Toxicology Reports 2 (2015) 1396–1403

Contents lists available at ScienceDirect

Toxicology Reports

journal homepage: www.elsevier.com/locate/toxrep

Preclinical rodent toxicity studies for long term use of ceftriaxone

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ARTICLE INFO

Article history:
Received 21 April 2015
Received in revised form
27 September 2015
Accepted 28 September 2015
Available online 9 October 2015

Keywords:
Ceftriaxone
Amyotrophic lateral sclerosis (ALS)
Rodent
Toxicology
Pharmacokinetics
Preclinical study

ABSTRACT

A 6-months rodent toxicology and pharmacokinetic (PK) study was performed to provide supportive safety data for long-term use of intravenous ceftriaxone in a clinical trial in patients with amyotrophic lateral sclerosis (ALS). Ceftriaxone was administered by subcutaneous injection at up to 2 g/kg/day to Sprague-Dawley Crl:CD (SD) rats. Ceftriaxone was found to be safe and well tolerated. Specifically, no significant differences in body weight and food consumption were observed between the treatment and control groups. With the exception of in red cell parameters decrease, there were no ceftriaxone-related changes in hematology, coagulation, clinical chemistry and urinalysis parameters.

Injection site trauma and associated reversible anemia, likely due to chronic blood loss at the injection site, were all attributable to subcutaneous route of administration. Cecum dilatation and some skin changes were reversible after recovery period, while bile duct dilatation, observed only in a few animals, persisted. Changes in the non-glandular stomach do not have a human correlate. The no-observed-adverse-effect dose level (NOAEL) was 0.5 g/kg/day ceftriaxone in both sexes.

Ceftriaxone showed rapid absorption with half-life values ranging between 1 and 1.5 h. Additionally, there was no evidence of accumulation and a virtually complete elimination by 16 h after the last dose. Overall there were no toxicologically meaningful drug-related animal findings associated with the long-term administration (6 months) of ceftriaxone. These results support safety of long-term use of ceftriaxone in human clinical trials.

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1. Introduction

Ceftriaxone is a bactericidal 3rd generation cephalosporin that inhibits bacterial cell wall mucopeptide synthesis [3]. This beta-lactam antibiotic is approved by the federal drug administration (FDA) for intramuscular or intravenous (IV) delivery at maximum doses of 2–4 g daily [1] for up to 4–6 weeks [2].

ALS is a neurodegenerative disease characterized by progressive degeneration of motor neurons leading to progressive muscle paralysis and death within 3–5 years from onset [24], with riluzole as the only one FDA-approved treatment [12,19,7]. A large *in-vitro* drug screen using multiple models identified ceftriaxone as a promising candidate therapy for ALS [13,26]. Subsequent studies demonstrated that ceftriaxone up-regulates the excitatory amino acid transporter 2 (EAAT2; mouse-analog GLT-1) transporter [22], potentially reducing glutamate excitotoxicity. Administration of ceftriaxone also prolonged survival in a mouse model of ALS [22]. In addition, ceftriaxone has neuroprotective effects in models of spinal muscular atrophy [20], Huntington's disease [18], multiple sclerosis [17] and ischemia [8,15,23]. Based on the promising preclinical data in ALS, a large randomized, placebo- controlled, phase I–III adaptive design human trial of IV ceftriaxone for the treatment of ALS was conducted [4,10,28]. In order to support the safety of a long-term (up to 52 weeks) use of IV ceftriaxone in clinical trials in ALS patients, further to a 14-day preliminary rodent study (unpublished data), a 6-months chronic toxicological and pharmacokinetic study in rodents was designed and executed.

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http://dx.doi.org/10.1016/j.toxrep.2015.09.010
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2. Material and methods

2.1. Overall design

A 6-months toxicology and pharmacokinetic (PK) study to evaluate the long term safety of ceftriaxone was performed following established international guidelines for evaluating medicinal products [14,9] by Charles River Laboratories Preclinical Services (Spencerville, OH and Worcester, MA). The study was approved by the Institutional Animal Care and Use Committee (IACUC). To guide design and dose selection, a preliminary toxicology and PK 14-day study of daily subcutaneous (SC) ceftriaxone administration was conducted in rodents (unpublished data). The 6-months administration was followed by 1-month recovery period to allow evaluation of reversibility of potential toxicological findings.

