White Paper – Toxicity Study (Oral & Nasal Route) of Bio-Immune™

Research & Development Centre (DSIR RECOGNIZED)

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Acute and sub-acute toxicity assessment of andrographolide-2-hydroxypropyl-*β*-cyclodextrin complex via oral and inhalation route of administration in Sprague-Dawley rats

Abstract

Objective: Acute and sub-acute toxicity analysis of AND-2-HyP-*β*-CYD complex was conducted in Sprague Dawley (SD) rats following oral and inhalation route of administration. **Methods and Results:** Single dose acute toxicity was carried out at 2000-mg/kg of AND-2-HyP-*β*-CYD complex, while the doses of 200, 400 and 666 mg/kg were administered, over a period of 28 days under repeated dose oral toxicity study. Hence, LD50 (Lethal dose) was found to be >2000-mg/kg in addition to NOAEL (No-Observed Adverse Effect Level) of 666 mg/kg. Correspondingly, single dose acute inhalation toxicity of AND-2-HyP-*β*-CYD complex was carried out at 5 mg/L/4h/Day and sub-acute inhalation toxicity at 0.5, 1, and 1.66-mg/L/4h/Day over a period of 28 days. The NOAEL and LOAEL (Lowest Observed Adverse Effect Level) was estimated to be 0.5- mg/L/4h/Day and 1-mg/L/4h/Day, respectively. **Conclusion:** The findings of the present study would further be useful in assessing and utilizing the medicinal and therapeutic benefits of AND-2-HyP-*β*-CYD complex.

Keywords: Andrographolide; Acute toxicity, Sub-acute toxicity, Oral Toxicity, Inhalation toxicity

1. Introduction

Andrographolide (AND) is a diterpenoid with multiple biological activities, but most commonly employed for its anti-inflammatory action [1]. It is abundantly present in leaves and stems, followed by the seeds of plants belonging to Andrographis genus, commonly known as “Creat” or “Green Chiretta” [2]. The purified form of AND has been investigated for its anti-inflammatory effects in various stressful conditions, such as liver disorders, ischemia, arthritis, cancer, and oxidative stress [3-8]. Besides anti-inflammatory activity, AND also displays immunostimulatory action by efficaciously increasing CD4+ and CD8+ cells population [9]. All these properties of AND form the foundation for its clinical application against viral infections. Further, these studies necessitate the development of a bio-pharmaceutically effective dosage form for oral and inhalation administration.

Previously, we have synthesized and characterized andrographolide-2-hydroxypropyl-*β*-cyclodextrin (AND-2-HyP-*β*-CYD) complex to augment the bioavailability of phytomolecule [10]. Thus, the evaluation of toxicity profile of AND-2-HP-*β*-CYD complex through oral and inhalation routes seems to be important and needs to be further studied.

Therapeutic entity mediated hepatotoxicity and nephrotoxicity are the foremost important reasons for the pharmaceutical withdrawals of promising chemical entities in clinical trials. In order to evaluate drug induced hepatoxicity, alanine aminotransferase (ALT) biomarker plays most important role followed by alkaline phosphate (ALP), Albumin (ALB), and Bilirubin (BIL). On the other hand, urea, phosphorous (PHOS) and serum creatinine (CREJ) level are the commonly used end point indicators for the assessment of renal functions [11]. Therefore, evaluation of at-least four serum parameters (hepatocellular and hepatobiliary serum biomarkers) has been recommended for toxicity profiling of therapeutically active

compounds [12]. Hence, single dose acute (14 days) and repeated dose sub-acute (28 Days) toxicity of AND-2-HyP-*β*-CYD complex was assessed following oral and inhalation route of administration in Sprague Dawley (SD) rats under a set of stringent *in vivo* parameters.

2. Materials and Methods

2.1. Ethics Statement

All animal studies were performed in accordance with the guidelines of Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), Ministry of Fisheries, Animal Husbandry, and Dairying, Government of India, New Delhi. The study was approved by the Institutional Animal Ethics Committee (IAEC). The animals were examined and allowed to adapt the new environmental conditions for a week before the commencement of experiments.

