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Toxicity Studies on 4-Chloro-5-Sulfamoylanthranilic Acid the Degradation Product of a Loop Diuretic Furosemide

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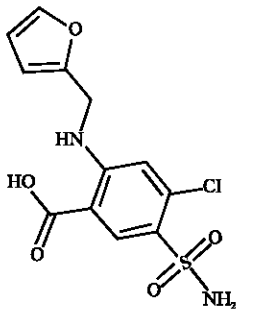
Abstract: Concerns about quality of a drug product and its maintenance during the shelf life is voiced by many health authorities. Usually the degradation products of a drug lead to toxicity and/or unexpected clinical results. 4-Chloro-5-Sulfamoylanthranilic Acid (CSA) is the major degradation product of the well established loop diuretic Furosemide (FM). Acute, subacute and chronic toxicity studies were conducted on CSA and the parent drug FM for comparison using mice as experimental model. General toxicity symptoms, body weight changes, effect on vital organs, hematological and biochemical changes and effects on the bone marrow cells were recorded following established experimental protocols. In each case, the results obtained were sustanciated by histopathological investigations. During acute treatment, FM 100 mg kg^{-1} dose significantly increased the levels of AST, creatinine and urea, while CSA treatment caused increase only in AST and creatinine levels. FM treatment induced clastogenic effect $p<0.05$ on the femoral cells of mice. In subacute study, both CSA and FM treatment significantly increased ($p<0.001$) AST and ALT levels. In addition, CSA treatment significantly reduced glucose levels as well. There were no appreciable changes in hematological indices during acute treatment. However, subacute and chronic treatment with CSA higher dose, caused significant changes in hematological and biochemical indices. No clastogenic activity was observed during subacute and chronic toxicity studies. Mortality rate was found higher in FM higher dose treatment group in both subacute and chronic toxicity studies. Histopathological investigations after acute treatment revealed inflammatory change and congestion in liver of mice; while only mild congestion in heart and kidney were also noticed. Histopathological studies on CSA treated mice after subacute and chronic treatment induced interesting changes. The over all results of current study shown CSA to possess toxic potential.

Key words: 4-chloro-5-sulfamoylanthranilic acid, furosemide, acute toxicity, subacute toxicity, chronic toxicity, Swiss albino mice

INTRODUCTION

Concern about the quality of drugs and their maintenance during the shelf life, has been voiced by many health authorities because of their complex chemical and physical stability kinetics (Harnischfeger, 1985; WHO, 2004; Majed *et al*., 2005). Loss of drug through a chemical reaction results in a fall in potency. Loss of potency indicates 'poor product quality'. Based on their chemical structures, several drugs degraded into toxic substances due to improper storage and handling (Khalil *et al*., 1993; Kopp, 2006). Therefore, it is not enough to study how much drug was lost during storage but also to investigate what are its degradation products and their toxicity (Qureshi *et al*., 1993; Yoshioka and Stella, 2002; Isidori *et al*., 2006; Williams *et al*., 2007).



Chemical formula; $C_{12}H_{11}ClN_2O_5S$

Furosemide (4-chloro-N-furfuryl-S-sulphamoyl-anthranilic acid), $C_{12}H_{11}ClN_2O_5S$, is a potent diuretic which acts primarily by inhibiting electrolyte absorption in the loop of Henle. It may be effective in patients who do not respond to Thiazide diuretics, including those with

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impaired renal function and patients of cardiorenal syndrome (Jerie, 2007; Kociol *et al*., 2009). Frusemide (FM) is also, used for the treatment of edema associated with congestive heart failure, pulmonary disorder and nephrotic syndrome (Brater, 1983; Snopek *et al*., 2008; Kapur *et al*., 2009; Kociol *et al*., 2009). It can produce adverse effects on fluid and electrolyte balance leading to hyponatremia, hypokalemia and hypochloremic alkalosis (Reynolds and Parfitt, 1989; Fadel *et al*., 2009). However, to reduce the incidence of chronic lung disease in extremely low birth weight infants, an effective response to acute FM challenge was observed. There was an increase in urine volume, urinary electrolytes and urinary aldosterone excretion without any significant change in creatinine clearance (Chemtob *et al*., 1989; Costta *et al*., 2009).

FM is susceptible to acid-catalyzed hydrolysis to yield the hydrolytic product: 4-Chloro-5-Sulfamoylanthranilic Acid (CSA). The hydrolysis rate is influence by adverse storage conditions and pH of the medium (Cruz *et al*., 1979). The USP Monograph of FM contains limits for the level of the degradation product CSA, indicating its possible toxic potential. In view of the hazards of the degradation products formed during drug storage and distribution, there is a growing need to evaluate toxicity of such products and their comparison to the parent drug.

Keeping in view the possible toxic effects of the degradation products, CSA was subjected to acute, subacute and chronic toxicity studies in mice following standard toxicity protocols and the results are presented in the current communication.

MATERIALS AND METHODS

Animal stock: Toxicity studies were conducted in male Swiss Albino mice (SWR), aged 6-7 weeks and weighing 22-26 g. The animals were bred and maintained at the Animal House, Central Laboratory for Drug and Food Analysis, Ministry of Health, Riyadh, Saudi Arabia under standard conditions of humidity, temperature and light (12 h dark/12 h light). The animals were fed with purina chow diet and had free access to water. The standard official protocol for animal experiments was followed. Unless, otherwise specified the following parameters were studied in acute, subacute and chronic toxicity evaluation (Robin *et al*., 1982; Hayes, 2001; Al-Ashban *et al*., 2005).

General toxicity symptoms: The general toxic symptoms were observed within 24 h for acute and subacute studies while these observations were continued for 2 weeks for chronic study. The observations were allowed sufficient time for the onset of signs, recovery and the time to death. The different toxic symptoms observed were autonomic responses, motor activity and CNS excitation, etc. (Chan *et al*., 1982; Hayes, 2001).

Incidence of mortality: In subacute and chronic toxicity the animals were observed for mortality daily and at the end of the treatment to analyze the impact of the treatment.

Body weight: The body weights of individual animals were taken before the beginning of the treatment and at the end of the treatment. The difference was computed statistically to examine the impact of the degradation product on body weight of animals.

Organ weight: At the end of the treatment the vital organs including heart, lungs, kidney, spleen, liver and testes were weighed and computed on a per 100 g body weight basis.

Hematology: The blood was analyzed on a coulter counter for the quantification of different hematological indices including White Blood Cells (WBC), Red Blood Cells (RBC), Hemoglobin (Hb), Haematocrit (HCT) and Mean Cell Volume (MCV).

Biochemical indices: The blood was collected, serum samples were separated, stored at -20°C and Analyzed for Alanine Aminotransferase (ALT/GPT), Aspartate Aminotransferase (AST/GOT), isoenzyme MB of Creatine Kinase (CK-MB), creatinine, urea and glucose. The parameters were analyzed by enzymatic colorimetric method using test combination reagents (Boehringer Mannheim GmbH, Diagnostic, Germany). The measurements were carried out in a spectrophotometer Ultrospec II (LKB).

Genotoxic studies: The adhering soft tissues and epiphyses of both the tibiae were removed. The marrow was aspirated from each femur in fetal calf serum and transferred to centrifuge tubes. After centrifugation at 1000 rpm for 5 min, the supernatant was discarded and the residual cells were spread on slides and air dried. The slides were fixed in methanol, stained in May-Gruenwald solution followed by Giemsa stain. The coded slides were screened for the presence of micronuclei in polychromatic erythrocytes, which indicated non-disjunction, chromosomal breaks and structural or numerical changes in the chromosomes. The bone marrow depression (mitotic index) was evaluated on the basis of the ratio of Polychromatic to Normochromatic

Erythrocytes (PCE/NCE ratio) as described earlier (Al-Harbi *et al*., 1994). Adriamycin was used as a standard cytotoxic drug for reference (Zeiger, 1998; BEST, 2006).

Histopathological procedures: Tissue samples of liver, heart and kidney were preserved in 10% buffered formalin and processed for routine paraffin block preparation. Using an American Optical Rotary Microtome, sections of thickness about 5 μm were cut and stained with haematoxylon and eosin. These were examined under the microscope for the following histopathological changes (Al-Ashban *et al*., 2005; Ibrahim *et al*., 2009).

Liver: Abnormal portal spaces, abnormal duplication of Kupffer cells, changes in number of binuclear cells, abnormal medullary erythropoiesis, presence of megakaryocyte, mitotic division, lymphocytic infiltration, necrosis, congestion and edema.

Heart: Inflammatory changes, lymphocytosis, necrosis and congestion.

Kidney: Abnormal peripheral fats, abnormal cellularity, abnormal glomeruli, abnormal cortex, abnormal medulla, congestion and necrosis.

In chronic toxicity studies in addition, lungs and spleen were also, investigated for histopathological changes.

Statistical analysis: For the evaluation of results obtained during acute, subacute and chronic toxicity studies χ^2-test and Student's t-test were used to assess the significance of the values obtained in the treatment groups as compared to controls.

Acute toxicity evaluation

Dose selection and mode of administration: The dose levels selected were in the range of those already investigated (Nakahama *et al*., 1987). One tenth of the dose determined for the parent drug FM, was selected as a dose for the degradation product CSA. Both, the parent drug FM and the degradation product CSA were suspended separately and homogeneously in 1% Carboxymethyl Cellulose solution (CMC) and administered intra peritoneally (i.p.) in a single acute dose. The experimental mice were randomly allotted to different groups as follows:

- Control (CMC, 1%, i.p.)
- FM (100 mg kg^{-1}, i.p.)
- CSA (10 mg kg^{-1}, i.p.)

