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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☆

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Abstract

Simultaneous separation and quantification of ezetimibe (EZM) and its phase-I metabolite i.e., ezetimibe ketone (EZM-K) and phase-II metabolite i.e., ezetimibe glucuronide (EZM-G) in various matrices was accomplished by gradient HPLC with UV detection. The assay procedure involved deproteinization of 500 μL of either incubation or bile sample containing analytes and internal standard (IS, theophylline) with 75 μL acetonitrile containing 25% perchloric acid. An aliquot of 100 μL supernatant was injected onto a C_{18} column. The chromatographic separation was achieved by gradient elution consisting of 0.05 M formic acid:acetonitrile:methanol:water at a flow rate of 1.0 mL/min. The detection of analyte peaks were achieved by monitoring the eluate using an UV detector set at 250 nm. Nominal retention times of IS, EZM-G, ezetimibe ketone glucuronide (EZM-KG), EZM and EZM-K were 9.39, 24.23, 27.82, 29.04 and 30.56 min, respectively. Average extraction efficiencies of EZM, EZM-G and IS was >75–80% and for EZM-K was >50% from all the matrices tested. Limit of quantitation (LOQ) for EZM, EZM-K and EZM-G was 0.02 μg/mL. Due to the lack of availability of reference standard of EZM-KG, the recovery and LOQ aspects for this metabolite were not assessed. Overall, the method is suitable for simultaneous measurement of EZM, and its phase-I and phase-II metabolite (EZM-G) in *in vitro* and *in vivo* studies.

Keywords: Ezetimibe; Ezetimibe ketone; Ezetimibe glucuronide; Ezetimibe ketone glucuronide; HPLC; Metabolism

1. Introduction

Ezetimibe (EZM, Zetia®, Fig. 1), chemically 1-(4-fluorophenyl)-3(*R*)-[3-(4-fluorophenyl)-3(*S*)-hydroxypropyl]-4(*S*)-(4-hydroxyphenyl)-2-azetidinone is the first member of novel class of lipid-lowering drug that inhibits intestinal uptake of dietary and biliary cholesterol and related phytosterols. EZM does not appear to compromise the absorption of fat-soluble vitamins, triglycerides and bile acids [1–3]. It is postulated that EZM inhibits cholesterol uptake by binding to a specific transport protein (NPC1L1) located in the wall of the small intestine [4–6]. In 2002, EZM obtained marketing approval in USA for use as monotherapy or in combination with statins for the treatment of primary hypercholesterolaemia. EZM reduces total cholesterol, low-density lipoprotein cholesterol, and apolipoprotein B and increases high-density lipoprotein cholesterol in patients with hypercholesterolaemia [2,7,8].

Following oral administration, EZM is completely absorbed and the extensive metabolic fate is characterized by a phase-II metabolism involving intestinal UDP-glucuronosyltransfereases (UGT) before entering systemic circulation. Additionally, EZM is readily taken up by the liver from the portal blood and rapidly excreted into bile, which results in low peripheral blood concentrations. It has been

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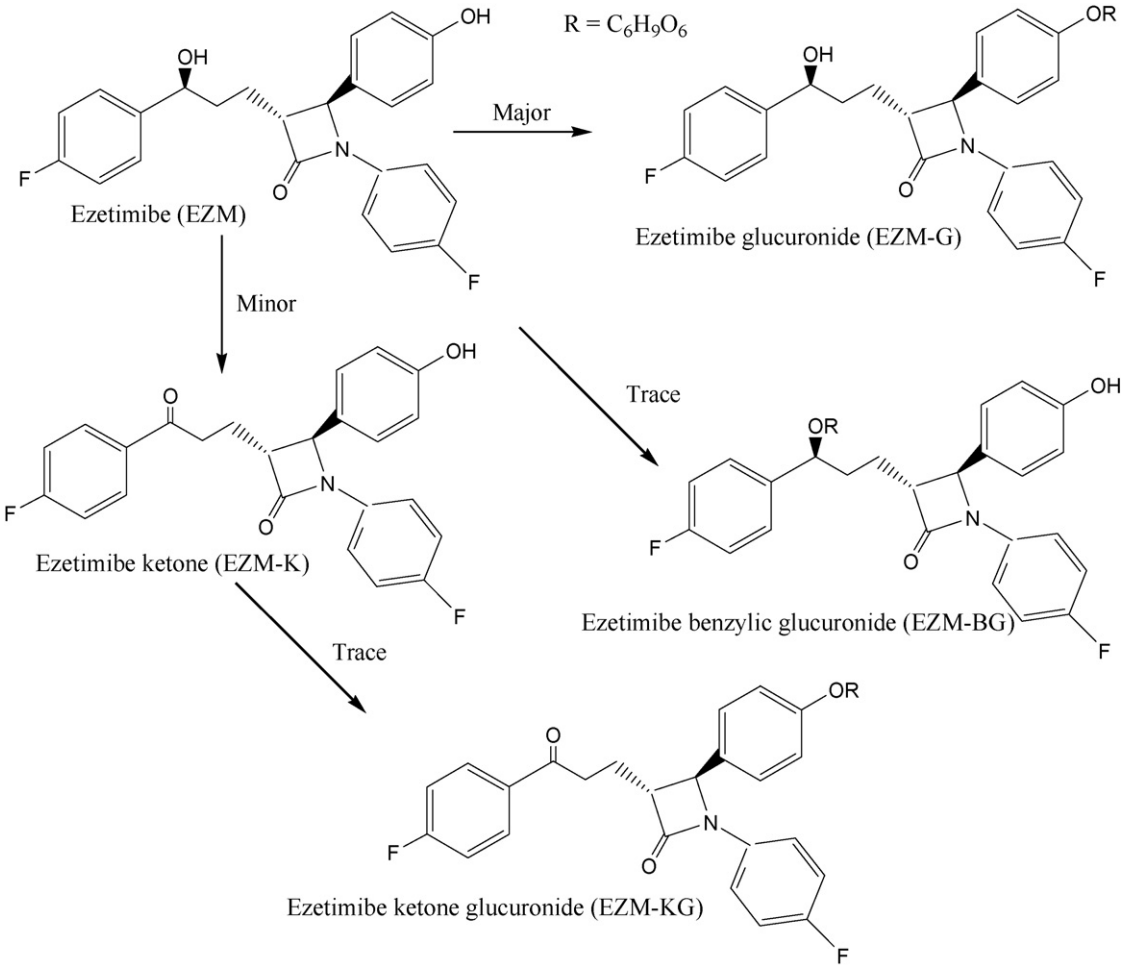

