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Single and Repeat Dose Toxicity Study of 7-Hydroxycoumarin, Ethanol, and Their Mixture in Rats

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Abstract: Acute and repeat-dose toxic effects of HOC (7-hydroxycoumarin), ethanol and their mixture were studied in rats. Single oral administration of HOC (5,000 mg/kg) caused transitional glycosuria associated with lowered serum glucose levels, decreased urea clearance. HOC given orally during 28 days (200 mg/kg) decreased serum glucose, and increased serum triglyceride concentrations. No enhancement of acute toxic effect of HOC (5,000 mg/kg) and ethanol (6,000 mg/kg) mixture was found in the acute toxicity study phase. Effect of HOC (200 mg/kg) and ethanol (750 mg/kg) during 28 days of exposure was less pronounced in comparison with HOC effect only, as far as neither decrease of glucose, nor increase of triglyceride serum concentrations were found.

Key words: 7-hydroxicoumarin, ethanol, mixture toxicity.

1. Introduction

Coumarins are naturally occurring benzopyrone derivatives, a lot of them have been identified from plants. These compounds found in vegetables, fruit, seeds, tea, have attracted interest in recent years because of their diverse pharmacological properties and expected low toxicity. HOC (7-hydroxycoumarin) is phyto-constituent found in eatable and officinal plants belonging to umbelliferae family. Besides, HOC is the main, low toxic metabolite of coumarin in a human body [1]. A wide range of pharmacological activities of HOC is demonstrated in animal studies. The compound lowers serum GLU (glucose) level in rats with streptozotocin diabetes, exhibit antioxidant, antinociceptive and anti-inflammatory effects, is used as derivative in the synthesis of drugs [2-7]. The recent study showed that HOC is α-amylase and α-glucosidase inhibitor in vitro [8], causes neuroprotective effects [9, 10], attenuate alcohol-induced fatty liver in rats [11]. The abovementioned points that HOC might be considered either as a promising pharmaceutical agent, or as a biologically active food additive. Furthermore, HOC as suspected low toxic plant derived compound, and because of its fluorescent abilities [12] was considered as a budding fluorescent dye for flavored alcoholic beverages with relatively low alcohol content (maximum 8% alcohol by volume). Although constituents of traditionally used plants are preliminary admitted as a low toxic, the data of the short-term and subchronic toxicity studies of the compounds should be obtained in order to increase the confidence in their safety for humans.

Our previous investigations [13-15] showed that HOC demonstrates low acute toxicity when administered orally to rats, and mice (LD50 > 10,000 mg/kg). No substantial interspecies difference in lethality, clinical signs, and postmortem examination results were noted. Dermal acute toxicity assessed in rats was also low (LD50 > 2,000 mg/kg). The substance did not cause a skin irritation, slightly irritated eye, when studied in rabbits. Neither sensitizing properties in Buehler test, nor suppression of delayed type hypersensitivity reaction to sheep red blood were revealed. Weak cumulative properties, characterized by functional impairment of liver and kidney, slight irritation/inflammatory histopathology changes of gastrointestinal tract mucosa were shown

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for HOC. The similar changes were observed during subchronic toxicity study, when the impaired function and histopathology changes of liver and kidney, lesion in small intestine mucosa observed in rats of both sexes at dose range 50-500 mg/kg were considered as the main treatment-related effects. Changes of serum GLU, and serum TG (triglyceride) levels found in the subchronic toxicity study were considered as early shifts indicating HOC effect. As it is known, histopathological changes are meaningful to distinguish adverse/non-adverse effects. The key histopathology findings observed in liver, kidney, and intestine after 3 months of HOC exposure may indicate rather adaptive response leading to dystrophic reversible changes, then intensive alteration. Indeed, the slight fine lipid vacuolation, and hypertrophy of hepatocytes observed in the liver did affect neither the organ histostructure, nor cellular wholeness, reflecting a functional reaction to the HOC induced metabolic disorder. The kidney tubular epithelia impairment may reflect the organ function overloading due to elimination of the excessive amount of HOC [16]. The observed glomeruli contraction reflecting the restricted blood flow might be an adaptive reaction, aimed to the tubular epithelia off-loading due to xenobiotic elimination, as it was shown by Gozhenko [17]. Toxicological meaning of the observed dystrophic changes of small intestine mucosa remains questionable, because it's not clear either they reflect a result of the found metabolic disorder, or, in a contrary, the dystrophic changes lead to disturbance of the digestive absorption of glucose, causing serum GLU decrease. Taking account of the reversibility of repeat dose toxicity alterations observed at the highest studied dose, the dose 500 mg/kg might be considered as no adverse effect level, although this statement remains discussed. NOEL (no observed effect level) in the subchronic HOC study in rats was determined to be 20 mg/kg/day.