In each case animals were scarified 30 h after the treatment and used for the conduct of different experiments (Chan and Hayes, 2007).

Subacute toxicity evaluation: The study on subacute treatment involved repeated dose exposures for a short term. It is recommended to provide information on cumulative effects, latency period for development of toxicity, reversibility of toxicity and dose response relationship (Chan *et al*., 1982; Zeiger, 1998; BEST, 2006). The purpose of this investigation was to evaluate the effects of short term treatment of the degradation product CSA, as well as to determine the maximum tolerable dose and the nature of toxic reactions in order to design suitable experiments for chronic toxicity. The parameters included in this study were based on standard toxicological screening programs and included screening on general toxicity symptoms, mortality, body weight and organ weight changes, hematology, biochemistry, genotoxicity and histopathological investigations (Chan *et al*., 1982; Robin *et al*., 1982; Al-Ashban *et al*., 2005; Ibrahim *et al*., 2009).

Dose selection and mode of administration: The subacute treatment included two doses in order to evaluate the dose-response relationship. The lowest dose selected was that used in the acute treatment study. The second dose was double of the dose used for acute treatment study. The dose selected for the degradation product CSA, was one tenth of the dose used for the parent drug FM. In each case the test compound was homogeneously suspended in 1% CMC separately and administered (i.p.) daily for 7 days. All parameters were studied and compared with the respective controls. The experimental groups were as follows:

- Control (CMC, 1%, i.p.)
- FM (100 mg/kg/day, i.p.)
- FM (200 mg/kg/day, i.p.)
- CSA (10 mg/kg/day, i.p.)
- CSA (20 mg/kg/day, i.p.)

The subacute treatment was continued for 7 days. In each case group of animals were scarified 30 h after the last treatment and used for the conduct of different experiments mentioned earlier (Ibrahim *et al*., 2009).

Chronic toxicity evaluation: In chronic toxicity evaluation mice were treated with the parent drug FM and its degradation product CSA for 90 days which was suggested to be sufficient in the shorten lived rodents in order to predict the hazard of long term low dose exposure

(Mosberg and Hayes, 1989; Shah *et al*., 1998). The purpose of the study was to evaluate the effects of prolonged treatment on the target organs and the physiological and metabolic tolerance of the compounds at low doses. All the standard prolonged toxicity parameters were included (Chan *et al*., 1982; Robin *et al*., 1982; Mosberg and Hayes, 1989; BEST, 2006) and the findings were substantiated by histopathological investigations (Ibrahim *et al*., 2009).

Dose selection and mode of administration: The regulatory guidelines on selection of dose for long term treatment require minimal toxicity to allow meaningful evaluation of the data (Mosberg and Hayes, 1989). The doses selected for FM was in the range of human therapeutic dose (Mosberg and Hayes, 1989; Hayes, 2001). Accordingly, the doses were calculated for 7 days a week and then administered on 2 days a week basis. The dose of CSA was one tenth of the dose of the parent drug FM. Both the low and high doses calculated for FM and CSA were suspended in 0.01% CMC and administered intraperitoneally (i.p.). Adriamycin was used as a standard cytotoxic drug for comparison.

The experimental groups and treatment during chronic toxicity studies were as follows:

- Control (CMC, 0.01%, 1 mg kg^{-1}, i.p.)
- FM (1.5 mg kg^{-1}, i.p.)
- FM (3.0 mg kg^{-1}, i.p.)
- CSA (0.15 mg kg^{-1}, i.p.)
- CSA (0.30 mg kg^{-1}, i.p.)

During chronic toxicity studies, the general behaviour of the animals was observed daily for the first 2 weeks of the treatment. The body weight of the individual animal was recorded weekly throughout the study. The animals were observed for mortality daily till the end of the treatment.

RESULTS AND DISCUSSION

Acute toxicity: The results of current acute toxicity study are presented in Table 1-6. During acute toxicity studies, general signs of toxicity such as: autonomic responses, motor activities and effects on central nervous system, etc. were recorded (Table 1). FM acute treatment (100 mg kg^{-1}, i.p.) induced pilo erection, micturition, writhing, deification, Waltzing movements and sedation in animals. These results are in agreement with the earlier reported weakness, dry mouth, thirst, dizziness, confusion, restlessness after FM use (Amdur *et al*., 1991).

Table 1: Effect of FM and its degradation product CSA on general toxicity symptoms observed within 24 h of acute treatment in Swiss Albino Mice

	Treatment and dose		
Toxic symptoms under observation	Control (CMC, 1%)	FM (100)	CSA (10)
Autonomic responses			
Respiration	-	-	-
Hypothermia	-	-	-
Hyperthermia	-	-	-
Pilo erection	-	+	+
Salvation	-	-	-
Micturition	-	+	-
Writhing	-	++	-
Defection	-	+	+
Motor activity			
Staggering	-	-	-
Rightening reflex	-	-	-
CNS activity			
Straub tail	-	-	-
Tremors	-	-	-
Convulsions	-	-	-
Aggression	-	-	-
Twitches	-	-	-
Excitation/hyperactive	-	-	-
Itching	-	-	-
Waltzing movements	-	+	+
Other symptoms			
Drowsy/calm/sedation	-	+	-
Momentary limbs paralysis	-	+	-
Blood accumulation (Ear veins)	-	-	-

-: Absent; +: Present; ++: Severe effect

Table 2: Effect of FM and its degradation product CSA on mortality induced in Swiss Albino mice after acute treatment

Treatment and dose (mg/kg/day) (i.p.)	Initial no. of animals	No. of animals dead	Mortality (%)
Control (CMC, 1%, i.p.)	10	1	8.33
FM (100)	10	1	8.33
CSA (10)	10	0	0.00

Statistically non-significant (Chi-square test); FM: Furosemide, CSA: 4-Chloro-5-Sulfamoylanthranilic acid

Acute treatment with the degradation product CSA (10 mg kg^{-1}, i.p.) shown pilo erection, deification, micturition and Waltzing movements in the treated animals. These results indicated that the parent compound FM is relatively more toxic as compared to its degradation product CSA. However, no significant mortality occurred both in the FM and CSA treatment groups as compared to the control (Table 2).

The analysis of different biochemical indices after acute treatment with FM (Table 3), revealed a significant increase ($p<0.05$) in the serum creatinine and urea levels, which shown nephrotoxic potential of FM (Asaeda *et al*., 1984; Hardman *et al*., 2001; Hsu *et al*., 2005; Metra *et al*., 2008). The results of current study are in agreement with earlier studies (Eshaghian *et al*., 2006) where higher dosages of loop diuretics, were associated with increased serum blood urea nitrogen and elevated creatinine levels, which could help identifying patients with heart failure and high risk for mortality (Chemtob *et al*., 1989;

Amdur *et al*., 1991; Eshaghian *et al*., 2006). In a clinical setting, high free water intake and low serum urea and uric acid favours acute hyponatremia, which is an emergency situation. Therefore, for differential diagnosis of acute hyponatremia, a detailed history of the patient was considered essential (Hsu *et al*., 2005).

During current acute toxicity study, FM acute treatment in mice, elevated the blood levels of AST/GOT and ALT/GPT indicating some hepatotoxic activity of FM, however, these values were statistically significant only for AST levels (Liu *et al*., 1995). In the group of mice treated with CSA, there was a significant increase ($p<0.05$) in the levels of AST and ($p<0.01$) creatinine. These findings indicated possible hepatotoxic potential of FM-treatment in mice observed in the current experiment. the findings were substantiated by an earlier report showing FM treatment to induce hepatic necrosis in mice (Williams *et al*., 2007). The increase in creatinine levels by FM treatment is in agreement with the reports on nephrotoxicity of furosemide (NTP, 1989; Hori *et al*., 2007). There were no significant hematological changes observed in FM as well as in CSA treatment groups as compared to the control (Table 4). The histopathological investigations confirmed congestion in the liver and heart of FM treatment group as compared to the control (Table 5).

In CSA treatment group, there were inflammatory changes and congestion in the liver after acute treatment as compared to the control. Furthermore, histopathological studies also revealed congestion in kidneys of the CSA-treated animals (Table 5). FM acute treatment induced statistically significant $p<0.05$ cytotoxicity in the femoral cells of mice (Table 6). These results are in agreement with the results of earlier studies on the cytological effects and carcinogenic activity of FM in mice, which needs further confirmation (Subramanyam and Jameela, 1977; Jameela *et al*., 1979; NTP, 1989).

Based on some reports in the scientific literature, FM was demonstrated to possess antioxidative properties (Lebedev and Petrenko, 1996; Vargas *et al*., 1998). FM is also used to control hypertension and reduce the swelling and fluid retention caused by various medical problems, including heart or liver disease (Chemtob *et al*., 1989; Snopek *et al*., 2008; Kapur *et al*., 2009; Kociol *et al*., 2009). It causes the kidneys to get rid of un-needed water and salt from the body into the urine (Costta *et al*., 2009; Fadel *et al*., 2009). Keeping view the results obtained during present acute toxicity study (Table 1-6) and existence of limited toxicological data on CSA and FM in scientific literature, it was considered essential to conduct subacute and chronic toxicity studies to evaluate the toxic potential of the degradation product CSA as compared to the parent drug.