Fig. 1. Metabolic pathway for ezetimibe in humans and pre-clinical species.

shown that EZM is conjugated to a major phenolic glucuronide and only traces to a benzylic and ketone glucuronide (Fig. 1). Although many UGT isoforms have been shown to catalyze the phase-II conjugation reaction of EZM (UGT 1A1, 1A3 and 2B15), UGT 1A1 is the most active isoform [9,10]. After a single 10 mg oral dose of EZM tablets to fasted humans, mean total EZM (EZM + EZM glucuronide) and EZM peak plasma concentrations (C_{max}) achieved within 1–2 and 4–8 h, respectively. Additionally, due to the involvement of enterohepatic circulation, the pharmacokinetics of these analytes are characterized by multiple peaks [9,11]. EZM has shown linearity and dose proportionality in AUC (area under the plasma concentration–time curve) in human subjects receiving single oral doses at 5, 10 and 20 mg [12].

Till date there are only few analytical methods reported for the estimation of EZM and EZM-G. The reported bioanalytical methods were based either on HPLC coupled to UV detector [4,13] / radiodetector [10] or LC–MS/MS [9,14,15]. Although Van Heek et al. [4] have reported the HPLC method for simultaneous estimation of EZM and EZM-G, the method primarily served the need of assessing the formation of EZM-G qualitatively without furnishing some important details such as limit of quantitation (LOQ) and applicability to other laboratory experiments. To the best of our knowledge, currently there is no bioanalytical method reported in literature describing the full details of a complete methodology for simultaneous estimation of EZM and its phase-I metabolite (EZM-K) and two phase-II metabolites viz., EZM-G and EZM-KG using HPLC. In this manuscript, we are presenting a method, which has utility for multiple applications viz., to quantify the phase-I metabolite of EZM (EZM-K) in rat and human liver microsomes; to quantify the major phase-II metabolite of EZM i.e., EZM-G in rat and human liver microsomes fortified with UDPGA and qualitative analysis of bile collected from rats for the presence of phase-I (EZM-K) and phase-II metabolites (EZM-G and EZM-KG) of EZM with full details on intra- and inter-day precision and accuracy, absolute recovery and LOQ for each analyte in all matrices. In this paper, we present a simple, sensitive and reproducible HPLC–UV assay with commercially available IS for the simultaneous determination of EZM, EZM-K, EZM-KG and EZM-G from different matrices and its application to various *in vitro* studies.

2. Experimental

2.1. Chemicals and reagents

EZM, EZM-K and EZM-G (Fig. 1) were synthesized by the Discovery Chemistry Group, Discovery Research, Dr. Reddy's

Laboratories Ltd. (DRL), Hyderabad and were characterized using chromatographic (HPLC, LC–MS/MS) and spectral techniques (IR, UV, Mass, ^{1}H and ^{13}C NMR) by the Analytical Research Group, Discovery Research, DRL, Hyderabad. Purity was found to be more than 98.5% for all three compounds. Acetonitrile, methanol, ethyl acetate (HPLC grade), formic acid (analytical reagent grade), potassium phosphate buffer and magnesium chloride were purchased from Qualigens, Mumbai, India. Theophylline (IS) and $NADP^{+}$ were purchased from Spectrochem, Mumbai, India. UDP-glucuronic acid (UDPGA), glucose-6-phosphate, and glucose-6-phosphate dehydrogenase were obtained from Sigma–Aldrich (Milwaukee, WI, USA). All aqueous solutions, including the buffer for the HPLC mobile phase, were prepared with Milli Q (Millipore, USA) grade water. Pooled human liver microsomes were obtained from In Vitro Technologies, Baltimore, MD and male Wistar rat liver microsomes were prepared in-house.

2.2. Animals

Male Wistar rats (bred in DRL animal facility), ~3 months of age and weighing between 190 and 200 g were used in the present study. Animal experiments were approved by the DRL Institutional Animal Ethics Committee and were in accordance with the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), Ministry of Social Justice and Environment, Government of India. Animals were maintained on normal laboratory chow (National Institute of Nutrition, Hyderabad, India), with water ad libitum, and a 12 h light/dark cycle. Before dosing, the animals were fasted overnight (~14 h) and had free access to water throughout the experimental period.

2.3. HPLC operating conditions

The HPLC system consisted of a Waters 2695 Alliance (Milford, MA, USA) separation module attached with a Waters® 2996 photodiode array detector. A C_{18} Symmetry shield column (4.6 mm × 250 mm, 5 μm, Waters) was used for the analysis. The ternary mobile phase system consisted of A (0.05 M formic acid, pH 3.0), B (Milli Q water: acetonitrile; 10: 90, v/v) and C (Milli Q water: methanol; 10: 90, v/v) and was run as per the gradient program with a total flow rate of 1.0 mL/min (Table 1) through the column to elute the analytes. The eluate was monitored by the PDA detector (set at 250 nm) and data integration was carried out by Millennium^{32} software (version 4).

