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Determination of Rosuvastatin and Dabigatran Simultaneously in Human Plasma for Application to Pharmacokinetic Parameters in Healthy Jordanian Subjects by LC-MS/MS- Method

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Abstract

This research aimed to develop a simple validated a sensitive, selective, high accurate and efficient assay for the simultaneously evaluation of Rosuvastatin and Dabigatran in human plasma using liquid chromatography tandem mass spectrometry (LC-MS/MS). Rosuvastatin-Dabigatran combines a competitive inhibitor of HMG-CoA reductase oral anticoagulant drug that acts as a direct thrombin (factor II-a) inhibitor. The design of this study was to develop special method for Rosuvastatin and Dabigatran in human plasma by a sensitive and robust (LC-MS/MS) that would result into a concurrent appraisal of Rosuvastatin and Dabigatran avoiding conversions from sample collections to assay on the LC-MS/MS-technique. The collection of sample and it procedure were optimized for Rosuvastatin influence good for Dabigatran, thus occur into an assessment simultaneously, of Rosuvastatin and Dabigatran. Liquid chromatography, Liquid-liquid extraction and coupled to confident ion mode was used according to US- FDA guidelines to evaluate and develop - validated this method. The validation parameters for calibration curves for two analytes were linear ($R2 \geq 0.9951$, n = 4) over the concentration range of 0.20 – 30.0 ng/mL for Rosuvastatin and 1 - 250 ng/mL for Dabigatran. Average extraction recoveries (improvement) 80.34 ± 9.43 for Rosuvastatin and 88.19 ± 7.13 for Dabigatran. Intra- day and inter-day run mean percent accuracy was 'tween 85.0% - 115.0% and % imprecision was ≤15.0%. Stability studies declare that Rosuvastatin and Dabigatran were stable in plasma during bench top (11 h at room temperature), in Injector (48 h), at the end of three successive freeze and thaw cycles and long term at −65.0°C ± 15.0°C for three months. The method was successfully tested to the pharmacokinetics parameters of Rosuvastatin and Dabigatran in healthy subjects. The simultaneously estimated method of Rosuvastatin and Dabigatran is low-cost effective, reduce analysis time and analysis cycle, enables effective utilization of resources and reduces in human volunteers bleeding burden.

Keywords: Rosuvastatin; Dabigatran; Pharmacokinetic; LC-MS/MS; Validation; Human; Plasma

1. INTRODUCTION

Rosuvastatin (RSVN), known as selective and competitive inhibitor of - (3-hydroxy-3-methyl-glutaryl-co-enzyme, A reductase known as HMG-CoA reductase. Chemically, it is bis-[(E)-7-[4-(4-fluoro-phenyl)-6-isopropyl-2- [methyl-(methyl-sulfonyl-) amino] pyrimidin-5-yl] (3R,5S)-3,5-dihydroxyhept-6-enoic acid] (figure 1) [1].It fit to a group of drugs labelled statins, which lower hyper-cholesterolemia and related conditions and avoid cardiovascular diseases. Rosuvastatin is effective in lowering low-density lipoprotein (LDL) and cholesterol [1]. It is widely used for primary avoidance in populations at high risk of cardiovascular disease, as well as n secondary avoidance or prevention for those who have matured cardiovascular disorders. Even small increase in LDL, literally plays a portrayal aspect in heart disorders, and accordingly will be manage is controversial, with statins [2].

Dabigatran (DBGN), which used as etexilate salt is the oral/vocal, prodrug of the active API- moiety Dabigatran. The DBGN etexilate prod rug was developed due to the narrow availability of DBGN, and it is transformed into DBGN in (vivo) via esterase's enzyme. The drug substance is the mesylate salt form of the pro- drug, called DBGN etexilate mesylate (figure 2). The IUPAC name of DBGN etexilate (mesylate) is [Ethyl-N-{[2-({[4-((E)-amino -{[(hexyloxy) -carbonyl-] amino} methyl) phenyl-] amino} methyl)-1-methyl-1H-benz imidazol-5-yl-] carbonyl}-N-pyridin-2-yl-β-alaninate methane-sulfonate], [3] It molecular formula $C_{35}H_{45}N_7O_8S$. DBGN is an oral anti-coagulant medicine, that action as a forthright thrombin (factor II-a) inhibitor [4].

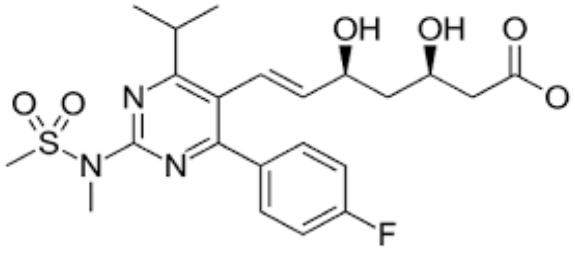

Figure 1. Rosuvastatin (RSVN), chemical structure

Petty methods of analysis for determination RSVN and DBGN were reported using UV [5]. LC/MS [6] and UPLC MS/MS [7] in bulk and/or plasma. Also, two stability indicating assay methods are cited in the literature using HPLC in bulk [8.9] and in formulations [10]. The