Subacute toxicity studies: The subacute toxicity studies on CSA and FM were conducted according to the protocol described earlier. FM and CSA subacute treatment results provided information on cumulative effects, latency period for development of toxicity, reversibility of toxicity and dose response relationship (Chan *et al*., 1982; BEST, 2006). The results of the present Subacute treatment in mice (Table 7-16) demonstrated that FM treatment (100 and 200 mg kg^{-1}) caused a dose dependent increase in writhing, pilo erection, micturition, defection; Waltzing movements and partial paralysis in animals within 24 h post-treatment (Table 7). These results are in agreement with the earlier reported side effects of FM-treatment causing weakness, dry mouth, dizziness and restlessness (Amdur *et al*., 1991).

The FM-degradation product CSA, was found to cause pilo erection, defection and Waltzing movements. The results on the mortality after subacute treatment (Table 8), FM treatment induced statistically significant ($p<0.01$) lethality. Furthermore, the pre-treatment and post-treatment body weight changes in FM treated groups as well as CSA treatment groups were statistically non-significant (Table 9). These results clearly indicated

Table 3: Effect of FM and its degradation product CSA) on the biochemical parameters in Swiss Albino mice after acute treatment

	Treatment and dose		
Parameters	Control CMC (1%)	FM (100)	CSA (10)
AST/GOT (u L^{-1})	15.96±0.810	17.45±0.800*	18.95±2.2100*
ALT/GPT (u L^{-1})	10.19±1.180	13.79±2.890	12.86±1.8400
CK-MB (u L^{-1})	150.13±14.14	146.19±16.01	140.63±14.520
Creatinine (µmol L^{-1})	147.60±2.030	195.19±4.910**	220.51±3.9600**
Urea (mmol L^{-1})	5.84±0.510	10.63±0.490*	6.01±0.5600
Glucose (mmol L^{-1})	5.75±0.150	6.13±0.190	5.62±0.0900

*p<0.05; **p<0.01 (students t-test); Treatment groups were compared with the control group; Five animals were used in each group; FM: Furosemide, CSA: 4-Chloro-5-Sulfamoylanthranilic acid

Table 4: Effect of FM and its degradation product CSA on hematological indices of Swiss albino mice after acute treatment

	Hematological indices				
Treatment/dose (mg/kg/day) (i.p.)	WBC×10^9/L	RBC×10^{12}/L	Hb. (g dL^{-1})	HCT % ratio	MCV fl
Control (CMC, 1%)	5.24±0.69	5.55±0.61	10.73±0.88	28.38±2.77	49.86±0.79
FM (100)	4.57±1.42	5.48±0.40	10.36±0.72	26.75±1.78	50.68±0.87
CSA (10)	4.19±0.84	5.70±0.37	10.27±0.57	28.22±1.96	49.65±0.67

p>0.05 (students' t-test); Five mice were used in each group; FM: Furosemide; CSA: 4-Chloro-5-Sulfamoylanthranilic acid

that FM was more toxic as compared to its degradation product (CSA) in the given dose levels. The weight of vital organs were not affected except the weight increase of liver after subacute treatment with higher dose of FM (Table 10). It is worth mentioning that in higher dose, FM treatment might have caused hepatic necrosis in animals due to its metabolic bioactivation to a chemically reactive metabolite that binds to hepatic proteins (Williams *et al*., 2007).

The differences in the effects of FM and CSA treatments and lack of their metabolic relation is supported by the hematological indices (Table 11). FM treatment was generally associated with reduced hemoglobin levels and elevated creatinine and urea levels. Long-term administration of FM was also reported to increase the BUN and plasma creatinine (Hori *et al*., 2007). The results obtained during the current subacute experiment, shown a non-significant decrease in hemoglobin levels and an increasing trend in creatinine and urea levels after subacute treatment. However, all these changes were statistically insignificant. On the other side, CSA high dose treatment was found to reduce significantly ($p<0.05$) the levels of WBC, RBC, Hb and HCT. These findings are supported by earlier researchers reporting that the use of loop diuretics might be

Table 5: Effect of FM and its degradation product CSA on the histopathological changes in Swiss Albino mice after acute treatment

Organs studied	FM (100)	CSA (10)
Liver		
Inflammatory changes	-	+
Fatty changes	-	-
Necrosis	-	-
Congestion	+	+
Heart		
Inflammatory changes	-	-
Necrosis	-	-
Congestion	+	-
Kidney		
Inflammatory changes	-	-
Necrosis	-	-
Congestion	-	+

-Normal; +Little effect; ++Appreciable effect

Table 6: Clastogenic effect of FM and its degradation product CSA on the femoral cells of Swiss Albino mice after acute treatment (Mean±SE)

Treatment and dose (mg kg^{-1}), i.p.	Polychromatic Erythrocytes (PCE) screened	Micro nucleated polychromatic erythrocytes (%)	Normochromatic Erythrocytes (NCE) screened	PCE/NCE ratio
Control (CMC, 1%)	5475	0.31±0.03	5341	1.02±0.03
FM (100)	4697	0.41±0.07	5100	0.92±0.02*
CSA (10)	4819	0.35±0.02	5000	0.96±0.02
Adriamycin (8)	5350	6.18±0.38**	5547	0.68±0.07*

*$p<0.05$; **$p<0.01$ (Student's t-test); Five mice were used in each group; Each group was compared with group 1 (Control); FM: Furosemide; CSA: 4-Chloro-5-Sulfamoylanthranilic Acid

Table 7: Effect of FM and its degradation product CSA on general toxicity symptoms observed within 24 h after subacute treatment in Swiss Albino mice

Toxic symptoms under observation	Treatment/dose (mg/kg/day)				
	Control	FM (100)	FM (200)	CSA (10)	CSA (20)
Autonomic responses					
Respiration	-	-	-	-	-
Hypothermia	-	-	-	-	-
Hyperthermia	-	-	-	-	-
Pilo erection	-	+	++	+	+
Salvation	-	-	-	-	-
Micturition	-	+	+	-	-
Writhing	-	+	++	-	-
Defecation	-	+	++	+	+
Motor activity					
Staggering	-	-	-	-	-
Rightening reflex	-	-	-	-	-
CNS activity					
Straub tail	-	-	-	-	-
Tremors	-	-	-	-	-
Convulsions	-	-	-	-	-
Aggression	-	-	-	-	-
Twitches	-	-	-	-	-
Excitation/hyperactive	-	-	-	-	-
Itching	-	-	-	-	-
Waltzing movements	-	+	+	+	-
Other symptoms					
Drowsy/calm/sedation	-	+	+	-	-
Momentary limbs paralysis	-	+	+	-	-
Blood accumulation (Ear veins)	-	-	-	-	-

-; Absent; +: Present; ++: Severe effect; FM: Furosemide; CSA: 4-Chloro-5-Sulfamoylanthranilic acid

associated with decrease in hemoglobin levels and increase in blood urea nitrogen and creatinine levels (Eshaghian *et al*., 2006; Hori *et al*., 2007). Current findings are supported by an earlier observation that only prolonged furosemide use, could lead to severe nephropathy in experimental animals (NTP, 1989).

The data of the current study indicated, the degradation product CSA to be relatively more toxic then the parent drug FM, as shown by the hematological parameters. There is no study report available on the subacute toxicity evaluation of CSA in the scientific literature for comparison. However, these results might by justified by taking into consideration the possible toxic potential of anthranilic acid moiety present in the structure of CSA and also, by the results of an earlier experiment (Black, 1986).

The biochemical analysis after FM treatment shown a significant increase in AST and ALT concentration in the serum (Table 12) thus, confirming the results obtained during the present acute toxicity experiments. It is worth mentioning that the raise in urea and creatinine levels was statistically non-significant. The histopathological investigations on liver shown extramedullary erythropoiesis (Table 13). On the other side, CSA subcute treatment was found to significantly ($p<0.001$) increase the concentrations of AST and ALT in the serum. The increase in liver enzymes may be attributed to the known hepatotoxic effects of FM which caused hepatic necrosis in mice (Astashkin *et al*., 1988; Williams *et al*., 2007). These toxic effects appeared to be caused by known prostaglandin suppression activity of the drug and may be attributed to the anthranilic acid moiety in the structure of CSA (Black, 1986). However, histopathological examination of vital organs including liver, during the current study, shown the presence of duplicated Kupffer cells and binucliated cells in liver of the treated animals. The presence of binucleated cells clearly demonstrated a process of repair following the toxic implications of CSA.

