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Research Article

A Validated LC-MS/MS Method for Pharmacokinetic Study of Edoxaban in Healthy Rabbits

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ABSTRACT

A liquid chromatography-tandem mass spectrophotometric (LC–MS/MS) method was developed to quantify Edoxaban in rabbit plasma employing liquid liquid extraction with ethyl acetate. Developed method was validated for specificity, precision, accuracy, recovery, and stability characteristics. Chromatographic separation was achieved on Chromolith C18column (100 mm x 4.6 mm x 5 µm) with 70:30 ratio of methanol and 0.1% formic acid as an isocratic mobile phase with a flow rate of 0.80 mL/min. The developed LC-MS method was applied to assess pharmacokinetics parameters of edoxaban in healthy rabbits. Six Male albino rabbits weighing 2.0-2.5 Kg were randomly selected for the pharmacokinetic study. Blood samples (1-mL) were withdrawn from the marginal ear vein from 0 to 24 hours after administration (1.2 mg/kg). Plasma was separated by centrifugation and the plasma concentrations of edoxaban at various times were determined by LC-MS/MS. Pharmacokinetic parameters was calculated. Edoxaban showed T_{max} of 2.0 and mean $C_{max,}$ $AUC_{0@t}$ and $AUC_{0@a}$ for Test formulation is 213.83 ± 10.46, 945.13 ± 24.32 and 986.135 ± 19.31, respectively. A highly specific, rugged, and rapid method with sufficiently low LLOQ was developed to analyze routine samples of single dose or multiple-dose pharmacokinetics with any marketing formulation of edoxaban.

Introduction

Edoxaban is a direct, oral factor Xa (FXa) inhibitor with linear and predictable pharmacokinetics (PK),[1] indicated for the prevention of stroke in patients with nonvalvular atrial fibrillation (NVAF) and the treatment of venous thromboembolism (VTE).[2] Large phase III studies have demonstrated that direct oral anticoagulants (DOACs), including edoxaban, are at least as efficacious as warfarin and are associated with less major bleeding in patients with NVAF and VTE.[3-9] Edoxaban (EXN), chemically known as *N'*-(5-chloropyridin-2-yl)-*N*-[(1*S*,2*R*,4*S*)-4-(dimethylcarbamoyl)- 2- [(5-methyl-6,7-dihydro-4*H*- [1,3] thiazolo [5,4-c] pyridine-2-carbonyl) amino] cyclohexyl] oxamide, is an oral anticoagulant that acts as highly specific direct factor Xa inhibitor.[10] So far, EXN is not official in any pharmacopeias. The literature is poor regarding the reports on the assay of EXN[11] analyzed EXN and its four metabolites in human plasma, urine, and fecal samples, after oral administration of [14C] EXN to 6 healthy male subjects by either high-performance liquid chromatography/tandem mass spectrometry or a liquid chromatography radiometric method. This study was used to determine the mass balance and pharmacokinetics of EXN in humans. A validated turbulent flow liquid chromatography with high-resolution mass spectrometry was developed for assay of EXN in human plasma, which was time-consuming because of long chromatographic analysis (6 minutes). This method was applied for the therapeutic drug monitoring of EXN.[12] A highly sensitive, selective, and rapid UPLC–MS/MS method was developed to simultaneously estimate rivaroxaban, apixaban, and edoxaban in rat plasma. The quantitative analysis of EXN in bulk and tablet dosage forms.[13] In the current article, we describe a highly sensitive, selective, and rapid LC–MS/

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MS method developed and fully validated to estimate edoxaban in rabbit plasma. After oral administration, this method is also successfully applied to the pharmacokinetic study in rabbits. It could be easily extended to analyze edoxaban in other biological matrix or other species of animals, even in humans.

Materials and Methods

Apparatus and Software

The HPLC system with an auto sampler was a Shimadzu LC- 20ADvp (Shimadzu, Japan) coupled with Applied Biosystem Sciex (MDS Sciex, Canada) API 4000 Tandem mass spectrometer. The auto sampler was SIL-HTC from Shimadzu, Japan. The solvent delivery module was LC-20AD from Shimadzu, Japan. The chromatographic integration was performed by Analyst software (version: 1.4.2; Applied Biosystems).

