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ORIGINAL ARTICLE

Non-clinical safety evaluation of a novel pharmaceutical salt, rosuvastatin ethanolamine, in Wistar rats

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ABSTRACT

Rosuvastatin, a second generation 3-Hydroxy-3-Methyl Glutaryl Coenzyme-A reductase inhibitor, is widely used for the management of hypercholesterolemia. Rosuvastatin ethanolamine, developed by Cadila Healthcare Ltd., is a novel, chemically stable, and pharmaceutically acceptable salt, having better physiochemical properties than commercially available Rosuvastatin salt. The objective of the present study is to evaluate safety, tolerability, and toxicokinetic profile of novel salt. Therefore, four weeks repeated dose oral (gavage) toxicity and toxicokinetic study of Rosuvastatin ethanolamine was carried out. The drugs were administered once daily at salt corrected dose of 15, 40, and 100 mg/kg for four weeks. No signs of toxicity were observed during repeated (four weeks) oral administrations of Rosuvastatin ethanolamine in rats up to 40 mg/kg. Single male mortality was observed at 100 mg/kg dose. Microscopy finding in liver was minimal to mild bile ductular proliferation, single cell necrosis, and hepatocellular vacuolation of cytoplasm with associated statistically significant serum elevation of transaminase enzymes; AST, ALT, ALP, and/or liver functional marker; total bilirubin with at ≥40 mg/kg. The systemic exposures (AUC_{0-24} and C_{max}) were not markedly different between males and females, or between the administration periods (except high dose, where exposure on day 28 was approximately 2 to 3 fold higher than that of day 1. In conclusion, Rosuvastatin ethanolamine exhibited toxicities to liver as the target organ at ≥40 mg/kg in this study. These adverse effects with associated exposures should be taken into consideration for the future assessing of potential Rosuvastatin toxicities.

KEY WORDS: Rosuvastatin ethanolamine, toxicity study, toxicokinetic study, Wistar rat

INTRODUCTION

Rosuvastatin (Crestor; licensed to AstraZeneca) markedly reduces low-density lipoprotein (LDL) cholesterol levels, increases high-density lipoprotein (HDL) cholesterol levels, and improves other parameters of the atherogenic lipid profile (Olsson *et al.,* 2002). The mechanism of action of statin class drugs is to competitively inhibit 3-Hydroxy-3-Methyl Glutaryl Coenzyme-A (HMG-CoA) reductase which catalyzes the rate limiting step in cholesterol synthesis, HMG-CoA to mevalonate (Buse, 2003). This *de-novo* decreases in hepatic cholesterol synthesis leading to an up-regulation of hepatic LDL receptors with subsequent increases in LDL uptake and resulting to reduced plasma LDL levels. In addition to reducing LDL levels, statins can also decrease triglyceride (TG), perhaps, by reducing the rate of very low-density lipoprotein (VLDL) synthesis and increasing its clearance (Buse, 2003). Intensive lipid-lowering therapy with rosuvastatin 40 mg per day provides greater LDL lowering efficacy than atorvastatin 80 mg per day, enabling more patients to achieve goal LDL level. Therefore, Rosuvastatin may improve achievement of goal LDL level in high-risk patients with hypercholesterolemia (Leiter *et al.,* 2007).

Rosuvastatin ethanolamine, developed by Cadila Healthcare Ltd., is a novel, chemically stable, and pharmaceutically acceptable salt of rosuvastatin. Rosuvastatin ethanolamine reveals better purity and physiochemical properties like melting point, solubility, and improved

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stability under various stress conditions than currently marketed rosuvastatin salt (Patent number – WO 2012/073256 A1, Cadila Healthcare Ltd., 2012). As it is quite imperative to evaluate safety of novel salt, repeated dose toxicity and toxicokinetics study of rosuvastatin ethanolamine (test article) was evaluated in Wistar rats at salt corrected equivalent dose of 15, 40, and 100 mg/kg. Findings from this study provide insights into the design and interpretation of data derived for toxicology studies with rosuvastatin.

Materials and methods

Drugs

Rosuvastatin ethanolamine was supplied by Cadila Healthcare Ltd., India. Dose formulations were prepared at salt corrected equivalent rosuvastatin concentrations of 1.5, 4.0, and 10.0 mg/ml in the vehicle composition of 5.0% polyethylene glycol 400 (PEG 400)/ 5.0% polyoxyethylene sorbitanmonooleate (Tween™ 80)/ 90.0% of 0.5 % (w/v) methylcellulose in reverse osmosis-treated water, which was dispensed into amber glass bottles. Dose formulations were prepared freshly on each day prior to the dosing.