UV method reported using acetonitrile (AcN)as solvent which is costly. The aim of this work is to report a sensitive , cheap and rapid liquid chromatography for quantitative speculation of DBGN and RSVN in human plasma and validate the method as per ICH guidelines. [11-22]

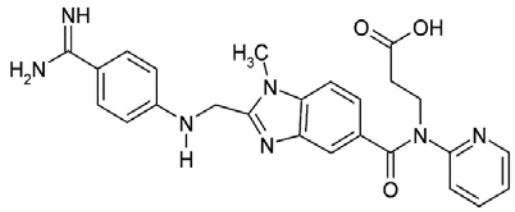

Figure 2. Dabigatran (DBGN), chemical structure

2. EXPERIMENTAL

2.1. Chemicals Reagents & Pharmaceutical Materials

RSVN and DBGN were achieved from Dar Al Dawa/ Pharma-Amman/Jordan as a generous gift. Rosuvastatin (RSVN- IS), Dabigatran (DBGN-IS) were purchased from Syntho - Labs United, Amman. Methanol (Me-OH), acetonitrile(AcN) formic acid as buffer and diethyl-ether manufactured by Merck Labs (Darmstadt- Germany) purchased from (Arabs Co. Jordan, was purchased from (Arabs Chemicals –Jordan), hydrogen sodium phosphate was obtained from (Arabs Chemicals -Jordan), human plasma K_2EDTA was collected from a registered Blood Bank, Al Bashir Hospital –Amman, and kept at –20°C till to use and all chemicals or reagent were HPLC grad.

2.2. Instrumentation: Liquid Chromatography-Mass Spectrometry (LC/MS-MS)

A HT - Shimadzu (Shimadzu Scientific Instruments-Japan) Liquid Chromatography system equipped with (DGU-20A5) degasser, (LC01-20AD) binary –pump, along with(SIL-HTC), auto-sampler which used to inject 10.0 µl diluted aliquots of the prepared samples on a ACE-5/C18 (50.0 × 4.60 mm, ACE, Edinburgh -Scotland) which was maintained at 40.0°C ± 2.0°C in column oven (CTO/10-AS). The mobile phase isocratic, combined with a mixture of 0.10% (HCOOH)formic acid: acetonitrile(AcN) (30.0:70.0, V/V) was filtered through a 0.45 µm membrane filter (XI-5522050-0) (United State – Millipore or similar) and then degassed ultrasonically for 4-6 minutes was delivered at a flow rate of 0.50 ml/min into the mass spectrometer electrospray ionization room/chamber. Quantitation was accomplished by using MS/MS-detection in positive - ion mode for analytes and IS using a MDS- Scitex (CA-USA) API-4000.0 MS-Mass Spector, equipped with a Turboion-spray interface at 400.0°C. The common parameters viz., curtain gas, (GS1) nebulizer gas, (GS2) auxiliary gas and (CAD) collision gas were set at 25.0, 30.0, 40.0 and 5.0 psi, commonly. The compounds parameters viz., (CE)collision energy, (CXP) collision exit potential, (DP) decluttering potential, and (EP) entrance potential for RSVN, DBGN, RSVN-IS and DBGN-IS were 70.0, 27.0, 12.0, 10.0 V; 50.0, 19.0, 10.0, 10.0 V and 70.0, 27.0, 12.0, 10.0 V and 50.0, 19.0, 10.0, 10.0 V reciprocally. Ions detection was behaved in the (MRM) multi reaction monitoring system, transition of the *m/z* 481.4 precursor ion to the *m/z* 479.1 product ion for RSVN, *m/z* 630.0 precursor ion to the *m/z* 627.4 merchandise ion for DBGN, *m/z* 481.4 precursor ion to the *m/z* 483.1 product ion for RSVN-IS and *m/z* 630.0 precursor ion to the *m/z*629.7 merchandise ion for DBGN-IS. Quadrupole Q1 and Q3 were set on entity resolution*(Rs)*. The dwell time was 200 msec. A software for analytical data were handled by Analyst -A. Software (version 1.5.5.11).

2.3. Preparation of Stock, and Standard Solutions

Stock solutions of RSVN (1000 µg/which is primary) and DBGN also (1000 µg/ml) were prepared in methanol separately. The RSVN-IS and DBGN-IS stock solution of 1000 µg/ml were prepared in methanol also. All stock solutions were stored at –65.0°C ± 15.0°C, which were found to be stable for one month (data not shown) and successively diluted with 50% methanol to prepare working stock dilutions. Samples calibration standards and quality control (QC) were prepared by spiking 1.0% total volume of blank plasma with working- stock dilutions of analytes. Stock solutions for working procedure were stored at –65.0°C ± 15.0°C for seven days. The determination of precision and accuracy, samples were prepared by spiking into interference free control plasma containing in bulk with RSVN and DBGN at appropriate concentrations [for RSVN: 0.210 (LLOQ), 0.620 (LQC), 13.440 (MQC) and 24.790 (HQC) ng/ml; for DBGN: 1.030 (LLOQ), 3.060 (LQC), 113.300 (MQC) and 211.380 (HQC) ng/ml] and 100.0 µl aliquots were distributed into disparate tubes, of 5.0 µl of 1.0 M sodium phosphate(Na_2HPO_4) buffer was added to avert the lactone alteration or conversion of RSVN depend on the well-established conditions of RSVN stability in plasma with and without addition of 1.0 M sodium phosphate buffer(Na_2HPO_4) to monitor the acid to lactones inter alteration or conversions. Working and analytical samples were stored at –65.0°C ± 15.0°C.