Chemicals and Reagents

Edoxaban and apixaban (IS) were procured from Lara Drugs Pvt. Ltd, Nalgonda, Telangana, Mumbai, India, water used was collected from water purification systems (Milli Q, Milli Pore, USA) installed in the laboratory, formic acid analytical grade, and supplied by J. T. Baker, USA. Hyderabad. The study was approved by Institutional Ethical committee no: VCP/IAEC/2017-47.

Calibration Standard Solutions

Stock solutions of edoxaban and apixaban internal standard (IS) were prepared in methanol. Further dilutions were carried out in 70% methanol. Calibration standards of eight concentration levels were prepared freshly by spiking drug-free plasma with Edoxaban stock solution to give the concentrations of 1.0, 2.0, 5.0, 10, 20, 50, 100, and 200 ng/mL.

Quality Control Standards

The lowest quality control standards, Median quality control standards, and highest quality control standards were prepared by spiking drug-free plasma with edoxaban to give a solution containing 2.0, 40, and 160 ng/mL. They were stored at –20°C till the time analyzed.

Chromatographic Conditions

Chromatographic separation was performed on a Chromolith C18 column (100 mm x 4.6 mm x 5 µm) with 70:30 ratio of methanol and 0.1% formic acid as an isocratic mobile phase with a flow rate of 0.80 mL/min. Total analysis time of single injection was 3 minutes. Column oven temperature and auto sampler temperature was set to 40°C and 5°C, respectively.

Mass Spectrometric Conditions

The LC eluent was split (75%), and approximately 0.25 mL/min was introduced and Quantitation was achieved with MS/MS detection in negative ion mode for the analytes and IS using a MDS Sciex API-4000 mass spectrometer (Foster City, CA, USA) equipped with Turboion spray™ interface at 400ºC. The ion spray voltage was set at 5500 V. The source parameters viz., the nebulizer gas, curtain gas, CAD gas were set at 40, 40 and 5 psi, respectively. The compound parameters viz. the declustering potential (DP), collision energy (CE), entrance potential (EP) and collision cell exit potential (CXP) for MT and MT-*D3* were similar and are -55, -25, -10, -6 V. For Edoxaban and Edoxaban D3 the DP, CE, EP and CXP were -55, -24, -10 and -18 V. A Turbo ion spray interface (TIS) operated in negative ionization mode was used for the detection. Detection of the ions was carried out in the multiple reaction monitoring mode (MRM), by monitoring the transition pairs of m transitions of m/z 548.2/366.1 for Edoxaban and m/z 460.0 -443.1for apixaban. Quadrupoles Q1 and Q3 were set on unit resolution.

Study Design

Six Male albino Rabbits (weighing about 2.5 kg) were selected as the animal model. The age of the rabbits was 8–12 weeks. The Rabbits selected for the study had no medication for two weeks prior to the study. Twelve hours before drug administration, food was withdrawn from the rabbits until 24 hr post-dosing, while water was available for rabbits throughout the study. The 1.2mg/kg were administered to rabbits. Blood samples (0.6 ml) were withdrawn from the marginal ear vein before dosing (zero time) and at time intervals of 0.25, 0.5, 1, 1.5, 2, 3, 4, 6, 8, 12 and 24 hours after administration. EDTA disodium salt was used as an anticoagulant. Plasma was separated by centrifugation at 5000 rpm for 10 minutes and the resulting plasma sample from each blood sample was divided into two aliquots and stored in suitably labeled polypropylene tubes at -20°C until used. All the plasma samples were analyzed under the construction of standard calibration curve of edoxaban in rabbit's plasma. The edoxaban concentrations in the rabbit plasma samples were calculated using the calibration curve, obtained after linear regression of the peak area ratio (edoxaban/apixaban) versus edoxaban concentration. The study was approved from the IAEC with ref no: VCP/IAEC/2016-45.