Experimental animals and housing conditions

The study was designed to use minimum number of animals to meet scientific objectives, goals, and considerations of applicable regulatory requirements. Healthy young adult Wistar rats (age ~7 weeks; body weight range ~170–230 g for male and 120–165 g for female on day of receipt) were obtained from Animal Research Facility of Zydus Research Centre and were acclimatized for a minimum period of seven days. Animals were housed in individually ventilated cages in environmentally controlled rooms (temperature of 18–26 °C; relative humidity of 30–70%; 12 h light/dark cycle) with feed and water provided *ad libitum*. The experimental animals were provided with UV treated Teklad global diet supplied by Harlan Laboratory, USA and filtered drinking water (Reverse osmosis water filter system followed by UV treatment). Proximate analysis of nutrient content and microbial contaminant of feed was analyzed batch wise. Quality of water was periodically checked to ensure acceptable limits of total dissolved solute and microbial contamination. On the first day of dosing, rats were approximately 8 weeks of age; males weighed ~200–250 g and females weighed ~140–180 g. Animal used was in accordance with Committee for the Purpose of Control and Supervision of Experiments on Animals and protocol was approved by the Institutional Animal Ethics Committee. The study was conducted in AAALAC (Association for Accreditation and Assessment of Laboratory Animal Care) accredited facility.

Experimental design

The animals were randomized into different study groups (10/group/sex) to maintain the parity in different groups as per body weight. The difference between and within the groups was not more than 20% of mean body weight. The animals from the respective groups were administered for four weeks by oral gavage using a dose volume of 10 ml/kg with rosuvastatin ethanolamine at 15, 40, 100 mg/kg (test article). The animals from the control group (vehicle control) were administered with vehicle alone. An additional 6 animals/sex/group were added to each dose group for toxicokinetic evaluation.

In-life observations

Detailed clinical and mortality observations were performed daily on all animals throughout the treatment phase. Body weights were recorded during acclimatization, on first day of dosing as well as every week thereafter during the treatment phase. Food consumption was measured weekly during the treatment phase to coincide with body weight measuring.

Toxicokinetics

Toxicokinetic evaluation was carried out on day 1 and day 28 after treatment for evaluating plasma drug concentration. A serial blood collection was carried out at different time intervals such as pre-dose, 15 min, 30 min, 1, 2, 4, 8, 12, 18, and 24 h post dosing. The vehicle control group's animals were bled only for 2 time points (pre-dose and 1 h post dose). The blood samples were collected in saline diluted sodium heparin and placed on wet ice bath, followed by centrifugation (3 000 rpm, 15 min) in cold condition to obtain plasma. The plasma samples were stored frozen at –75±10 °C until analysis.

Bioanalysis

The estimation of rosuvastatin in the plasma samples were employed using a high performance liquid chromatography coupled with tandem mass spectrometry with turbo ion source (LC-MS/MS). The analytical method was validated in accordance to in-house procedure and guidance for industry on bioanalytical method validation (Available from: https://www.fda.gov/downloads/Drugs/Guidance/ucm070107.pdf). The alprazolam was used as an internal standard (0.1 µg/ml). The chromatographic separation of rosuvastatin and internal standard from endogenous matrix was carried on analytical column ACE 100, C18 50×4.6 mm, 5 µ (ACE, ML9 2QS, Scotland) using gradient elution with flow rate of 1.0 ml/min. The mobile phase was solvent mixture of; (A) 100 mg ammonium acetate in 1 l purified water and 100 µl TFA, (B) acetonitrile 100% v/v. The purification of plasma samples for analyte and IS was achieved using the protein precipitation extraction with methanol, followed by 5 µl injection of clear supernatant for analysis. The quantitative measurement was performed in multiple reaction monitoring (MRM) in positive ion mode with mass transition pair of m/z 482.1–258.1 for rosuvastatin and m/z 309.1–281.0 for internal standard. The calibration standard curve was linear over 1 to 1000 ng/ml with limit of quantitation (LOQ) 1.0 ng/ml. During the analysis of plasma samples, a quality control (QC) samples at low, medium, and high

levels were analyzed and distributed across the samples. The inter-run back calculated results of the QC samples indicated accuracy 100.45%, 99.52%, and 101.00% and precisions (%CV) 10.31%, 5.24%, and 5.50% at low, medium, and high QC levels, respectively. The unknown sample concentrations below the LOQ were set to 'zero' for evaluation of toxicokinetic parameters.

Ophthalmic examination

Ophthalmic examination was performed by a veterinarian prior to the initiation of dosing and during fourth week of the treatment phase by using an indirect ophthalmoscope and a slit lamp. Prior to examination, a mydriatic agent (Tropicamide ophthalmic solution 1%, Sunways Ltd.) was instilled into each eye.