Both FM as well as CSA treatment did not induce any significant effects on the serum concentrations of CK-MB, creatinine and urea. These results are supported by the histopathological studies (Table 14 and 15) where, no drastic changes in the cardiac and renal tissue were observed. The results of the present subacute experiment showing no effect on creatinine levels are not in full agreement with the results obtained during current acute toxicity study. FM was earlier reported to possess significant nephrotoxicity (Asaeda *et al*., 1984; NTP, 1989;

Table 8: Effect of FM and its degradation product CSA on mortality induced in Swiss Albino mice after subacute treatment

Treatment and dose (mg/kg/day), i.p.	Initial No. of animals	No. of animals dead	Mortality (%)
Control (CMC, 1%, i.p.)	12	1	8.33
FM (100)	12	1	8.33
FM (200)	12	5	41.67*
CSA(10)	12	0	0.00
CSA (20)	12	2	16.67

*p<0.05; **p<0.01 (Chi-square test); FM: Furosemide; CSA: 4-Chloro-5-Sulfamoylanthranilic acid

Table 9: Effect of FM and its degradation product CSA on weight gain induced in Swiss Albino mice after subacute treatment (mean±SE)

Treatment and dose (mg/kg/day) (i.p.)	Initial No. of mice	Body weight	
		Pre-treatment	Post-treatment
Control (CMC, 0.01%)	14	24.7±1.2	26.6±1.4
FM (1.5)	13	25.9±1.4	26.8±1.5
FM (3.0)	14	25.2±1.1	26.5±1.9
CSA (0.15)	13	24.9±1.3	27.2±1.8
CSA (0.3)	14	26.2±1.5	27.4±1.5

p>0.05 (statistically not significant) (student's t-test); Post-treatment body weight of the surviving mice was statistically compared with the pre-treament body weight; FM: Furosemide; CSA: 4-Chloro-5-Sulfamoylanthranilic acid

Table 10: Effect of FM and its degradation product CSA on the organ weight of Swiss Albino mice after subacute treatment

Treatment/dose (mg/kg/day) i.p.	Mean weight/100 g body weight±SE					
	Heart	Lungs	Liver	Kidney	Spleen	Testes
Control (CMC, 1%)	0.57±0.02	0.89±0.06	6.76±0.21	1.47±0.04	0.63±0.08	0.64±0.05
FM (100)	0.51±0.03	0.85±0.06	6.68±0.19	1.52±0.05	0.78±0.09	0.58±0.05
FM (200)	0.53±0.02	0.87±0.08	7.41±0.15*	1.44±0.07	0.64±0.03	0.72±0.05
CSA (10)	0.58±0.02	0.80±0.02	6.62±0.38	1.50±0.06	0.61±0.11	0.74±0.04
CSA (20)	0.55±0.01	0.75±0.07	6.67±0.17	1.45±0.01	0.69±0.07	0.60±0.02

*p<0.05 (students' t-test); FM: Furosemide; CSA: 4-Chloro-5-Sulfamoylanthranilic acid

Table 11: Effect of FM and its degradation product CSA on hematological indices of Swiss Albino mice after subacute treatment

Treatment/dose (mg/kg/day), i.p.	Hematological indices				
	WBC×10^9/L	RBC×10^{12}/L	Hb (g dL^{-1})	HCT ratio (%)	MCV fl
Control (CMC, 1%)	5.00±0.75	5.52±0.47	10.62±0.88	27.80±2.90	50.94±0.87
FM (100)	4.77±1.31	5.41±0.38	10.26±0.85	27.80±2.00	52.32±0.88
FM (200)	4.30±0.17	5.19±0.63	9.87±0.37	25.20±3.10	49.92±0.28
CSA (10)	3.98±0.48	5.66±0.30	10.64±0.53	28.40±1.60	51.32±0.72
CSA (20)	3.25±0.19*	4.25±0.23*	8.17±0.54*	20.00±1.50*	50.77±0.57

*p<0.05 (students' t-test); Five mice were used in each group; FM: Furosemide; CSA: 4-Chloro-5-Sulfamoylanthranilic acid

Table 12: Effect of FM and its degradation product CSA) on the biochemical parameters in Swiss Albino mice after subacute treatment (Mean±SE)

	Treatment and dose (mg/kg/day)				
Parameters	Control (CMC, 1%)	FM (100)	FM (200)	CSA (10)	CSA (20)
AST/GOT (u L^{-1})	14.50±00.83	29.80±2.550***	26.80±2.240**	23.80±1.650***	23.80±1.590***
ALT/GPT (u L^{-1})	10.12±01.20	19.28±1.800***	24.72±1.920***	21.98±1.260***	25.18±0.900***
CK-MB (u L^{-1})	165.60±15.95	218.40±19.44	259.44±42.43	154.20±11.69	231.60±26.12
Creatinine (µmol L^{-1})	145.80±03.84	141.30±4.240	156.70±4.430	140.09±1.860	159.09±6.210
Urea (mmol L^{-1})	5.70±00.42	5.75±0.170	4.44±0.510	5.77±0.430	5.34±0.250
Glucose (mmol L^{-1})	5.64±00.11	5.89±0.070	5.35±0.120	5.25±0.060*	4.98±0.080**

*p<0.05; **p<0.01; ***p<0.001 (Students' t-test); Treatment groups were compared with the individual control group; Five animals were used in each group; FM: Furosemide; CSA: 4-Chloro-5-Sulfamoylanthranilic acid

Table 13: Effect of FM and its degradation product CSA on the histopathological changes in Liver of Swiss Albino mice after subacute treatment

Organs studied liver	Control (CMC, 1%)	FM (100 mg kg^{-1})	FM (200 mg kg^{-1})	CSA (10 mg kg^{-1})	CSA (20 mg kg^{-1})
Fatty changes	-	-	-	-	-
Abnormal portal space	-	-	-	-	-
Kupffer cells (abnormal duplication)	-	-	+	+	++
Change in number of binucleated cells	-	-	+	+	++
Abnormal medullary erythropoiesis	-	+	+	-	-
Presence of megakaryocyte	-	-	-	-	-
Mitotic division	-	-	-	-	-
Inflammatory changes	-	-	+	-	-
Lymphocytic infiltration	-	-	+	-	-
Necrosis	-	-	-	-	-
Congestion	-	-	-	-	+

-: Normal; +: Little effect; ++: Appreciable effect; FM: Furosemide; CSA: 4-Chloro-5-sulfamoylanthranilic acid

Table 14: Effect of FM and its degradation product CSA on the histopathological changes in heart of Swiss Albino mice after Sub-Acute treatment

Organs studied liver	Control (CMC)	FM (100 mg kg^{-1})	FM (200 mg kg^{-1})	CSA (10 mg kg^{-1})	CSA (20 mg kg^{-1})
Inflammatory changes	-	-	-	-	-
Lymphocytosis	-	-	-	-	-
Congestion	-	-	-	-	+
Necrosis	-	-	-	-	-

-: Normal; +: Little effect; FM: Furosemide; CSA: 4-Chloro-5-sulfamoylanthranilic acid

Table 15: Effect of FM and its degradation product CSA on the histopathological changes in Kidney of Swiss Albino mice after Sub-Acute treatment.

Organs studied liver	Control (CMC)	FM (100 mg kg^{-1})	FM (200 mg kg^{-1})	CSA (10 mg kg^{-1})	CSA (20 mg kg^{-1})
Abnormal peripheral fats	-	-	-	-	-
Abnormal cellularity	-	-	-	-	-
Abnormal glomeruli	-	-	-	-	++
Abnormal cortex	-	-	-	-	+
Abnormal medulla	-	+	+	-	-
Congestion	-	-	-	-	-
Necrosis	-	-	-	-	-

-Normal; + Little effect; ++Appreciable effect, FM: Furosemide, CSA: 4-Chloro-5-Sulfamoylanthranilic acid

Saways and Robertson, 1992; Hardman *et al*., 2001) and induced a significant increase in creatinine levels. Loop diuretics are commonly used in critically ill patients with acute renal failure, but their effect on clinical outcome remains uncertain (Bagshaw *et al*., 2007). FM is also used in treating congestive heart failure, however, there is a possibility of depletion of electrolytes which must be monitored carefully (Schwinger and Erdmann, 1992).

The discrepancy in the results on the current acute and subacute treatments may be attributed to the possible reversibility and latency of toxic implications during the short term treatment (Chen *et al*., 1982; Al-Harbi *et al*., 1994; BEST, 2006), which may be confirmed in the detailed chronic toxicity experiments. Earlier studies on FM (Sandstrom, 1986; Sandstrom and Schin, 1988) have shown increased hyperglycemic activity in mice within 2 h of treatment with FM, however, the levels of glucose were fully recovered within 24 h. In the current subacute study, there was no effect on the glucose levels of FM treated animals as compared to the control. It is worth mentioning that CSA subacute treatment, was found to reduce serum glucose levels (Table 12), which needs confirmation with the data of chronic toxicity experiment, which is a part of current study.