2.4. LC–MS/MS operating conditions

Analyses of the metabolites were performed for their identification with the HPLC conditions described above. An interface was established between a Agilent (Agilent Technologies, Waldbronn, Germany) 1100 series LC system equipped with degasser, isopump, auto-sampler, colcom and a PE Sciex (Foster City, CA, USA) API 2000 triple quadruple mass spectrometer, equipped with a Turboionspray™. Samples were either injected (50 μL volume) onto a C_{18} Symmetry shield column (4.6 mm × 250 mm, 5 μm) kept at ambient temperature or infused with a peristaltic pump (at a rate of 5 μL/min) into the mass spectrometer. Mass spectra were acquired in full scan (from $m/z = 200$ to 800 at 1/scan/s) and product ion scan mode. Negative ion monitoring with scanning mode was performed at a capillary voltage of 3500 V. The operating conditions were as follows: ionization voltage, 3.5 kV; nitrogen curtain gas setting, 15; collision energy, ~45 eV; declustering and focusing potentials, 80 and 260 V, respectively. CID (collision induced dissociation) studies were performed using collision energy of 45 eV and a collision cell of argon gas with the pressure at 2×10^{-3} mbar. Authentic standards of EZM [$(M-H)^{-}$ ion at m/z 408], EZM-K [$(M-H)^{-}$ ion at m/z 406] and EZM-G [$(M-H)^{-}$ ion at m/z 584] were available to support the standard chromatographic retention time, UV spectra and MS results in structural assignment.

Table 1
Gradient-time program for the HPLC method with a ternary mobile phase system

Time (min)	A (%)	B (%)	C (%)
0	100	0	0
2	90	0	10
9	50	20	30
12	50	25	25
25	30	50	20
28	10	85	5
35	10	85	5
36	100	0	0
40	100	0	0

A: 0.05 M Formic acid.
B: Milli Q water:acetonitrile::10:90.
C: Milli Q water:methanol::10:90.

2.5. Preparation of stock and standard solutions

Primary stock solutions of EZM, EZM-K and EZM-G for preparation of standard and quality control (QC) samples were prepared from separate weighing. The primary stock solutions were prepared in methanol (1000 μg/mL). The IS solution of 1000 μg/mL was prepared in methanol. The stock solutions of EZM, EZM-G and IS were stored at 0 °C, which were found to be stable for 1 month (data not shown) and successively diluted with methanol to prepare working solutions to prepare calibration curve (CC). Another set of working stock solutions of EZM, EZM-K and EZM-G were made in methanol (from primary stock) for preparation of QC samples. Working stock solutions were stored at approximately 5 °C for a week (data not shown). Individually, three-in-one working QC and CC stock solutions of EZM, EZM-K and EZM-G were made before spiking them into QC and CC samples accordingly. A working IS solution (100 μg/mL) was prepared in methanol.

2.6. Specificity and selectivity

The lack of chromatographic interference from endogenous liver microsomal components was investigated using pooled rat

and human liver microsomes samples and as well as the bile collected from rats.

2.7. Calibration curves

Calibration curves were acquired by plotting the peak area ratio of EZM or EZM-G or EZM-K: IS against the nominal concentration of calibration standards. The concentrations used were 0.02, 0.05, 0.20, 0.5, 1.00, 2.00, 5.00 and 10.0 μg/mL. The results were fitted to linear regression analysis without the introduction of weighing factors.

2.8. Precision and accuracy

The intra-day assay precision and accuracy were estimated by analysing four replicates containing EZM, EZM-K and EZM-G at four different QC levels, i.e., 0.02, 0.06, 4.00 and 8.00 μg/mL. The inter-assay precision was determined by analyzing the four levels QC samples on four different runs. The criteria for acceptability of the data included accuracy within ±15% deviation (DEV) from the nominal values and a precision of within ±15% relative standard deviation (RSD), except for LLOQ, where it should not exceed ±20% of RSD [16,17].

2.9. Recovery

The absolute recovery (extraction efficiency) of EZM, EZM-K and EZM-G from rat and human liver microsomes and rat bile was evaluated at two different concentrations (0.20 and 2.00 μg/mL). This was established by comparing the peak area ratio between analyte and IS from the spiked matrices and bile with those of unextracted standards prepared in methanol, which represents 100%. The recovery of IS was established at a single concentration of 10.0 μg/mL.

2.10. Phase-I enzyme assay

The incubation mixtures of EZM or EZM-K with liver microsomes (rat and human) contained the following at the indicated final concentrations (final volume of 500 μL): 100 mM potassium phosphate buffer (pH 7.4), microsomes (1 mg/mL), 1 mM $NADP^+$, glucose-6-phosphate (10 mM), glucose-6-phosphate dehydrogenase (2 units/mL) and $MgCl_2$ (5 mM). The mixtures were pre-incubated at 37 °C for 5 min. The metabolic reaction (in triplicate) was initiated by addition of EZM or EZM-K (10 μM, added as methanolic solution with a final methanol concentration of 1%). After 120 min incubation, the reaction was terminated by addition of 75 μL of acetonitrile containing 25% perchloric acid, to this 10 μL of IS working stock solution was added, vortexed and centrifuged at 13,000 rpm for 4 min. Clear supernatant (100 μL) was separated and analyzed on HPLC as mentioned above. Both rat and human liver microsomes viability was confirmed with positive control i.e., testosterone (data not shown).

2.11. Phase-II enzyme assay

The incubation mixtures of EZM or EZM-K with rat or human liver microsomes contained the following at the indicated final concentrations (final volume of 500 μL): 50 mM Tris–HCl buffer (pH 7.4) containing 0.02% (w/v) Brij-58, a non-ionic surfactant, microsomes (1 mg/mL) and EZM or EZM-K (50 μM, added as methanolic solution). The mixtures were pre-incubated at 37 °C for 5 min. The metabolism reaction (in triplicate) was initiated by addition of UDP-glucuronic acid (UDPGA, 2 mM). After 120 min incubation, the reaction was terminated by addition of 75 μL of acetonitrile containing 25% perchloric acid, to this 10 μL of IS working stock solution was added, vortexed and centrifuged at 13,000 rpm for 4 min. An aliquot of 100 μL was injected on to a column and analyzed by HPLC as mentioned above.