Clinical pathology

Detailed clinical pathology investigations were carried out for all the animals immediately before scheduled necropsy, except animals which died during the treatment phase. All animals were fasted overnight (water allowed) before the blood collection. Blood samples were drawn from the retro-orbital plexus under a mild anaesthetic condition (Isoflurane). Following blood collection, samples were immediately placed on wet ice and centrifuged. Samples for hematologic analysis were collected into tubes containing plasma EDTA and the following parameters were measured: red blood cell count (RBC), hematocrit (HCT), hemoglobin (HGB), platelet count (PLT), absolute reticulocyte count (RET), absolute differential leukocyte count, total leukocyte count, mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), and mean corpuscular hemoglobin concentration (MCHC). Samples for coagulation were collected into sodium citrate tubes, centrifuged and the resultant plasma used to measure prothrombin time (PT) and activated partial thromboplastin time (APTT). Samples for serum clinical chemistry were collected in tubes containing no anticoagulant, centrifuged and the resultant serum was used to measure the following parameters: glucose (GLU), triglyceride (TG), total cholesterol (TCHOL), low-density lipoprotein (LDL), high-density lipoprotein (HDL), aspartate amino transferase (AST), alanine amino transferase (ALT), alkaline phosphatase (ALP), urea nitrogen, creatinine (CRE), total bilirubin (TBIL), total protein (TP), albumin (ALB), calcium (Ca^{2+}), inorganic phosphorus (IP), sodium (Na^{+}), potassium (K^{+}), chloride (Cl^{-}), and creatinine kinase (CPK). Urine samples were collected overnight at the end of the treatment phase by placing animal individually in metabolic cages to evaluate the following parameters: volume/quantity, appearance/color, pH, glucose, blood, protein, bilirubin, urobilinogen, nitrite, ketones, specific gravity, and sediment examination.

Terminal procedures

Complete necropsies and gross pathology examinations were performed on animals that died during the treatment phase and all surviving animals at the conclusion of the study. At necropsy, major organs were evaluated for grossly visible lesions and various organs were weighed. Tissues from all major organs were fixed and preserved in 10% neutral buffered formalin, processed, trimmed, embedded in paraffin, and stained with hematoxylin and eosin for microscopic examination. The histopathological evaluation was performed by board certified toxicopathologist (Diplomat Indian Association of Toxicologic Pathologist affiliated to Society of Toxicologic Pathologist). All the tissues/organs collected from control and high dose groups were subjected initially to histopathological evaluation. The grading of the histological lesions was performed by following criteria. Severity of lesions was graded depending on the approximate percentage of tissue involved i.e. less than 20% as minimal, 21% to 50% as mild, 51% to 75% as moderate and 76% to 100% as severe. The treatment related changes observed at high doses were evaluated from next lower dose for the all respective tissue. All treatment related histopathological changes were peer reviewed by Board Certified Veterinary Pathologist (Diplomat Indian College of Veterinary Pathologist). Organs weighed included adrenals, heart, kidneys, liver, spleen, thymus, testes/ovaries with oviduct, epididymides/uterus with cervix, brain, prostate, and seminal vesicles with coagulating glands.

Statistical methods

Statistical analysis was performed using GraphPad Prism Software version 5.04 (GraphPad Software, Inc., CA, USA). Data were analyzed for dose wise comparison. Analysis of Variance (ANOVA) was used for the comparison of different dosage groups with the control group for different parameters. Comparison of dosage groups with the control group was done on the basis of individual group data. Bartlett's test for equal variances was performed for each parameter. *Post-hoc* test to analyze data after ANOVA was done using Dunnett's test. Feed consumption was analyzed using two way ANOVA procedure. Comparison was done on the basis of individual group data. All data are presented as the mean ± SD (n=10 for toxicity study group, n=6 for toxicokinetics group). TK parameters such as T_{max}, C_{max}, AUC_{0-24}, and $T_{½}$ were calculated using non-compartmental analysis model (NCA) of Win-Nonlin Software 5.3 (Pharsight, Mountain View, CA, USA).

Results

Mortality and in-life observation

Single case of male mortality was observed during the study at high dose of 100 mg/kg dose on day 14 which showed adverse signs of toxicity such as lean body condition, chromodacryorrhea, decreased motor activities, hunched back, and lethargy for few days prior to death.

There were no other adverse clinical signs noticed in the study. The weekly body weight recording revealed statistically significant and marginally lower group mean body weight (<10%) in females at 100 mg/kg dose from the

