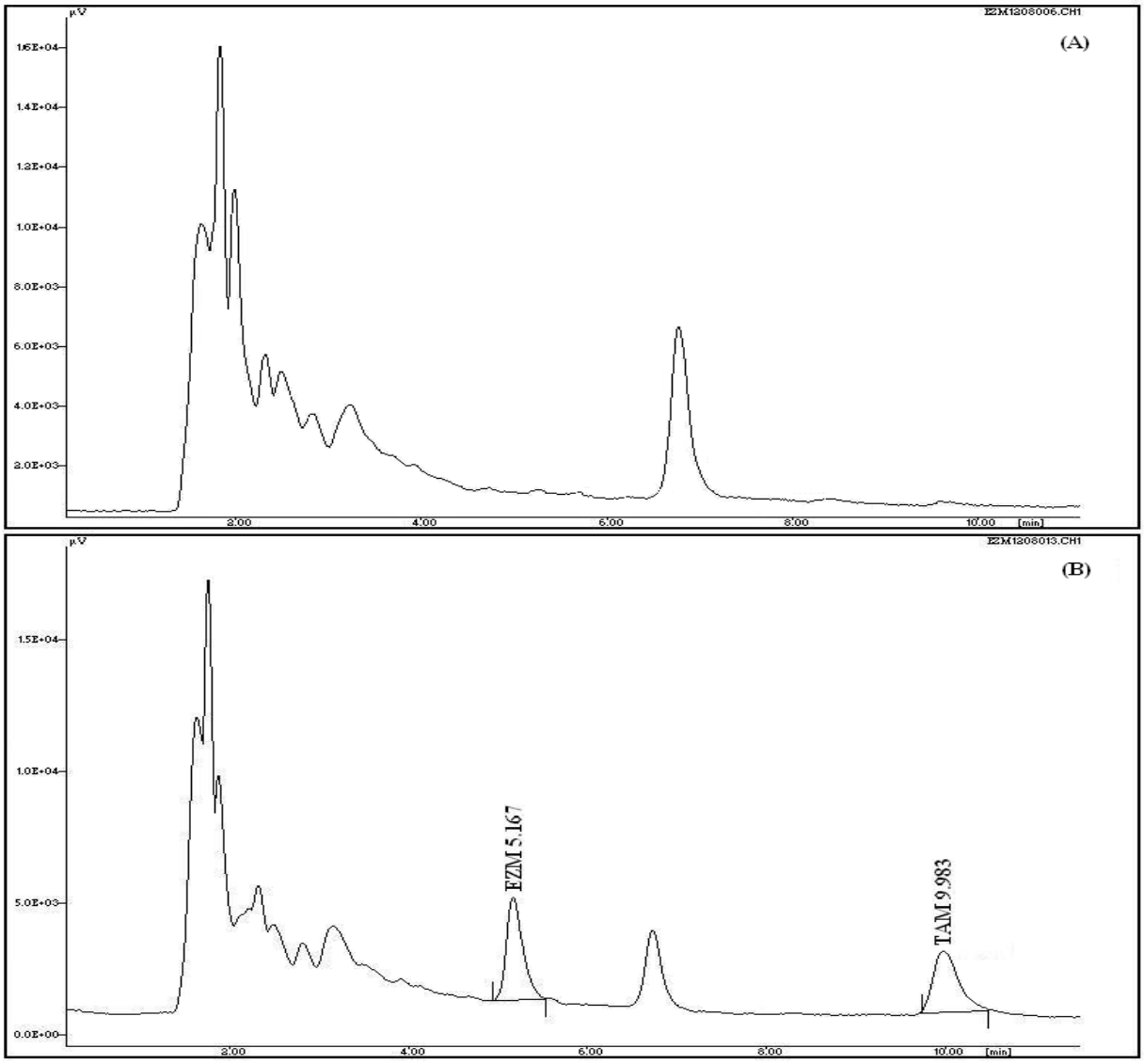


Fig. 3. Representative mass spectra of plasma extracts from LC-MS/MS (A) Plasma extract from a subject at pre-dose; (B) Plasma extract from a subject 1.67 hrs post dose (10 mg). Ezetimibe – 0.80 min

