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Development and Validation of Bio-Analytical Method for Simultaneous Estimation of Metformin, Vildagliptin and Remogliflozin in Rabbit Plasma by using RP-HPLC

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Abstract

The current investigation was intended to develop and validate bio-analytical RP-HPLC method for the simultaneous analysis of Metformin, Vildagliptin and Remogliflozin in rabbit plasma employing Saxagliptin as an internal standard. Protein precipitation method was used to extract the analytes from spiked plasma samples. Chromatographic separation of extracted analytes was achieved on X Bridge C18, (150 mm X 4.6 mm and 3.5 µm) column using the mobile phase consists of 0.01N KH_2PO_4: acetonitrile (70: 30 v/v, pH 5.4). Isobestic point of 235 nm wavelength was selected for quantification of drugs. The peaks eluted at 2.262 min, 3.850 min and 5.903 min were recognized as Metformin, Vildagliptin and Remogliflozin respectively. The present method showed desirable proportional response in the range of 25-2500 ng/mL for Metformin, 5-1000 ng/mL for Vildagliptin and 25-2500 ng/mL for Remogliflozin. Low variance was observed (%CV) in the results of precision and accuracy and excellent and reproducible recoveries were obtained with spiked plasma samples. Stability studies such as long term, short term and freeze thaw stability were performed and produced inconsistent results. The results revealed the proposed method can be appropriate for the simultaneous bio-analysis of Metformin, Vildagliptin and Remogliflozin in rabbit plasma and successfully employed for pharmacokinetic studies.

Keywords: Metformin, Vildagliptin, Remogliflozin, Saxagliptin, Validation.

Introduction

Metformin HCl (MET) is a biguanide derivative that is recommended as a preferred choice to reduce the high glucose levels in diabetic patients. Chemically it is known as 1,1-dimethylbiguanidine hydrochloride (Figure 1). It exerts its action by lowering hepatic glucose production and increasing the insulin sensitivity (1-3).

Vildagliptin (VLD) belongs to a dipeptidyl peptidase inhibitor class, which shows their action by decreasing postprandial triglyceride rich lipoprotein levels. Chemically it consists of cyanopyrrolidine moiety and works by preventing the inactivation incretin hormones (Figure 2) (4-5).

Chemically Remogliflozin (REM) is benzylpyrazole glucoside (Figure 3) and it is an inhibitor of sodium dependent glucose co-transporter-2 which blocks reabsorption of glucose into kidney (6-7).

This combined dosage form would improve patient compliance and offer better glycemic control to patients with uncontrolled Type 2 diabetes.

A survey of literature revealed that many methods (8-14)were developed for individual estimation of Metformin, Vildagliptin, Remogliflozin and in combination with other drugs. However, few analytical methods (15-18) were developed for the quantification of the above three drugs in fixed dose combination. This research work emphasizes developing a bioanalytical method for the quantification of all three components, Metformin, Vildagliptin, and Remogliflozin, in rabbit plasma by using RP-HPLC.

Materials and Methods

Chemical & Solvents

The pure samples of Metformin, Vildagliptin, Remogliflozin and Saxagliptin were acquired from Biocon, Bangalore. The

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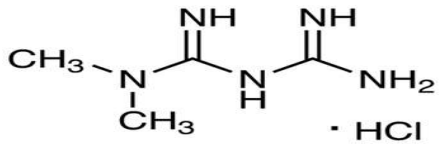

Figure 1: Structure of Metformin

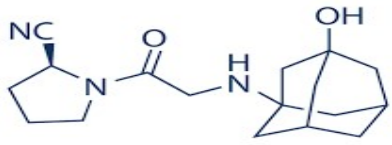

Figure 2: Structure of Vildagliptin

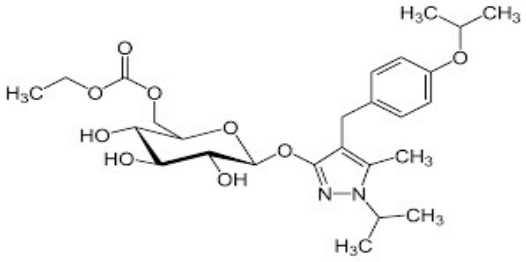

Figure 3: Structure of Remogliflozin

reagents of HPLC grade were bought from Merck chemical division, Mumbai and utilized for chromatographic analyses.