The subacute treatment with FM was found to increase the frequency of micro nucleated PCE and reduced the PCE/NCE ratio (Table 16). However, all these findings were statistically non-significant, but sufficient enough to support the data obtained during the acute toxicity experiment. These results indicated a weak clastogenic and cytotoxic potential of FM and these findings are in agreement with the earlier reports on the

Table 16: Clastogenic effect of FM and its degradation product CSA on the femoral cells of Swiss Albino mice after subacute treatment

Treatment and dose (mg kg^{-1}, i.p.)	Polychromatic Erythrocytes (PCE) screened	Micro nucleated polychromatic erythrocytes (%)	Normochromatic Erythrocytes (NCE) screened	PCE/NCE ratio
Control (CMC)	5201	0.28±0.03	5143	1.01±0.03
FM (100)	4969	0.33±0.02	5361	0.93±0.05
FM (200)	5112	0.35±0.04	6016	0.88±0.07
CSA (10)	5065	0.29±0.03	4923	1.04±0.06
CSA (20)	5265	0.29±0.06	4696	1.15±0.07
Adriamycin (8)	5216	5.60±0.38**	6664	0.78±0.09*

*p<0.05; **p<0.001 (student's t-test); Five mice were used in each group; Each group was compared with group 1 (control); FM: Furosemide; CSA: 4-Chloro-5-Sulfamoylanthranilic acid

Table 17: Effect of FM and its degradation product CSA on general toxicity symptoms observed within 24 h after chronic treatment in Swiss Albino mice

Toxic symptoms under observation	Treatment/dose (mg/kg/day) Control	FM (1.5)	FM (3.0)	CSA (0.15)	CSA (0.3)
Autonomic responses					
Respiration	-	-	-	-	-
Hypothermia	-	-	-	-	-
Hyperthermia	-	-	-	-	-
Pilo erection	-	++	++	+	++
Salvation	-	-	-	-	-
Micturition	-	+	+	+	+
Writhing	-	+	++	-	-
Defecation	-	+	++	+	+
Motor activity					
Staggering	-	-	-	-	-
Rightening reflex	-	-	-	-	-
CNS activity					
Straub tail	-	-	-	-	-
Tremors	-	-	-	-	-
Convulsions	-	-	-	-	-
Aggression	-	-	-	-	-
Twitches	-	-	-	-	-
Excitation/hyperactive	-	-	-	-	-
Itching	-	-	-	-	-
Waltzing movements	-	+	++		-
Other symptoms					
Drowsy/calm/sedation	-	+	+	-	-
Momentary limbs paralysis	-	+	+	-	-
Blood accumulation (Ear veins)	-	-	-	-	-

-: Absent; +: Present; ++: Severe effect; FM: Furosemide; CSA: 4-Chloro-5-Sulfamoylanthranilic acid

clastogenic and mitodepressive effect of FM (Subramanyam and Jameela, 1977; Jameela *et al*., 1979; Isidori *et al*., 2006). Subacute treatment with CSA could not affect the frequency of micronuclei in PCE and the ratio of PCE/NCE as compared to the control (Table 16). These results confirmed the data obtained during acute toxicity studies. A comparison of the genotoxic effects of CSA with that of FM revealed the parent drug FM to be more toxic than its degration product CSA. In some previous studies, FM induced sister chromatid exchanges and chromosomal aberrations in CHO cells, both in the presence and absence of exogenous metabolic activation (NTP, 1989).

Chronic treatment: The general symptoms of toxicity observed during chronic treatment revealed FM treatment to induce symptoms such as writhing and waltzing movements in dose-dependent manner. While pilo erection, micturition and paralysis of limbs were found to be present in both treatment groups. Calmness and itching were observed only in group of treated with higher doses (Table 17). The chronic treatment with the degradation product CSA, induced pilo erection in a dose dependent manner. Symptoms like defection and micturition were observed at both dose levels. The results on the mortality revealed that both CSA and FM treatment increased the rate of mortality (Chuen and MacFadyen, 2006). However, the mortality rate was statistically significant ($p<0.05$) only in the group of mice treated with higher dose of the parent drug FM (Table 18). These data confirmed the previous observations during subacute toxicity studies and demonstrated that FM is more toxic than its degradation product CSA (Beerman *et al*., 1975; Smith and Benet, 1983; Smith and Orrenius, 1984). FM chronic treatment at both dose levels was found to increase body weight only during first 2 weeks; whereas CSA treatment influenced the body during first week of treatment. However, at the end of chronic treatment the

body weight increase in all the treatment groups was statistically significant ($p<0.01$). There is no explanation in the literature on the observed body weight changes induced by FM and CSA treatments. The weight of the vital organs after chronic treatment with FM and CSA were not affected except the increase ($p<0.05$) in the weight of liver observed only by high dose of FM treatment (Table 19). These results confirmed the data obtained during the subacute toxicity studies done as part of current investigations.

Chronic treatment with CSA and FM failed to induce any significant changes in the levels of different hematological indices analyzed (Table 21). However, HCT was significantly ($p<0.05$) reduced in FM high dose treatment group, as well as in the CSA treatment groups as compared to the control. It is worth mentioning that during the current subacute toxicity studies, CSA treatment was found to be relatively more toxic to these indices analyzed in the blood. The discrepancy observed in these studies appears to be due to possible reversibility of toxicity upon prolonged treatment (Chen *et al*., 1982; Qureshi *et al*., 1993; Shah *et al*., 1998; Williams *et al*., 2007).

Furosemide treatment is known to cause hepatic necrosis in mice (Williams *et al*., 2007). In the current chronic treatment, FM failed to significantly alter serum AST, ALT, CK-MB, creatinine, urea and glucose levels at both doses used (Table 22). Earlier reports suggested FM-treatment to raise creatinine levels, which could not be demonstrated during the current chronic toxicity studies. However, an increase ($p<0.05$) in the serum creatinine levels was observed in CSA higher dose treatment group (Table 22). There is equipoise and need for higher-quality evidence to better characterize the role of loop diuretics in kidney injury (Hori *et al*., 2007; Bagshaw *et al*., 2008). On the other side, in CSA chronic treatment groups, at both dose levels, serum glucose levels were significantly ($p<0.05$) decreased. CSA chronic treatment failed to significantly alter serum levels of AST, ALT, CK-MB and urea at both dose levels (Table 22). Some results of FM treatment could not corroborate well with the finding of the subacute treatment studies (Table 12). The histopathological examination (Table 23) on hepatic tissue shown the presence of abnormal erythropoiesis, megakaryocyte, lymphocytic infiltration, necrosis, congestion, edema and pigmentation of Kupffer cells. The discrepancy in the current chronic toxicity and subacute toxicity results, appears to be due to the gross pathological damage of the hepatic tissue, which might have distorted the membranes to retain the enzymes. The observed hepatotoxicity may be attributed to the already reported prostaglandin-stimulating activity of FM (Brater, 1983; Hardman *et al*., 2001) and to the effects of some of its toxic metabolites (Liu *et al*., 1995; Hardman *et al*., 2001). The results of histopathological investigations shown no drastic changes in lungs and spleen of the chronically treated animals (Luellman *et al*., 2005).

The CSA treatment also did not affect serum concentration of ALT. However, the results on subacute toxicity experiment revealed an increase in the concentration of ALT. This difference in the results of subacute and chronic toxicity studies, may be attributed to the possible reversibility of toxicity upon prolonged treatment (Chen *et al*., 1982; Robin *et al*., 1982; Hardman *et al*., 2001; Williams *et al*., 2007). The increase in binucleated cells in liver, clearly demonstrated a

Table 18: Effect of FM and its degradation product CSA on mortality induced in Swiss Albino mice after Chronic treatment

Treatment and dose (mg/kg/day) (i.p.)	Initial no. of animals	No. of animals dead	Mortality (%)
Control (CMC, 0.01%)	14	2	14.28
FM (1.5)	13	3	23.08
FM (3.0)	14	6	42.86*
CSA (0.15)	13	4	28.57
CSA (0.3)	14	4	30.77

*$p<0.05$ (χ^2-test); FM: Furosemide, CSA: 4-Chloro-5-Sulfamoylanthranilic Acid

Table 19: Effect of FM and its degradation product CSA on weight gain induced in Swiss Albino mice after Chronic treatment (mean±SE)

Treatment and dose (mg/kg/day), i.p.	No. of animals	Body weight	
		Pre-treatment	Post- treatment
Control (CMC, 0.01%)	14	24.7±1.2	38.4±1.5*
FM (1.5)	13	25.9±1.4	36.8±1.2*
FM (3.0)	14	25.2±1.1	35.6±1.9*
CSA(0.15)	13	24.9±1.3	37.4±1.4*
CSA (0.3)	14	26.2±1.5	36.0±1.5*

*$p<0.01$ (Student's t-test); Post-treatment body weight of the surviving mice was statistically compared with the Pre-treament body wight; FM: Furosemide; CSA: 4-Chloro-5-Sulfamoylanthranilic acid

Table 20: Effect of FM and its degradation product CSA on the organ weight of Swiss Albino mice after chronic treatment

Treatment/dose (mg/kg/day) i.p.	Mean weight/100 g body weight±SE					
	Heart	Lungs	Liver	Kidney	Spleen	Testes
Control (CMC, 0.01%)	0.62±0.02	0.71±0.06	6.51±0.29	1.53±0.06	0.50±0.04	0.66±0.03
FM (1.5)	0.58±0.03	0.72±0.04	6.37±0.42	1.59±0.02	0.57±0.09	0.59±0.05
FM (3.0)	0.64±0.03	0.76±0.05	7.21±0.16*	1.38±0.11	0.71±0.18	0.58±0.02
CSA (0.15)	0.58±0.05	0.79±0.06	6.64±0.29*	1.52±0.07	0.50±0.06	0.64±0.05
CSA (0.3)	0.63±0.06	0.68±0.05	6.60±0.17	1.62±0.11	0.48±0.06	0.63±0.03

*$p<0.05$ (students' t-test); All treatment groups were compared to the control; FM: Furosemide; CSA: 4-Chloro-5-Sulfamoylanthranilic acid

Table 21: Effect of FM and its degradation product CSA on hematological indices of Swiss Albino mice after chronic treatment