2.2. Animals

Healthy male and female SD rats with average weight of 168.9 g were purchased from the certified suppliers. All the toxicity studies were conducted by strictly following Organization for Economic Cooperation and Development (OECD) Guidelines. All the animals were housed separately in plastic cages according to their sex and maintained for 12-h light/day cycle at 19.1-22.7˚C with relative humidity (RH) of 39-65%. All the animals were caged with ready availability of food and water.

2.3. Toxicity Experiments

2.3.1. Single Dose Acute Oral Toxicity Analysis

Three female rats with average age of 6-7 weeks were used for single dose acute oral toxicity analysis. In brief, AND-2-HyP-*β*-CYD complex [10] at the dose of 2000-mg/kg was administered to female rats through oral route of administration according to OECD guideline 423 [13]. Animals were closely observed initially every 4 h, followed by once a day for a period of 14 days for any signs of toxicity or mortality, according to established criteria [14].

In addition, food and water consumption were recorded at alternate days. On the other hand, body weight was recorded weekly. On the 14th day of experiment, animals were anaesthetize using ketamine (0.35 mL/kg) and xylazine (0.10 mL/kg) intraperitoneally and subjected for thorough observation of clinical signs, gross behavioral changes and mortality along with assessment of food intake and gain in body weight.

2.3.2. Repeated Dose Sub-Acute Oral Toxicity Analysis

Seventy-two rats (36 males and 36 females) with average age of 6-7 weeks were randomly selected and grouped into low dose (1/10 of LD50 dose~200-mg/kg), medium dose (1/5 of LD50~400 mg/kg), high dose (1/3 of LD50~666 mg/kg), reversal control of high dose (666 mg/kg), reversal control and normal control. Rats were administered once daily the solution of AND-2-HyP-*β*-CYD complex by oral gavage as per the schedule throughout the experiment for 28 consecutive days according to OECD guideline 407. All the animals were strictly observed for mortality and morbidity in addition to clinical signs for a period of 28 days followed by 14 additional days for evaluating reversal effects. Additionally, food and water consumption were recorded at alternate days whereas, body weight was documented weekly.

In order to carry out the haematological and biochemical evaluation, blood samples were collected on 28th and 42nd day through retro-orbital vein. Furthermore, animals were sacrificed for gross necropsy and subjected to fastidious evaluation of external body surface including all the orifices, cranial, thoracic and abdominal cavities along with their contents. Following analysis of gross necropsy, liver and kidney in addition to other organs were removed surgically, weighed and stored at -40˚C in 10% formalin solution. Liver and kidney were studied for further histopathological examination.

2.3.3. Single Dose Acute Inhalation Toxicity Analysis

Thirty SD rats with average age of 6-7 weeks were randomly selected and grouped into 3 groups and each group was constituted with 5 males and 5 females as per OECD guideline 403. The animals in control group did not receive any vehicle or treatment, while citrate buffer (pH~6.5) was administered in vehicle control group through nebulization as liquid aerosols. The animals in treatment group were exposed to AND-2-HyP-*β*-CYD complex in citrate buffer (pH~6.5) through nebulization at the dose of 5 mg/L for 4 h. All the animals were examined cautiously for any clinical signs related to gross behavioral changes and mortality along with the recording of food and water consumption at alternate days whereas, change in body weight was plotted weekly.

2.3.4. Repeated Dose Sub-Acute Inhalation Toxicity Analysis

Sixty SD rats with average age of 6-7 weeks were randomly selected and differentiated into 2 groups (30 males and 30 females). The two groups were further subdivided into: normal control group, vehicle control group (citrate buffer, pH~6.5), low dose (1/10 of MTD dose i.e. 0.5 mg/L/4 h), medium dose (1/5 of MTD dose i.e. 1 mg/L/4 h), and high dose (1/3 of MTD i.e. 1.66 mg/L /4 h) group. Rats were exposed once daily AND-2-HyP-*β*-CYD solution by nebulization as per the schedule throughout the experiment for 28 consecutive days according to OECD guideline 412. All the animals were observed for mortality and morbidity in addition to clinical signs for a period of 28 days in addition to recording of food and water consumption at alternate days whereas, body weight was assessed weekly.