Mixture toxicity is one of the challenges of modern toxicology. Taking into account that HOC is proposed as a prospective food dye for alcohol containing beverages, and the fact that ethyl alcohol is commonly used as a solvent in the oral liquid dosage formulations [18], the mixture toxicity of HOC and ethanol assessment is relevant.

The current study is purposed to carry out the comparative simultaneous evaluation of HOC, ethanol, and their mixture acute and repeat dose toxicity in rats.

2. Materials and Methods

Materials. HOC (99.0% purity) obtained from Sigma-Aldrich (USA), ethanol (96.5%) obtained from GP Krasnoslobodsky distillery (Ukraine) were used in the study.

Animals. Experimental animals were handled according to the guidance [19]. Healthy SPF (specific-pathogen-free) Wistar Han rat females of 9-10 weeks old were obtained from nursery of "L. I. Medved's Research Center of Preventive Toxicology Food and Chemical Safety" (Ukraine). Animals were provided with standard pelleted diet (Altromin, Germany) and UV-treated deionized water *ad libitum*. All animals were acclimatized during at least 3 days, randomly assigned by groups of 5 rats of each sex, housed in plastic cages. In the course of experiments, they were maintained at temperature 23 ± 2 °C, humidity 50-70%, with a 12 h light/dark cycle.

Preparations and dosing. HOC, ethanol, and their mixtures were given orally using purified deionized water as a vehicle. Ethanol dosing was calculated as an absolute ethyl alcohol. Control animals received vehicle only. For all animals the daily administered volume of liquid was equal to 10 mL/kg.

In the acute toxicity study phase HOC at dose 5,000 mg/kg, and ethanol at dose 6,000 mg/kg were used. The doses were chosen as sublethal ones for each of the substances.

In the subacute toxicity study phase ethanol was used at dose 750 mg/kg, which is corresponding to its upper level in low alcohol beverages. HOC was used at doses 20-200 mg/kg (NOEL, and middle effective dose

respectively, based on the results of subchronic toxicity study). Mixtures of ethanol with HOC at each mentioned dose were studied.

Experimental design. In the acute toxicity study phase 4 groups of 20 animals received vehicle (control group), ethanol, HOC, mixture of ethanol and HOC. Five animals of each group were euthanized in 3, 24, 72, 196 h after administration. Mortality and clinical signs were recorded daily. Body weights were measured in 72 and 196 h in groups of animals terminated 196 h after exposure. Blood samples were collected from femoral vein of animals anaesthetized by CO_2 inhalation. Following biochemical parameters were measured in blood serum: ALT (alanine aminotransferase), AST (aspartate aminotransferase), ALP (alkaline phosphatase), TP (total protein), UR (urea), GLU, TG, TCH (total cholesterol) using diagnostic kits "Phyllis-diagnosis" (Ukraine). Diuresis for 24 h was measured after water loading (2.5% of animal body weight), except animals terminated 3 h after dosing, when 3 h diuresis was measured. Urinalysis included pH, SG (specific gravity), urine GLU, PRO (protein), UR concentrations determined by "Phyllis-diagnosis" diagnostic kits. The blood UR clearance was calculated using routine formula [20]. Liver and kidney of animals necropsied after 24, 72, 196 h exposure were weighed.