2.12. Animal study

EZM was administered *intravenously* at a dose of 10 mg/kg through tail vein and the animals were anaesthetized with pentobarbitone sodium (50 mg/kg, *i.p*) throughout the experimental period. During the experimental period body temperature of the rats was maintained at 37 °C with a heating pad. Bile was collected from rats at 1 h intervals for 6 h directly into microcentrifuge tubes (∼1 mL at each time interval) containing 100 μL of 0.1 M ammonium acetate buffer, pH 4.5 and stored at −20 °C till analysis. At the time of analysis bile was allowed to thaw at ambient room temperature unassisted. To a 500 μL of bile sample, 10 μL of IS working stock was added and mixed for 15 s on a cyclomixer (Remi Instruments, Mumbai, India); followed by precipitation with 75 μL of acetonitrile containing 25% perchloric acid. The mixture was vortexed; centrifuged at 13,000 rpm for 4 min. An aliquot of 100 μL was injected on to a column for analysis.

3. Results

3.1. Method development

In order to select optimum conditions of separation, preliminary tests were performed with EZM, EZM-K and EZM-G. Parameters such as detection of wavelength, optimal mobile phase and their proportions, optimum pH and concentration of the buffer were studied. Based on the wavelength of maximal absorption for all analytes, 250 nm was set for the quantification by HPLC. Feasibility of various mixture(s) of solvents such as acetonitrile and methanol using different buffers such as ammonium acetate, phosphate and formic acid with variable pH range of 3.0–5.0, along with altered flow-rates (in the range of 0.7–1.0 mL/min) was tested for complete chromatographic resolution of EZM, EZM-K, EZM-G and IS (data not shown). Finally, the use of formic acid (pH 3.0) based buffer along with acetonitrile and methanol provided adequate peak separation. Versatility, suitability, and robustness of the method was checked with several C_{18} columns from various manufacturers viz., Kromasil C_{18} (250 mm × 4.6 mm, 5 μm, Hichrom, Berkshire, UK), Symmetry shield C_{18}, (250 mm × 4.6 mm, 5 μm, Waters corporation, Milford, Ireland) and C_{18} Inertsil® ODS 3 V column (4.6 mm × 250 mm, 5 μm, GL Sciences Inc., Tokyo, Japan) by running four replicates of each combination

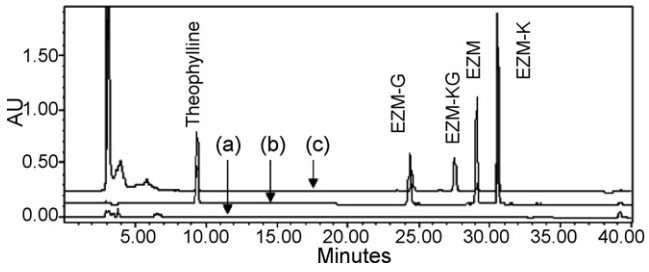

Fig. 2. HPLC chromatograms of a 100 μL injection of (a) blank incubation mixture; (b) incubation mixture spiked with EZM, EZM-K and EZM-G at LOQ (0.02 μg/mL) and 1 μg/mL of IS and (c) an *in vitro* phase-II metabolism incubation sample terminated at 0.5 h.

set comprising EZM, EZM-K, EZM-G and IS under identical HPLC conditions (data not shown). It was found that chromatographic resolution, selectivity, and sensitivity were good with Symmetry shield C_{18} column.

3.2. Specificity and chromatography

In the chosen chromatographic conditions, specificity was indicated by the absence of any endogenous interference at retention times of peaks of interest as evaluated by chromatograms of blank incubation mixture, liver microsomes (rat and human) and rat bile and incubation mixture, liver microsomes (rat and human) and rat bile spiked with EZM, EZM-K, EZM-G and IS. All the analytes viz., EZM-G, EZM-KG, EZM, EZM-K and IS were well separated with retention time of 24.23, 27.82, 29.04, 30.56 and 9.39 min, respectively. Fig. 2 shows a typical overlaid chromatogram for the incubation mixture (free of analytes and IS), incubation spiked with IS, EZM-G, EZM and EZM-K at the LOQ (0.02 μg/mL) and an *in vitro* incubation sample containing EZM and EZM-K mixture subjected for phase-II metabolism. Fig. 3 shows an overlaid chromatogram for the rat and human liver microsomes (free of analytes and IS) and rat liver microsomal samples spiked with IS, EZM-G, EZM and EZM-K, whereas Fig. 4 shows an overlaid chromatogram for the rat bile (free of

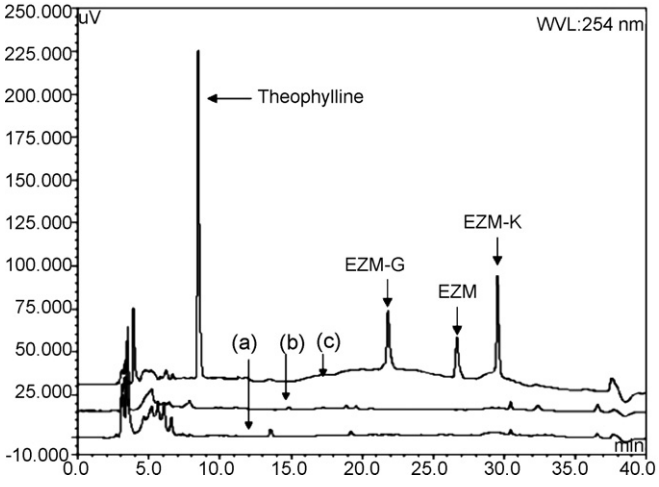

Fig. 3. HPLC chromatograms of a 100 μL injection of (a) blank RLM; (b) blank PHLM; (c) blank RLM spiked with IS, EZM-G, EZM and EZM-K at LOQ (0.02 μg/mL).