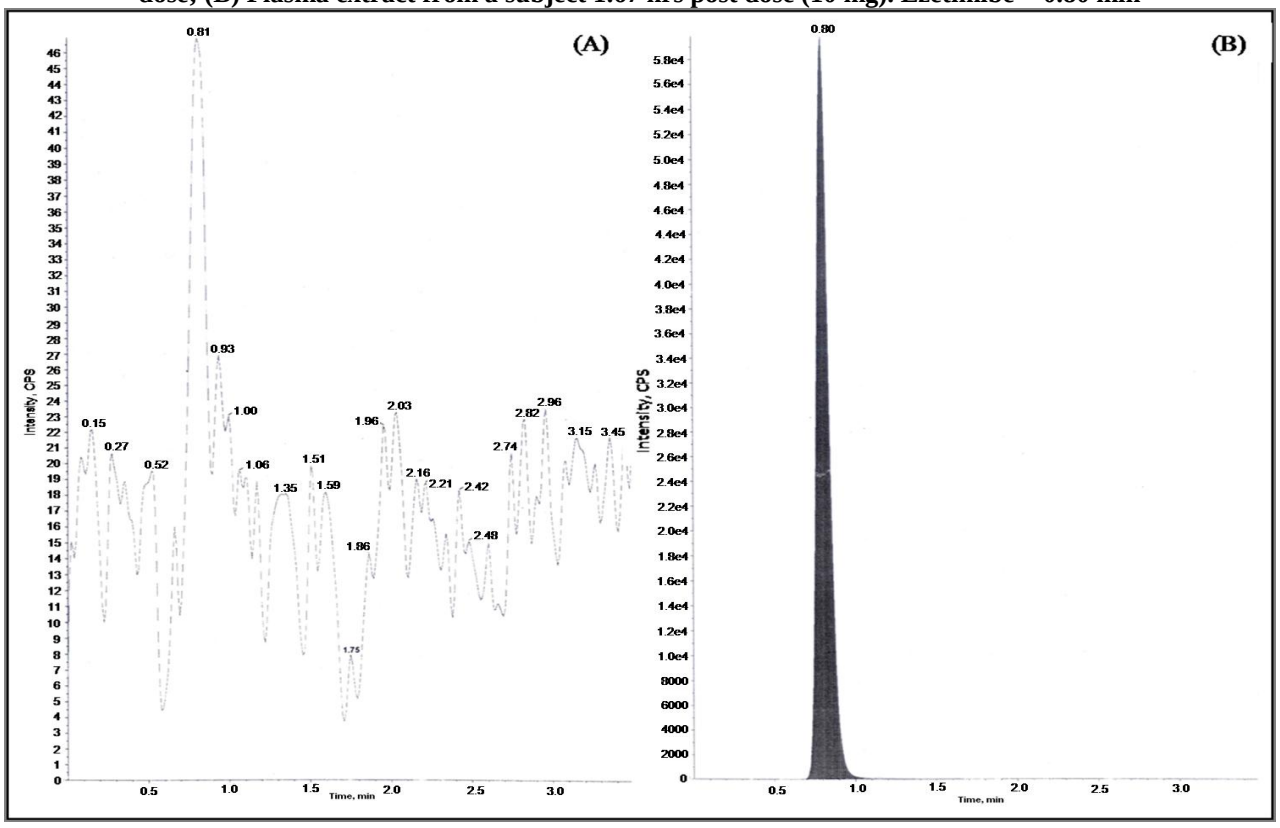
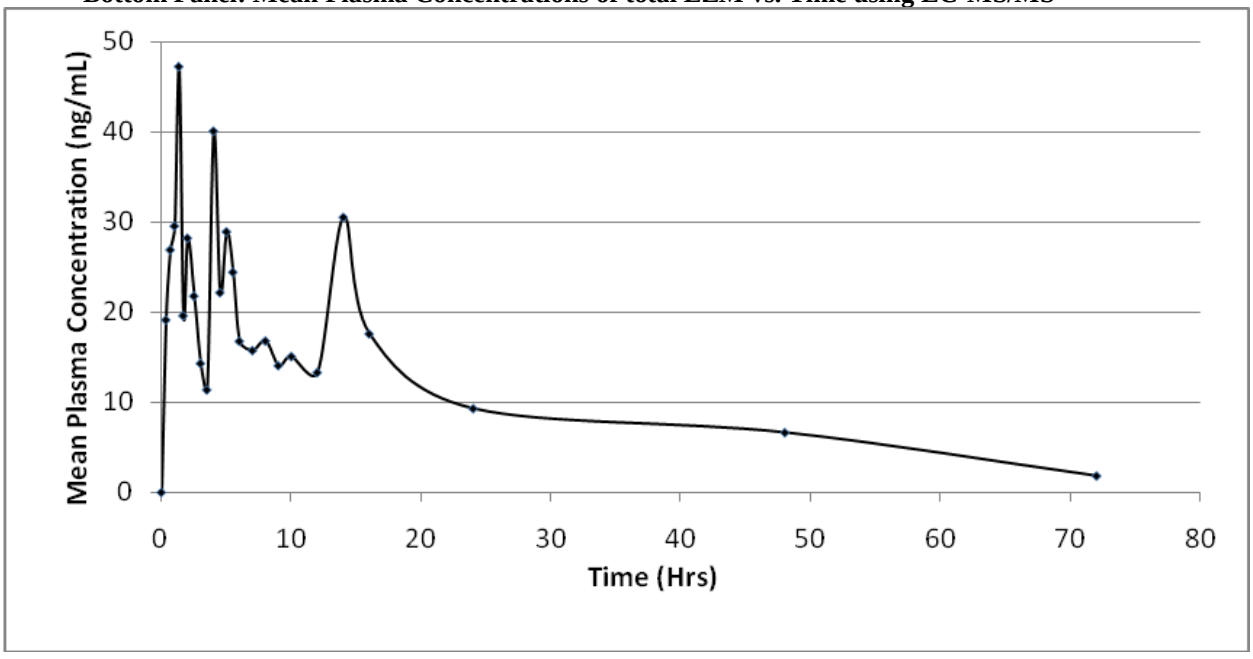