Treatment/dose	Hematological indices				
(mg/kg/day), i.p.	WBC×10^9/L	RBC×10^{12}/L	Hb. g dL^{-1}	HCT % ratio	MCV fl
Control (CMC, 0.01%)	5.05±1.81	6.24±0.44	11.62±0.82	32.72±1.50	54.50±5.22
FM (1.5)	4.32±1.34	5.59±0.51	10.78±0.68	29.82±2.79	50.32±2.16
FM (3.0)	4.50±1.16	5.29±1.01	8.74±1.49*	20.87±4.20*	43.74±5.32
CSA (1.5)	4.57±1.18	5.67±0.54	9.88±1.22	26.80±1.17*	47.56±4.76
CSA (0.3)	3.92±1.43	4.87±0.71	9.65±0.62	26.07±2.42*	48.64±1.08

*p<0.05 (students' t-test); Five mice were used in each group; Statistically significant as compared to the control; FM: Furosemide; CSA: 4-Chloro-5-Sulfamoylanthranilic acid

Table 22: Effect of FM and its degradation product CSA on the biochemical parameters in Swiss Albino mice after chronic treatment (Mean±SE)

Parameters	Treatment and dose (mg/kg/day)				
	Control (CMC, 0.01%)	FM (1.5)	FM (3.0)	CSA (0.15)	CSA (0.3)
AST/GOT (u L^{-1})	13.0±2.5	12.4±1.7	13.6±2.24	17.2±6.3	18.7±5.5
ALT/GPT (u L^{-1})	11.0±2.2	12.4±1.7	10.6± 1.2	15.4±2.5	17.8±4.5
CK-MB (u L^{-1})	94.0±12.0	93.0±5.0	77.0± 9.0	98.0±16.0	123±12.0
Creatinine (µmol L^{-1})	64.0±8.0	63.0±9.0	46.0±11.0	68.0±14.0	102.0±12.0*
Urea (mmol L^{-1})	3.9±0.6	2.9±0.9	3.1±0.8	3.5±0.9	2.6±0.6
Glucose (mmol L^{-1})	5.9±1.0	4.4±0.6	4.0±0.6	3.4±0.5*	3.1± 0.4*

*p<0.05; **p<0.01 (Students' t-test); Treatment groups were statistically compared with the individual control group; Five animals were used in each group; FM: Furosemide; CSA: 4-Chloro-5-Sulfamoylanthranilic acid

Table 23: Effect of FM and its degradation product CSA on the histopathological changes in Swiss Albino mice after acute treatment

Histopathological organs	Histopathological changes, treatment and dose (mg kg^{-1})				
studied investigations	CMC (0.01%)	FM (1.5)	FM (3.0)	CSA (0.15)	CSA (0.30)
Liver					
Inflammatory changes	-	-	+	-	-
Abnormal erythropoiesis	-	-	+	-	-
Kupffer cells - pigmented	-	-	+	-	-
Binucleated cell	-	-	+	-	-
Necrosis	-	-	+	+	-
Congestion	-	-	++	-	-
Heart					
Inflammatory changes	-	-	+	-	-
Necrosis	-	-	-	-	-
Congestion	-	-	+	-	+
Kidney					
Inflammatory changes	-	-	-	-	-
Necrosis	-		-	-	-
Congestion	-		-	-	+
Lungs					
Edema	-	-	+	-	-
Abnormal alveoli	-	-	-	-	-
Abnormal bronchioles	-	-	-	-	-
Abnormal blood vessels	-	-	-	-	-
Abnormal lumen	-	-	-	-	-
Capillary congestion	-	-	-	-	+
Spleen					
Abnormal white pulp	-	-	-	-	-
Abnormal red pulp			-	-	-
Increased Magakaryocytes	-	-	+	-	-

-Normal, +Mild effect, ++Appreciable effect

Table 24: Clastogenic effect of FM and its degradation product CSA on the femoral cells of Swiss Albino mice after chronic treatment

Treatment and dose (mg kg^{-1}, i.p.)	Polychromatic Erythrocytes (PCE) screened	Micro nucleated polychromatic erythrocytes (%)	Normochromatic Erythrocytes (NCE) screened	PCE/NCE ratio
Control (CMC)	5190	0.28±0.02	5270	0.99±0.07
FM (1.5)	5218	0.30±0.04	6348	0.87±0.09
FM (3.0)	5890	0.28±0.03	7612	0.81±0.01
CSA (0.15)	5483	0.28±0.03	6251	0.95±0.11
CSA (0.30)	5148	0.26±0.04	5546	0.93±0.05
Adriamycin (8)	5230	4.16±0.32*	8714	0.63±0.06*

*p<0.001 (Student's t-test); Five mice were used in each group; Each group was compared with group 1 (Control); FM: Furosemide; CSA: 4-Chloro-5-Sulfamoylanthranilic acid

process of repair following the toxic implication of FM and CSA. Both FM as well as CSA treatment shown no significant alteration in serum CK-MB by FM as well as CSA treatments. These results are in full agreement with the findings during subacute treatment and were further substantiated by histopathological changes in the cardiac tissue. However, chronic administration of furosemide was reported to induce thiamine deficiency, which can aggravate myocardial dysfunction (Table 20) (Da Cunha *et al*., 2007).

Similarly, histopathology results on the renal tissues after treatment with FM, shown no significant nephrotoxic effect of FM chronic treatment, which is not in agreement with the previous studies (Saarela *et al*., 1999). In the biochemical results, it was also found that no significant increase in serum creatinine levels occurred. Although, the observed effects are in agreement with the data of the current subacute treatment and the acute treatment studies; but is not in full agreement with the reports about the nephrotoxic potential of FM treatment recorded by earlier researchers (NTP, 1989; Hori *et al*., 2007) and increase in BUN and plasma creatinine levels by long-term administration of furosemide was observed (Asaeda *et al*., 1984; Sawaya and Robertson, 1992; Hardman *et al*., 2001; Hori *et al*., 2007). This discrepancy after chronic treatment, might be due to the possible reversibility of toxic implication due to prolonged treatment (Chen *et al*., 1982). It is worth mentioning that renal calcification (but not nephrocalcinosis) is a known complication of furosemide therapy. Renal calculi can complicate FM treatment in children after repair of congenital heart disease. Therefore, it was suggested to do frequent urine analysis and to monitor urine calcium/ceatinine ratio for the early detection of such complications (Ali, 2006).

It is worth mentioning that earlier nephropathy signs were found in the kidney of mice fed with diets containing furosemide (NTP, 1989; Hayes, 2001). In the current chronic toxicity studies, CSA treatment was found to increase the levels of creatinine, however, histopathological investigations shown no significant changes in the renal tissues, except mild congestion due to edema. These results failed to show any significant impact on the increased serum creatinine levels and indicated the latency of toxic implications (Chen *et al*., 1982; BEST, 2006).

Unlike, the results of acute and subacute toxicity studies, FM chronic treatment failed to affect the serum concentration of glucose during chronic toxicity experiments. Earlier reports on FM treatment have shown increased hypoglycemic activity in mice, however, the levels of glucose were fully recovered within 24 h (Sandstrom, 1986; Sandstrom and Schlin, 1988). The discrepancy in these results indicated the time bound effect of FM on the serum levels of glucose. On the other hand, a comparison of the results of subacute treatment and findings of CSA-chronic treatment revealed CSA to reduce the serum levels of glucose. Compounds containing anthranilic acid moiety in their chemical structure, are known to decrease prostaglandin levels (Black, 1986). The observed hypoglycemic effect induced by CSA after chronic treatment, appears to be possibly caused by the known prostaglandin suppression property of such compounds. The cases and reports on furosemide allergy have also, been reported, alarming about the careful use of diuretics (Schreuder *et al*., 2008).

The treatment with FM failed to induce any significant changes in the frequency of micro nucleated PCE and in the ratio of PCE/NCE (Table 24). These results confirmed the previous findings during subacute toxicity studies. However, these results contradict the findings of Subramanyam and Jameela (1977), Jameela *et al*. (1979), Zeiger (1998) and Isidori *et al*. (2006) on the mutagenic effects of FM on human leucocytes in culture and evidence of carcinogenic activity of FM in female mice, as shown by an increase in malignant tumors of the mammary gland in female mice (NTP, 1989). The current results do not correspond well with earlier observations on the clastogenic and mitodepressive potential and toxicity of FM and its degradation product CSA. The discrepancy in these results may be due to the exposure of the drug to pharmacokinetics and pharmacodynamic processes involved in the whole organ, which is different from the partial heterogenicity of cells *in vitro* (Smith and Orrenius, 1984; Lullmaun *et al*., 2005).

Furthermore, the properties of FM such as inhibition of lipid peroxide, electrolyte depletion may have played a role leading to differences in current acute, subacute and chronic toxicity experiments (Lebedev and Petrenko, 1996; Jerie, 2007). In another experiment, Zila *et al*. (2007), it was concluded that hypercapnia during heat stress in both normo-volemic and hypo-volemic animals may be associated with altered cardiorespiratory responses (Zila *et al*., 2007).

The results of the current toxicity studies provided basic toxicological data on CSA, the main degration product of FM formed during adverse storage conditions. Current study added significant information for planning future investigations and experimental trails where, not only the toxicity of the parent drug rather its proper storage and stability aspects should also be taken into consideration which might lead to the formation of toxic degradation products.

REFERENCES

Al-Ashban, R.M., D.A. Barrett and A.H. Shah, 2005. Effect of chronic treatment with ethanolic extract of *Teucrium polium* in mice. J. Herb. Spices Med. Plants, 11 (4): 27-36. INIST-CNRS, Cote INIST: 26084, 35400013935326.0040. DOI: 10.1300/J044v11n04_04.