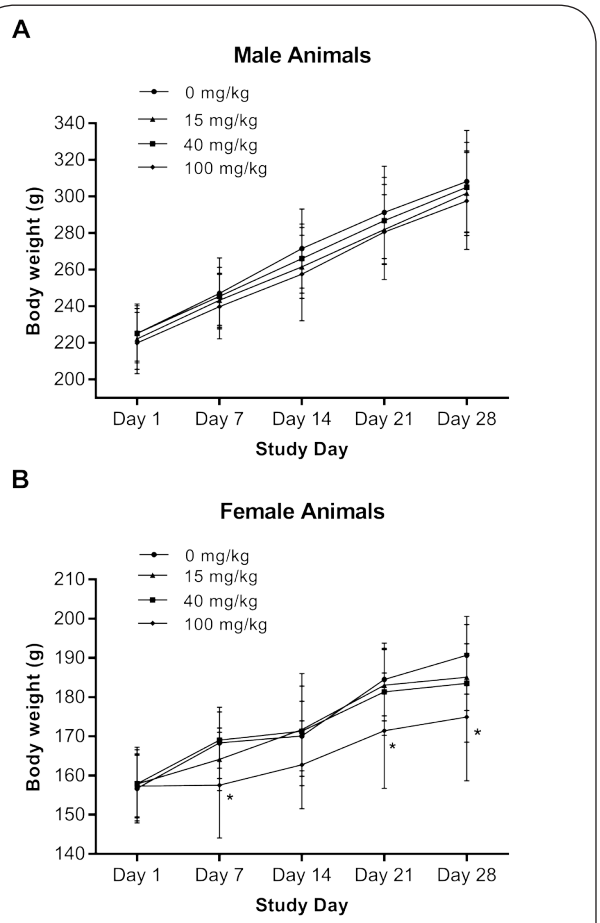

Figure 1. Body weight. Each value represent mean ± SD (n= 10), $p<0.05$ vs. vehicle control.

day 7 of treatment when compared with the concurrent control group (Figure 1B). Group mean feed consumption of each treatment groups was found to be comparable with the concurrent control group in both sexes. No drug related ophthalmic lesions were observed in all rats in any of the dose groups during the study.

Clinical pathology

Hematology

There were no drug treatment related significant changes observed in hematology and coagulation parameters in any dose group treated with rosuvastatin ethanolamine in the both sexes. The non-dose and/or sex dependent minor variations were noticed and are presented in the Table 1 & 2.

Clinical chemistry

Rosuvastatin ethanolamine treatment at 15 mg/kg dose resulted in minimal elevation of ALT (~39 %) in males, and minimal elevation of total bilirubin (~25–40%) in females. The treatment at 40 mg/kg in males resulted in a mild elevation of AST and ALT and decline in total protein albumin. The females treated at 40 mg/kg with rosuvastatin ethanolamine exhibited mild elevation of total bilirubin and albumin when compared with concurrent control group. In high dose group (100 mg/kg) of ethanolamine salt treatment showed moderate enzymatic elevation of AST, ALT, ALP, total bilirubin, and decline in total protein in males. In females, rosuvastatin ethanolamine treatment raised levels of AST, ALP, and total bilirubin as compared to control. All other statistically significant observations are presented in Tables 3 & 4.

Table 1. Group Mean Hematological Analytes (Sex: Male)

Analytes	Vehicle	Rosuvastatin Ethanolamine			Reference Values
	0 mg/kg	15 mg/kg	40 mg/kg	100 mg/kg	Lower Limit-Upper Limit
WBC (10^3/µl)	8.4±1.7	8.6±2.7	8.8±1.0	9.9±1.8	3.7–12.3
RBC (10^6/µl)	8.3±0.2	8.3±0.4	8.2±0.6	8.5±0.4	6.6–9.1
HGB (g/dl)	14.9±0.3	14.7±0.7	14.9±1.2	15.1±0.6	12.7–16.1
HCT (%)	49.0±0.9	47.8±2.1	48.6±3.7	49.2±2.1	40.0–49.9
MCV (fL)	58.9±1.3	57.9±2.0	59.5±1.7	57.8±2.6	51.1– 63.7
MCH (pg)	17.9±0.5	17.8±0.6	18.3±0.6	17.8±0.6	16.3–20.3
MCHC (g/dl)	30.3±0.6	30.8±0.2*	30.7±0.4	30.8±0.4	30.6–33.3
PLT (10^3/µl)	582.1±58.1	613.6±45.8	563.4±29.9	602.8±72.2	508–1045
NEU (10^3/µl)	1.23±0.29	1.62±0.91	1.30±0.32	1.35±0.37	0.43–2.15
LYM (10^3/µl)	6.88±1.74	6.38±1.98	7.09±1.07	8.05±1.48	2.56–10.30
MONO (10^3/µl)	0.17±0.14	0.34±0.24	0.25±0.15	0.25±0.15	0.013–0.545
EOS (10^3/µl)	0.055±0.02	0.082±0.03	0.079±0.04	0.090±0.03*	0.029–0.232
BASO (10^3/µl)	0.095±0.04	0.133±0.07	0.108±0.05	0.092±0.05	0.029–0.265
RET (10^3/µl)	296.4±64.8	289.0±66.7	237.8±38.4	459.1±98.5*	121–622
PT (sec)	12.59±0.6	11.98±0.7	13.60±0.2**	13.04±0.9	9.5–15.4
APTT (sec)	18.0±2.2	18.1±2.6	18.1±3.9	17.2±2.5	10.9–30.0

* Significant at 5% level ($p<0.05$), ** Significant at 1% level ($p<0.01$)

Urinalysis

Treatment related changes were not observed in the parameters of urine analysis of animals treated with rosuvastatin ethanolamine. (Data not shown)

Pathology

Gross pathology examination at the completion of study did not reveal any gross lesions across all doses. The single male animal found dead on day 14 treated by high dose

Table 2. Group Mean Hematological Analytes (Sex: Female)