Sample Preparation Method

To 300 µL of plasma, 50 µL of apixaban (1-µg/mL) was added and vortexed. The drug was extracted with 3 mL of ethyl acetate followed by centrifugation at 4000 rpm/min on a cooling centrifuge for 15 minutes at 4°C. The organic phase was withdrawn and dried using lyophiliser. To the residue, 250 µL of mobile phase was added, transfer appropriate volume of samples into pre-labeled Auto sampler vials, and inject by using HPLC-ESI-MS/MS.

Pharmacokinetic Analysis

Single dosage pharmacokinetic parameters were calculated with PK Solver tool from plasma drug concentration-time data by non-compartmental methods.

The maximum plasma concentration (C_{max}) and time to maximum plasma concentration (Tmax) were obtained directly from the observed concentration-time profiles. Linear trapezoidal rule was used to estimate the area under the plasma concentration versus time curve (AUC) from 0 to the last measurable concentration (AUC_{0-t}). The area under the plasma concentration versus time curve from 0 to infinity ($AUC_{0-\infty}$) was calculated as $AUC_{0-t}+ C_{t/ke}$, where C_t was the last measurable concentration. Ke was the elimination rate constant. The terminal elimination half-life ($t_{1/2}$) was calculated as 0.693/Ke.

Validation

Specificity

A solution containing 1.0ng/ml was injected on to the column under optimized chromatographic conditions to show the separation of Edoxaban from impurities and plasma. The specificity of the method was checked for interference from plasma.

Linearity

Spiked concentrations were plotted against peak area ratios of Edoxaban to internal standard and the best fit line was calculated. Wide range calibration was determined by solutions containing 1-ng/mL to 200 ng/mL.

Recovery Studies

The %mean recoveries were determined by measuring the responses of the extracted plasma Quality control samples at HQC, MQC, and LQC against unextracted Quality control samples at HQC, MQC, and LQC.

Precision and Accuracy

Intraday precision and accuracy were determined by analyzing quality control standards (20, 40 and 160 ng/mL) and LLOQ Quality control standard (1-ng/mL) five times a day randomly, interday precision and accuracy was determined from the analysis of each quality control standards (20, 40and 160 ng/mL) and LLOQ Quality Control standards (1.0-ng/mL) once on each of five different days.

Matrix Effect

The matrix effect for the intended method was assessed by using chromatographically screened human plasma. Concentrations equivalent to LQC and HQC of edoxaban were prepared with six different lots of plasma and are injected.

Results and Discussion

Results of Method Validation

The chromatography observed during validation was acceptable and representative chromatograms of standard blank, HQC, MQC, and LQC samples are shown in Fig. 1 to 4.

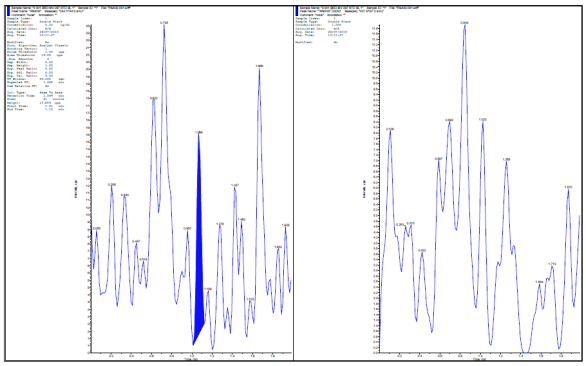

Fig. 1: Representative blank chromatograms of edoxaban and IS in blank plasma

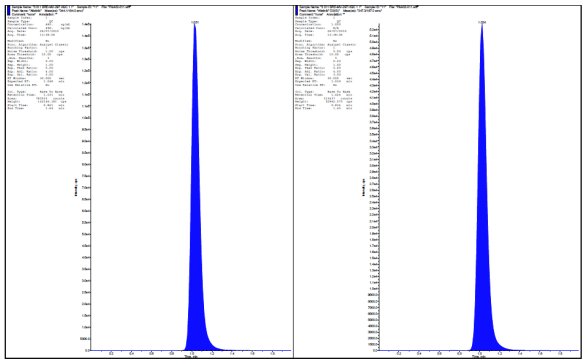

Fig. 2: Representative HQC-chromatograms of edoxaban in plasma with internal standard