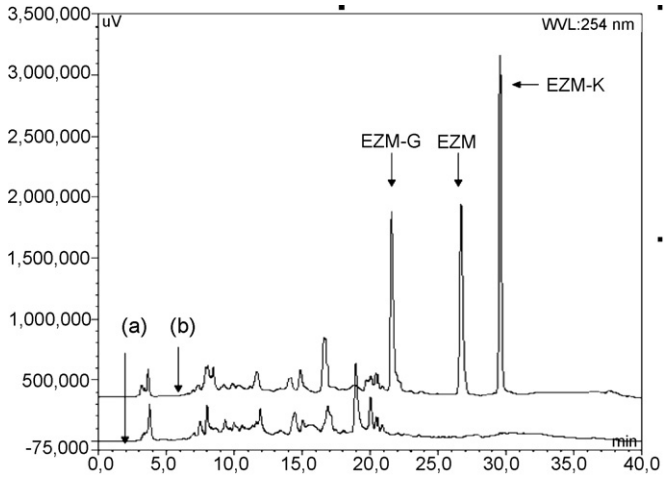

Fig. 4. HPLC chromatograms of a 100 μL injection of (a) blank rat bile; (b) blank rat bile spiked with EZM-G, EZM and EZM-K at LOQ (0.02 μg/mL).

analytes and IS) and rat bile spiked with EZM-G, EZM and EZM-K.

3.3. Calibration curve

Peak area ratios of EZM or EZM-G or EZM-K to the IS were measured and used for quantitation. A representative calibration graph of peak-area ratio of EZM or EZM-G or EZM-K to IS was linear ($r^2 > 0.998$) and reproducible in the concentration range of 0.02–10.0 μg/mL.

3.4. Precision and accuracy

Precision and accuracy data for intra- and inter-day for EZM, EZM-G and EZM-K in rat liver microsomes (RLM) and pooled human liver microsomes (PHLM) are presented in Tables 2–4, respectively.

3.5. Extraction recovery

The results of the comparison of neat standards versus incubation mixture/bile-precipitated standards were estimated at 0.2 and 2.0 μg/mL concentrations. The absolute recoveries ranged from 70 to 85% for EZM and EZM-G, whereas for EZM-K it was ∼50% across the concentrations. The absolute recovery of IS at 10 μg/mL was >80%.

3.6. Phase-I enzyme assay

In vitro metabolism experiments carried out with EZM in both rat and human liver microsomes supplemented with NADPH generating system and subsequent HPLC analysis of incubation mixture extracts revealed the formation of one metabolite (**EZM-M1**), which eluted at 30.56 min matched with retention time of synthetic standard i.e., EZM-K. LC–MS analysis of **EZM-M1**, which was formed in both rat and human liver microsomes indicated a deprotonated molecule $(M-H)^-$ at *m/z* 406, suggesting oxidation product of the parent compound. Further,

Table 2
Intra and inter-day precision data of EZM quality controls in RLM and PHLM

Theoretical concentration (μg/mL)	Run	Measured concentration (μg/mL)							
		RLM				PHLM			
		Mean	SD	RSD	Accuracy (%)	Mean	SD	RSD	Accuracy (%)
Intra day variation (Four replicates at each concentration)									
0.02	1	0.018	0.000	1.11	90.0	0.019	0.000	1.33	95.0
	2	0.018	0.000	0.67	90.0	0.020	0.000	0.80	100.0
	3	0.019	0.000	0.58	95.0	0.019	0.000	0.81	95.0
	4	0.019	0.000	0.67	93.0	0.019	0.000	0.80	93.0
0.06	1	0.058	0.001	1.35	96.7	0.059	0.000	0.71	98.3
	2	0.061	0.001	1.29	101.7	0.057	0.001	0.97	95.0
	3	0.057	0.000	0.71	95.0	0.060	0.001	1.39	100.0
	4	0.059	0.000	0.54	98.3	0.060	0.001	0.85	100.0
4.00	1	4.288	0.250	5.84	107.2	4.128	0.120	2.91	103.2
	2	4.149	0.269	6.49	103.7	4.019	0.309	7.70	100.5
	3	4.199	0.249	5.94	105.0	3.991	0.143	3.59	99.8
	4	4.054	0.186	4.59	101.4	4.151	0.136	3.28	103.8
8.00	1	8.228	0.211	2.56	102.9	8.041	0.107	1.34	100.5
	2	8.349	0.234	2.80	104.4	8.145	0.131	1.60	101.8
	3	8.053	0.364	4.52	100.7	8.163	0.236	2.90	102.0
	4	8.141	0.254	3.12	101.8	7.941	0.154	1.94	99.3
Inter day variation (sixteen replicates at each concentration)									
0.02		0.018	0.001	5.85	94.0	0.019	0.001	5.47	96.5
0.06		0.057	0.001	1.50	96.4	0.059	0.000	0.70	98.5
4.00		4.211	0.253	6.02	105.3	4.018	0.183	4.56	100.5
8.00		8.210	0.301	3.67	102.6	8.111	0.263	3.24	101.4

RSD: Relative standard deviation (SD × 100/mean).
RLM: Rat liver microsomes.
PHLM: Pooled human liver microsomes.