Analytes	Vehicle	Rosuvastatin Ethanolamine			Reference Values
	0 mg/kg	15 mg/kg	40 mg/kg	100 mg/kg	Lower Limit-Upper Limit
WBC (10^3/µl)	5.9±1.7	4.8±1.6	5.2±1.6	5.1±1.3	2.37–9.36
RBC (10^6/µl)	7.6±0.7	7.6±0.4	7.6±0.3	8.0±0.6	6.42–8.42
HGB (g/dl)	13.9±1.3	13.9±0.4	13.9±0.6	14.4±1.0	12.8–15.2
HCT (%)	15.0±4.0	45.1±1.3	44.8±2.2	46.5±3.3	39.7–47.5
MCV (fL)	59.4±1.3	59.2±1.8	58.7±1.4	58.3±1.7	53.7–63.8
MCH (pg)	18.3±0.5	18.2±0.6	18.2±0.5	18.0±0.5	17.4–20.5
MCHC (g/dl)	30.8±0.4	30.8±0.3	31.0±0.5	30.9±0.2	30.5–33.6
PLT (10^3/µl)	602.6±75.5	610.7±63.1	606.7±75.7	602.4±80.6	545–1057
NEU (103/µl)	0.53±0.10	0.60±0.24	0.65±0.27	0.63±0.28	0.353–1.499
LYM (10^3/µl)	4.98±1.67	3.84±1.36	4.20±1.34	4.06±1.15	1.350–8.260
MONO (10^3/µl)	0.20±0.09	0.17±0.09	0.20±0.1	0.24±0.13	0.014–0.389
EOS (10^3/µl)	0.055±0.02	0.071±0.02	0.054±0.02	0.073±0.04	0.026–0.169
BASO (10^3/µl)	0.102±0.04	0.075±0.04	0.071±0.04	0.072±0.03	0.016–0.179
RET (10^3/µl)	400.0±152.1	458.2±176.3	434.9±119.5	529.2±154.8	139–936
PT (sec)	12.7±0.5	11.3±0.4	10.6±0.3	12.9±0.6	9.3–13.1
APTT (sec)	19.4±2.9	19.0±2.7	21.2±4.5	18.5±4.0	10.8–24.6

Table 3. Group Mean Clinical Chemistry Analytes (Sex: Male)

Analytes	Vehicle	Rosuvastatin Ethanolamine			Reference Values
	0 mg/kg	15 mg/kg	40 mg/kg	100 mg/kg	Lower Limit-Upper Limit
GLU (mg/dl)	86.2±11.9	78.2±12.6	89.0±13.1	84.1±7.1	42.3–150.7
TG (mg/dl)	104.2±57.8	85.4±27.5	74.5±28.4	73.6±15.7	33.0–152.0
TCHOL (mg/dl)	52.6±6.5	62.4±14.8	62.3±6.0	64.4±10.2*	37.2–87.5
HDl (mg/dl)	21.0±2.3	21.8±4.5	22.0±2.3	23.4±2.9	12.7–33.8
LDl (mg/dl)	3.8±1.0	3.3±1.0	4.8±1.8	6.2±1.9**	1.4–7.1
AST (U/l)	94.3±5.6	118.5±17.0	127.5±34.3**	148.7±23.5**	74.6–197.0
ALT (U/l)	31.4±3.6	43.5±3.7**	46.4±14.8**	51.0±13.7**	20.6–48.8
ALP (U/l)	110.0±35.7	129.3±28.3	136.7±31.7	176.8±41.8**	53.5–246.8
TBIL (mg/dl)	0.16 ±0.03	0.16±0.02	0.19±0.02	0.23±0.04**	0.02–0.27
TP (g/dl)	6.35±0.27	6.12±0.25	6.06±0.23*	5.82±0.14**	5.3–6.9
ALB (g/dl)	3.69±0.14	3.50±0.11**	3.49±0.09**	3.59±0.08	3.2–4.0
UREA (mg/dl)	34.6±4.1	35.9±4.4	32.7±2.6	32.1±4.2	23.2–49.6
CRE (mg/dl)	0.71±0.04	0.65±0.05	0.69±0.06	0.70±0.04	0.42–0.82
CPK (U/l)	818.0±198.1	980.1±278.4	991.3±561.3	739.9±148.7	286.1–2098.8
Ca^{2+} (mg/dl)	10.3±0.3	10.1±0.3	9.8±0.3	10.2±0.2	9.0–11.0
IP (mg/dl)	6.5±0.4	5.7±0.4	6.0±0.5	6.2±0.4	4.2–8.3
Na^+ (mmol/l)	144.8±1.0	145.8±1.4	144.7±1.2	144.1±1.6	137.4–146.8
K^+ (mmol/l)	3.6±0.1	3.7±0.1	3.6±0.3	3.5±0.2	3.4–5.0
Cl^- (mmol/l)	102.7±0.9	104.6±1.1	102.7±1.0	103.1±1.5	99.7–107.6

* Significant at 5% level ($p<0.05$), ** Significant at 1% level ($p<0.01$)

Table 4. Group Mean Clinical Chemistry Analytes (Sex: Female)