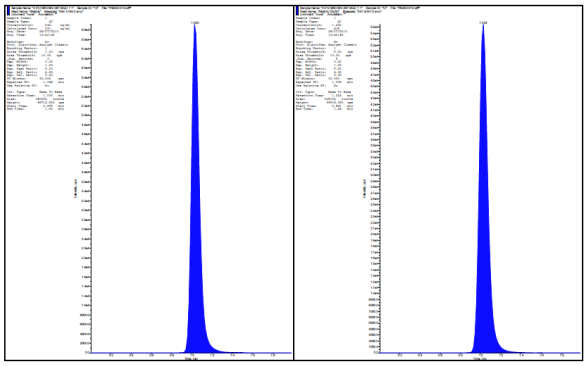

Fig. 3: Representative MQC- chromatograms of edoxaban & its internal standard

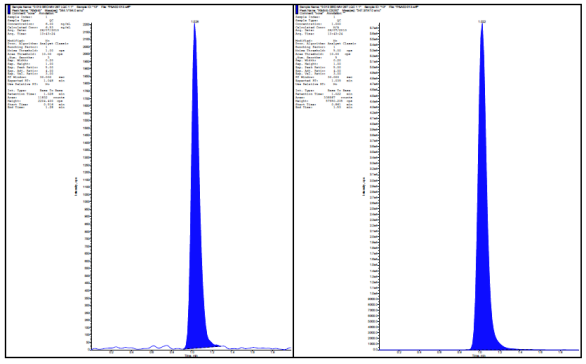

Fig. 4: Representative chromatograms of edoxaban & its internal standard at LQC Level

Table 1: Linearity standards of edoxaban

Actual conc. (ng/mL)	*1*	*2*	*5*	*10*	*20*	*50*	*100*	*200*	*Slope*	*Intercept*
1	0.99	1.93	5.01	10.18	20.54	50.11	103.17	195.50	0.984	0.689
2	0.97	1.93	5.05	9.99	20.11	49.56	105.17	196.25	0.991	0.557
3	0.99	1.97	5.09	9.85	19.75	49.22	102.33	194.00	0.977	0.505
Mean	0.98	1.94	5.05	10.01	20.13	49.63	103.56	195.25	0.984	0.584
±SD	0.01	0.02	0.04	0.17	0.39	0.45	1.46	1.15	0.007	0.095
%CV	1.32	0.99	0.83	1.67	1.96	0.90	1.41	0.59		
% Accuracy	98.33	97.22	100.96	100.07	100.67	99.26	103.56	97.63		
LOD	0.32									
LOQ	0.96									

Table 2: The% Mean Recovery of edoxaban for LQC, MQC and HQC

	LQC			*MQC*			*HQC*		
ID	*Unextracted*	*Extracted*		*Unextracted*	*Extracted*		*Unextracted*	*Extracted*	
	(Area ratio)	*(Area ratio)*	*% Recovery*	*(Area ratio)*	*(Area ratio)*	*% Recovery*	*(Area ratio)*	*(Area ratio)*	*% Recovery*
1	0.138	0.131	94.928	0.746	0.546	73.19	1.077	1.058	98.24
2	0.131	0.126	96.183	0.76	0.56	73.68	1.117	1.044	93.46
3	0.137	0.126	91.971	0.767	0.567	73.92	1.114	1.014	91.02
4	0.137	0.133	97.080	0.712	0.572	80.34	1.148	1.066	92.86
5	0.136	0.131	96.324	0.717	0.587	81.87	1.089	1.071	98.35
6	0.143	0.131	91.608	0.722	0.597	82.69	1.073	1.061	98.88
Mean	0.137	0.130	94.682	0.737	0.572	77.615	1.103	1.052	95.468
±SD	0.004	0.003	2.348	0.023	0.018	4.469	0.029	0.021	3.411
%CV	2.81	2.27	2.48	3.19	3.22	5.76	2.60	1.98	3.57