In the subacute toxicity study phase the preparations were administered daily during 28 days to groups of five rat females. Six groups of animals received orally following preparations: vehicle (control group), ethanol (750 mg/kg), HOC (20 mg/kg), HOC (200 mg/kg), ethanol (750 mg/kg) + HOC (20 mg/kg), ethanol (750 mg/kg) + HOC (200 mg/kg). All animals were observed daily for mortality and clinical signs. Quantitative non-automated assessment of behavior in "open field" and "hole board" tests [21] performed for each animal of groups at day 26 of treatment. Time of beginning of movement, ambulatory activity (assessed by square crossing), number of rearings, grooming, defecation were counted in the "open field" test; the ambulatory activity and rearing number were calculated differentially in inner and outer areas. Number of head-dips was counted in the "hole board" test. Duration of each test was 3 m. Blood clinical chemistry determination and urinalysis was performed at 28 day of exposure in the same scope as in the acute toxicity phase study.

Statistical analysis. Obtained data have been subjected to statistical evaluation for the significance of any observed changes relatively to control group. The analysis was carried out by the Fisher-Student's test. Statistical comparisons were evaluated at the 5% significant level. The data in tables are given as "mean ± standard error of the mean". Only those of studied parameters are presented in the tables, which were statistically different.

3. Results and Discussion

The acute toxicity study showed that HOC given orally at 5,000 mg/kg caused no death. Clinical observation detected slightly decreased general activity during the first hours after administration, wet urogenital zone was observed during the second day. Ethanol at 6,000 mg/kg caused sleep of rats continuing 47.6 ± 7.3 min, general activity of animals was meaningfully decreased during the day of administration. When ethanol with HOC mixture was administered, the duration of ethanol induced sleep, compared to rats undergone to ethanol administration only, shortened by 60% accounting for 18.7 ± 6.7 m. During the day of administration general activity of animals decreased, during the second day wet urogenital zone was observed. No statistically significant changes of body weight were found in all treatment groups to compare with control. Body weight gain over a period of 196 h after exposure was significantly less ($p < 0,05$) in all treated groups: (–) 0.50 ± 6.44 g for the group received HOC, (+) 2.00 ± 4,72 g for the group received ethanol, (–) 0.30 ± 2.15 g for the group received mixture of both substances, whereas the control group body weights gain amounted

15.00 ± 3.22 g.

Blood biochemistry and urinalysis parameters changes are provided in Table 1. Administration of HOC caused mostly signs of kidney dysfunction. diuresis reduced by 50% and heightened SG was found at 3 h after exposure. Moderate glycosuria was accompanied by lowered serum GLU (by 16%) at 24 h; serum UR was elevated by 22% at this time point and remained by 14% at 72 h. No changes of parameters of blood clinical biochemistry and urinalysis were observed in 196 h after administration of HOC.

In group of animals treated with ethanol, after 3 h diuresis was 87% lower, and SG was greater than in control group as well as in the case of HOC administration; TP were lowered by 18%; slightly elevated serum ALT and AST indicated mild liver dysfunction at this time point. Later changes were lowered by 10% TP, and increased levels of TG (by 31%) and serum TCH (by 24%) were found only 196 h after administration.

Effect of mixture of ethanol and HOC 3 h after exposure comprised of diuresis decrease (by 80%), and SG increase in the same way as it was after separate exposure of studied compounds. The most prominent changes were found in 24 h: slightly elevated AP, moderately heightened serum UR (by 42%), lowered serum GLU (by 13%), slight decrease of urine UR concentration (by 16%), and higher SG. A glycosuria was not revealed to compare with effect of HOC only. At 72 h after exposure of the mixture negligible increase of AP was found only. At 196 h no changes of studied parameters of blood biochemistry and urinalysis were detected.