Analytes	Vehicle	Rosuvastatin Ethanolamine			Reference Values
	0 mg/kg	15 mg/kg	40 mg/kg	100 mg/kg	Lower Limit-Upper Limit
GLU (mg/dl)	66.4±17.2	89.4±10.4**	79.2±7.6	45.0±12.8**	53.3–135.2
TG (mg/dl)	33.5±10.1	42.0±13.1	32.8±7.3	25.0±7.5	22.0–81.3
TCHOL (mg/dl)	40.5±6.7	56.2±11.6**	45.9±10.6	39.7±13.5	26.0–71.9
HDl (mg/dl)	16.6±2.77	21.8±4.1*	17.9±3.4	18.1±6.1	10.1–21.7
LDl (mg/dl)	1.2±0.4	1.8±0.7	2.4±0.7**	2.0±1.3	0.50–4.40
AST (U/L)	131.0±41.1	106.5±30.1	105.4±28.5	218.2±101.2**	72.0–200.3
ALT (U/L)	27.9±4.3	28.1±4.6	27.0±5.5	35.1±11.9	16.6–38.8
ALP (U/L)	50.6±18.4	68.5±13.3	66.6±18.6	128.9±62.3**	19.1–162.7
TBIL (mg/dl)	0.16±0.02	0.20±0.03*	0.23±0.02**	0.29±0.06**	0.08–0.31
TP (g/dl)	6.4±0.4	6.5±0.5	6.6±0.2	6.2±0.3	5.4–7.3
ALB (g/dl)	3.73±0.20	3.84±0.25	3.95±0.12*	3.69±0.14	3.4–4.4
UREA (mg/dl)	38.7±4.3	37.7±3.9	36.3±2.7	37.0±4.5	31.5–61.4
CRE (mg/dl)	0.70±0.04	0.73±0.05	0.71±0.07	0.67±0.07	0.51–0.89
CPK (U/l)	1609.1±838.8	1065.4±918.7	808.9±517.9*	1272.6±188.9	520.6–1976.8
Ca^{2+} (mg/dl)	10.2±0.3	10.2±0.4	10.4±0.3	10.1±0.3	8.84–11.20
IP (mg/dl)	4.3±0.8	4.5±0.6	5.0±0.5*	5.2±0.7**	3.10–7.20
Na^{+} (mmol/l)	145.5±1.6	145.5±1.1	145.3±0.9	143.4±1.5**	137.4–146.4
K^{+} (mmol/l)	3.4±0.2	3.6±0.4	3.6±0.2	3.5±0.2	3.03–4.64
Cl^{-} (mmol/l)	104.6±1.3	104.8±1.1	104.8±1.0	103.6±0.9	101.1–108.2

* Significant at 5% level ($p<0.05$), ** Significant at 1% level ($p<0.01$)

Table 5. Microscopic Examination

Dose (mg/kg)		Vehicle		Rosuvastatin Ethanolamine					
		0		15		40		100	
Sex		M	F	M	F	M	F	M	F
Unscheduled Deaths (Number of Animals)		0	0	0	0	0	0	1	0
Liver									
Single cell necrosis	Minimal	0	0	0	0	3	5	2	5
	Mild	0	0	0	0	1	0	5	2
Bile ductular proliferation	Minimal	0	0	0	0	0	0	7	7
	Mild	0	0	0	0	0	0	0	0
Vacuolated cytoplasm	Minimal	0	0	2	2	4	6	4	3
	Mild	0	0	0	0	1	0	3	4
Thymus									
Atrophy and lymphoid depletion	Mild	0	0	0	0	0	0	1	0
	Moderate	0	0	0	0	0	0	0	0

showed, histopathologically, mild single cell necrosis, minimal bile ductular proliferation and cytoplasmic vacuolation of liver, mild atrophy and lymphoid depletion in thymus, mild single cell necrosis and vacuolation in pancreas, and minimal inflammatory cells and hyperkeratosis in the fore stomach.

Absolute and relative organ weight estimation revealed no treatment related adverse changes in animals treated with test and reference substance in both sexes (data not presented). The minor variations of organ weights such as higher weight of spleen, seminal vesicles with coagulating glands at 40 mg/kg of rosuvastatin ethanolamine, lower adrenals weight at 100 mg/kg of rosuvastatin ethanolamine in females were neither dose dependent nor of any pathological significance. Histopathological examination did not reveal any adverse changes in any of the major organs examined in this study except for treatment related adverse effects in liver at ≥40 mg/kg in the both sexes of rosuvastatin ethanolamine treatment groups (Table 5). Treatment and dose-related adverse effects noticed in liver were: minimal to mild bile ductular proliferation, single cell necrosis, and hepatocellular vacuolation of cytoplasm (Figures 2, C & D). The dose related relationship was clearly observed during the histopathology examination of liver, especially in terms of increased observation of sever findings. Incidence of stress related changes was noticed, such as minimal atrophy and lymphoid depletion of thymus in single male at 100 mg/kg dose.