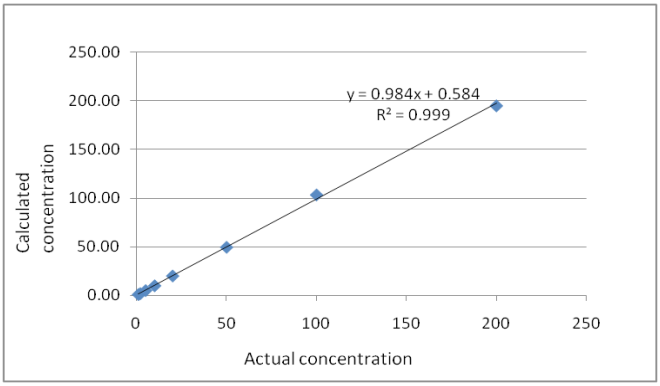

Fig. 5: Calibration curve

The method developed was validated for linearity, accuracy & precision, and stability as per ICH guidance (Q1A(R2) ICH, 2005; Q2(R1) ICH, 2005).[14,15] The results of validating parameters are given below.

Linearity

The three calibration curves (Calculated concentration Vs Actual Concentration) were linear over the working range of 1-ng/mL to 200 ng/mL with eight-point calibration used for quantification by linear regression (Fig 5). The regression equation for the analysis was Y=0.984x+0.584 with the coefficient of correction (r^2) = 0.999. The precision (%CV) observed for the calibration curve standards was found to be ≤ 1.96 for Edoxaban (Table 1).

Recovery

The %mean recovery for Edoxaban in LQC (20 ng/mL), MQC (40 ng/mL) and HQC (160 ng/mL) was 94.68%, 77.61% and 95.46%, respectively (Table 2).

Intra- and Inter-day Precision

The method's mean intraday and inter-day precision were found to be 0.78 to 3.95% for the quality control samples. This is within the acceptable limits of precision is 15%. The lower limit of quantification was found to be 1ng/ml. at such concentration the mean inter day and intraday precision was found to be 0.87% and 2.91%, respectively. Which are within the acceptable limits of precision is 20% (Table 3).

Matrix Effect

The % CV for HQC and LQC samples was observed 2.42% and 3.69%, respectively (Table-4), within 15% as per the acceptance criteria.

Results of Pharmacokinetic Studies

The Pharmacokinetic parameter of edoxaban was calculated from the plasma concentration-time curves

Table 3: Intra-day and Inter-day quality control samples for edoxaban

Edoxaban (ng/mL)				
Intra-batch	*LLOQ QC (1-ng/mL)*	*LQC (20 ng/mL)*	*MQC (40 ng/mL)*	*HQC (160 ng/ml)*
Mean	1.05	20.10	38.08	163.86
SD	0.03047	0.67	0.81	1.28
%CV	2.91	3.33	2.11	0.78
Mean	1.05221	17.13	39.07	168.32
SD	0.0092	0.68	0.80	1.97
%CV	0.87	3.95	2.06	1.17
Mean	1.06888	19.23	38.36	164.49
SD	0.01355	0.74	0.75	2.47
%CV	1.27	3.84	1.95	1.50
Inter-batch	LLOQ QC (1-ng/mL)	LQC (20 ng/mL)	MQC (40 ng/mL)	HQC (160 ng/mL)
Mean	1.06679	19.41	36.89	158.04
SD	0.01742	0.244	1.160	2.343
%CV	1.63274	1.26	3.15	1.48