2.4. Preparation of Samples

Fifty microliters (50.0 µl) of IS solution then 50.0 µl of 1% (V/V) ortho-phosphoric acid in water were added to an aliquot each of 100 µl collected human plasma sample and mixed by vortex for 30 secs on a cyclo-mixer, with speed of0-5500 PRM (Labline Equipments Pvt. Ltd-India)

The extension of phosphoric acid(H_3PO_4) convert the proteins that lunched in the human plasma from the matrix which helps recovery in the liquid- liquid extraction. 2 mL of diethyl ether were added to sample mixture, vortexed for 5.0 minutes., then by centrifuged for 5 minutes. at 5000 PRM on multi-fuge 3S/R (Heraus Fresenius, Germany). 1.80 mL of organic layer has been removed, evaporated to dehydration and dryness at 40.0°C by gentle stream of nitrogen(N) (turbo200 vap®N, Zy- mark®, Tennessee, USA). The 500.0 µl of mobile phase residue was reconstituted and 10.0 µl was precisely injected in instrumentation system mentioned above.

Table 1. Stability data of RSVN and DBGN quality controls Q.C in human plasma

Nominal conc. (ng/ml)	Stability	ROSUVASTATIN			DABIGATRAN		
		Mean ± SD[a] n = 6 (ng/ml)	Accuracy (%)[b]	Precision (% CV)	Mean ± SD[a] n = 6 (ng/ml)	Accuracy (%)[b]	Precision (% CV)
RSVN 0.60 DBGN 3.06	0 h (for all)	0.62 ± 0.0424	102.80	6.87	3.11 ± 0.05	101.69	1.51
	10.50 h (bench-top)	0.62 ± 0.0353	103.33	5.69	3.12 ± 0.07	101.94	2.36
	47.50 h (in-injector)	0.62 ± 0.0301	102.76	4.88	3.01 ± 0.147	98.29	4.91
	114 days at –65˚C ± 15˚C	0.63 ± 0.0242	105.56	3.82	3.38 ± 0.049	110.46	1.45
	Freez-thaw stability (3 cycles at –65˚C ± 15˚C)	0.62 ± 0.0458	103.33	7.39	3.01 ± 0.148	98.49	4.92
RSVN 24.88 DBGN 211.38	0 h (for all)	24.74 ± 0.4659	99.43	1.88	203.43 ± 2.75	96.24	1.35
	10.50 h (bench-top)	24.69 ± 0.5050	99.25	2.04	202.97 ± 3.54	96.02	1.75
	47.50 h (in-injector)	24.62 ± 0.5365	98.93	2.18	198.36 ± 4.05	93.84	2.05
	114 days at –65˚C ± 15˚C	21.47 ± 0.3141	96.68	1.49	191.51 ± 3.125	90.59	1.63
	Freez-thaw stability (3 cycles at –65˚C ± 15˚C)	23.69 ± 0.5234	95.22	2.21	205.53 ± 2.81	97.23	1.37

[a]Back-calculated plasma concentrations; [b](Mean assayed concentration/mean assayed concentration at 0 h) × 100.

2.5. Validation Procedures

The methodology design was developed and validated to match the acceptance criteria of guidelines [11.21.22.24].

2.5.1. Specificity of Matrix Effect, and Sensitivity

The consequence of human plasma ingredients over ionization of RSVN, DBGN, RSVN-IS and DBGN-IS was quantified by analyze the replay of the post obtained plasma QC (n = 6) samples, with response of analytes target from accurately standard samples (10.0 µl in 90.0 µl) adapted by testing aqueous recovery dilutions at concentrations in equivalent. Matrix or sample effect has been determined at low- level and high –level of concentrations (RSVN; 0.620 and 24.790 ng/mL, DBGN; 3.060 and 211.380 ng/mL), forasmuch as the matrix or sample effect on RSVN-IS and DBGN-IS determined at a separate concentration of 50.0 ng/mL. Specificity of RSVN, it was determined by screening and obscure a six diverse batches or assortment of blank human plasma, spiked with DBGN -ULOQ. Sensitivity was by injection of six LLOQ samples from six different lots of human plasma by spiking the concentration of DBGN-ULOQ. The specificity of the DBGN was determined by screening six different batches of human blank plasma spiked with RSVN-ULOQ. The sensitivity was determined by injectable of six- LLOQ sampling from six contrasting parts of concentration of RSVN-ULOQ [12-18,23-25] by *spiking* with human plasma

2.5.2. Recovery

Recovery of RSVN, DBGN, RSVN-IS and DBGN-IS was purposeful by correlating the responses of the set extracted standard plasma QC -samples (n = 6) with the replay of analytes from precisely equivalent samples concentrations [20,25]. Recoveries was detected at low-level, medium-level and high-level concentrations for quality control, although , the recovery of the RSVN-IS and DBGN-IS was obtained at a single original concentration of 50.0 ng/mL Internal standard (IS) was obtained at a single original concentration of consequences effect of plasma components over the ionization of target or analytes and internal standard was resolved by comparing the replay or responses of the post extracted QC -samples plasma standard (n = 6) with response of target of analyte against orderly samples at equivalent proportional concentrations [20,25].