To conduct the haematological and biochemical evaluation, blood samples were collected on 28^{th} day through retro-orbital vein. In addition, animals were sacrificed post blood collection and subjected to gross necropsy including a careful examination of the external surface. Following analysis of gross necropsy, liver, kidney and lungs in addition to other organs were removed surgically, weighed and stored at -40˚C in 10% formalin solution. Liver, kidney and lungs tissues were studied for further histopathological examination.

2.4. Haematological Analysis

Blood samples collected in heparinized tubes were examined using an automated haematology system at a commercial diagnostic laboratory. The blood samples were evaluated for leukocytes (WBC), erythrocytes (RBC), haemoglobin (Hb), haematocrit (HCT), mean corpuscular volume (MCV), mean corpuscular haemoglobin (MCH), mean corpuscular haemoglobin concentration (MCHC), platelet count (PLT), neutrophils (NEUT), monocytes (MONO), eosinophils (EOS) and basophils (BASO).

2.5. Serum Biochemical Analysis

To obtain samples for serum analysis, blood samples were collected into sterile tubes without any anticoagulant coating and allowed to stand for 30 min. The samples were centrifuged at 1500 *g* for 10 min at 4 ^{0}C. The supernatant was collected and stored at 4˚C till further processing. Serum samples were analyzed for albumin (ALB), alkaline phosphatase (ALP), alanine aminotransferase (ALT), aspartate aminotransferase (AST), bilirubin (BIL), calcium (CA), cholesterol (CHO), creatinine (CREJ), phosphorous (PHOS), total protein (TP), urea, and glucose (GLU) level by using standard diagnostic test kits on a semi-automated clinical biochemistry analyzer at a commercial laboratory.

2.6. Histopathological Assessment

The organs collected for histopathology analysis were embedded in paraffin wax, sectioned with microtome and stained by haematoxylin and eosin (H&E) dye. Blinded histological analysis was performed by a trained pathologist.

2.7. Statistical Analysis

Data obtained for various studies were expressed as mean value along with standard deviation (mean ± SD). All the statistical analysis were performed by one-way analysis of variance (ANOVA) and the graph was drawn by using GraphPad Prism 5.0 (GraphPad Prism, San Diego, USA) and p value < 0.05 was considered statistically significant.

3. Results and Discussion

3.1. Acute and Sub-Acute Oral Toxicity analysis of AND-2-HyP-β-CYD Complex

A large population in developing countries depends on herbal medicines for their treatment. However, very limited scientific literature is available regarding the safety and efficacy of traditional medicines [15]. This necessitates the conduction of toxicological studies to serve two purposes i.e. establishment of dose ranges in preclinical studies, and disseminate the data on the safety profile of herbal drug prior to product development [16]. The first sign of toxicity over repeated exposure of any substance is aberrant alteration in body and organ weights; and therefore, they are considered as vital indicators for adverse effects [17].

The single dose acute oral toxicity analysis at 2000 mg/kg of AND-2-HyP-*β*-CYD complex indicated that all the animals were in somnolence condition with decreased motor activity. No abnormality was detected in gross pathology of rats **(Suppl. Table 1)**. Hence, LD50 (Lethal dose) was found to be >2000 mg/kg as per the OECD 423 guidelines. Apart from this, there was no remarkable ($P>0.05$, One way ANOVA test followed by Dunnett's Multiple Comparison Test) fluctuation in the body weights of treated animal group as compared to control group post 28-days treatment period in sub-acute oral toxicity analysis **(Suppl. Table 2)**. The data collected for food-water intake and weight gain shows no significant changes in the body weight, thereby inferring the non-toxic effect of the complex on the growth of the animals. Moreover, there was no remarkable difference in the organs weight ($P>0.05$, One way ANOVA test followed by Dunnett's Multiple Comparison Test) of control and treatment groups in sub-acute oral toxicity analysis **(Suppl. Table 3)**. Organ weight indicates the pathological and physiological status of animals, and are beneficial parameters in toxicity studies as they play important role in toxicity prediction, enzyme induction, physiologic perturbations and acute injury; correlation to any histopathological changes; and little inter-animal variability [18].

