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Sub-acute Toxicity Studies of a Metabolite of *Streptomyces* Species

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Abstract: The subacute toxicity study of a brown antimicrobial metabolite Di (2-ethyl hexyl) Phthalate (AK_2), isolated from the culture filtrate of a *Streptomyces* species, was carried out on long Evan's rats. The studies include the gross general observations such as changes in body weight, haematological profiles [such as total count of red blood cells (RBC) and white blood cells (WBC), differential count of WBC, platelet count and haemoglobin (Hb) percentage], biochemical parameters of blood such as serum glutamate oxaloacetate transaminase (SGOT), serum glutamate pyruvate transaminase (SGPT), serum alkaline phosphatase, uric acid and creatinine and histopathology of the liver, kidney, heart, lung and spleen of both control and experimental groups of rats. The change of haematological and biochemical parameters were statistically insignificant. No abnormalities were found in the histopathology of the liver, kidney, heart, lung and spleen in the experimental group of rats at a dose of 200 µg/rat/day for 14 consecutive days, when compared with the control group.

Key words: *Streptomyces* sp., sub-acute toxicity, AK_2, SGOT, SGTP

Introduction

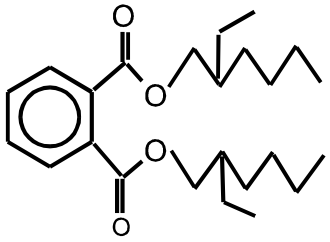
The concept of antibiosis opened a new field of research for the isolation of antibiotics from microorganisms and so far more than 4,000 of such antibiotics are known. In the United State and Japan between 1953 to 1970 approximately 85% of the antibiotics are produced by Actinomycetes, 11% by fungi and 4% by bacteria (Reiner, 1982). Among the *Actinomycetes,* most of the research work has been carried out on the *Streptomyces* species to search for antibiotics. Based on this concept, the field of research for newer antibiotics was broadened. As a part of our continuing search for microbial metabolites from soil samples (Sathi *et al.,* 2001), soil samples were collected from different parts of Bangladesh and a strain having antimicrobial activity was isolated from the soil of Upashahar, Rajshahi, Bangladesh and was identified as *Streptomyces* species (Holt *et al.,* 1994). From the chloroform extract of the yeast-extract glucose broth culture filtrate of the organism, an antimicrobial agent was isolated by preparative thin layer chromatographic technique (PTLC) (Egon and Stahl, 1969) and was identified as Di (2 ethyl hexyl) phthalate i.e. AK_2 by spectral analysis (Chowdhury, 2000). The antibacterial and cytotoxic activity of the compound was conducted by Chowdhury *et al.* (2001). In continuation of this study we herein report the subacute toxicity of the antibiotic AK_2.

The aim of these studies was to evaluate the safety margin of AK2 prior to clinical trial, as all drugs are toxic at higher doses (Goldstein, 1974). Moreover, even at therapeutic blood level some drugs have unavoidable side effects.

Materials and Methods

Collection of the organism: The organism was isolated from soil samples, collected from Upashahar, Rajshahi, Bangladesh, during July, 1999 at the depth of 1.0 meter using "crowed plate technique" (Hammond and Lambert, 1978) and was identified as *Streptomyces* species by morphological and biochemical study. The experiment was carried out in "Microbiology Research Laboratory" Department of Pharmacy, Rajshahi University, Bangladesh.

Extraction, isolation and characterization of the compound: For the collection of metabolites, the Streptomyces species was grown optimally in yeast extract glucose broth media at 37.5°C in order to optimum production. The liquid broth was separated through filtration. Then the filtrate was extracted with chloroform and concentrated. By the use of PTLC technique, a pure compound AK_2 was isolated from the chloroform extract and identified as Di (2 ethyl hexyl) phthalate on the basis of its spectral data.



Di-(2 ethyl hexyl)-Phthalate

Collection and maintenance of rats: Long Evan's rats of same age (adult) and sex (male) were collected from the Animal Resources Branch, ICDDR'B, Mohakhali, Dhaka, Bangladesh. They were kept in properly numbered iron cages in a clean animal house and supplied with isocaloric food. The animals are maintained in this way for 15 days before drug administration and continued up to the end of the experiment.