Instrument

Chromatographic process was performed with Waters Alliance HPLC-e2695 with the type of PDA detector and data integration was performed with Empower software 2.0 version.

Preparation of stock solutions

Preparation of mobile phase: 30 volumes of acetonitrile (ACN) combined with 70 volumes of 0.01N KH_2PO_4 to prepare the mobile phase.

Preparation of Standard solutions: 25 mg of Metformin, 10 mg of Vildagliptin and 25 mg of Remogliflozin were dissolved in 10 mL diluent to prepare 2500 µg/mL, 1000 µg/mL and 2500 µg/mL solutions respectively**.** From the standard stock solution eight spiked plasma calibration samples were prepared in the concentration ranging from 25-2500 ng/mL for both Metformin & Remogliflozin and 5-1000 ng/mL for Vildagliptin.

Preparation ofInternal Standard stock solution (ISTD): Saxagliptin stock solution was prepared in diluent to obtain concentration of 500 ng/mL.

Extraction Procedure

Simple protein precipitation approach was used for extraction of drugs from plasma samples. To the 750 µL of blank plasma, add 250 µL internal standard and 250 µL spiked plasma of each drug solution and diluted with 2 mL of extraction solvent. The mixture should be centrifuged for five minutes with a speed of 4000 rpm, at 4^0C and supernatant layer was separated.

Results

Method development

The current study described an effective bio-analytical RP-HPLC method for simultaneous determination of Metformin, Vildagliptin & Remogliflozin in rabbit plasma All the components are well resolved on Water X-bridge (150 x 4.6 mm, 3.5 µ) analytical column using combined mobile phase of ACN and 0.01N KH_2PO_4 in the proportion of 30: 70 v/v, with a flow of 0.8 mL/min. Peak response was detected at 235 nm. Optimized chromatogram of developed method was represented in Figure 4.

Validation

System suitability

System suitability is a parameter which is used to assess the performance of an instrument by injecting six homogenous samples of MQC into HPLC system. %RSD values were calculated for all analytes and

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internal standard. The %CV of retention time and area ratio for MET, VLD, REM and ISTD areas were found to be in the range of 0.0048-1.82% and 0,068-0.285 respectively. %CV values were submitted in Table 1.

Specificity and Selectivity

The specificity and selectivity of HPLC method was assessed to know the interference of blank matrix with elution times of analytes and internal standard by spiking of screening 6 different lots of rabbit plasma to LLOQ samples. The data and specific chromatograms revealed that there was no interference of blank plasma with samples.

Sensitivity

Sensitivity of the method was assessed by obtaining the responses of 6 LLOQ replicates and % CV was calculated. 25 ng/mL of MET, 5 ng/mL of VLD and 25 ng/mL of REM was used to evaluate the sensitivity of the method. The % CV was found to be 1.825, 5.447, 0.384 for MET, VLD and REM respectively which can be found within the limits of acceptance criteria. Sensitivity data was shown in Table 2.

Matrix effect

QC samples were made from 6 distinct matrix sources at HQC and LQC levels and showed no significant effect. The %CV data

Table 1: Results for system suitability

Drug	%CV	
	Retention time	Area ratio
Metformin	0.285	0.182
Vildagliptin	0.117	0.048
Remogliflozin	0.068	1.825
ISTD	0.0040	-

Table 2: Sensitivity results

Sample Name	Metformin	Vildagliptin	Remogliflozin
	Nominal Conc. (25ng/mL)	Nominal Conc. (5ng/mL)	Nominal Conc. (25ng/mL)
	Cal Conc.(ng/mL)		
LLOQ-1	25.22	4.89	24.94
LLOQ-2	24.65	4.76	25.13
LLOQ-3	25.07	5.18	24.87
LLOQ-4	24.94	4.59	25.05
LLOQ-5	25.54	5.26	24.91
LLOQ-6	25.95	4.72	24.97
Mean	25.228	4.9	24.978
SD	0.4605	0.266	0.096
%CV	1.825	5.447	0.384
% Mean Accuracy	100.91	98	95.88