Assessment of UR clearance (see Table 2) showed that after 3 h exposure it significantly dropped in all treated groups of rat females, obviously due to depressed diuresis. At later time points, HOC acute intoxication caused UR clearance decrease in 72 h, its mixture with ethanol caused similar effect earlier in 24 h after exposure.

Changes of liver and kidney weights were found in 24 h in all treated groups (see Table 3). Administration

Table 1 Acute toxicity study phase.

Parameter		Control	7-HOC	Ethanol	Ethanol + HOC
3 h					
I	TP (g/L)	69.17 ± 1.42	70.00 ± 1.42	56.41 ± 1.94*	69.02 ± 2.02
	ALT (mM/h·L)	0.36 ± 0.02	0.37 ± 0.03	0.46 ± 0.03*	0.40 ± 0.03
	AST (mM/h·L)	0.89 ± 0.02	0.92 ± 0.03	1.02 ± 0.04*	0.98 ± 0.04
II	Diuresis (mL)	8.52 ± 0.90	4.24 ± 1.07*	1.12 ± 2.83*	1.74 ± 1.76*
	SG (g/mL)	1.0040 ± 0.0005	1.0100 ± 0.0011*	1.0450 ± 0.0053*	1.0100 ± 0.0021*
24 h					
I	UR (mM/L)	4.84 ± 0.08	5.88 ± 0.29*	4.63 ± 0.33	6.90 ± 0.59*
	AP (mkM/sec·L)	3.79 ± 0.06	3.84 ± 0.27	4.04 ± 0.15	4.58 ± 0.18*
	GLU (mM/L)	5.03 ± 0.13	4.25 ± 0.19*	4.81 ± 0.12	4.39 ± 0.11*
	SG (g/mL)	1.0182 ± 0.0006	1.0344 ± 0.0049*	1.0169 ± 0.0011	1.0271 ± 0.0037*
II	UR (mM/L)	394.62 ± 9.74	324.67 ± 23.73*	390.98 ± 11.37	329.92 ± 15.99*
	GLU (mM/L)	0.00 ± 0.00	5.17 ± 0.64*	0.00 ± 0.00	0.00 ± 0.00
72 h					
I	UR (mM/L)	4.58 ± 0.15	5.24 ± 0.24*	4.10 ± 1.05	4.43 ± 0.24
	AP (mkM/sec·L)	3.26 ± 0.10	3.58 ± 0.11	2.98 ± 0.18	4.24 ± 0.26*
196 h					
I	TP (g/L)	69.72 ± 0.63	74.39 ± 4.86	62.91 ± 1.82*	71.52 ± 1.11
	TCH (mM/L)	2.42 ± 0.22	2.59 ± 0.15	3.00 ± 0.06*	1.91 ± 0.24
	TG (mM/L)	0.55 ± 0.041	0.56 ± 0.040	0.72 ± 0.030*	0.57 ± 0.045

* $p \leq 0.05$. Acute toxicity study phase. I: Blood clinical chemistry; II: urinalysis parameters.

Table 2 Acute toxicity study phase.

Time (h)	UR clearance (mL/min)			
	Control	7-HOC	Ethanol	Ethanol + HOC
3	4.66 ± 0.44	1.78 ± 0.15*	0.65 ± 0.46*	0.96 ± 0.45*
24	0.37 ± 0.02	0.29 ± 0.05	0.45 ± 0.06	0.20 ± 0.03*
72	0.38 ± 0.05	0.17 ± 0.02*	0.30 ± 0.06	0.31 ± 0.06
196	0.39 ± 0.03	0.51 ± 0.07	0.43 ± 0.05	0.32 ± 0.03

* $p \leq 0.05$. Acute toxicity study phase. UR clearance.

Table 3 Acute toxicity study phase.