2.5.3. Calibration Curve

Calibration curve was constructed by nine points (0.20, 0.41, 1.02, 1.50, 4.53, 15.11, 22.55, 27 and 30 ng/mL for RSVN and 1, 1.99, 4.98, 12.46, 37.76, 125.87, 187.87, 224.99 and 249.99 ng/mL for DBGN). Peak area ratio of each analyte was then plotted against the concentration. Following the evaluation of different weighing factors, the results were fitted to linear regression analysis with the use of 1/X2 (X: con- centration) weighting factor. The calibration curve had to have a correlation coefficient (r) of 0.99 or better. The acceptance criteria for each back-calculated standard concentration were ±15% deviation from the nominal value except at LLOQ, which was set at ±20% [17-25].

2.5.4. Precision and Accuracy

The precision and accuracy were predicted to be calculated in intra/ inter—assay by analyzing six repeats trails, which containing RSVN and DBGN at four distant Q-C levels RSVN: 0.210 (L-LOQ), 0.60 (LQC), 13.44 (M-QC) and 24.88 (H-QC) ng/mL; for DBGN: 1.030 (LLOQ), 3.06 (LQC), 113.30 (M-QC) and 211.38 (H-QC) ng/mL] in plasma. The inter-assay precision was determined by analyzing the four levels QC samples on four different runs. The principle for acceptability in this criteria , of the results including, accuracy within ±15.0%, standard deviation (SD) from the nominal ostensible values and a precision of interior of ±15.0%, relative standard deviation (RSD) except for L-LOQ, where it should not exceed ±20.0%, of SD [17, 25].

2.5.5. Stability Experiments

The stability of IS and of two analytes in injected solvent was detected, periodically by inserts a replicate of processed samples up to 47.50 hours, (in auto- sampler) after the initial injection. The peak-space areas of the two analytes and IS's pick up at introductory, cycle were use, as the allusion to quantify the relatively stability of the two analytes at subsequent marks point. Stability of the two analytes in the bio- matrix later 10.50 hours' liability (bench top) was detected at two concentrations in six repeated replicates trails. Freezing stability of the two mentioned analytes in bio matrix was assessing by testing the Q.C samples saved and stored at – 65.0°C ± 15.0°C for at the minimum of 4 months. stability of the detected two analytes in bio matrix repeated in three freeze-thaw rounds (stored and saved at –65.0°C ± 15.0°C throw rounds) was estimated by Q.C samples with analytes spiked. Samples were processed as mentioned in section 2.4., and were treated for stability, if the assay parameters or values were in or into the advised limits of accuracy (±15.0 % S.D) and precision (15.0% R.S.D) [17, 25]. Table 1. Shows the Stability parameters values for the two analytes RSVN and DBGN. Stability data for both RSVN and DBGN, presented in Table 1.

2.5.6. Stability of RSVN in Plasma

Statins as, RSVN molecules known to be affected to inter alteration, of lactone and acidic derivate, extremely, it necessary to contemplate this circumstances all along methodology of validation and development too. Rosuvastatin RSVN, executed as the open-ring hydrox-yl acid, although, established dose sampling consist of both acidic and lactonizedic forms [17,25]. For the set of samples of [OH]-yl acid the chemical formula and analogous lactone mode, it is essential to retain pH 'tween 4.0 and 5.0 in order rule, to curtail (inter conversions) modifications [24]. This was proved by performing operating bench top -stability trail at L-QC and H-QC parameters of RSVN with / without added of 1.0 M sodium phosphate (Na_2HPO4) buffer, for maintaining the pH scale around 4.0 – 5.0 units in plasma, so that inter conversed can be seized. Data obtained shown 130.0% higher and more accuracies (131.2 - 137.8) with a precision (% CV) average (3.78 - 5.69) for L-QC and H-QC parameter levels for RSVN in neutral or evenhanded plasma (without added 1.0M phosphate buffer) which, illustrate that inter conversed, in neutral or evenhanded plasma. L-QC and H-QC inspected samples, spiked with 1.0M, Na- phosphate buffer are precised and accurated parameters, RSVN within 96.41 - 108.90, while the precision (% CV) values ranged 2.620 - 6.780 for RSVN.

2.6. Pharmacokinetic Study

A study of pharmacokinetic, in healthy volunteer's male subjects was performed in Jordan. The study ethics was allowed by committee, in the protocol and the volunteers provided with knowledgeable reported consent. The samples blood was collected, succeeding oral administrated of 20.0/1.0 mg of RSVN /DBGN tablet into polypropylene tubes containing K_2 -EDTA solution as anti-coagulant which contains a pre-dose 0.250,0.50, 0.750,1.00, 1.250, 1.50, 1.750, 2.0, 2.330, 2.670, 3.00, 3.50, 4.0, 4.50, 5.0, 5.50, 6.0, 7.0, 8.0, 10.0, 12.0, 16.0, 24.0, 36.0, 48.0 and 72.0 hours. The harvested plasma by centrifuged, using Biofuge-H (Hereaus- BL120, Germany) at 1800 rpm for 4-6 minutes, then transferred of 1.0 mL of plasma to vial tube which, containing 50.0 µl of 1.0 M sodium phosphate(Na_2HPO4) buffer, after that the samples frozen by stored at –65.0°C ± 15.0°C till investigation and analysis.