Ali, S.K.M., 2006. Renal calculi complicating short-term furosemide therapy after congenital heart surgery. Congenit. Heart Dis., 1 (5): 251-253. PMID: 18377534. DOI: 10.1111/j.1747-0803.2006.00044.x.

Al-Harbi, M.M., S. Qureshi, M.M. Ahmed, S. Rafatullah and A.H. Shah, 1994. Effect of *Commiphora molmol* (oleo-gum-resin) on cytological and biochemical changes induced by cyclophosphamide in mice. Am. J. Chin. Med. 22: 77-82. DOI: 10.1142/5019241 5x940-00103. PMID: 7518189.

Amdur, M.O., J. Doull, D. Curtis, C.D. Klaassen and L.J. Casarett, 1991. Casrett and Boull's Toxicology. The Basic Science of Poisons. 4th Edn. McGraw-Hill Co., New York. ISBN: 0071052399/0-07-105239-9.

Asaeda, N., M. Shinoda, S. Tamano, E. Ikawa, T. Yoshiyasu, H. Iwai, N. Nagai, Y. Tagawa and M. Koide, 1984. Effect of diuretic, azosemide (SK-110) in combination with antibiotic cephaloridine on kidney. J. Toxicol. Sci., 9 (1): 73-88. PMID: 6492 216.

Astashkin, E.I., V.I. Sarbash, M.G. Glezer, N.A. Kosheleva and I.F. Tishchenkova, 1988. Clinical and experimental studies of the combined use of dibasol and furosemide. Farmakol, Toksikol (Farmakologiia i toksikologiia, USSR), 51 (1): 60-62. PMID: 3360110.

Bagshaw, S.M., A. Delaney, M. Haase, W.A. Ghali and R. Bellomo, 2007. Loop diuretics in the management of acute renal failure: A systematic review and meta-analysis. Crit. Care Resurc., 9 (1): 60-68. DOI: 10. 1093/ndtplus/sfn199. PMID: 17352669.

Bagshaw, S.M., R. Bellomo and J.A. Kellum, 2008. Oliguria, volume overload and loop diuretics. Crit. Care Med., 36 (4 Suppl.): S172-178. DOI: 10.1097/ CCM.0b013e318168c92f. PMID: 18382190.

Beerman, B., E. Dalen, B. Lindstrom and A. Rosen, 1975. On the fate of furosemide in man. Eur. J. Clin. Pharmacol., 9 (1): 51-61. PMID: 1233253.

Board on Environmental Studies and Toxicology (BEST), 2006. Toxicity testing for assessment of environmental agents: Interim report. The National Academics Press, USA. ISBN: 10-0-309-10092-5, 13-978-0-309-10092-2.

Black, H.E., 1986. Renal toxicity of non-steroidal antiinflammatory drugs. Toxicol. Pathol., 14 (1): 83-90. DOI: 10.1177/019262338601400110. PMID: 3487106.

Brater, D.C., 1983. Pharmacodynamic considerations in the use of diuretics. Annu. Rev. Pharmacol. Toxicol., 23: 45-62. PMID: 6347052. IDS No. QN055.

Chan, P.K. and A.W. Hayes, 2007. Acute Toxicity and Eye Irritancy. 5th Edn. In: Hayes, A.W. (Ed.). Principles and Methods of Toxicology. New York: Raven Press, pp: 579-647. ISBN: 10-084933778X, 13-97808 49337789.

Chan, P.K., G.P. O'hara and A.W. Hayes, 1982. Principles and Methods of Acute and Sub-Chronic Toxicity. 2nd Edn. In: Hayes, A.W. (Ed.). Principles and Methods of Toxicology. Raven Press, NY, pp: 1-51. ISBN: 08900-44708.

Chemtob, S., B.S. Kaplan, J.R. Sherbotie and J.V. Aranda, 1989. Pharmacology of diuretics in the newborn. Pediatr. Clin. North Am., 36 (5): 1231-1250. PMID: 2677940. CODEN PCNAA8. INIST-CNRS. Cote INIST: 9064.

Chuen, M.J. and R.J. MacFadyen, 2006. Dose-dependent association between use of diuretics and mortality in advanced systolic heart failure. Am. J. Cardiol., 97, 98 (12, 10): 1759-1764, 1416-1417. PMID: 17134642.

Costta, S., F. Gallini, M.P. De Carolis, C. Latella, L. Manggio, C. Zecca and C. Romagnoli, 2009. Urinary aldosterone excretion and renal function in extremely-low-birth-weight infants following acute furosemide therapy. Neonatology, 96 (3): 171-174. PMID: 19332997. DOI: 10.1159/000210090.

Cruz, J.E., D.D. Maness and G.J. Yakatan, 1979. Kinetics and mechanism of hydrolysis of furosemide. Int. J. Pharm. Amsterdam, 2: 275-281. ISBN: 0090-9556/98/ 2605-0401-0407$02.00/0.

da Cunha, S., J. Cunha-Bastos, J.B. Salles, M.C. Silva, V.L. Cunha-Bastos and C.A. Mandarim-de-Lacerda, 2007. Cardiac alterations in furosemide-treated thiamine-deprived rats. J. Card. Fail., 13 (9): 774-784. DOI: 10.1016/j.cardfail.2007.06.729. PMID: 17996828.

Eshaghian, S., T.B. Horwich and G.C. Fonarow, 2006. Relation of loop diuretic dose to mortality in advanced heart failure. Am. J. Cardiol., 98 (10): 1416-1417. DOI: 10.1016/j.amjcard.2006.08.007. PMID: 17134642.

Fadel, S., R. Karmaili and E. Cogan, 2009. Safety of furosemide administration in an elderly woman recovered from thiazide-induced hyponatremia. Eur. J. Int. Med., 20 (1): 30-34. DOI: 10.1016/j.ejim.2008. 04.006. PMID: 19237089. PII: S0953-6205(08)00135-0. http//www.ejinme.com/article/S0953-6205(08)00135-0/ abstract.

Hardman, J.G., L.E. Limbird and A.G. Gilman, 2001. Goodman and Gilman's the Pharmacological Basis of Therapeutics. 10th Edn.: McGraw-Hill, New York, ISBN: 0-07-135469-7.

Harnischfeger, G., 1985. Qualitatskontrolle von Phytopharmaka/Quality Control of Herbal Drugs. In: Sucker, H. and P. Fuchs (Eds.). Arbeitstechniken der Pharmazeutischen Industrie/Working Techniques of Pharmaceutical Industry, Band 6, Georg Thieme Verlag Stuttgart, Germany. ISBN: 3-13-673401-7.

Hayes, A.W., 2001. Principles and Methods of Toxicology. 4th Edn. Taylor and Francis, Philadelphia, PA 19106, USA, pp: 1887. ISBN: 1-560-814-2,RA1211,742000,615.9.dc21.00-037719.

Hori, Y., F. Takusagawa, H. Ikadai, M. Uechi, F. Hoshi and S. Higuchi, 2007. Effects of oral administration of furosemide and torsemide in healthy dogs. Am. J. Vet. Res., 68 (10): 1058-1063. DOI: 10.2460/ajvr.68.10.1058, PMID: 17916010.

Hsu, Y.J., J.S. Chiu, K.C. Lu, T. Chau and S.H. Lin, 2005. Biochemical and etiological characteristics of acute hyponatremia in the emergency department. J. Emerg. Med., 29 (4): 369-374. PMID: 16243191.

Ibrahim, K.E., R.M. Al-Ashban and S.A. El-Sammani, 2009. A study of the toxicity of cat's claw herbal medicine. Res. J. Pharmacol., 3 (3): 52-57. http://medwelljournals.com/fulltext/rjp/2009/52-57.pdf.

Isidori, M., A. Nardelli, A. Parrella, L. Pascarella and L. Previtera, 2006. A multi species study to assess the toxic and genotoxic effect of pharmaceuticals: Furosemide and its photoproduct. Chemosphere, 63 (5): 785-793. DOI: 10.1016/j.chemosphere.2005.07.078. PMID: 16213548.

Jerie, P., 2007. Milestones of cardiovascular therapy diuretics. Cas. Lek. Cesk., 146 (11): 858-862. PMID:18069212.http://www.find-health-articles.com/rec_pub_18069212-milestones-cardiovascular-therapy-v-diuretics.htm.

Jameela, M., S. Subramanyam and G. Sadasivan, 1979. Clastogenic effects of furosemide on human leukocytes in culture. Mutat. Res., 66 (1): 69-74. PMID: 423907, 0400763.

Kapur, G., R.P. Valentini, A.A. Imam and T.K. Mattoo, 2009. Treatment of severe edema in children with nephrotic syndrome with diuretics alone-a prospective study. Clin. J. Am. Soc. Nephrol., 4 (5): 907-913. DOI: 10.2215/CJN.04390808. PMID: 19406963.

Khalil, S.H.A., A.H. Shah and A.H. Al-Shareef, 1993. A reverse phase HPLC method for the determination of chloramphenicol and its hydrolytic product in ophthalmic solutions. Analytical. Lett., 26 (6): 1163-1179. DOI: 10.1080/00032719308019895.