Table 3
Intra and inter-day precision data of EZM-G quality controls in RLM and PHLM

Theoretical concentration (μg/mL)	Run	Measured concentration (μg/mL)							
		RLM				PHLM			
		Mean	SD	RSD	Accuracy (%)	Mean	SD	RSD	Accuracy (%)
Intra day variation (Four replicates at each concentration)									
0.02	1	0.020	0.001	5.47	99.5	0.019	0.001	4.62	94.5
	2	0.019	0.001	3.06	95.0	0.020	0.000	1.16	100.0
	3	0.019	0.001	4.36	94.4	0.020	0.001	2.74	101.0
	4	0.019	0.001	4.76	94.0	0.019	0.000	1.78	97.0
0.06	1	0.058	0.001	1.31	96.7	0.057	0.001	1.23	95.3
	2	0.062	0.001	1.11	102.9	0.061	0.001	1.06	101.2
	3	0.057	0.001	2.41	95.7	0.058	0.001	2.43	96.9
	4	0.059	0.002	4.07	98.3	0.060	0.002	3.13	100.5
4.00	1	4.138	0.118	2.86	103.4	4.016	0.110	2.74	100.4
	2	4.228	0.106	2.52	105.7	4.121	0.104	2.53	103.0
	3	4.007	0.150	3.73	100.2	4.016	0.117	2.90	100.4
	4	4.184	0.214	5.11	104.6	3.986	0.112	2.83	99.7
8.00	1	8.164	0.240	2.94	102.0	8.113	0.143	1.76	101.4
	2	8.033	0.157	1.96	100.4	8.086	0.192	2.38	101.1
	3	7.968	0.328	4.11	99.6	7.981	0.267	3.35	99.8
	4	8.214	0.254	3.09	102.7	8.011	0.202	2.52	100.1
Inter day variation (Sixteen replicates at each concentration)									
0.02		0.019	0.001	4.50	96.2	0.019	0.000	2.37	97.5
0.06		0.058	0.002	3.86	96.8	0.059	0.002	3.71	99.2
4.00		4.195	0.230	5.47	104.9	4.038	0.161	3.99	101.0
8.00		8.120	0.317	3.90	101.5	8.062	0.202	2.50	100.8

RSD: Relative standard deviation (SD × 100/mean).
RLM: Rat liver microsomes.
PHLM: Pooled human liver microsomes.

Table 4
Intra and inter-day precision data of EZM-K quality controls in RLM and PHLM

Theoretical concentration (μg/mL)	Run	Measured concentration (μg/mL)							
		RLM				PHLM			
		Mean	SD	RSD	Accuracy (%)	Mean	SD	RSD	Accuracy (%)
Intra day variation (Four replicates at each concentration)									
0.02	1	0.018	0.001	4.71	92.5	0.019	0.001	3.59	94.5
	2	0.021	0.001	3.57	104.4	0.020	0.000	2.20	101.4
	3	0.020	0.001	6.53	102.1	0.020	0.001	5.12	100.8
	4	0.020	0.001	5.16	99.3	0.020	0.001	4.59	98.2
0.06	1	0.058	0.002	3.38	96.0	0.060	0.002	2.75	99.5
	2	0.057	0.001	1.84	94.9	0.057	0.001	1.80	94.3
	3	0.061	0.001	2.04	101.1	0.060	0.001	1.69	100.7
	4	0.061	0.002	3.98	100.8	0.060	0.002	3.22	100.4
4.00	1	4.130	0.204	4.93	103.3	4.102	0.191	4.67	102.5
	2	4.100	0.229	5.59	102.5	4.101	0.204	4.98	102.5
	3	3.898	0.103	2.63	97.5	3.974	0.102	2.56	99.3
	4	3.962	0.118	2.98	99.1	3.987	0.107	2.70	99.7
8.00	1	8.238	0.263	3.20	103.0	8.207	0.244	2.97	102.6
	2	8.156	0.334	4.09	102.0	7.995	0.336	4.21	99.9
	3	8.206	0.467	5.69	102.6	8.174	0.360	4.41	102.2
	4	8.119	0.311	3.83	101.5	8.049	0.247	3.07	100.6
Inter day variation (Sixteen replicates at each concentration)									
0.02		0.019	0.263	5.97	94.8	0.019	0.001	5.13	99.3
0.06		0.059	0.334	2.11	98.5	0.059	0.001	1.85	99.6
4.00		4.151	0.467	0.05	103.8	4.101	0.002	0.05	102.5
8.00		8.280	0.311	2.16	103.5	8.110	0.146	1.80	101.4

RSD: Relative standard deviation (SD × 100/mean).
RLM: Rat liver microsomes.
PHLM: Pooled human liver microsomes.

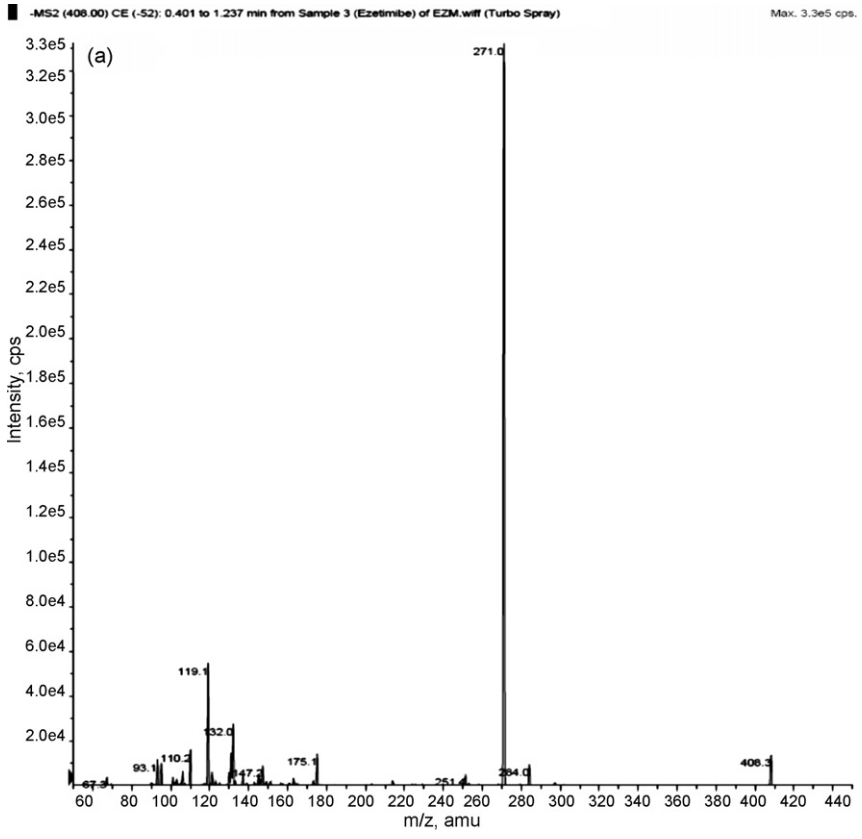

Fig. 5. Representative CID spectrum of EZM (upper panel), EZM-G (middle panel) and EZM-K (lower panel).