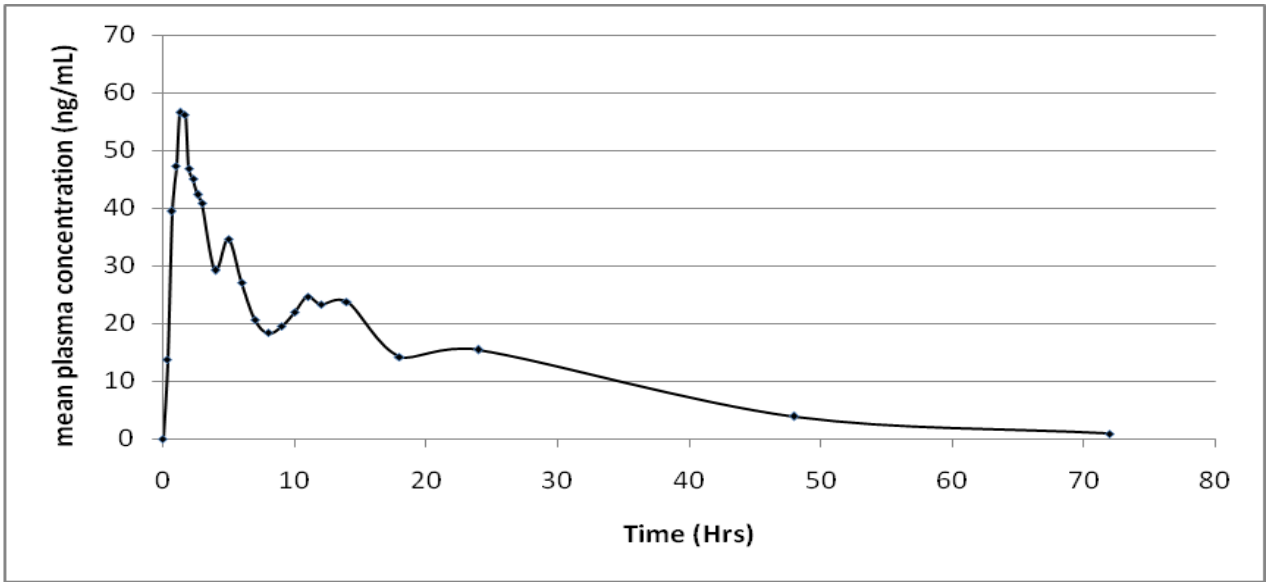