Group	Liver		Kidney	
	(g)	(%)	(g)	(%)
Control	6.33 ± 0.37	3.01 ± 0.11	1.29 ± 0.06	0.61 ± 0.02
HOC	6.58 ± 0.46	3.34 ± 0.22	1.30 ± 0.06	0.76 ± 0.03*
Control	6.32 ± 0.11	3.16 ± 0.13	1.25 ± 0.06	0.63 ± 0.02
Ethanol	6.85 ± 0.45	3.69 ± 0.10*	1.29 ± 0.07	0.70 ± 0.03*
Control	5.65 ± 0.06	2.73 ± 0.61	1.25 ± 0.06	0.61 ± 0.03
HOC+ethanol	6.50 ± 0.14*	3.20 ± 0.93*	1.28 ± 0.06	0.63 ± 0.04

* $p \leq 0.05$. Acute toxicity study phase. Absolute and relative organ weights (24 hours after exposure).

of HOC caused the increase of relative kidney weight in 25%, ethanol effect consisted of increase of relative weights of liver, and kidney in 17%, and in 11% respectively. When HOC with ethanol mixture was administered, liver weights (absolute, and relative) were higher rather due to low control group meanings, then due to the preparation effect.

In the subacute mixture toxicity study of HOC and ethanol, following results were obtained (see Table 4). Assessment of behavior of rat females revealed that HOC at both studied doses did not lead to significant changes of studied parameters in comparison to control group of animals. Ethanol at 750 mg/kg subacute exposure caused depressive effect on central nervous system of rat, comprised elongation of the time of beginning of movement in "open field" test, decrease of ambulatory activity in inner and outer area of "open field", decrease of numbers of rearings, and head-dips.

When mixture of HOC at each of studied dose was administered with ethanol, changes of rat behavior were similar to ethanol only effect, except of no elongation of time of beginning of movement, and decrease of rearing number in the "open field" was found.

Evaluation of clinical biochemistry and urinalysis parameters after 28-day of exposure of rats to ethanol at 750 mg/kg (see Table 5) revealed decrease of urine UR concentration in 14% only. HOC at 200 mg/kg caused decrease of GLU by 33%, and increase of TG level in 52% in blood serum, no statistically significant changes were found after administration of HOC at 20 mg/kg.

The mixture of ethanol with the higher studied dose of HOC caused mild increase of serum UR (in 18%), decrease of urine UR (in 12%) concentration, and increased SG. Noteworthy is that no significant changes of serum GLU, and TG were found when compared with control group. Mixture of ethanol with HOC at the low dose did not affect the assessed parameters.

Thus, as a result of the study of number of in-life, physiological, clinicochemical parameters during 196 h after single oral administration of HOC at 5,000 mg/kg, mostly symptoms of kidney dysfunction were found. They comprised clinical signs of diuresis disorder, glycosuria leading to drop of serum GLU, lowered UR clearance, and increased weight of kidney. The changes were transient, possibly indicating an emergency load of the organ due to renal excretion of HOC and its conjugates.

Table 4 Subacute toxicity study phase.

Parameter	Control	Ethanol, 750 mg/kg	HOC, 20 mg/kg	HOC, 200 mg/kg	Ethanol, 750 mg/kg +HOC, 20 mg/kg	Ethanol, 750 mg/kg +HOC, 200 mg/kg
Time of beginning of movement (sec)	3.1 ± 0.8	7.5 ± 1.6*	5.2 ± 0.4	4.2 ± 0.9	4.4 ± 0.8	3.2 ± 0.54
Inner square crossed (unit)	8.2 ± 1.5	3.4 ± 0.6*	6.4 ± 1.4	10.1 ± 2.01	4.4 ± 0.3*	4.1 ± 0.75*
Outer square crossed (unit)	39.7 ± 3.1	11.9 ± 5.7*	29.6 ± 4.2	35.5 ± 3.87	27.8 ± 1.9*	19.3 ± 5.36*
Inner rearing (unit)	4.9 ± 0.9	1.5 ± 0.6*	5.4 ± 0.4	4.1 ± 1.10	4.1 ± 1.4	4.3 ± 1.61
Head-dip (unit)	12.2 ± 2.04	10.4 ± 3.12	8.60 ± 198	6.4 ± 0.54*	5.7 ± 0.54*	7.2 ± 0.64*

* $p \leq 0.05$. Subacute toxicity study phase. Behavior of rats in "open field", and "hole board" tests.