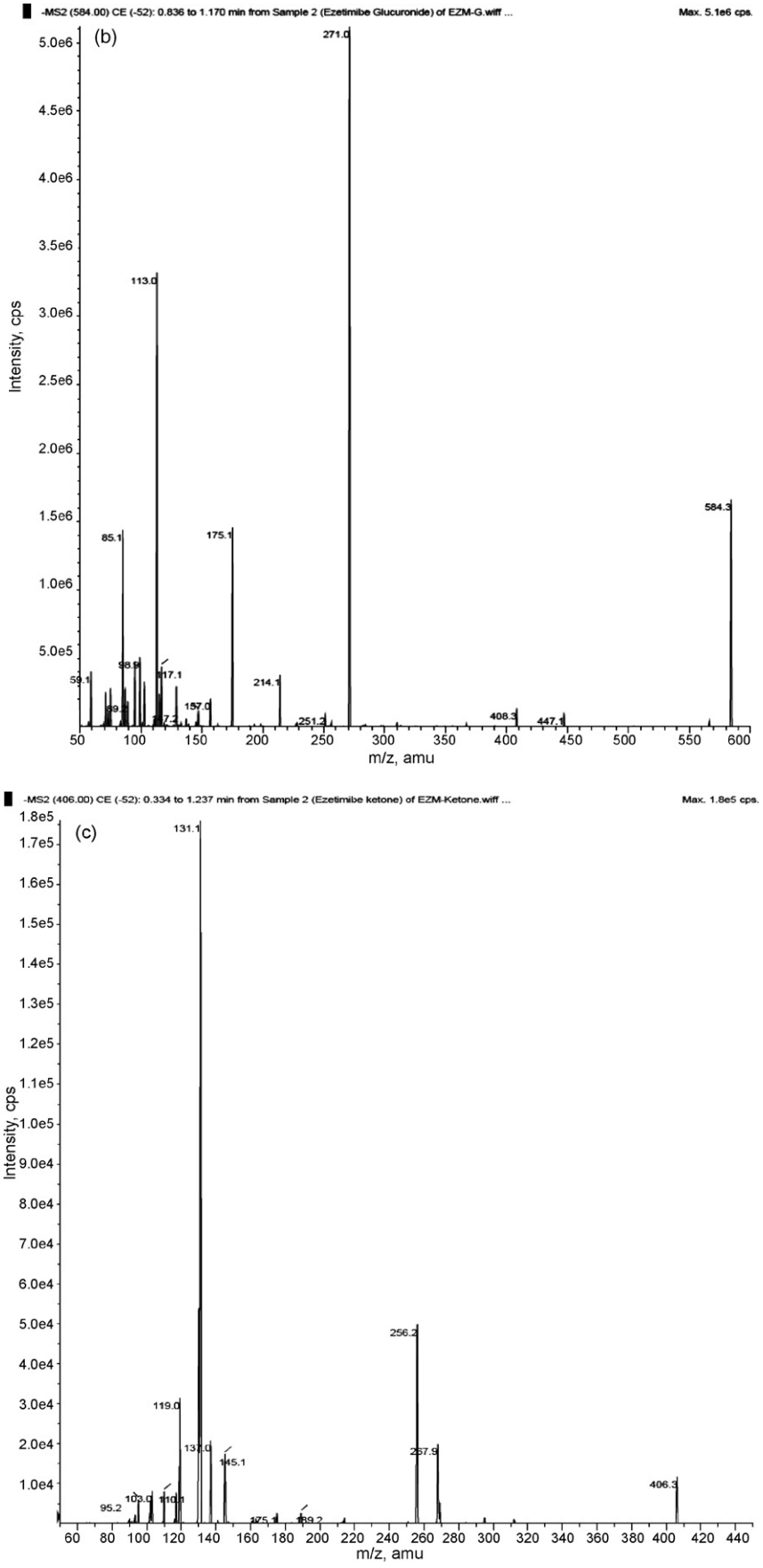

Fig. 5. (*Continued*).

the structure of the metabolite was confirmed by comparison of chromatographic and MS data with the synthetic standard material i.e., EZM-K. The CID spectrum of *m/z* 406 generated a series of product ions at *m/z* 137, 131.1, 118.9 and 95.1, which matched with the mass spectrum of authentic synthetic standard (EZM-K) (Fig. 5c). Following 2 h incubation it was found that EZM-K was metabolically stable in human liver microsomes, whereas in rat liver microsomes around 30% disappearance of EZM-K was seen. Efforts are going on to identify the minor metabolites of EZM-K.

3.7. Phase-II enzyme assay

When EZM or EZM-K was used as substrate for glucuronidation (in rat and human liver microsomes in presence of UDPGA) one peak in each reaction mixture was detected by HPLC, namely **EZM-M2** and **EZM-K-M1**, respectively, which disappeared after β-glucuronidase hydrolysis (data not shown). LC–MS analysis of **EZM-M2** and **EZM-K-M1** revealed a deprotonated molecule $(M-H)^-$ at *m/z* 585 (176 Da higher than EZM) and $(M-H)^-$ at *m/z* 583 (176 Da higher than EZM-K), respectively suggesting monoglucuronide formation for both EZM and EZM-K. On HPLC the retention time of synthetic standard of EZM glucuronide i.e., EZM-G matches with the retention time of **EZM-M2**. The CID spectrum **EZM-M2** generated a series of product ions at *m/z* 408.3, 284.1, 271.2, 175, 146.9, 130, 112.9, 84.9 and 58.9, which matched with authentic synthetic standard i.e., EZM-G (Fig. 5b). The product ion at *m/z* 408.3 (corresponding to *m/z* of EZM) was generated through neutral loss of 176 Da, a characteristic cleavage for glucuronide conjugate. The sequential loss of 124 (C_7H_5OF, fluorobenzaldehyde), 13 (–CH), 96 (C_6H_5F), 28 (–CO) from *m/z* 408.3 generated ions at *m/z* 284.1, 271.2, 175, 146.9, respectively (Fig. 5a). Based on these data, **EZM-M2** was identified as EZM-G. Similarly, the CID spectrum of **EZM-K-M1** generated a series of product ions *m/z* 406.1, 137, 131.1, 118.9 and 95.1. The product ion at *m/z* 406.1 (corresponding to *m/z* of EZM-K) was generated through neutral loss of 176 Da, a characteristic cleavage for glucuronide conjugate. All the product ions after *m/z* 406.1 (from **EZM-K-M1**) were similar to those in CID spectrum of EZM-K. Based on these data **EZM-K-M1** was identified as EZM-KG.