2.2. Dosage and administration

Dose levels tested in this study included: 0.25, 0.5, 1 g/kg/day and 1 g/kg BID, given approximately 8 h apart (Table 1). These were selected based on the toxicity and TK data from a previous 14-day study in rats using the same formulation and route of injection (unpublished data). The dose of 1 g/kg administered subcutaneously twice daily (BID) to rats was considered to produce ceftriaxone exposure comparable to or exceeding that observed in patients after intravenous injection at the approved clinical therapy in human. Therefore, to achieve maximum ceftriaxone systemic exposure compatible with reasonable survival, the dose of 1 g/kg SC BID was chosen as the highest dose level for this 6-months study.

Subcutaneous dosing was used because other routes of administration were considered problematic for long-term administration in rodents. IV administration in rats is poorly tolerated, causing tail necrosis and infection. Intraperitoneal (IP) administration was also thought to carry a risk of infection. The SC injection sites were rotated for each dose into four separate quadrants (left scapular, right scapular, right mid-dorsal thoracic, and left mid-dorsal thoracic).

2.3. Test material and control substance

Ceftriaxone for injection was provided in the form of a white powder by Baxter ACC. Ceftriaxone was mixed with sterile water and stored refrigerated in amber glass bottles for up to a week. The control material was sterile water.

2.4. Animals

Sprague-Dawley rats Crl:CD (SD) were provided by Charles River Laboratories Inc. and used in all arms and phases of the studies. Animals were housed individually in suspended stainless steel cages in a controlled environment (temperature: 22 ± 4 Celsius; humidity $50 \pm 20\%$; light cycle: 12-h light/12-h dark cycle; air changes: 10 or more per hour with 100% fresh air), provided free access to chow and water. They were acclimated to the controlled laboratory environment for 6 days prior to dosing, and weighted and examined before randomization. A total of 228 male and 228 female rats participated in the study. Animals were approximately 8 weeks of age at the time of randomization, with body weights ranging from 238 to 274 g for males and 171 to 200 g for females.

2.5. Study groups and randomization

Animals were divided into two main arms: toxicology and pharmacokinetic (PK) groups (Table 1).

The toxicology arm included 120 male and 120 female animals divided into Groups 1–5 and receiving placebo (Group 1) or study drug at one of four dosage levels (Groups 2–5) (Table 1). Animals underwent intermittent observations, and ultimately were evaluated by necropsy.

The PK arm included 84 male and 84 female rats randomized to placebo or test material. These animals were divided into subgroups and blood and urine samples for PK analysis were collected according to the sample collection schedule (Supplementary Tables 1 and 2).

2.6. Clinical observations and examination schedule

2.6.1. Animal observation

General health/mortality and moribundity checks were performed twice daily. Detailed clinical observations including weight were performed prior to treatment, weekly and prior to euthanasia. Cage-side observations were also performed following dose administration and daily during the recovery period. Food consumption measurements were recorded weekly. Ophthalmology examinations were performed by a board-certified veterinary ophthalmologist prior to randomization, during week 26, and prior to necropsy.

2.6.2. Blood and urine sample collection procedures

Blood and urine samples were collected on the day of necropsy for all animals and on day 90 and 177 from 10 animals/sex/group that were ultimately sacrificed on day 211. Animals were fasted overnight prior to sample collections, but had *ad libitum* access to water. Blood samples for hematology, coagulation, and chemistry were obtained via the orbital plexus while the animals were under light isoflurane anesthesia. Blood sampling for ceftriaxone concentration was obtained through jugular vein puncture under isoflurane anesthesia per sample collection schedule (Supplementary Tables 1 and 2). Urine samples for urinalysis were collected by cage pan drainage overnight.

2.6.3. Necropsy and histopathology

All animals surviving to the planned necropsy day were euthanized by exsanguination while under deep anesthesia induced with carbon dioxide inhalation. Organs were weighted and selected organs were collected and preserved. Slides from tissues of interest were examined microscopically by a board-certified veterinary pathologist.

2.7. Pharmacokinetics procedures

A volume of approximately 0.0250 mL was collected into tubes containing K_3EDTA as anticoagulant. Samples were analyzed according to a method including sample extraction by protein precipitation and final extract analyses by liquid chromatography (LC)/mass spectroscopy (MS). The method was validated for analysis of ceftriaxone in 0.0250 mL sample volumes of K_3EDTA rat plasma over a concentration range of 1.00–500 μg/mL.