Kociol, R., J. Rogers and A. Shaw, 2009. Organ cross talk in the critically ill: The heart and kidney. Blood Purif., 27 (4): 311-320. DOI: 10.1159/000207198. PMID: 19270450.

Kopp, S., 2006. Stability testing of pharmaceutical products in a global environment. WHO policy on stability testing. The Secretariats of the World Health Organization's Expert Committee on Specifications for Pharmaceutical Preparations. Informal UK Ltd. www.Rajpharma.com. Regulatory Feature, WHO, pp: 291-294. http://www.who.int/medicines/areas/quality_safety/quality_assurance/RAJ2006WHOStability.pdf.

Lebedev, A.A. and N.A. Petrenko, 1996. The antioxidative properties of furosemide in renal ischemia. Eksp. Klin. Farmakol. Russia, 59 (4): 28-30. PMID: 9026184. http://www.find-health-articles.com/rec_pub_9026184-the-antioxidative-properties-furosemide-renal-ischemia.htm.

Liu, J., Y. Liu, P. Bullock, and C.D. Klassen, 1995. Suppression of liver cytochrome P 450 by α-hederin: Relevance to hepatoprotection. Toxicol. Applied Pharmacol., 134: 124-131. PMID: 7676446. http://content.nhoindemed.com.

Luellmann, H., K. Mohr, L. Hein and D. Bieger, 2005. Color Atlas of Pharmacology, Thieme International. 3rd Revised Exp. Edn. ISBN: 10-158890332X, 13-978-1588903327. http://www.amazon.com/Color-Atlas-Pharmacology-Heinz-Luellmann/Dp/158890332X/ref=pd_sim_b_2/182-8133607-2845059.

Majed, A.A., A.A. Al-Yahya, A.M. Al-Bekairi, Al-Shabanah and S. Qureshi, 2005. Genetic and biochemical effects of *Ginkgo biloba* on somatic and germ cells of mice. Saudi Pharmaceutical. J., 13 (4): 148-157. http://www.ksu.edu.sa/colleges/pharm/SPJ/1304/130403.pdf.

Metra, M., S. Nodari, G. Parrinello, T. Bordonali, S. Bugatti, R. Danesi, B. Fontanella, C. Lombardi, P. Milani, G. Verzura, G. Cotter, H. Dittrich and B.M. Massie, 2008. Worsening renal function in patients hospitalized for acute heart failure: Clinical implications and prognostic significance. Eur. J. Heart Fail., 10 (2): 188-195. DOI: 10.1016/j.ejheart.2008.01.011. PMID: 18279773.

Mosberg, A.T. and A.W. Hayes, 1989. Sub-chronic Toxicity Testing. 2nd Edn. In: Wallace, H.A. (Ed.). Principles and Methods of Toxicology. Raven Press, NY, pp: 221-236. ISBN: 08900-44708.

Nakahama, H., Y. Miwa, A. Yamaji, Y. Fukuhara, M. Yanase, T. Kamada, T. Sonoda, M. Ishibasi and Y. Ichikawa, 1987. The urinary excretion of furosemide and its metabolites on kidney transplant patients. Eur. J. Clin. Pharmacol., 32: 313-316. PMID: 3297734.

NTP (National Toxicology Program), 1989. Toxicology and carcinogenesis studies of furosemide (CAS No. 54-31-9) in F344/N rats and B6C3F1 mice (feed studies). Natl. Toxicol. Program Tech. Rep. Ser., 356: 1-190. PMID: 12695785. http://ntp.niehs.nih.gov/go/6972. http://ntp.niehs.nih.gov/ntp/htdocs/LT_rpts/tr356.pdf. http://ntp.niehs.nih.gov/?objected=07089914-FDE4-C583-1F13CC110E963972.

Qureshi, S., M.M. Al-Harbi, M.M. Ahmed, M. Raza, A.B. Giangreco and A.H. Shah, 1993. Evaluation of genotoxic, cytotoxic and antitumor properties of *Commiphora molmol* using normal and *Ehrlich ascites* carcinoma cell-bearing Swiss albino mice. Cancer Chemother. Pharmacol., 33: 130-138. DOI: 10.1007/BF00685330. PMID: 8261571.

Reynolds, J.E.F. and K. Parfitt, 1989. Martingale. The Extra Pharmacopoeia. 29th Edn. The Pharmaceutical Press, London, pp: 987-991. ISBN: 0-85369-210-6.

Robin, J.F., J.J. Jomer and R.R. Schueler, 1982. Principles and Methods of Acute Ad Sub-chronic Toxicity. In: Wallace, H.A. (Ed.). Principles and Methods of Toxicology. Raven Press, NY, pp: 79-105. ISBN: 08900 44708.

Saarela, T., P. Lanning, M. Koivisto and T. Paavilainen, 1999. Nephrocalcinosis in full-term infants receiving furosemide treatment for congestive heart failure: A study of the incidence and 2 years follow up. Eur. J. Pediatr., 158 (8): 668-672. PMID: 10445348.

Sandstrom, P.E., 1986. Probenecid potentiates the hyperglycemic effect, but reduces the diuretic effect of furosemide in mice. Br. J. Pharmacol., 89: 307-312. PMID: 3779212. PMCID: PMC1917023.

Sandstrom, P.E. and J. Schlin, 1988. Furosemide causes acute and long-term hyperglycemia and reduces glucose tolerance in mice. Acta. Physiol. Scand, 132: 75-81. PMID: 3066121.

Sawaya, G.F. and P.A. Robertson, 1992. Hepatotoxicity with the administration of nifedipine for treatment of preterm labour. Am. J. Obstet. Gynecol., 167 (2): 512-513. PMID: 1497061.

Schreuder, M.F., J.A. van Wijk, H.J. van der Horst and A. Bökenkamp, 2008. A boy with cyclic vomiting and an alleged furosemide allergy on the basis of a subpelvic stenosis. Ned. Tijdschr. Geneeskd., 152 (5): 275-277. PMID: 18333543, SID: 78573161.

Schwinger, R.H. and E. Erdmann, 1992. Heart failure and electrolyte disturbances. Methods Find. Exp. Clin. Pharmacol., 14 (4): 315-325. PMID: 1507935.

Shah, A.H., A.H. Al-Shareef, S. Qureshi and A.M. Ageel, 1998. Toxicity studies on some common spices: *Cinnamomum zylanicum* and *Piper longum*. Plant Foods Hum. Nutr., 52: 231-239. DOI: 10.1023/A:1008088323164. PMID: 9950084.

Smith, D.E. and L.Z. Benet, 1983. Biotransformation of furosemide in kidney transplant patients. Eur. J. Clin. Pharmacol., 24: 787-790. PMID: 6350024.

Smith, M.T. and S. Orrenius, 1984. Studies on Drug Metabolism and Drug Toxicity in Isolated Mammalian Cells. In: Mitchell, J.R. and M.G. Horning (Eds.). Drug Metabolism and Drug Toxicity. American Society for Pharmacology and Experimental Therapeutics, Michigan University, Raven Press, New York, pp: 71-98. ISBN: 0890049971, 9780890049976.

Snopek, G., J. Kotlarska, D. Daniewska, T. Zelem, W. Drewniak, A. Krol-Jaskulska and M. Dabrowski, 2008. Use of intermittent ultrafiltration in decompensated, diuretic-resistant heart failure 6 case reports. Kardiol. Pol., 66 (11): 1202-1204. PMID: 19105097.

Subramanyam, S. and M. Jameela, 1977. Studies on cytological effects of fursemide on meiotic cells of male mice. Indian. J. Med. Res., 66 (1): 104-113. PMID: 924576.

Vargas, F., I. Martinez-Volkmar, J. Sequera, H. Mendez, J. Rojas, G. Fraile, M. Velasquez and R. Medina, 1998. Photo degradation and phytotoxicity studies of furosemide. Involvement of singlet oxygen in the photoinduced hemolysis and lipid per oxidation. J. Photochem. Photobiol. B., 42 (3): 219-225. DOI: 10.1016/S1011-1344(98)00074-8. PMID: 9595711.

WHO (World Health Organization), 2004. Stability testing for hot and humid climates. WHO Drug Information, 18 (2): 113FF. www.who.int/druginformation/vol18num2_2004/D118-2.pdf.

Williams, D.P., D.J. Antoine, P.J. Butler, R. Jones, L. Randle, A. Payne, M. Howard, I. Gardner, J. Blagg and B.K. Park, 2007. The metabolism and toxicity of furosemide in the Wistar rat and CD-1 mouse: A chemical and biochemical definition of the toxicophore. J. Pharmacol. Exp. Ther., 322 (3): 1208-1220. DOI: 10.1124/jpet.107.125302. PMID: 17556636.

Yoshioka, S. and V.J. Stella, 2002. Stability of Drugs and Dosage Forms. Springer Verlag. New York. ISBN: 0306464047.

Zeiger, E., 1998. Identification of rodent carcinogens and noncarcinogens using genetic toxicity tests: Premises, promises and performance. Regul. Toxicol. Pharmacol., 28 (2): 85-95. DOI: 10.1006/rtph.1998.1234. PMID: 9927558.

Zila, I., A. Brozmanova, M. Javorka, A. Calkovska and K. Javorka, 2007. Effects of hypovolemia on hyper-capnic ventilatory response in experimental hyper-thermia. J. Physiol. Pharmacol., 58 (Suppl. 5 Pt 2): 781-790. PMID: 18204192. www.jpp.krakow.pl.