The haematopoietic system is one of the highly sensitive sites for toxicity and is a vital indicator of the pathological and physiological conditions in humans as well as animals [19]. Marginal fluctuations in haematological parameters provide greater prognostic factors for drug-induced toxicity [20]. Likewise, oral consumption of AND-2-HyP-*β*-CYD complex had no undesirable consequences on the circulating blood cells as well as on their production **(Table 1),** except slight reduction in WBCB ($x10^3$cells/µL) and MCH (pg) as compared to reference values in male rats. However, previous studies indicated that WBCB, MCH and % MONO values in normal rats were found to be 3.10±0.27 ($x10^3$/µl), 19.06±0.42 (pg), 5.10±0.91% respectively [21], almost complying with the WBCB (1.53 ±0.17 $x10^3$/µl to 3.17 ± 0.29$x10^3$/µl), MCH (14.00 ± 0.73 pg to 16.13 ± 0.34 pg) and MONO (3.08 ± 0.14 to 4.20 ± 0.62%) values of AND-2-HyP-*β*-CYD complex treatment in male rats (**Table 1**). Correspondingly, identical observations were also noticed in female rats following oral treatment with AND-2-HyP-*β*-CYD complex in sub-acute oral toxicity analysis (**Table 1**). Following this, biochemical parameters were also estimated in AND-2-HyP-*β*-CYD complex treatment groups in sub-acute toxicity analysis in both male and female SD rats (**Table 2**). AND-2-HyP-*β*-CYD complex treatment did not induce any fluctuation in the ALB, ALP, ALT, and AST level (**Table 2**). Moreover, a slight increase in PHOS (mg/dL) level **(Table 2)** was noticed in AND-2-HyP-*β*-CYD complex treatment groups with no significant difference (One way ANOVA test, $P>0.05$). Similar alternations were also exhibited by female rats following oral administration of AND-2-HyP-*β*-CYD complex at specified doses **(Table 2)**. Subsequently, histopathological analysis was carried out under sub-acute oral toxicity analysis for liver and kidney as presented in **Figure 1** in both male and female rats. Photomicrographs of histopathology of liver of male and female rats indicated inflammatory changes with overall unremarkable lesion score of +1 **[22, 23]** (**Figure 1 A-F**). Moreover, no degenerative and necrotic changes were observed in all treated and normal group of male

rats. In addition, histopathology of kidney of male and female rats treated with AND-2-HyP-*β*-CYD complex through oral route of administration did not exhibit any alterations in terms of vascular, degenerative and necrotic changes of renal tubules (**Figure 1 A-F**). Hence, male and female rats treated with oral doses of 200, 400 and 666 mg/kg of AND-2-HyP-*β*-CYD complex did not exhibit any noteworthy signs of abnormalities. The NOAEL (No Observed Adverse Effect Level) was found to be 666 mg/kg of AND-2-HyP-*β*-CYD complex.