2.8. Statistical analysis

Inferential statistical analyses were performed for the toxicology and recovery phase animals, analyzing body weight, change in body weight, food consumption, hematology, coagulation, clinical chemistry, urinalysis, and organ weights. The data were initially analyzed for homogeneity of variance using Levene's test ($p < 0.01$) followed by the Shapiro–Wilk test for normality ($p < 0.05$). If both assumptions were fulfilled, a single-factor (animal grouping) ANOVA was applied, otherwise the Kruskal–Wallis ANOVA was applied. If the ANOVA was significant at $p < 0.05$, Dunnett's test (parametric ANOVA) or Dunn's test (K–W ANOVA) was used to compare the control group to each test article-treated group at

Table 1
Dosage level groups.

Group no.	Toxicology arm (male/female)	PK[a] arm (male/female)	Test material	Dose and frequency days 0–49 (g/kg)	Adjusted frequency days 50–182 (g/kg)	Recovery days (no drug delivered)	Day of necropsy
1	20/20	6/6	Sterile water	0 bid[b]	sid[c]	–	182–4
1 Recovery	10/10	–	Sterile water	0 bid	sid	182–211	211
2	20/20	18/18	Ceftriaxone	0.25 sid	No adjustment	–	182–4
3	20/20	18/18	Ceftriaxone	0.5 sid	No adjustment	–	182–4
4	20/20	18/18	Ceftriaxone	1 sid	No adjustment	–	182–4
4 Recovery	10/10	–	Ceftriaxone	1 bid	sid	182–211	211
5	15/15	24/24	Ceftriaxone	1 bid	–	–	50
5 Recovery	5/5	–	Ceftriaxone	1 bid	–	50–78	78

[a] PK: pharmacokinetics.
[b] bid: twice a day, approximately 8 h apart.
[c] sid: once per day.

the 0.05, 0.01, and 0.001 levels of significance. Mean ceftriaxone concentration-time data for males and females separately were subjected to non-compartmental PK analysis using WinNolin 1.5 with nominal sampling times.

3. Results

3.1. Mortality

Mortality was limited and was not deemed related to ceftriaxone. Five animals died by accidental death prior to 3 weeks. Four of these animals died on day 1 in the pharmacokinetic arm of the study. The fifth belonged to the toxicology arm and was replaced on day 15. One Group 1 (control group) male was found dead on study day 177. The cause of death was considered to be severe inflammation of the kidney and lower urinary tract.

3.2. Clinical observations

No overt clinical signs of toxicity were noted in males and females in the control or treated groups. An increased incidence of swelling was observed during the treatment period at the injection site of Groups 1, 4 and 5 animals, more frequently in males. The irritation at the injection site was particularly relevant in Group 5, therefore, BID dosing was discontinued after Day 49. On day 50, 15 animals per sex from Group 5 were terminated. Another five animals per sex from Group 5 were allowed a 4-week recovery period and necropsied on Day 78. The remaining 10 animals per sex from Group 5 continued receiving study drug at a reduced frequency (once daily 1 g/kg/day) for a minimum of 182 days, followed by a 4-week recovery period and necropsy on Day 211 (Table 1). No remarkable clinical signs were observed during the recovery period, including swelling at the injection site that had almost completely resolved during this time.

3.3. Body weight and food consumption

There were no toxicologically meaningful differences in mean body weights or body weight gains for animals in the treated groups compared to controls (Fig. 1). Mean body weights of males in Groups 4 and 5 were statistically lower than controls during the treatment period (Fig. 1a). However, the differences were not considered toxicologically meaningful since the Group 4 and 5 mean body weights remained within approximately 10% of controls throughout the treatment period. Mean body weights of females in the treated groups were comparable or slightly higher than controls throughout the study (Fig. 1b).

There were no toxicologically meaningful differences in mean food consumption for animals in the treated groups compared to controls. The few statistically significant differences in mean food consumption were not considered toxicologically meaningful since they did not follow a consistent pattern and, often, the mean values were higher, rather than lower, in treated groups compared to controls.

3.4. Clinical chemistry, urinalysis and hematology

Several clinical chemistry parameters in the treated groups were found significantly different from the control group. However, all the differences were considered slight and the absolute values remained within the limits of normal for this species. The differences found in the urinalysis were not considered toxicologically meaningful since they did not follow a consistent pattern or dose response.