Toxicokinetics

Mean plasma toxicokinetic parameters following oral administration of rosuvastatin ethanolamine in male and female rats are presented in Table 6. In the both males and females, the mean systemic exposure (C_{max} and AUC_{0-24})

Table 6. Toxicokinetic Parameters

Sex	Dose (mg/kg)	Day	T_{max} (h)	C_{max} (µg/ml)	$T_{1/2}$ (h)	AUC_{0-24} (h.µg/ml)	Fold increased exposure of low dose
Male	15	1 28	2.46±4.68 0.71±0.70	0.15±0.12 0.11±0.02	8.37±4.39 8.44±2.27	0.39±0.09 0.66±0.18	–
	40 (2.67 fold of low dose)	1 28	0.50±0.00 2.42±4.70	0.97±1.13 0.77±0.61	5.41±3.00 5.17±2.36	1.47±0.95 2.01±0.59	3.77 3.05
	100 (6.67 fold of low dose)	1 28	0.67±0.38 0.38±0.14	11.63±13.69 21.62±13.93	4.98±1.53 5.12±1.89	17.80±13.69 30.12±18.93	45.64 45.63
Female	15	1 28	0.50±0.27 0.29±0.10	0.12±0.04 0.21±0.10	7.56±3.51 3.90±0.48	0.37±0.13 0.73±0.09	–
	40 (2.67 fold of low dose)	1 28	0.33±0.13 0.46± 0.10	0.85±0.40 2.18±1.27	7.67±5.46 4.65±1.71	2.07±1.14 2.95±1.49	5.59 4.04
	100 (6.67 fold of low dose)	1 28	0.30±0.11 0.25±0.00	9.08±4.65 49.86±28.20	5.12±1.86 4.17±1.83	9.81±4.53 32.13±10.24	26.51 44.01