Grouping of the rats: Individual weights of the rats were taken and they were grouped into two, A and B. Group-A received the vehicle only and Group-B received the antibiotic AK_2. Detail is as follows:

Group	No. of rats	Average body weight (g)	Sex	Average age (weeks)	Dose (i.p) rat/day
A	4	145.375	Male	6-8	200 µl (vehicle)
B	4	146.75	Male	6-8	200 (AK_2)

Preparation and administration of AK_2 solution: The antibiotic AK_2 was dissolved in distilled water with the help of tween-20 so that 0.2 ml contained 200 µg of the antibiotic. The compound (AK_2) was administered intraperitoneally to each of the experimental rats of Group-B according to the experimental schedule. Rats of Group-A received vehicle only.

Gross general observation after drug administration: During the whole experimental period their behaviour, central nervous system (CNS) excitation, CNS depression, reflexes, muscular weakness, salivation, diarrhoea and food intake were observed. The body weight of each rat of group A and B were measured before administration of the drug and after the completion of the treatment prior to scarify them.

Haematological profiles of blood: For haematological studies, blood was drawn from the tail veins of all the rats in group A and B before the commencement of drug administration. Then blood smears were made on glass slides and stained with "Leishmen reagent" to perform total count (TC), differential count (DC) and platelet count. With the use of capillary tubes blood was drawn from each of the rat to estimate the hemoglobin percentage by a haemocytometer. This was the pre-haematological study on normal rats. Post haematological studies were done on 7th and 14th days after the commencement of drug administration following the same procedure used for normal rats.

Biochemical parameters of blood: For the study of biochemical parameters, the rats of all the groups were sacrificed with the help of a surgical blade No. 22 on the 14th day of treatment with AK2, and the blood were collected in plastic centrifuge tubes. These were then allowed to clot at 40°C for 4 hour. After clotting, the blood samples were centrifuged at 4000 rpm for 15 minutes using a WIFUNG centrifuge LABO-50M. The clear straw colour serum was then collected in vials with Pasteur pipette and stored at −20°C. Then the enzymes SGOT, SGPT, serum alkaline phosphatase and serum creatinine, uric acid and urea were determined by using the procedures reported by Reitman and Frankel (1957), and Coulombe and Favreau (1963).

Histopathological study of liver, kidney, heart, lungs and spleen: The liver, kidney, heart, lungs and spleen of all of the rats of group A and B were collected after sacrificing them at 14th day of observation. The tissues were sliced into pieces and immersed in 10% formalin for three days, processed, stained with "Harris Hematoxylin and eosin reagent", mounted on glass slides with diphenyl xylene mounting fluid and observed under microscope at the "Bangladesh Sericulture Research Institute", Rajshahi, Bangladesh.

Results and Discussion

Gross general observation: The rats of group A and B showed no signs of tremor, convulsions and reflex abnormalities. No muscular numbness of the hind and forelegs, salivation and diarrhoea was observed. The food intake per day was also to be found normal. The body weights of all the rats (Table 1) were recorded before and after the treatment. In each rat (both control and experimental) body weight was found to be increased. This is because the rats were in the growing stage. Thus body weight increased with time but the group-A showed more growth rate than the group-B indicating that compound AK_2 has got some effect on normal growth of rat.

Haematological profiles: Haematological profiles were studied on normal rats (before treatment) and after 7 and 14 days of treatment. Each time the value of the parameters in each rat was changed slightly. However, the parameters remained within the normal range. The results of hematological profiles are shown (Tables 2 and 3).

Table 1: Effect of the antibiotic AK_2 on body weight of rats after intraperitoneal administration

Group	Dose level µg/rat/day	Body weight (g) before drug treatment $M_1 \pm SD_1$ n= 4	Body weight (g) after drug treatment $M_2 \pm SD_2$	% Change	Calculated t value	Remarks
A (Control)	200 µg of vehicle	145.375± 4.6 (138,145,148,150)	161.375± 1.88 (155,160,159,158.5)	+ 11.00	6.439	NS
B (AK_2)	200 µg of AK_2	146.75± 4.8 (139.5,145.5,150,152)	158.625± 5.06 (150, 160,162.5,162)	+ 8.09	3.405	NS

M_1 and M_2 = Sample mean value, SD_1 and SD_2 = Standard deviations, n = No. of rats
+ = increase, − = decrease, NS = Not significant