Hematology analysis showed decreased red cell parameters (erythrocyte count, hemoglobin, hematocrit, mean corpuscular hemoglobin concentration (MCHC)) and increased reticulocyte count and fibrinogen in Group 5 animals terminated early (Day 50) (Supplementary Table 3). Similar hematology parameters changes were also observed in Group 4 and Group 4 recovery on days 90 and 177. These findings were reversible in Group 5 recovery animals euthanized on Day 78 (Supplementary Table 3). In addition, changes in red cell parameters in the Group 4 female recovery group as well as reticulocytes counts (Supplementary Table 4) and fibrinogen normalized on day 211. These hematologic findings were consistent with iron deficiency anemia due to slow blood loss, which was attributed to irritation at the injection site.

3.5. Gross necropsy

Gross necropsy findings included enlarged cecum in treated animals at scheduled euthanasia at the end of the treatment period and thickened and discolored injection site in animals belonging to both the control and treated groups at the end of the treatment and recovery periods (Supplementary Table 5).

The enlarged ceca appeared dose-related as did the discolored skin injection sites. Skin thickening was observed in all groups, including the control group. Virtually all animals in Group 5 had an enlarged cecum on day 50 as well as thickened and discolored skin at the injection sites. The enlarged ceca and thickened skin had almost completely resolved at the end of the recovery period for this group (day 78).

At the end of the recovery period on day 211, cecum size appeared normal, while, thickened and discolored skin remained a frequent finding.

In the stomach, discoloration, foci, or thickening were observed in the non-glandular stomach of some Group 4 animals (Supplementary Table 5). The discoloration and foci correlated microscopically with hyperplasia, and occasionally, with degeneration or ulcer of the stratified squamous epithelium, while

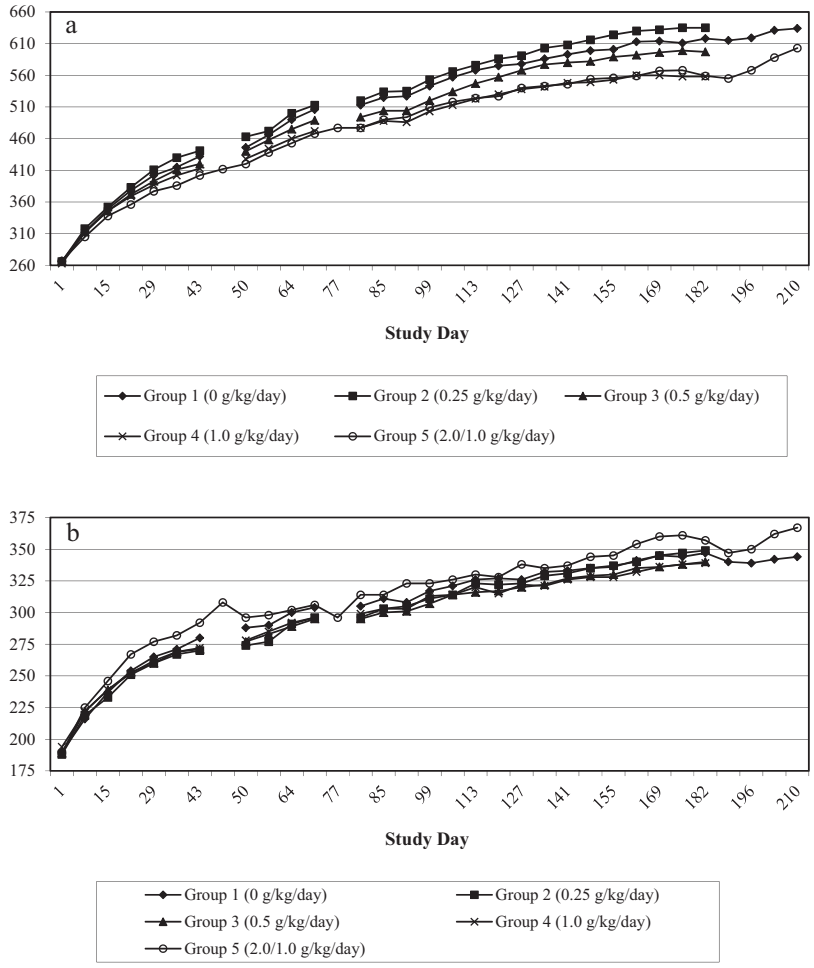


Fig. 1. Mean body weights. (a) Male group mean body weights (g), (b) female group mean body weights (g).

thickening correlated either with epithelial hyperplasia or edema of the wall of the non-glandular stomach.