Fig. 4. Top Panel: Mean Plasma concentrations of total EZM vs. Time using LC-UV;
Bottom Panel: Mean Plasma Concentrations of total EZM vs. Time using LC-MS/MS





RESULTS AND DISCUSSION

Even though EZM is not a new drug, there is no analytical method describing the use of High Performance Liquid Chromatography, to be used for the pharmacokinetic studies of total EZM in human volunteers. Therefore, we aimed at developing a new analytical method to quantitate total EZM in human plasma. The developed method should be comparable to the LC-MS/MS method so that it can be applied for studying the pharmacokinetic parameters of total EZM. The first problem encountered in the development of the analytical method was to separate the analyte peak from endogenous interfering components of enzyme in addition to plasma components. In order to minimize the interference, the enzyme concentration was reduced and optimized the enzymatic reaction parameters so as to have optimal recovery to be able to detect by High Performance Liquid Chromatography. Thus the enzyme used per analysis was reduced by approximately 75% as compared to the reported methods (Li *et al*., 2006; Oliveira *et al*., 2006; Oswald *et al*., 2006); thereby allowing increased number of samples to be analysed by commercially available concentrated form of Enzyme. A further problem was the ability of the analytical method to be able to quantitate the nanogram levels of total EZM in real samples. This problem was overcome by spiking a constant concentration of 50 ng/mL of total EZM in real samples. Thus, modifying the concentration of real samples allowed us to quantitate in the concentration range of 30 – 500 ng/mL of EZM. The concentrations obtained, underwent subtraction of 39.5 ng/mL (accounting for the recovery of the method) and the final concentrations were subjected to pharmacokinetics and statistical analysis. The results obtained were compared with those obtained from LC-MS/MS without modifying the concentration of EZM.

Method validation

Selection of LC and LC-MS/MS conditions

For the LC-UV method, initial development was conducted on a C18 5 µm, 250 × 4.6 mm column, operated at ambient temperature. This column provides efficient and reproducible separations of non-polar compounds. Consequently, it was selected for method development and remains the column utilized in the validated assay. Preliminary method development of suitable isocratic conditions to resolve EZM, on the C18 column, was conducted with ACN: H_2O:: 60: 40 as the mobile phase. A mobile phase of ACN: 1mM NH_4COOCH_3 (60:40) was found to provide a reproducible, resolved peak with an average peak tailing of 1.15. The retention times for EZM and Tamsolusin (IS) were 5.3 and 10.3 min respectively. Fig. 2 shows representative chromatograms of extracted plasma samples.