A portion of aliquot which it 100.0 µl, of defrost plasma samples for inspection were spiked with internal standard (IS), handled as specified in section sample preparation (2.4). Forward with consideration samples, Q-C samples at low L-QC, medium M-QC, and high-QC concentrations were appraisal in duplicate and were assigned betwixt anonymous samples in analytic detailed run. The acceptable criteria for of the analytic detailed runs compasses the not higher than 33.0% of the Q.C samples were highest than ± 15.0% of the mentioned concentration which no more or less than 50.0 % for each Q.C concentration parameters level which must meet the acceptance compliance criteria. The time data of plasma-concentration of RSVN and DBGN was examined by non-apportioned method using Win- Nonlin.16 - Version 5.1 (Pharsight£ XS. Corp., Mounta-in View, CA).

3. RESULTS & DISCUSSION

3.1. Methodology design by LC-MS/MS (Liquid Chromatography and Mass Spectroscopy)

The usefulness of deferments mixture(s) of solutions(solvents) like acetonitrile(AcN) and methanol (MeOH) using divergent buffers solutions alike ammonium acetate $CH_3COONH^{4+,}$ ammonium format $HCOONH^{4+}$ and formic acid HCOOH forward with modified flow/rates (in the average of 0.10 - 0.60 ml/min) were verified for a exhaustive chromatography resolution of the two analytes DBGN , RSVN with IS .It was achieved the resolution of peaks which it is with 0.10% HCOOH: Acetonitrile(AcN) (30.0:70.0 v/v) and flow rate of 0.50 ml/minute, on a ACE column C18.0 (50.0 × 4.60 mm, ACE, Aberdeen-Scotland) it was retained around 40.0°C ± 2.0°C which was formed to be applicable for the assured determinate of electrospray response for RSVN, DBGN, RSVN-IS and DBGN-IS. For optimization of ESI conditions for RSVN, DBGN, RSVN-IS and DBGN-IS, mass- spectrometry sensitivity of detection that accomplished in positive (+) electro-spray ionization model, with multi reactions monitoring scanning. During a direct infusion experiment, the mass spectra for RSVN, DBGN, RSVN-IS and DBGN-IS revealed peaks at *m/z* 559.30, 491.20, 564.20 and 356.30, commonly, as molecular ions which protonated, $[M + H]^+$. Subsequently, optimized details of mass spectrometry MS-MS circumstances: *m/z* 560.10-precursor (harbinger) ion to the *m/z* 441.04 used for quantification of RSVN and *m/z* 489.98-precursor ion to the *m/z* 351.97 used for quantification of DBGN. correspondingly, for RSVN-IS *m/z* 565.14 precursor ion to the *m/z* 444.94and *m/z* 496.32 precursor (harbinger) ion to the *m/z* 355.96 was used for quantification of DBGN-IS was used for quantification desired. The combination of ACE- C18.0 column use for

chromatography disengagement separation and mode positive ion for quantifications of RSVN and DBGN on mass spectrometry MS-MS, achievement for simultaneously estimation of the two analytes in low L-LOQ and lower run time correlated to the prior described independent methodology for each two analytes, in run period time and L-LOQ parameter values.

3.2. Effect Matrix, Sensitivity and Specificity

In retention time there is no potential intrusion or interferences of the RSVN was detected in six diverse, human blanks plasma that were spiked onward with the DBGN /U-LOQ for RSVN blanks. The RSVN/ L-LOQ's sensitivities in presence of DBGN/U-LOQ was accurate and precise with % Relative Standard Deviation (RSD) of 3.20. No possible interference impact at the retention (t_r) time of DBGN was attended in six diverse, human blanks that spiked onward /along with RSVN ULOQ for DBGN blanks. Sensitivity of the DBGN/L-LOQ's in existence of RSVN/U-LOQ was accurate and precise with % RSD of 2.60. The mean matrix factor values (matrix factor = response of post spiked concentrations/response of neat concentrations) obtained were –0.090 (CV: 2.571%, n = 6.0) and –0.07 (CV: 4.830%, n = 6.0) for RSVN and +0.250 (CV: 8.880%, n = 6.0) and +0.160 (CV: 7.020%, n = 6.0) for DBGN at Q.C low and Q.C high concentrations, commonly. The peak area differences were recognized with no significant parameters. The effect matrix on RSVN-IS was established to be –0.070 (CV: 3.400%, n = 12.0) and DBGN-IS was established to be +0.210 (CV: 7.720%, n = 12.0) at examined concentration of 500.0 ng/mL. Long –term it was initiated that the extract of plasma has a poor brunt on the ionization of each analyte and the IS.