Discoloration, foci or thickening were each found in the glandular stomach of one Group 4 animal in association with non-glandular stomach changes in two out of the three animals considered. In these rare cases, glandular stomach discoloration and thickening correlated microscopically with edema of the stomach wall, while black foci correlated with erosion. At the recovery sacrifices, gross findings related to ceftriaxone administration were observed at the injection site, in the cecum, and common bile duct. Gross dilation of the common bile duct with spherical contents was observed in a few treated males. The dilation was confirmed microscopically. No correlate to the spherical content was observed but these were presumably choleliths which were lost during tissue processing.

3.6. Organ weights

Absolute cecum weights and cecum weights relative to body weight were significantly increased for males in Groups 2–4 compared to controls at study termination (days 183–185) (Fig. 2).

Absolute adrenal weights and adrenal weights relative to body weight were significantly increased for males in Group 4 compared to controls at study termination (days 183–185) (Fig. 2). The increase in adrenal weights in Group 4 males may have been related to the stress of injections, which proved to be highly irritating.

3.7. Histopathology

Histopathologic effects related to ceftriaxone administration were observed at the injection site, and in the cecum, common bile duct, and stomach (glandular and non-glandular) (Table 2).

Findings at the injection site at the terminal necropsy included fibrosis, hemorrhage, and pigmentation. Fibrosis occurred with similar incidence across groups of control and treated animals. The average severity of fibrosis was also similar across control and treated groups of males, while in females it was greater in treated groups than in control and was greatest in the two highest dose female groups (Groups 4 and 5).

Hemorrhage occurred commonly across all groups but the average severity was greater in treated groups than in controls. Incidences and average severities were somewhat less in Group 5 high-dose animals than in other treated groups, presumably because these animals were sacrificed earlier than animals from the other treated groups and therefore received fewer injections.

Pigmentation was observed frequently in Groups 3–5 animals with an average severity greater in Groups 3 and 4 animals compared to Group 5, presumably because Group 5 animals were sacrificed earlier than others. Microscopically, pigmentation consisted of variable numbers of macrophages, filled with dark brown/black granular material, scattered within the dermal fibrous tissue. In some cases, as in the control female, the pigment may have been hemosiderin resulting from hemorrhage. In most cases, however, the nature of the pigment was uncertain.

Table 2
Summary of relevant histopathology at terminal sacrifice.

(a) Summary of relevant histopathology in males at terminal sacrifice

Organ	Lesion	Group 1[b]	Group 2	Group 3	Group 4	Group 5
Kidney	Nephropathy	9/20	NE[a]	0/1	5/20	12/15
	Minimal	9			5	11
	Mild	0			0	1
Bile duct	Hyperplasia	0/20	NE	NE	0/20	2/15
	Minimal	0				1
	Mild	0				1
	Dilatation	1/18	0/17	0/20	1/19	1/1
	Minimal	0/18			1/19	0/1
	Mild	1/18			0/19	0/1
	Marked	0/18			0/19	1/1
	Cholelithiasis	0/18	0/17	0/20	0/19	1/1
	Marked					1/1
Cecum	Dilatation	0/20	5/20	8/20	11/20	15/15
	Minimal		0	0	5	0
	Mild		5	6	6	3
	Moderate		0	2	0	12
STomach	Hyperplasia	0/20	1/20	3/20	4/20	0/15
non-glandular	Minimal		1	1	1	
	Mild		0	2	1	
	Marked		0	0	2	
	Edema	0/20	0/20	0/20	3/20	0/15
	Moderate				3	
	Marked				0	
	Inflammation	0/20	0/20	0/20	0/20	0/15
	Ulceration	0/20	0/20	0/20	0/20	0/15
Stomach glandular	Edema	0/20	0/20	0/20	1/20	0/15
	Mild				1	
	Inflammation	0/20	0/20	0/20	1/20	0/15
	Minimal				1	
	Erosion	0/20	0/20	0/20	0/20	0/15
Injection site	Fibrosis	20/20	19/20	20/20	20/20	15/15
	Minimal	2	7	3	0	0
	Mild	5	9	10	7	5
	Moderate	8	2	5	9	8
	Marked	5	1	2	4	2
	Hemorrhage	16/20	19/20	20/20	20/20	15/15
	Minimal	12	9	3	4	0
	Mild	4	9	7	7	13
	Moderate	0	1	7	5	2
	Marked	0	0	3	4	0
	Pigmentation	0/20	0/20	8/20	18/20	8/15
	Minimal			3	2	7
	Mild			3	7	1
	Moderate			2	6	0
	Marked			0	3	0