For the development of LC-MS/MS method, ESI was selected to detect total EZM in human plasma. To obtain an appropriate ionization mode in analysis, EZM and EZM D4 were scanned with negative and positive ion mode; negative mode gave higher peak intensity. In addition, a prominent fragment with *m/z* 271.100 was observed in the product ion scan with negative ESI. Two ion transition pairs for EZM and EZM D4 were presented at *m/z* 408.100/271.100 and 412.100/275.100, respectively. Excellent selectivity and sensitivity were observed under the present LC-MS/MS condition by combination with a liquid chromatographic separation. The retention time of both analyte and I.S. was approximately 0.80 min and total run time for each sample was 3 min 30s. Fig. 3 shows representative LC-MS/MS spectras for total EZM in plasma samples.

Selectivity and Sensitivity

No interference from endogenous matrix components was observed at the retention time or *m/z* of either EZM or the IS. LC-UV method was found to be sensitive at 30 ng/mL with accuracy of 93.41% and precision of 7.37%. The interference found at 30 ng/mL was 7.22% for EZM and 0.16% for TAM at 300 ng/mL. Sensitivity of 1.02 ng/mL with accuracy of 98.73% and precision of 2.14% was obtained for LC-MS/MS.

Matrix effect

No significant matrix effect was observed in all six sources of plasma at low (LQC) and high (HQC) concentrations of analyte. The IS normalized matrix factor was found to be 0.9952% at LQC and 1.018% at HQC, with coefficient of variations 0.23% and 0.21%, respectively, indicating very little difference in ionization efficiency of the total EZM and EZM D4 from different plasma sources.

Calibration curve

Calibration curves were linear in the concentration ranges 30 – 500 ng/mL for LC-UV and 1.02 – 303.07 ng/mL for LC-MS/MS. The eight-point calibration curves gave acceptable results for both LC-UV and LC-MS/MS. A correlation coefficient *(r)* of above 0.997 was obtained during method validation. Results of calibration curves of EZM in human plasma are presented in Table 2. For both LC-UV and LC-MS/MS, the CVs were less than 10.98%, accuracy ranged from 91.17% to 103.16%.

Precision and Accuracy

The accuracy and precision of both the assay are summarized in Table 3. The intra-day and inter-day precision of the assay was estimated by analyzing four different concentrations of EZM in plasma. The intra-day precision ranged from 6.78 to 9.81% (LC-UV) and 0.86 to 4.76% (LC-MS/MS), while inter-day precision ranged from 6.51 to 9.56% (LC-UV) and 0.20 to 3.52% (LC-MS/MS), respectively. The intra-day accuracy ranged from 94.32 to 99.30% (LC-UV) and 97.99 to 105.44% (LC-MS/MS), while inter-day accuracy ranged from 97.44 to 100.78% (LC-UV) and 96.43 to 109.90% (LC-MS/MS), respectively.

Recovery

The mean overall recovery of EZM was 79.12% with a precision of 11.04% by LC-UV. The mean overall recovery obtained by LC-MS/MS for EZM and EZM D4 was 44.71% with a precision of 1.54% and 64.65% with a precision of 1.00%. The variation in the recovery of EZM and EZM D4 in case of LC-MS/MS could be attributed to handling or processing error.

Bioequivalence study

The validated methods were successfully used to determine the Total EZM in human plasma samples from a pharmacokinetic study after a single oral dosing of EZM (10 mg) to 6 healthy human subjects. Fig. 4 shows mean plasma concentration-time profiles of total EZM in the 6 subjects using both the methods. The results obtained from HPLC method, employing standard addition, are in good agreement with the LC-MS/MS results without the use of standard addition method (Table 4). The plasma concentration-time profile exhibited significant multiple peaks suggesting enterohepatic recycling. The large means obtained for each of the pharmacokinetic parameter may be attributed to the large inter-individual variation shown by EZM (Hegele *et al*., 2005).