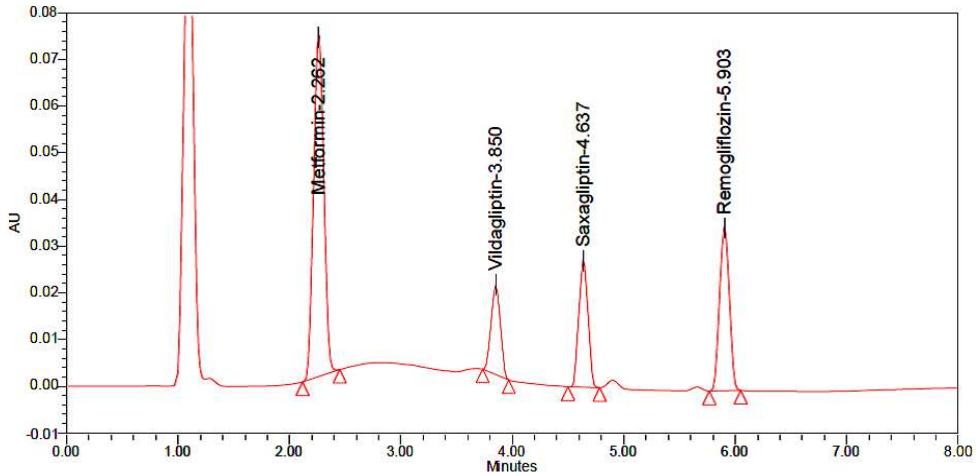

Figure 4: Optimized chromatogram

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presented in Table 3 was found to be less than 15% which satisfied acceptance limits.

Linearity

Linearity curve should be generated by recording the responses for blank, internal standard and 8 calibration standards including LLOQ. Calibration standards were prepared in the same biological matrix which is used for analysis. The present method brings about the linear response between the limits of 25-2500 ng/mL for MET, 5-1000 ng/mL for VLD & 25-2500 ng/mL for REM. The response ratio was calculated from the calibration graph. The linearity data was displayed in Table 4 and related graphs were depicted in Figures 5-7.

Table 3: Matrix effect results

S. No.	Plasma Lot No.	Metformin		Vlidagliptin		Remogliflozin	
		HQC	LQC	HQC	LQC	HQC	LQC
		Nominal Conc. (ng/mL)		Nominal Conc. (ng/mL)		Nominal Conc. (ng/mL)	
		2000	75	400	15	2000	75
		Nominal Concentration range(ng/mL)		Nominal Concentration range(ng/mL)		Nominal Concentration range(ng/mL)	
		1600.00-2400.00	60-90	320-480	2.64-3.36	1600.00-2400.00	60-90
1	Lot 1	1947.37	78.43	392.02	13.96	1935.74	76.22
		1968.77	74.81	406.94	13.95	2032.77	73.12
		2046.63	73.92	387.96	14.09	1959.31	69.92
2	Lot 2	2137.95	76.68	401.77	13.93	1929.52	68.19
		2149.86	75.66	392.02	13.55	2003.17	76.22
		2046.68	74.50	403.00	13.95	1992.56	75.79
3	Lot 3	1938.22	77.58	402.82	14.23	1972.21	75.53
		1984.11	74.12	396.79	13.39	2005.32	72.21
		1995.41	77.56	400.85	13.95	2123.72	72.68
4	Lot 4	1978.34	75.24	398.85	14.04	2129.66	71.27
		2038.43	76.32	389.75	13.30	1992.73	75.94
		2067.68	77.57	402.91	13.97	1989.55	71.67
5	Lot 5	2139.93	76.26	403.80	14.07	2039.88	74.93
		1956.87	74.69	385.83	13.97	2130.33	76.14
		1925.26	75.78	394.79	14.46	2023.32	77.89
6	Lot 6	1975.76	76.14	403.82	13.37	2039.55	76.06
		2029.67	77.97	395.83	12.99	1930.44	69.04
		2037.58	77.45	386.91	13.33	2030.29	72.61
n		18	18	18	18	18	18
Mean		2020.25	76.15	397.04	13.81	2014.45	73.64
SD		69.7599	1.4054	6.6602	0.3881	63.0844	2.8406
%CV		3.4530	1.8457	1.6775	2.8115	3.1316	3.8576
% Mean Accuracy		101.01%	101.53%	99.26%	92.04%	100.72%	98.18%