(b) Summary of relevant histopathology in females at terminal sacrifice

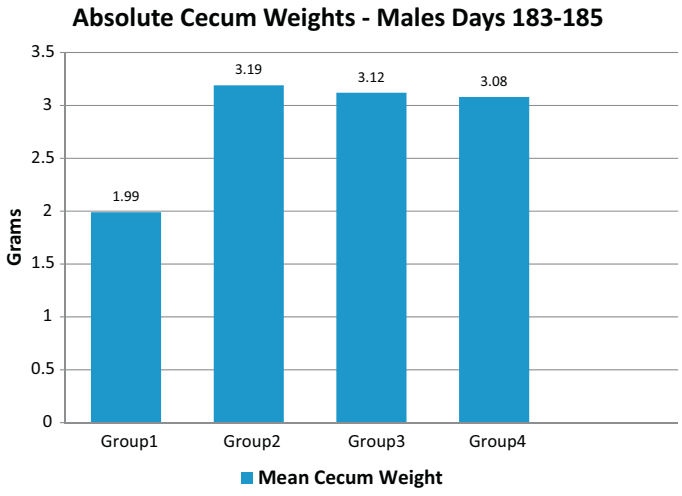
Organ	Lesion	Group 1[b]	Group 2	Group 3	Group 4	Group 5
Kidney	Nephropathy	1/20	NE[a]	0/2	0/20	3/15
	Minimal	1				2
	Mild	0				1
Bile duct	Hyperplasia	1/20	NE	NE	1/20	0/15
	Minimal	1			1	
	Dilatation	0/17	0/20	0/17	0/20	NE
	Cholelithiasis	0/17	0/20	0/17	0/20	NE
CECUM	Dilatation	0/20	7/20	9/20	14/20	15/15
	Minimal		1	1	3	0
	Mild		6	8	6	3
	Moderate		0	0	5	12
Stomach non-glandular	Hyperplasia	0/20	3/20	3/20	8/20	0
	Minimal		2	3	0	
	Mild		0	0	4	
	Marked		1	0	4	
	Edema	0/20	0/20	0/20	1/20	0/15
	Moderate				0	
	Marked				1	
	Inflammation	0/20	0/20	0/20	2/20	0/15
	Minimal				1	
	Mild				1	
	Ulceration	0/20	0/20	0/20	1/20	0/15
	Mild				1	

Table 2 (*Continued*)

(a) Summary of relevant histopathology in males at terminal sacrifice

Organ	Lesion	Group 1[b]	Group 2	Group 3	Group 4	Group 5
Stomach glandular	Edema	0/20	0/20	0/20	1/20	0/15
	Mild				1	
	Inflammation	0/20	0/20	0/20	0/20	0/15
	Erosion	0/20	0/20	0/20	1/20	0/15
	Mild				1	
Injection site	Fibrosis	19/20	17/20	20/20	20/20	15/15
	Minimal	13	13	4	0	1
	Mild	5	4	12	4	4
	Moderate	1	0	3	8	7
	Marked	0	0	1	8	3
	Hemorrhage	13/20	19/20	20/20	20/20	14/15
	Minimal	11	14	9	1	4
	Mild	2	5	8	5	9
	Moderate	0	0	2	12	1
	Marked	0	0	1	2	00
	Pigmentation	1/20	1/20	13/20	19/20	8/15
						8
	Minimal	1	1	10	4	8
	Mild	0	0	3	6	0
	Moderate	0	0	0	8	0
	Marked	0	0	0	1	0

[a] NE: not examined.
[b] Group 1 (0 g/kg/day); Group 2 (0.25 g/kg/day); Group 3 (0.5 g/kg/day); Group 4 (1.0 g/kg/day); Group 5 (2.0/1.0 g/kg/day).