Table 4: Matrix effect obtained with six different lots of plasma

QC ID	*LQC*	*HQC*
Actual conc.	20 (ng/mL)	160 (ng/mL)
1	20.37	161.53
2	20.12	160.57
3	19.20	159.08
4	19.87	159.48
5	21.28	154.60
6	19.45	166.55
Mean	20.05	160.30
± SD	0.74	3.88
% CV	3.69	2.42
% Accuracy	100.24	100.19

using pk solver software. Also, the area under the plasma concentration-time curve from 0 to 12 hours (AUC_{0-12}) was calculated using trapezoidal rule. Edoxaban showed T_{max} of 2.0 and mean C_{max}, $AUC_{0®t}$ and $AUC_{0®a}$ for Test

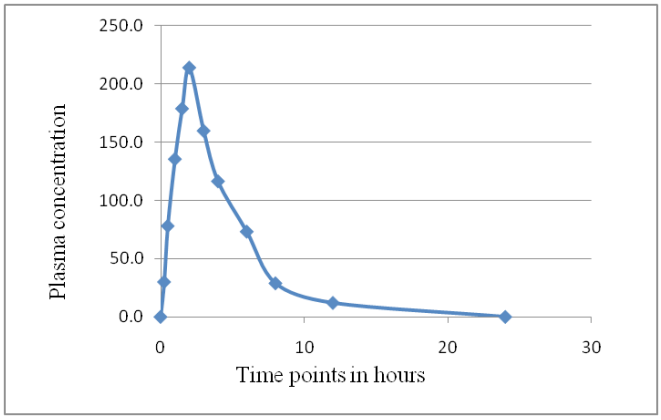

Fig. 6: Plasma time profile curves of test animals

Table 6: Calculated mean values of PK parameters for test animals

Parameter	*Unit*	*Avg value (n = 6)*	*SD*
Lambda_z	1/h	0.30084	0.03
$t_{1/2}$	h	2.32444	0.24
T_{max}	h	2	0.00
C_{max}	ng/ml	213.833	10.46
T_{lag}	h	0	0.00
Clast_obs/C_{max}		0.05662	0.02
AUC_{0-t}	ng/mL*h	945.125	24.32
AUC0-inf_obs	ng/mL*h	986.135	19.31
AUC0-t/0-inf_obs		0.95838	0.01
AUMC 0-inf_obs	ng/mL*h^2	4207.31	314.88
MRT 0-inf_obs	H	4.26564	0.30
Vz/F_obs	(mg)/(ng/ml)	0.00408	0.00
Cl/F_obs	(mg)/(ng/ml)/h	0.00122	0.00

Table 5: Calculated plasma concentrations in rabbits at each time point

Time points (Hours)	*Calculated concentrations (ng/mL)*							
	Rabbit 1	*Rabbit 2*	*Rabbit 3*	*Rabbit 4*	*Rabbit 5*	*Rabbit 6*	*Avg*	*SD*
0	0	0	0	0	0	0	0.0	0.0
0.25	28	21	36	22	32	41	30.00	7.87
0.5	84	77	84	74	68	81	78.00	6.29
1	122	129	127	146	150	138	135.33	11.17
1.5	173	166	175	183	194	181	178.67	9.65
2	206	199	212	216	227	223	213.83	10.46
3	171	152	158	145	163	169	159.67	10.03
4	112	105	117	124	112	128	116.33	8.50
6	71	77	68	83	61	79	73.17	8.06
8	38	31	24	33	28	19	28.83	6.74
12	11	16	14	12	11	8	12.00	2.76
24	0	0	0	0	0	0	0.0	0.0

formulation is 213.83 ± 10.46, 945.13 ± 24.32and 986.135 ± 19.31, respectively. The results were presented in Tables 5 and 6, and Fig. 6.

Conclusion

The bio-analytical methodology for the determination of Edoxaban described in this manuscript is highly specific, rugged and rapid for therapeutic drug monitoring both for analysis of routine samples of single dose or multiple dose pharmacokinetics and clinical trial samples with desired sensitivity, precision, accuracy, and high throughput. The method involved a simple and specific sample preparation by liquid-liquid extraction followed by isocratic chromatographic separation in 2 minutes. The overall analysis time is promising compared to other reported procedures for edoxaban. The established LLOQ is sufficiently low to conduct a pharmacokinetic study with any marketing formulation of edoxaban.

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