CONCLUSION

An analytical method for the determination of total EZM from human plasma by the use of LC-UV and LC-MS/MS is described in this article. Validation results are also presented to demonstrate the quality and suitability of the method for the intended use. The developed LC-UV method showed accuracy, precision and was found to be feasible in terms of cost per analysis. The applicability of this method was evaluated in the analysis of unknown samples taken from volunteers in a pharmacokinetic study and compared with the results obtained from LC-MS/MS. The results obtained by both the method of analysis were found to be comparable.

ACKNOWLEDGEMENT

The authors wish to thank C. B. Patel Research Centre, Mumbai-56, India for their technical assistance and Dr. Ashish Mungantiwar (Macleods Pharmaceuticals Ltd., Mumbai-59) for providing resources in carrying out this work.

REFERENCES

Andersen A, Holte H, and Slordal L. Pharmacokinetics and metabolism of doxorubicin after short-term infusions in lymphoma patients. *Cancer Chemotherapy and Pharmacology*. 1999; 44(5): 422-426.

Bahrami G, Mohammadi B, Khatabi PM, Farzaei MH, Majnooni MB, Bahoosh SR. Application of one-step liquid chromatography-electrospray tandem MS/MS and collision-induced dissociation to quantification of Ezetimibe and identification of its glucuronated metabolite in human serum: A pharmacokinetic study. *Journal of Chromatography B*. 2010; 878(28): 2789-2795.

Basha SJS, Naveed SA, Tiwari NK, Shashikumar D, Muzeeb S, Kumar TR, Kumar NV, Rao NP, Srinivas N, Mullangi R, and Srinivas NR. Concurrent determination of ezetimibe and its phase-I and II metabolites by HPLC with UV detection:

Quantitative application to various *in vitro* metabolic stability studies and for qualitative estimation in bile. *Journal of Chromatography B: Biomedical Sciences and Applications*. 2007; 853: 88-96.

Bressolle F, Bromet-Petit M, and Audran M. Validation of liquid chromatographic and gas chromatographic methods Applications to pharmacokinetics. *Journal of Chromatography B: Biomedical Sciences and Applications*. 1996; 686: 3-10.

Fish F and Hayes TS. Hydrolysis of Morphine Glucuronide. *Journal of Forensic Science*. 1974; 19(3): 676-683.

Ghosal A, Hapangama N, Yuan Y, Achanfuo-YeboahJ, Iannucci R, Chowdhury S, Alton K, Patrick JE, and Zbaida S. Identification of human UDP-Glucuronosyltransferase enzyme(S) responsible for the glucuronidation of Ezetimibe (Zetia). *Drug Metabolism and Disposition*. 2004; 32: 314-320.

Hegele RA, Guy J, Ban MR and Wang J. NPC1L1 haplotype is associated with inter-individual variation in plasma low-density lipoprotein response to Ezetimibe. *Lipids in Health and Disease*. 2005; 4: 16. DOI: 10.1186/1476-511X-4-16

Kadi AA and Hefnawy MM. Biological Fluids: Glucuronides from LC/MS, Encyclopedia of Chromatography, Third Edition, volume 2, Taylor and Francis, United Kingdom, 2009: 203-209.

Kaushik R, Levine B, and LaCourse WR. A brief review: HPLC methods to directly detect drug glucuronides in biological matrices (Part I). Analytica Chimica Acta. 2006; 556: 255-266.

Li S, Liu G, Jia J, Li X, Yu C. Liquid chromatography–negative ion electrospray tandem mass spectrometry method for the quantification of ezetimibe in human plasmaLiquid chromatography–negative ion electrospray tandem mass spectrometry method for the quantification of ezetimibe in human plasma Liquid chromatography–negative ion electrospray tandem mass spectrometry method for the quantification of ezetimibe in human plasma. *Journal of Pharmaceutical and Biomedical Analysis*. 2006; 40 (4): 987-992.

Matuszewski BK, Constanzer ML, Chavez-Eng CM. Matrix effect in quantitative LC/MS/MS analyses of biological fluids: A method for determination of finasteride in human plasma at pictogram per milliliter concentrations. *Analytical Chemistry*. 1998; 70: 882-889.