RP-HPLC

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Table 4: Results for Linearity

S. No.	Metformin		Vildagliptin		Remogliflozin	
	Conc. (ng/mL)	Area response ratio	Conc. (ng/mL)	Area response ratio	Conc. (ng/mL)	Area response ratio
1	0	0	0	0	0	0
2	25	0.0293	5	0.0069	25	0.0135
3	50	0.0528	10	0.0149	50	0.0319
4	75	0.0866	15	0.0205	75	0.0400
5	300	0.3592	120	0.1405	200	0.0985
7	1000	1.1162	400	0.4874	1000	0.5322
8	1500	1.6552	600	0.8529	1500	0.7933
9	2000	2.1645	800	1.1073	2000	1.0397
10	2500	2.8476	1000	1.4021	2500	1.2622
Slope	0.0011		0.0014		0.0005	
Intercept	0.0007		0.0106		0.0047	
R^2	0.999		0.9978		0.9993	

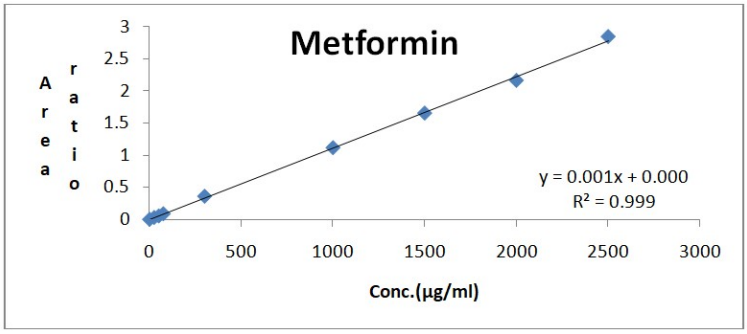

Figure 5: Calibration plot of Metformin

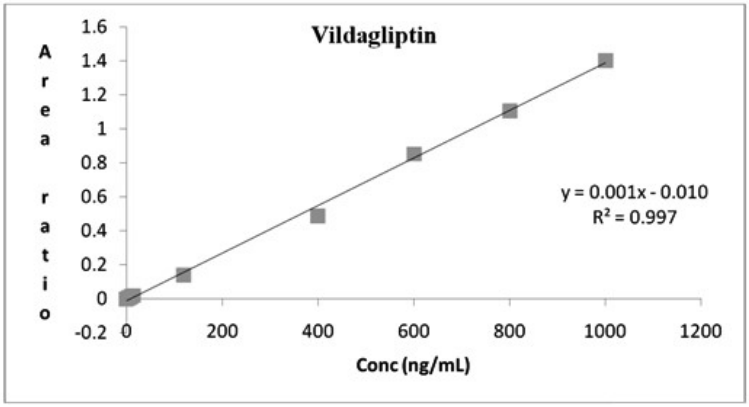

Figure 6: Calibration plot of Vildagliptin

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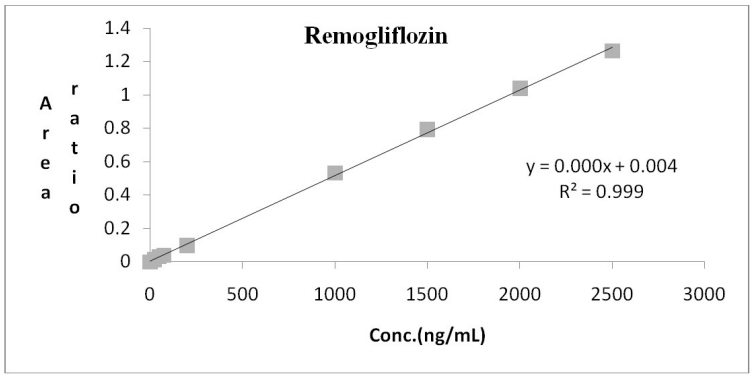