Table 2: Hematological profiles of group A rats

Hematological parameters	Normal rats	Rats treated with vehicle	
	1st day	7th day	14th day
Total RBC count (million cu. mm^{-1})	5.02± .0.353	5.30± 0.328	5.3± 0.189
Total WBC count (no cu. mm^{-1})	11480± 519	11620± 216	11660± 445
Differential count of WBC			
a) Neutrophil	41.0± 2.09	40.8± 1.16	39.4± 1.85
b) Lymphocyte	50.8± 1.72	50.0± 1.41	50.0± 2.44
c) Monocyte	3.0± 1.41	3.60± 1.01	3.6± 0.8
d) Eosinophil	5.40± 1.01	5.6± 0.49	6.6± 0.48
Platelet count (no cu. mm^{-1})	261000± 14966	300000± 14142	321000± 20099
Haemoglobin (%)	91.6± 2.89	91.2± 3.24	88.6± 4.22

Table 3: Hematological profiles of group B rats

Hematological parameters	Normal rats	Rats treated with AK_2	
	1st day	7th day	14th day
Total RBC count (million cu. mm^{-1})	5.1± 0.26	4.96± 0.403	5.14± 0.241
Total WBC count (no cu. mm^{-1})	12420± 457	12020± 667	12200± 400
Differential count of WBC			
a. Neutrophil	40.8± 2.13	41.2± 1.72	40.8± 1.16
b. Lymphocyte	52.2± 1.72	51.2± 2.31	51.2± 2.31
c. Monocyte	2.8± 0.74	3.6 ± 0.48	3.4± 0.8
d. Eosinophil	6.0± 0.89	6.0± 0.89	6.0± 0.63
Platelet count (no cu. mm^{-1})	305000± 138002	295000± 31622	307000± 22715
Haemoglobin(%)	89.6± 1.85	90.8± 2.48	88± 2.82

RBC= Red blood cells, WBC= White blood cells

Table 4: Effect of antibiotic (AK1) on biochemical parameters of rats blood after i.p. administration of 200 µg/rat/day for 14 consecutive days

Bio-chemical parameters	Group A n= 5 $M_1 \pm SD_1$	Group B n= 5 $M_2 \pm SD_2$	% change	t-value	t-value at 5% level of significance
SGOT (IU L^{-1})	44.6± 1.35	44.6± 1.8	0.00	0.00	
SGPT (IU L^{-1})	31.0± 1.78	32.6± 1.74	+ 5.16	1.437 (NS)	
SALP (IU L^{-1})	40.8± 2.03	42.8± 2.78	+ 4.90	1.299 (NS)	
Serum uric acid (mg dl^{-1})	6.8± 0.45	6.94± 0.40	+ 1.76	0.422 (NS)	2.306
Urea (mg dl^{-1})	54.0± 2.19	44.0± 2.82	-18.00	6.26 (S)	
Serum creatinine (mg dl^{-1})	1.4± 0.14	1.4± 0.08	0.00	0.00 (NS)	

SGOT= Serum glutamate oxaloacetate transaminase, SGPT = Serum glutamate pyruvate transaminase, SALP= Serum alkaline phosphatase, M_1 and M_2 = Sample mean value, SD_1 and SD_2 = Standard deviations, n = Number of rats, + = Increase, NS = Not Significant, Group A= Control rats, Group B= Experimental rats, t values at 5% level of significance 2.447

Table 5: Histopathological studies after treatment with antibiotic AK_2 at a dose level of 200 µg/rat/day for 21 consecutive days

Groups	Dose (µg/rat/day)	Body weight (g)	Histopathological changes observed			
			Liver	Kidney	Spleen	Heart
A	200 (H_2O)	150.4± 1.624 (150, 153, 148, 150, 151)	NDA	NDA	NDA	NDA
B	200 (AK_2)	151.6± 1.01 (152, 151, 153, 150 152)	NDA	NDA	NDA	NDA

NDA: No detectable abnormality

Biochemical parameters: Biochemical parameters were studied in normal rats (before treatment) and after 7 and 14 days of treatment (Table 4). The serum urea concentration was found to be decreased after AK_2 administration. The changes in other parameters were within the range and statistically insignificant. The reason of low urea concentration might be one of the followings: over hydration, inappropriate secretion of antidiuretic hormone, severe liver disease.

Histopathological studies: Histopathological studies of kidney, liver, heart, lung and spleen of the control and experimental rats were carried out after i.p. administration of the compound AK_2 for 14 days at a dose 200 µg/rat/day (Table 5). No detectable abnormality in the histopathological point of view was observed between the control and the drug treated rats when the tissue slides were examined under microscope.

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