Table 5 Subacute toxicity study phase.

Parameter		Control	Ethanol, 750 mg/kg	HOC, 20 mg/kg	HOC, 200 mg/kg	Ethanol, 750 mg/kg +HOC, 20 mg/kg	Ethanol, 750 mg/kg +HOC, 200 mg/kg
I	UR (mM/L)	7.86 ± 0.25	8.22 ± 0.46	7.48 ± 0.55	7.34 ± 0.50	8.42 ± 0.50	9.28 ± 0.57*
	TG (mM/L)	0.68 ± 0.03	0.77 ± 0.03	0.72 ± 0.04	1.03 ± 0.13*	0.78 ± 0.06	0.80 ± 0.10
	GLU (mM/L)	4.90 ± 0.19	4.61 ± 0.11	4.34 ± 0.17	3.31 ± 0.37*	4.92 ± 0.19	4.78 ± 0.21
II	SG (g/mL)	1.0240 ± 0.0005	1.0200 ± 0.0027	1.0220 ± 0.0018	1.0220 ± 0.0018	1.0225 ± 0.0022	1.0300 ± 0.0021*
	UR (mM/L)	439.3 ± 10.5	379.5 ± 8.6*	427.3 ± 8.7	421.2 ± 9.4	398.2 ± 18.2	384.8 ± 12.4*

* $p \leq 0.05$. Subacute toxicity study phase. I: Blood clinical chemistry; II: urinalysis parameters.

Acute toxicity study revealed no obvious enhancement of toxic effect of each test-substance, when administered as a mixture. The mixture of both compounds, administered at levels corresponding to sublethal dose of each one, did not cause death of animals. Body weight gain loss after the mixture gavage was similar to the decrease of body weight gain changes, caused by each compound. Clinical signs after the mixture exposure consisted of sum of clinical signs of each compound. Some shifts of the studied in dynamic of the mixture acute intoxication parameters (e.g. shortening of ethanol sleep, absence of HOC induced glycosuria, UR clearance drop revealed at relatively earlier time point in comparison with HOC effect only) indicate more likely toxicokinetic interaction of both substances, than toxicodynamic one.

The findings of the HOC and ethanol mixture subacute toxicity study did not reveal any amplification of toxicity of each compound as well. Not more than effect of ethanol was traced in course of behavior parameters assessment after its mixture with HOC at both studied doses administration. Blood chemistry parameters remained within normal limits, except the observed slight increase of serum UR concentration after ethanol with HOC at 200 mg/kg exposure. Although this mixture caused also decrease of urine UR concentration, no significant changes of UR clearance were found. It must be emphasized that no effect of HOC at dose 200 mg/kg on serum GLU, and TG levels were found when the substance was administered as a mixture with ethanol. The finding may reflect an antagonistic interaction of HOC at 200 mg/kg and ethanol at 750 mg/kg in the rat organism, leading to attenuation of the caused by HOC metabolic disturbance.

4. Conclusions

Thus, there is no toxic effect enhancement of HOC and ethanol, when used as mixture, it was observed in the acute and subacute toxicity study in rats. Moreover, effect of HOC at 200 mg/kg and ethanol at 750 mg/kg after 28 days of exposure was less pronounced in comparison with HOC effect only, as far as neither decrease of serum GLU, nor increase of serum TG concentrations were found.

Obtained results demonstrate that ethanol attenuates HOC effect in rats under condition of subacute oral exposure to the mixture of both compounds. Taking into account the wide use of ethanol in pharmaceutical, and food industry a further study of both compounds ineraction in vivo would be required in view of growing interest to biological activity of HOC and prospects for its application in pharmaceutical, and food industry.

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