3.2. Acute and Sub-Acute Inhalation Toxicity Analysis of AND-2-HyP-β-CYD Complex

The single dose (5-mg/L/4 h) acute inhalation toxicity analyses of AND-2-HyP-*β*-CYD complex in SD rats indicating no abnormality in 14 days study. Hence, MTD (Maximum Tolerated Dose) of AND-2-HyP-*β*-CYD complex was found to be >5 mg/L/4 h. Following this, sub-acute inhalation toxicity study (28 days) was conducted in SD rats with normal control group, vehicle control group (citrate buffer, pH~6.5), low dose (1/10 of MTD dose i.e. 0.5 mg/L/4 h), medium dose (1/5 of MTD dose i.e. 1 mg/L/4 h), and high dose (1/3 of MTD i.e. 1.66 mg/L/4 h) of AND-2-HyP-*β*-CYD complex. The body weight gain and organ weight was found to be normal in all groups of male and female rats treated with AND-2-HyP-*β*-CYD complex through nebulization (**Suppl. Table 4 and Suppl. Table 5**). Consumption of AND-2-HyP-*β*-CYD complex via inhalation route of administration slightly increased the RBC ($x10^6$cells/µL) level in all treated groups (**Table 3**) in both male and female rats. This effect may be attributed to presence of sodium citrate as excipient in citrate buffer in addition to variation in the normal range of RBCs according to age [24]. Moreover, a slight increase in MONO (%) and BASO (%) was also noticed in both the genders as compared to reference values (**Table 3**); however, it was complying with the normal % of MONO and BASO in rats [25]. **Table 4** represents the data of biochemical parameters in both male and female rats treated with AND-2-HyP-*β*-CYD complex via inhalation route of administration. A slight increase in ALP (IU/L), PHOS (mg/dL) and TP (g/dL) level was

noticed in all groups of male and female rats in comparison to reference values (**Table 4**). It was noticed that ALP (IU/L), PHOS (mg/dL) and TP (g/dL) level is raised with increase in the age of the rats [26]. The histopathological photomicrographs for sub-acute inhalation toxicity study are represented in **Figure 2.** Photomicrographs indicated overall lesion score of +3 in liver at high dose of 1.66 mg/L/4 h in comparison to +1 in liver of normal male and female rats. On the other hand, mid dose (1 mg/L/4 h) and low dose (0.5 mg/L/4h) exhibited the overall lesion score of +2 in liver of male and female rats with necrosis in hepatocytes (**Figure 2**). Correspondingly, identical results were also obtained in kidney and lungs tissues of both male and female rats with overall lesion score of +3 in high dose, +2 in mid dose and +1 in vehicle control group in addition to necrotic alterations in kidney and emphysema in alveoli of lungs. Emphysema refers to damage to the walls of the alveoli of the lungs (**Figure 2**). Hence, AND-2-HyP-*β*-CYD complex via inhalation route of administration exhibited mild to moderate toxicity at higher dose. Based on the results obtained from biochemical, haematological and histopathological analysis, the NOAEL was found to be 1/10 of MTC (0.5 mg/L/4hr/Day) and LOAEL (Lowest Observed Adverse Effect Level) was found to be 1/5 of MTC (1 mg/L/4hr/Day). Hence, the findings of the present study would further be useful in assessing and utilizing the medicinal and therapeutic benefits of AND-2-HyP-*β*-CYD complex.

4. Conclusion

In conclusion, administration of AND-2-HyP-*β*-CYD complex through oral and inhalation route of administration clarify the variations in histopathological, haematological and biochemical parameters. The results of acute and sub-acute toxicity analysis of AND-2-HyP-*β*-CYD complex provide valuable preliminary data on the toxic profile. Therefore, further assessments such genotoxicity and reproductive toxicity are required to proceed for clinical studies of AND-2-HyP-*β*-CYD complex. Eventually, it is mandatory to understand that

phytomolecules should be analyzed under a set of stringent parameters their toxicities and safeness.

Conflict of Interest Statement

Authors declares no conflict of interest

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Figure legends

Figure 1: Photomicrographs of liver and kidney of male and female rats in sub-acute oral toxicity of AND-2-HyP-*β*-CYD complex (A) Normal control (B) Low dose (1/10 of LD50 dose~200-mg/kg) (C) Medium dose (1/5 of LD50~400 mg/kg) (D) High dose (1/3 of LD50~666 mg/kg) (E) Reversal control of high dose (666 mg/kg), and (F) Reversal control

Figure 2: Photomicrographs of liver, kidney and lungs of male and female rats in sub-acute inhalation toxicity of AND-2-HyP-*β*-CYD complex (A) Normal control (B) Vehicle control (Citrate buffer, pH 6.5) (C) Low dose (0.5 mg/L/4 h), (D) Medium dose (1 mg/L/4 h), and (E) High dose (1.66 mg/L/4 h).