An ordinary chromatograms for the human plasma/ control and spiked human plasma with RSVN, DBGN, RSVN-IS, DBGN-IS at L-LOQ along with *in vivo* sample chromatogram are present in figure 3 to 8 respectively. peaks shows that no interfering from endogenous internal compositions or compounds are noticed at the retention times of each analytes and mentioned IS. Retention (t_r) time of RSVN and RSVN-IS was 1.910 min and DBGN and DBGN-IS was 2.280 minutes respectively. Chromatographic run time was 3.00 minutes.

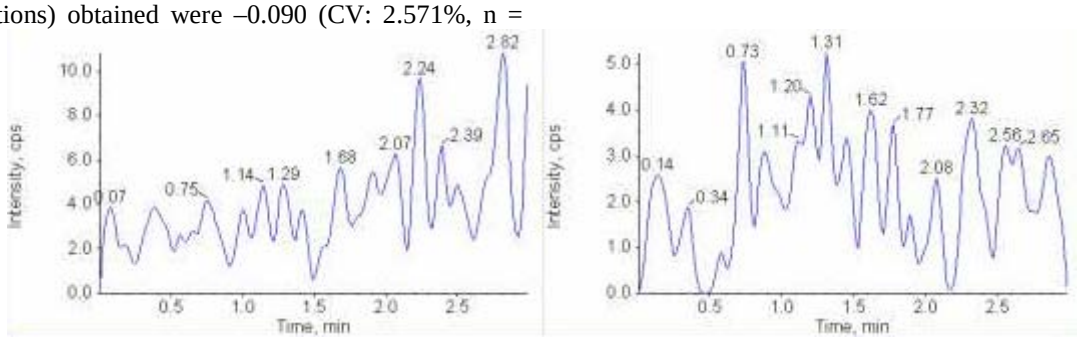

Figure 3. Chromatogram exhibits human plasma for (RSVN) and internal standard rosuvastatin (RSVN-IS)

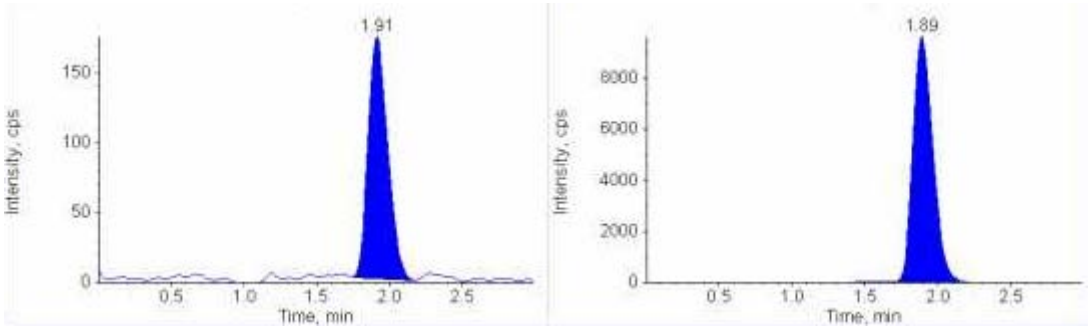

Figure 4. LC-MS/MS chromatogram exhibits0.2 ng/mL RSVN- LLOQ and 50.0 ng/mL human plasma containing internal standard rosuvastatin (RSVN-IS).

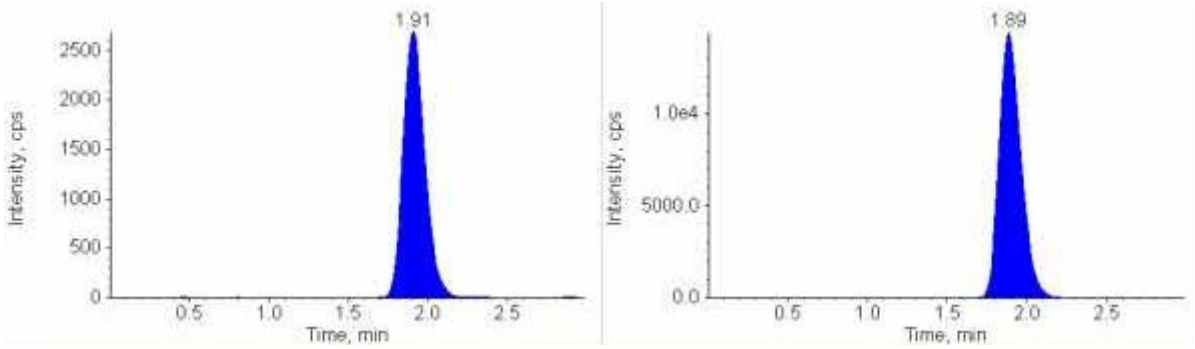

Figure 5. LC-MS/MS chromatogram exhibits human plasma sample containing 5.60 ng/mL RSVN and 50.0 ng/mL internal standard rosuvastatin (RSVN-IS). (4.50 hours)