Nobilis M, Holcapek M, Kolarova L, Kopecky J, Kunes M, Svoboda Z, and Kvetina J. Identification and determination of phase II nabumetone metabolites by high-performance liquid chromatography with photodiode array and mass spectrometric detection. *Journal of Chromatography A*. 2004; 1031: 229-236.

Oliveira PR, Brum L Jr., Fronza M, Bernardi LS, Masiero SMK, and Dalmora SL. Development and Validation of a liquid Chromatography-Tandem Mass Spectrometry Method for the Determination of Ezetimibe in Human Plasma and Pharmaceutical Formulations. *Chromatographia*. 2006; 63(7-8): 315-320.

Oswald S, Scheuch E, Cascorbi I, and Siegmund W. A LC–MS/MS method to quantify the novel cholesterol lowering drug ezetimibe in human serum, urine and feces in healthy subjects genotyped for SLCO1B1. *Journal of Chromatography B: Biomedical Sciences and Applications.* 2006; 830 (1): 143-150.

Patrick JE, Kosoglou T, Stauber KL, Alton KB, Maxwell SE, Zhu Y, Statkevich P, Iannucci R, Choudhury S, Affrime M, and Cayen MN. Disposition of the selective cholesterol absorption inhibitor Ezetimibe in healthy male subjects. *Drug Metabolism and Disposition.* 2002; 30(4): 430-437.

Rosenblum SB , Huynh T , Afonso A, Davis HR, Jr., Yumibe N , Clader JW , and Burnett DA. Discovery of 1-(4-Fluorophenyl)-(3*R*)-[3-(4-fluorophenyl)-(3*S*)- hydroxypropyl]-(4*S*)-(4-hydroxyphenyl)-2-azetidinone (SCH 58235): A Designed, Potent, Orally Active Inhibitor of Cholesterol Absorption. *Journal of Medicinal Chemistry.* 1998; 41(6): 973-980.

Shah VP, Midha KK, Dighe S, McGilveray IJ, Skelly JP, Yacobi A, Layloff T, Vishwanathan CT, Cook CE, McDowall RD, Pittman KA and Spector S. Analytical methods validation: bioavailability, bioequivalence and pharmacokinetic studies [conference report]. *European Journal of Drug Metabolism and Pharmacokinetics.* 1991; 16: 249-255.

Sistla R, Tata VSSK, Kashyap YV, Chandrasekar D, and Diwan PV. Development and validation of a reversed-phase HPLC method for the determination of ezetimibe in pharmaceutical dosage forms. *Journal of Pharmaceutical and Biomedical Analysis.* 2005; 39(3 - 4): 517-522.

Staimer N, Gee SJ, and Hammock BD. Development of a class-selective enzyme immunoassay for urinary phenolic glucuronides. *Analytica Chimica Acta.* 2001; 444 (1): 27-36.

Van Heek M, Farley C, Compton DS, Hoos L, Alton KB, Sybertz EJ, and Davis HR. Comparison of the activity and disposition of the novel cholesterol absorption inhibitor, SCH58235, and its glucuronide, SCH60663. *British Journal of Pharmacology*. 2000; 131(8): 1748-1756.

Xu X, Ziegler RG, Waterhouse DJ, Saavedra JE, and Keefer LK. Stable isotope dilution high-performance liquid chromatography-electrospray ionization mass spectrometry method for endogenous 2- and 4-hydroxyestrones in human urine. *Journal of Chromatography B: Biomedical Sciences and Applications*. 2002; 780(2): 315-330.

Zhai P, Eurell TE, Cotthaus RP, Jeffery EH, Bahr JM, and Gross DR. Effects of dietary phytoestrogen on global myocardial ischemia-reperfusion injury in isolated female rat hearts. *American Journal of Physiology, Heart and Circulatory Physiology*. 2001; 281: H1223-H1232.