3.8. LC–MS/MS identification of metabolites in bile

HPLC and LC–MS/MS analysis of bile collected following *intravenous* administration of EZM to male Wistar rats revealed the presence of large amounts of EZM-G and EZM-KG and trace amounts of EZM and EZM-K.

4. Discussion

The present day trend in bioanalysis calls for the analysis of multiple analytes in a single run and therefore, speed of discovery and development of drugs are not compromised [18]. In this work, a total of five analytes were separated in a single analytical run. Regardless of the polarity, functionality and optimized gradient flow conditions, it was possible to separate and quantify EZM, EZM-K (in rat and human liver microsomes) and EZM-G (in rat and human liver microsomes fortified with UDPGA); and qualitative analysis of EZM, EZM-K, EZM-G and EZM-KG in bile.

5. Conclusion

The assay developed is specific and reproducible for the qualitative determination of EZM, EZM-K, EZM-KG and EZM-G simultaneously in single HPLC run. The analytical method established in our laboratory has widened applicability viz., various *in vitro* metabolism studies (phase-I and II) and bile sample analysis.

References

[1] G. Watts, Clin. Sci. 103 (2002) 595.
[2] R.H. Knopp, H. Gitter, T. Truitt, H. Bays, C.V. Manion, L.J. Lipka, A.P. LeBeaut, R. Suresh, B. Yang, E.P. Veltri, Ezetimibe Study Group, Eur. Heart J. 24 (2003) 729.
[3] G. Salen, K. von Bergmann, D. Lutjohann, P. Kwiterovich, J. Kane, S.B. Patel, T. Musliner, P. Stein, B. Musser, Multicenter Sitosterolemia Study Group, Circulation 109 (2004) 966.
[4] M. Van Heek, C. Farley, D.S. Compton, L. Hoos, K.B. Alton, E.J. Sybertz, H.R. Davis, Br. J. Pharmacol. 129 (2000) 1748.
[5] M. Van Heek, C. Farley, D.S. Compton, L. Hoos, H.R. Davis, Br. J. Pharmacol. 134 (2001) 409.
[6] S.W. Altmann, H.R. Davis, L.J. Zhu, X. Yao, L.M. Hoos, G. Tetzloff, S.P. Iyer, M. Mauire, A. Golovko, M. Zeng, L. Wang, N. Murgolo, M.P. Graziano, Science 103 (2004) 595.
[7] H.E. Bays, P.B. Moore, M.A. Drehobl, S. Rosenblatt, P.D. Toth, C.A. Dujovne, R.H. Knopp, L.J. Lipka, A.P. LeBeaut, B. Yang, L.E. Mellars, C. Cuffie-Jackson, E.P. Veltri, Ezetimibe Study Group, Clin. Ther. 23 (2001) 1209.
[8] C.A. Dujovne, M.P. Ettinger, J.F. McNeer, L.J. Lipka, A.P. LeBeaut, R. Suresh, B. Yang, E.P. Veltri, Ezetimibe Study Group, Am. J. Cardiol. 90 (2002) 1092.
[9] J.E. Patrick, T. Kosoglou, K.L. Stauber, A.B. Alton, S.E. Maxwell, Y. Zhu, P. Statkevich, R. Iannucci, S. Chowdhury, M. Affrime, M.N. Cayen, Drug Metab. Dispos. 30 (2002) 430.
[10] A. Ghosal, N. Hapangama, Y. Yuan, J. Achanfuo-Yeboah, R. Iannucci, S. Chowdhury, K. Alton, J.E. Patrick, S. Zbaida, Drug Metab. Dispos. 32 (2004) 314.
[11] F. Ezzet, G. Krishna, D.B. Wexler, P. Statkevich, T. Kosoglou, V.K. Batra, Clin. Ther. 23 (2001) 871.
[12] L. Reyderman, T. Kosoglou, S.E. Maxwell, C. Chung, V. Batra, P. Statkevich, Clin. Pharm. Ther. 71 (2002) P97 (WPIII-79).
[13] R. Sistla, B.S.S.K. Tata, Y.V. Kashyap, D. Chandrasekar, P.V. Diwan, J. Pharm. Biomed. Anal. 39 (2005) 517.
[14] S. Oswald, E. Scheuch, I. Cascorbi, W. Siegmund, J. Chromatogr. B 830 (2006) 143.
[15] S. Li, G. Liu, J. Jia, X. Li, C. Yu, J. Pharm. Biomed. Anal. 40 (2006) 987.
[16] V.P. Shah, K.K. Midha, J.W. Findlay, J.D. Hulse, I.J. Mcgliveray, G. McKay, K.J. Miller, R.N. Patnaik, M.I. Powell, A. Tonelli, C.T. Viswanathan, J. Pharm. Sci. 17 (2000) 1551.
[17] The United States Pharmacopoeia, United States Pharmacopoeial Convention, Rockville, MD, 1995, p. 1982.
[18] N.R. Srinivas, Biomed. Chromtogr. 20 (2006) 383.