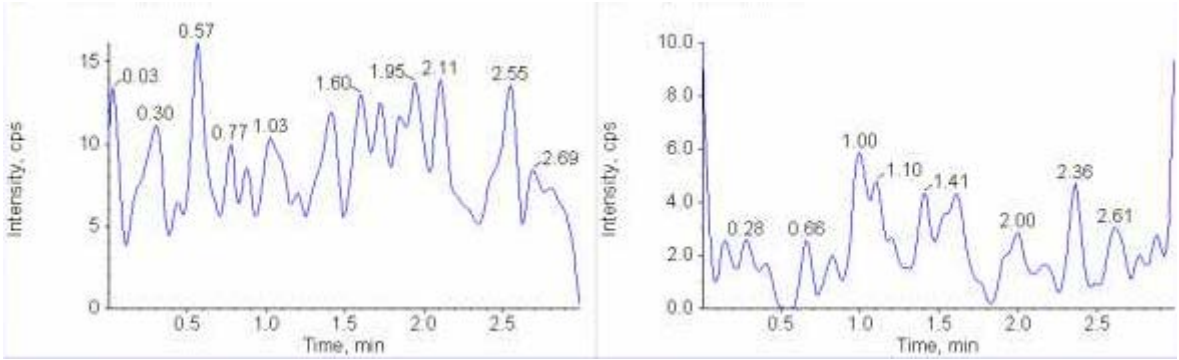

Figure 6. LC-MS/MS exhibits human plasma chromatogram for DBGN and internal standard dabigatran (DBGN-IS).

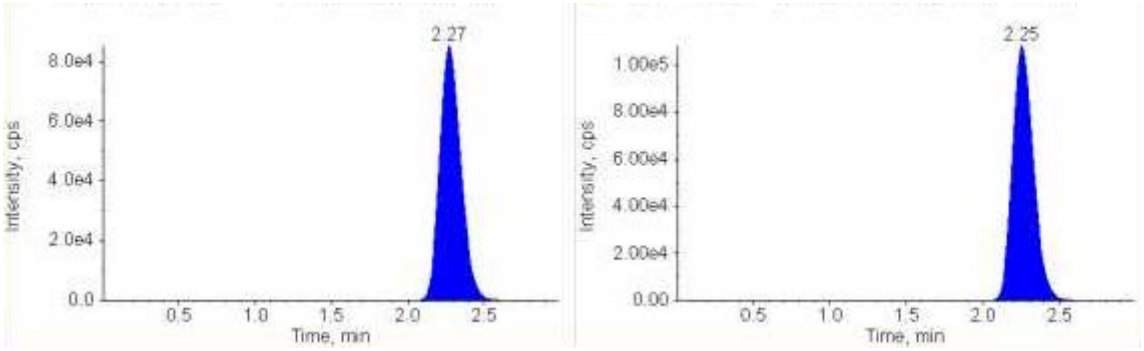

Figure 7. LC-MS/MS exhibits human plasma chromatogram containing 1.0 ng/mL DBGN/ L-LOQ and 50.0 ng/mL internal standard dabigatran (DBGN-IS).

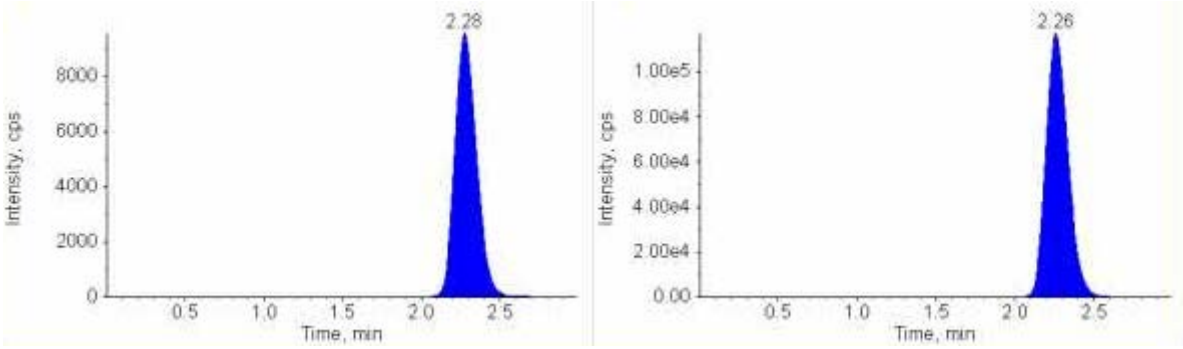

Figure 8. LC-MS/MS exhibits human plasma chromatogram sample containing 72.67 ng/mL DBGN and 50.0 ng/mL internal standard dabigatran (DBGN-IS). (3.50 hours)

Measured concentration (ng/ml)								
Intraday variation (Six replicates at each concentration)								
Theoretical concentration (ng/ml)	RSVN				DBGN			
	Mean	SD	RSD	Accuracy (%)	**Mean**	SD	RSD	Accuracy (%)
RSVN 0.21 DBGN 1.03	0.21	0.014	6.441	100.28	1.05	0.078	7.452	101.51
RSVN 0.60 DBGN 3.06	0.62	0.027	4.283	103.80	3.20	0.075	2.352	104.63
RSVN 13.44 DBGN 113.30	13.52	0.259	1.912	100.60	106.78	1.452	1.360	94.25
RSVN 24.88 DBGN 211.38	24.79	0.449	1.812	99.64	197.80	2.043	1.033	93.58
Inter day variation (Twenty four replicates at each concentration)								
RSVN 0.21 DBGN 1.03	0.20	0.02	10.52	93.76	1.06	0.09	8.93	103.19
RSVN 0.60 DBGN 3.06	0.61	0.03	4.48	100.94	3.10	0.12	3.91	101.20
RSVN 13.44 DBGN 113.30	13.43	0.39	2.94	99.90	108.25	2.73	2.52	95.54
RSVN 24.88 DBGN 211.38	24.61	0.64	2.61	98.91	199.23	3.59	1.80	94.25

R.S.D: Relative standard deviation (S.D × 100/Mean).