Figure 7: Calibration plot of Remogliflozin

Table 5: Precision & Accuracy data of Metformin					
QC sample	Spiked conc. (ng/mL)	Mean conc. (ng/mL)	SD	Mean Accuracy (%)	CV (%)
Intra-day					
LLOQ	25	24.54	0.77	98.15	3.14
LQC	75	74.16	0.79	98.89	1.07
MQC	1000	991.055	9.285	99.11	0.93
HQC	2000	1998.54	23.65	99.93	1.18
Inter-day					
LLOQ	25	24.57	1.07	98.27	4.38
LQC	75	74.025	1.641	98.7	2.21
MQC	1000	986.54	14.94	98.65	1.51
HQC	2000	2045.36	65.66	102.27	3.21

Table 6: Precision & accuracy data of Vildagliptin					
QC sample	Spiked conc. (ng/mL)	Mean conc. (ng/mL)	SD	Mean Accuracy (%)	CV (%)
Intra-day					
LLOQ	5	4.97	0.028	99.54	0.56
LQC	15	14.74	0.74	98.32	5.020
MQC	400	397.47	7.23	99.37	1.81
HQC	800	801.67	8.75	100.21	1.09
Inter-day					
LLOQ	5	4.96	0.131	99.2	2.64
LQC	15	14.47	0.537	96.47	3.71
MQC	400	395.76	8.49	98.94	2.15
HQC	800	802.69	7.52	100.34	0.94

Precision and Accuracy

Precision & accuracy can be established with four replicates of 3 individual batches at four different QC levels. For Intra-day batch the %CV values in the range of 0.93-3.14% for MET, 0.56-5.02% for VLD, 0.612-1.51% for REM and for inter-batch variation the %CV values in the range of 1.51-4.38% for MET, 0.94-3.71% for VLD, 1.32-2.73% for REM. The %mean

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Table 7: Precision & accuracy data of Remogliflozin					
QC sample	Spiked conc. (ng/mL)	Mean conc. (ng/mL)	SD	Mean Accuracy (%)	CV (%)
Intra-day					
LLOQ	25	24.82	1.33	99.3	1.33
LQC	75	74.21	1.12	100.29	1.51
MQC	1000	988.8	11.126	98.88	1.12
HQC	2000	1986.66	12.173	99.32	0.61
Intra-day					
LLOQ	25	1.93	0.04	96.96	2.44
LQC	75	5.8	0.09	98.28	1.56
MQC	1000	20.014	0.55	100.7	2.73
HQC	2000	29.568	0.39	98.56	1.32

Table 8: Recovery data			
Sample	% Mean Recovery		
	HQC	MQC	LQC
Metformin	80.25	76.85	74.15
Vildagliptin	75.28	78.23	76.25
Remogliflozin	70.82	73.46	72.54
Saxagliptin (ISTD)	75.29		

accuracy for all analytes at HQC and LQC levels were found between 85-115% of the acceptable range. The data was displayed in Tables 5-7.

Recovery

To determine the analyte recovery, compare the response of analyte in extracted and un extracted replicates at low, middle and high QC levels. The % mean values for analytes found to be within the range of 70.82-80.25 and 75.29% for ISTD. The recovery results were shown in Table 8.

Reinjection reproducibility

Reinjection reproducibility was carried out with the accepted batches of precision and accuracy. The same solutions of HQC, MQC, LQC and LLOQ levels were reinjected into HPLC system compare the obtained results with initial run CC standards.The %mean accuracy for Metformin was ranged from 98.75%-99.67%, for Vildagliptin it was 99.68%-97.2% and Remogliflozin 99.36-100.55% which are inside the acceptable range of 80-120%.

Stability

Stock solution stability: MET, VLD, REM and Saxagliptin(ISTD) stock solutions were freshly made and kept at ambient temperature for 6 hours to assess short term stability and kept at -20^{0}C for 30 days. Comparative samples were prepared and stored at a temperature below 10°C and mean responses should be compared.

Stability in Biological Matrix

Auto Sampler stability: The stability was assessed by storing spiked standards at HQC and LQC levels in auto samplers for 48 hours at 4^{0}C and compare the responses with control samples.