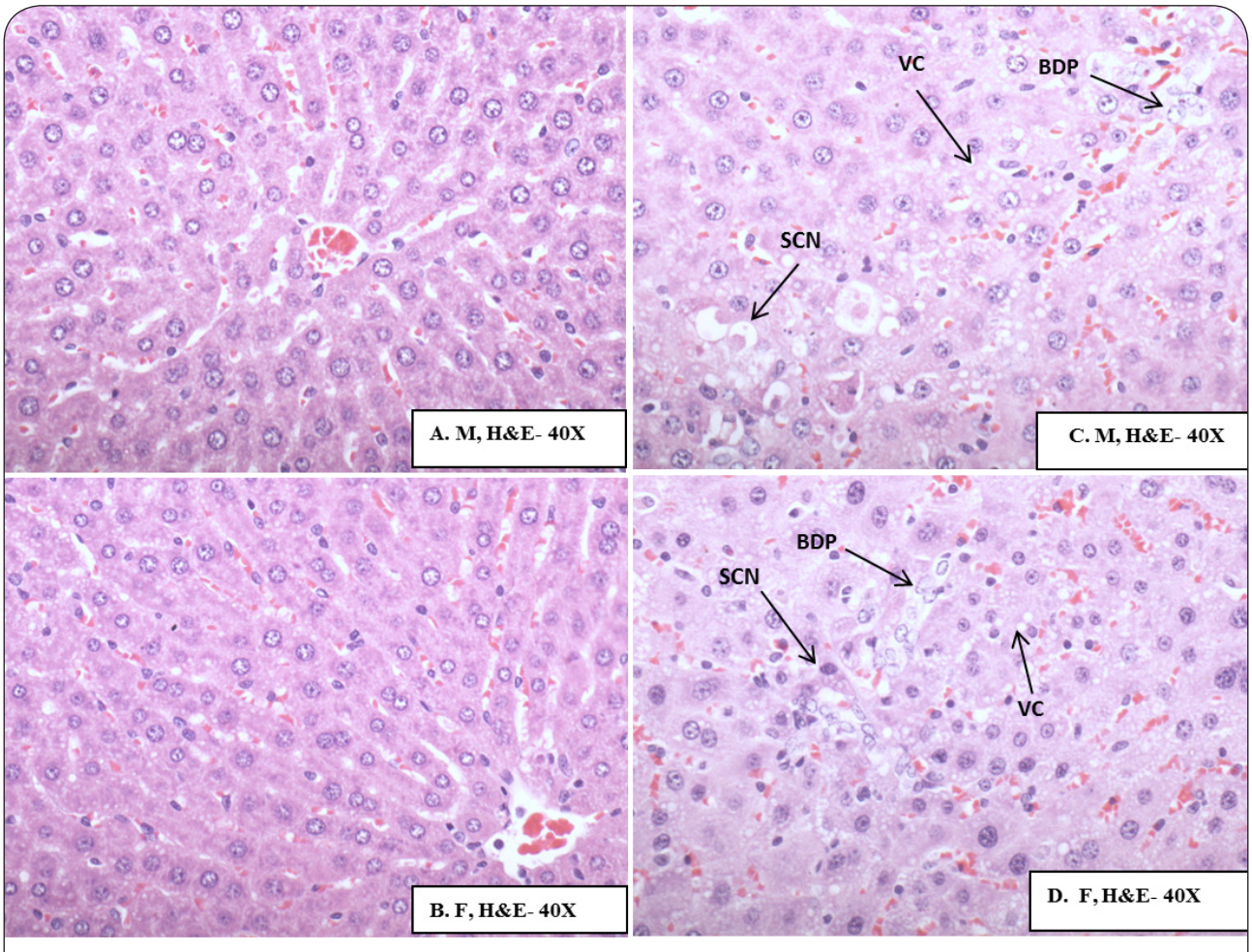

Figure 2. Adverse effects of Rosurvastatin ethanolamie in liver. A & B: control: normal histological appearance of liver; C & D: Rosrvastatin ethanolamine, 100 mg/kg. SCN – single cell necrosis; VC – vacuolated cytoplasm; BDP – bile duct proliferation; M – male; F – female.

to rosuvastatin following oral administration of ethanolamine salt was found to be more than a multiple of dose in both sexes on day 1 and 28 of treatment period. Significant gender specific differences were not noticed in this study. On day 28, the plasma exposure was higher by 2 and 3 folds approximately from that of day 1 in male and female rats respectively at 100 mg/kg. The median T_{max} ranged from 0.25 to 2.46 h following oral gavage administration of rosuvastatin ethanolamine with no apparent changes due to sex, day or dose level (Table 6). A second absorption peak was observed at about 8–12 h post dosing, which evident enterohepatic re-circulation of drug.

Discussion

We conducted toxicity and toxicokinetic studies in Wistar rats to evaluate safety, tolerability, and toxicokinetic of novel compound, rosuvastatin ethanolamine. The toxicity test was conducted over four weeks using oral route of administration, which is the intended route of administration in humans. The toxicokinetic test was done separately from the toxicity test. In this four week dose repeated oral toxicity study, the study results were comparable between male and female rats.

The single male animal at high dose of 100 mg/kg showed overt signs of toxicity such as lean body condition, chromodacryorrhea, decreased motor activities, hunched back, and lethargy, which eventually resulted into the death. The animals found dead exhibited single cell necrosis, bile ductular proliferation & cytoplasmic vacuolation of liver, atrophy and lymphoid depletion in thymus, single cell necrosis and vacuolation in pancreas, and inflammatory cells and hyperkeratosis in the fore stomach. The similar types of lesions were reported with the previously conducted study (Pharmacology review(s) of rosuvastatin calcium, available from: http://www.accessdata.fda.gov/drugsatfda_docs/nda/2003/21-366_Crestor.cfm). Hence, the cause of death of this animal could be attributed to the rosuvastatin treatment.

Rosuvastatin intends to reduce LDL-cholesterol and increase HDL-cholesterol levels by competitively inhibiting (HMG-CoA) reductase which catalyzes the rate limiting step in cholesterol synthesis, HMG-CoA to mevalonate, in hyperlipidemic conditions (Buse, 2003). But this action could not reflect in the clinical chemistry results because this study was carried out in the healthy test system having the normal levels of cholesterols.

Post-treatment clinical chemistry estimation revealed dose dependent enzymatic elevation of AST, ALT, ALP, and/or liver functional marker (total bilirubin) in the both sexes treated with rosuvastatin ethanolamine at doses from 40 mg/kg. Dose dependent findings of microscopic liver changes of the males and females, which included minimal to mild bile ductular proliferation, single cell necrosis, and hepatocellular vacuolation of cytoplasm correlated with the changes of clinical chemistry analytes. Elevated serum levels of transaminase enzyme and liver function marker (total bilirubin) along with liver histopathology are the standard biomarkers for the assessment of liver toxicity (Marrer & Dieterle 2010). Therefore based on the observed histopathological changes in liver and related clinical chemistry changes, liver was considered as target organ of toxicity for the rosuvastatin. This observed toxicity in liver is also a well-known safety concern with the statin therapy clinically (Famularo *et al.* 2007). Non-clinical toxicity for liver in rats treated with statin class of drugs was clearly categorized as exaggerated biochemical effect of HMG-CoA reductase inhibitors (Macdonald & Halleck 2004). The most serious adverse effects of statins are related to muscle toxicity (Pasternak *et al.* 2002 and Antons *et al.* 2006), contrastingly, in this study we did not observe any sign of muscle toxicity markers during either microscopic examination or serum creatinine kinase estimation. We concluded that this might be either due to short duration of treatment period or because the studied highest dose level was not sufficient to exhibit the muscle toxicity in rats under this experimental condition.

In conclusion, we investigated the standard parameters for characterizing the general toxicity and toxicokinetic profile of rosuvastatin ethanolamine. The novel salt, rosuvastatin ethanolamine, targeted liver as the main organ of its toxicity at ≥40 mg/kg in Wistar rats which was considered to be exaggerated biochemical effect of statin class of drugs.

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