3.3 Recovery

The recovery parameter, determined for each of analyte at high Q.C concentration and peak area response was use for the mathematically calculations. Recovery It was found that the recovery around 80.340% ± 9.430% and 88.200% ± 7.131% for RSVN and DBGN consecutively. The RSVN-IS and DBGN-IS shows an absolute recovery around 76.500% ± 5.701% and 91.97% ± 0.531%.

3.4. Calibration Curve

The standard calibration curve was a reliable reproducibility over the standard concentrations across the range of calibration. It was, qualified by determining the batter fit of peak -area- ratios (peak area of the analyte/ peak area of the IS) versus alternative concentration. The average regression (n = 4.0) was found to be >0.9950. The lowest concentration with the RSD < 20.0percentage was taken as L-LOQ and was found to be 0.210 and 1.030 ng/ml for RSVN and DBGN respectively. The % accuracy observed for the mean of back-calculated concentrations for four calibration curves for RSVN and DBGN was within 92.410 - 103.900 and 88.70- 98.540, respectively; while the precision (% CV) values ranged from 0.620 - 7.780 and 1.490 - 3.420 for RSVN and DBGN, respectively.

3.5. Accuracy and Precision

Accuracy and precision data for intra- and inter-day plasma samples for RSVN and DBGN are explained in table 2. The analysis values on both the opportunity (intra- and inter-day) established to be within the acceptable parameters of variable limits.

Table 2. Intra and inter-day precision determination of RSVN and DBGN quality controls in plasma.

3.6. Stability

The concentrations predicted for RSVN and DBGN at L-QC and H-QC deviated within ±15.0% of the nominal concentrations in a battery of stability tests in injector (47.50 hours), bench-top (10.5 hours) and freezer stability at –65.0°C ± 15.0°C for at least for 120 days (table 1).

3.7. Pharmacokinetic Study

This method was tested for the analysis samples of plasma collected from twenty healthy human been volunteers followed oral administration of 20.0/1.0 mg of RSVN/DBGN tablets or caplet as a portion of study for pharmacokinetic applications . The specificity and sensitivity of this work were found to be enough accurate characterized by the pharmacokinetics application and study in plasma of RSVN/DBGN in humans. Figure nine detailed the plasma concentration mean versus time (hours) profile of RSVN and DBGN in these volunteers subjects under fasted circumstances.

After the oral administration the average of maximum plasma concentrations (C_{max}), 5.62 ng/mL, were completed at around of 4.50 hours (T_{max}), although the $AUC_{(0-\alpha)}$ was 60.92 ng·hour/mL for RSVN and the average of maximum ,plasma concentrations (C_{max}), 72.669 ng/mL, were attained at around 5.57 hours (T_{max}), while the $AUC_{(0-\alpha)}$ was 515.210 ng·hour/mL for DBGN, respectively were coordinated with PK parameters in the previous study [20-25].

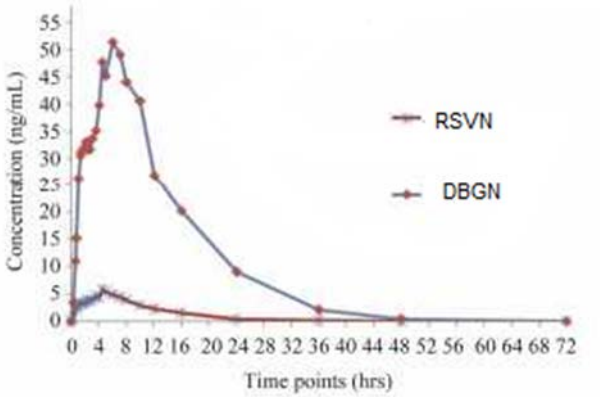

Figure 9. Plasma concentration-time profile of RSVN and DBGN in human plasma after oral dosing of 20.0/1.0 mg of RSVN/DBGN tablets.

4. CONCLUSION

In brief conclusion, this research it had been developed and validated an accurate precise sensitively, specifically, reproducibly and high-efficient LC- MS/MS bio-analytical methodology for quantification and estimation simultaneously of RSVN and DBGN. This work has been successfully used to characterization the concentrations of RSVN and DBGN in application to pharmacokinetic parameters studies. The benefits of the presented research method is fewer sample size volume, simple extraction, method and enables for estimation simultaneously of combination of order drugs; thence this research conduct for future bio-analytical assay will use this developed application for estimation and quantification of RSVN and DBGN in diversified biological and medicinal samples matrices with a limited or no adjustment

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