Freeze-Thaw stability: Six replicates of the high and low QC standards along with calibration standards were kept at -20°C and defrosted to normal temperature over the course of four freezing cycles and the mean concentrations were calculated.

Bench Top Stability: This type of stability should be evaluated through comparison of stability samples with at low and high QC standards. The mean concentration was calculated and all the stability results were shown in Table 9.

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Table 9: Stability results

	Metformin		Vlidagliptin		Remogliflozin	
	HQC	LQC	HQC	LQC	HQC	LQC
Nominated conc. (ng/mL) ±SD)	2000	75	800	15	2000	75
Short Term Stock Solution Stability (at Room temperature for 6 hours)						
Mean Conc. (ng/mL) ±SD)	2001. 49.±7.8	74.63±0.84	799.43±8. 08	14.92±0.27	2002.34±5. 52	75.3±1.36
%CV (n=6)	0.39	1.13	1.01	1.8103	0.296	1.81
Long Term Stock Solution Stability (at -20^{0}C for 30 days)						
Mean Conc. (ng/mL) ±SD)	1996.88±5. 38	73.92±1.09 5	796.83±2 .74	14.55±0.784	1995.648±4 .79	74.61±1.33
%CV (n=6)	0.269	1. 48	0.345	1.77	0.24	1.78
Auto Sampler Stability						
Mean Conc. (ng/mL) ±SD)	1999.93±8. 4	73.52±1.79	798.97±5 .02	14.89±.0.1	1999±7.03	73.85±1.92
%CV (n=6)	0. 42	2. 44	0.62	0.67	0.352	2.61
Freeze and thaw stability						
Mean Conc. (ng/mL) ±SD)	1999.97±3. 85	74.16±0.49	797.38±3 .53	14.81±0.17	1996.78±7. 2	74.50±1.21
%CV (n=6)	0.19	0.66	0.443	1.206	0.36	1.62
Bench Top Stability						
Mean Concentration (ng/mL) ±SD)	2002.44±6. 1	74.22±1.34	798.99±4 .98	14.87±0.069	2000.59±7. 9	74.81±1.35
%CV (n=6)	0.305	1.8	0.623	0. 464	0.398	1.81

Discussion

The current investigation described a simple, quick, precise and an effective bio-analytical method for the simultaneous analysis of MET, VLD and REM in rabbit plasma. Saxagliptin was used as internal standard at a conc. of 500 ng/mL. All the peaks were well resolved with the mobile phase consists of 0.01NKH_2PO4: ACN in the ratio of 70: 30 v/v in the X Bridge C18, with a flow of 0.8mL/min. Extraction of analyte was accomplished with simple protein precipitation method. The components were eluted at 2.262 minutes, 3.850 minutes and 5.903 minutes for MET, VLD & REM. The proposed method was validated for all parameters mentioned in US FDA guidelines. Linear regression analysis was performed in the limits of 25-2500 ng/mL for both Metformin & Remogliflozin & 5-1000 ng/mL for Vildagliptin. Specificity and matrix effect data demonstrated that the described method does not show any plasma interferences with analytes. % CV values for P & A batches were demonstrate the great reproducibility and accuracy of the method. Good recovery of analyte from plasma samples was achieved with this method. It was discovered during stability experiments that there is no notable degradation.

Conclusion

An effective RP-HPLC method was described for simultaneous bio-analysis of Metformin, Vildagliptin and Remogliflozin spiked with rabbit plasma using Saxagliptin as internal standard. Validation of method was done across the linear range of

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25–2500 ng/mL for Metformin, 5-1000 ng/mL for Vildagliptin and 25-2500 ng/mL for Remogliflozin. The described approach was assessed for matrix effect, sensitivity, precision, accuracy, and dilution integrity for validation and met the acceptance criteria with respect to US FDA guidelines. Stability studies demonstrated that insignificant degradation was observed at specified storage conditions and duration. The developed method resolved all the analytes with simple mobile phase and utilizes protein precipitation for samples extraction which makes this method for high-throughput bioanalysis. The validated method can be employed for estimation of MET, VLD and REM in rabbit plasma for bioequivalence studies.

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Conflict of interest

The authors declare that no conflict of interest.

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