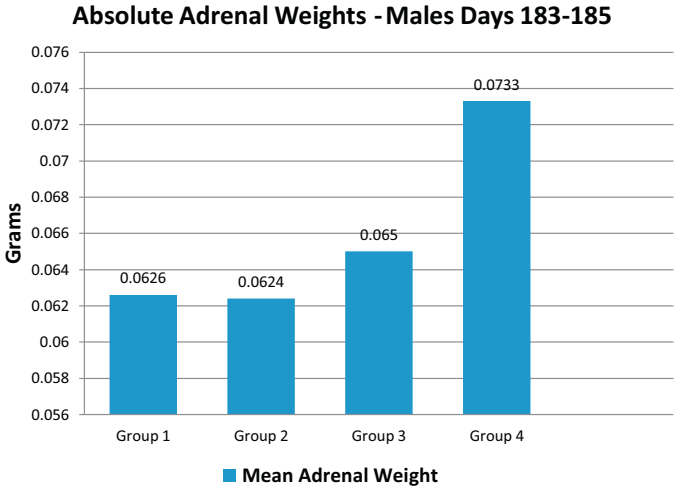


Fig. 2. Statistically significant organ weights changes.

Cecum dilatation occurred in all treated groups of males and females with a dose-related incidence and average severity. Grossly the affected ceca were enlarged up to approximately two times the normal size. Microscopically dilated ceca appeared only longer and with thinner mucosa.

Dilatation of the common bile duct was observed in one control male, one Group 4 male, and one Group 5 male. The latter also had cholelithiasis. Microscopically, the dilatation was characterized by an increase in the bile duct diameter, from approximately four times normal in the Group 1 male up to approximately 10 times normal size in the Group 5 male. The fibrous connective tissue wall of the dilated duct was variably thickened, while the biliary epithelium was generally hyperplastic.

In the non-glandular stomach, hyperplasia of the stratified squamous epithelium was observed in animals from Groups 2–4. In addition, edema of the non-glandular stomach was found in a few males and in one female from Group 4; inflammation in two Group 4 females; and ulceration in one Group 4 female. In the glandular stomach, edema was observed in one Group 4 male with epithelial degeneration of the non-glandular stomach, and in one Group 4 female with epithelial hyperplasia of the non-glandular stomach. Moreover, in the glandular stomach, inflammation was found in one Group 4 male and mucosal erosion was seen in one Group 4 female.

Nephropathy occurred with somewhat higher incidence in Group 5 males as compared with Group 1 males. Nephropathy is a common spontaneous change in male SD rats as they age [5], thus the higher incidence in the Group 5 males may simply represent a random variation in the normal background finding and it was deemed not to be toxicologically meaningful.

Based on the evaluation of the study results, the no-observed-adverse-effect dose level is considered to be 0.5 g/kg/day.

3.8. Pharmacokinetic

Pharmacokinetic findings are summarized in Table 3. Ceftriaxone was rapidly absorbed after subcutaneous injection; the maximum plasma level was reached in 1 h. Ceftriaxone was also very rapidly cleared, with a plasma half-life ranging between 1 and 1.5 h. Values for C_{max} and AUC_{last} were found similar for each dosage

Table 3
Summary of pharmacokinetics measures at day 182.

Group (N = 6 M and 6F)	Dosage (g/kg/dose)	Sex	C_{max} (mean ± SD) (μg/mL)	t_{max} (h)	t_{last} (h)	AUC_{last} (μg h/mL)	AUC (μg h/mL)	$t_{1/2}$ (h)
2	0.25 sid[b]	M	262 ± 38.4	1	8	831	861	1.6
		F	205 ± 142	2	8	906	NE[a]	NE[a]
3	0.5 sid	M	381 ± 53.6	1	8	1070	1090	1.2
		F	404 ± 26.5	1	8	1160	1190	1.3
4	1.0 sid	M	607 ± 144	1	8	1570	1610	1.5
		F	669 ± 119	1	8	1690	1740	1.5

[a] NE: not estimated, due to insufficient characterization of the terminal phase of mean concentration-time curve.
[b] sid: once daily.

level at each time point (days 1, 84, and 182) for Groups 1–4. No accumulation was observed.

Average urinary recovery was approximately 32% of the dose. The t_{max} was 2 h, with virtually all ceftriaxone excreted by 16 h (Supplementary Table 6).

4. Discussion

The study described was aimed at assessing the safety of ceftriaxone administered subcutaneously in rats at dosages up to 2 g/kg/day with a duration of 6 months in order to support a 52 weeks clinical trial of ceftriaxone for the treatment of ALS [4,10,28] .

In general, ceftriaxone was found to be safe and well tolerated. No toxicologically meaningful changes in body weight, food consumption and ocular findings between the treated and control groups were observed. In addition, apart from the decrease in red cell parameters, there were no ceftriaxone-related changes in hematology, coagulation, clinical chemistry and urinalysis parameters.

Injection site trauma and the associated reversible anemia most likely due to chronic blood loss at the injection site, were all attributable to subcutaneous route of administration. This route of administration was chosen in an attempt to avoid the challenges inherent to chronic intra-peritoneal or intravenous drug administration in rats. These findings are not relevant for human use of ceftriaxone, as it is administered intravenously.

Cecal enlargement in ceftriaxone-treated animals was a reversible finding without any corresponding histolpathological changes and was observed also in previous toxicological studies with ceftriaxone [25]. To our knowledge, an adult human correlate has never been reported.

Nephropathy was observed in both the control and treatment groups. Since nephropathy is a common spontaneous complication observed in male Sprague–Dawley rats as they age [5] and was also present in control males, its toxicological relevance was unclear. However, as renal complications associated with ceftriaxone are known in humans, in order to avoid renal adverse events, patients enrolled in the clinical trial receiving chronic ceftriaxone had regular urinalysis and blood tests for electrolytes, creatinine and BUN monitoring. If there was concern for renal dysfunction, and if serum creatinine rose of 25% or more from the screening visit level, the patients' drug administration was interrupted to allow further evaluation under the guidance of a nephrologist.

Previous preclinical studies showed that administration of ceftriaxone in dogs receiving 100 mg/kg/day for 4 weeks and in baboons receiving ≥335 mg/kg/day for six months was associated with calcium salt of ceftriaxone concretions in the gallbladder bile [1]. In the present study, bile duct dilatation was observed only in a few animals across all the study groups and cholelithiasis was confirmed only in one animal treated at the highest dose, while loss of dilated bile ducts content during the tissue processing cannot be excluded. Humans are known to develop pseudo-cholelithiasis, or sludging of the bile in the gallbladder and/or common bile duct, when treated with ceftriaxone for only three or ten days [6,21]. For this reason, in the trial of ceftriaxone for the treatment of ALS [4,10], liver function tests have been regularly assessed. Participants with symptoms underwent ultrasound to identify the development of bile sludge or stones, and ceftriaxone was held for participants with symptoms and/or signs of cholelithiasis. Since ursodeoxycholic acid might prevent or reduce the occurrence of pseudocholelithiasis [27,11] and significantly shortened event duration of biliary sludging and cholelithiasis in the first phases of the clinical trial [4], it was administered in all the patients treated with ceftriaxone in the Phase III of the trial to prevent biliary obstruction [10].

Finally, changes in the non-glandular stomach wall were observed. The non-glandular stomach is a common site of irritation in the rat and similar findings have been described by other authors [16]. Because humans lack the rat's non-glandular stomach, there is no human correlate to these changes. Only a small number of animals developed limited changes in the glandular stomach, which are unlikely to have any clinically significance in humans.

Overall there were no toxicologically meaningful drug-related animal findings associated with the long-term administration (6 months) of ceftriaxone. This study can then support the clinical long-term use of ceftriaxone in any future trials for indications that require chronic treatments and in particular has provided the preclinical toxicological support for the human trial of ceftriaxone for ALS [4,10] .

Acknowledgements

Malcolm Blair and Todd Merriman who directed the research team at Charles River Laboratories. Cheryl Bellamy and Todd Merriman who contributed to the preparation of some of the graphs reported.

This project was supported by grants from the National Institute of Neurological Disorders and Stroke (NINDS), National Institute of Health (U01 NS049640-05).

Lupin Limited Pharmaceuticals (Mumbai, India) provided ceftriaxone for the 14-day study; Phoenix Scientific, Inc. (St. Joseph, MO) provided sterile water (control material) for the 14-day study; Baxter ACC provided ceftriaxone for the 6-month study.

Elena Ratti was awarded from the National Institute of Neurological Disorders and Stroke (NINDS) with grant number R25NS065743, title: Neuroscience resident research program.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.toxrep.2015.09.010.

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