Investigation of Possible Toxicological Influences of Simmondsin after Subacute Administration in the Rat

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A 5-day administration of 250 mg of simmondsin/kg of body weight did not have any toxicological influences on liver, pancreas, and kidneys using several biochemical parameters. Anatomopathological investigation of kidney, liver, pancreas, stomach, intestine, testis, and seminal vesicle also did not demonstrate any pathological change. Since the concentrations of CN^- and of SCN^- in the blood are not elevated, it is concluded that there are no indications for a liberation of HCN during the metabolism of simmondsin in the rat. The food intake inhibition and weight-reducing effect of gastrally intubated simmondsin in rats has been confirmed, but there are no indications that HCN causes the food intake reduction or the weight loss after simmondsin intake in rats.

Simmondsin [2-(cyanomethylene)-3-hydroxy-4,5-dimethoxycyclohexyl β-D-glucoside], extracted from the jojoba plant (*Simmondsia chinensis*), has a powerful food intake inhibition effect (Booth et al., 1974; Cokelaere et al., 1992). Its emaciation effect was attributed not only to a reduction of food intake but also to a supplementary toxicological influence of simmondsin and its analogues (Booth et al., 1974). According to Williams (1980), mice to which simmondsin was administered demonstrated increased cyanide and thiocyanate in blood. According to this author, the liberated cyanide could be responsible for the weight loss. He could not find a specific histopathological influence on the internal organs in the experimental animals which could be ascribed to simmondsin. On the other hand, lambs had simmondsin mixed in their feed during 80 days. Five or ten percent of their feed consisted of nondetoxified deoiled jojobameal. The animals showed no morbid symptoms but were leaner than the controls at the end of this period. The authors could not find any organ damage. Blood urea nitrogen (BUN) was decreased, whereas γ-glutamyl transpeptidase was increased in ewes that took the simmondsin in their feed. This was not present in wethers, but here they found a decreased liver protein and a lower N-demethylase activity. They did not find an indication of mutagenic or promutagenic activity in the jojobameal (Manos et al., 1986).

In the following experiments the effect of a subacute administration of pure simmondsin on several biochemical parameters, indicative for liver, pancreas, and kidney function, was studied. Possible anatomopathological damage in liver, pancreas, kidney, stomach, intestine, seminal vesicle, and testis was monitored. The cyanide and thiocyanate in the blood of animals treated with

simmondsin and/or megadoses of hydroxocobalamin (vitamin B_{12a}) (HCB), which is able to capture free cyanide quickly (Baumeister et al., 1975; Mushett et al., 1952), were also measured. The influence of HCB on the food intake reduction caused by simmondsin was studied.

EXPERIMENTAL PROCEDURES

Extraction and Purification of Simmondsin. Simmondsin was purified following Soxhlet extraction with acetone. After crystallization, the mixture of simmondsin, its demethyl analogues, and simmondsin 2'-ferulate, diluted in water, was put on a Sephadex column for chromatography. The simmondsin fraction, pure on TLC and monitored by IR spectrography, was dried and lyophilized.

Animals. Male Wistar rats of about 150 g were housed by four in plastic cages at 22 °C and a relative humidity of 40–60%. They received light from 8 a.m. to 8 p.m. They received water and food ad libitum (except for the pair-fed group). During the experiment, body weights (BW) and food intake were measured daily.

Experiments. In a first experiment 40 rats were used. (a) Sixteen rats were used as controls (C). (b) Sixteen rats were given daily doses of 250 mg of simmondsin/kg of BW by gavage for 5 days (S). (c) Eight rats were pair-fed with S for 5 days (PF). They received only 47 g of food/4 animals at 5 p.m.; this is exactly the daily amount of food taken by the rats treated with simmondsin. After this period, they were asphyxiated with CO_2 at 8 a.m. Blood was taken by heart puncture, and afterward a full autopsy was done. Liver, left kidney, left testis, and seminal vesicles were weighed and, together with the stomach, intestines, and pancreas, fixed in formalin for histopathological examination (hematoxylin eosin and PAS coloration).

Biochemical blood values were measured for eight control rats (C), eight rats treated with simmondsin (S), and four pair-fed rats (PF). The blood of the other eight control rats (C) and of the other eight simmondsin-treated rats (S) was used for the measurement of the concentrations of cyanide and thiocyanate. Thiocyanate was measured by a colorimetric method, on deproteinized blood, at 460 nm with ferrinitrate; the limit of detection was 0.3 mg of SCN^-/100 mL of whole blood for the given experimental conditions.

Cyanide was measured by gas–liquid chromatography of the headspace of the blood after acidification with acetic acid which liberates the volatile HCN. Chromatography conditions: cap-

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Table I. Food Intake, Weight Evolution, and Weight of the Internal Organs (Expressed as Percent of Their BWs) of Controls (C), of Rats Treated during 5 Days with 250 mg of Simmondsin/kg of BW (S), of Rats Treated during 5 Days with 25 mg of HCB/kg of BW (HCB), of Rats Treated during 5 Days with 250 mg of Simmondsin/kg of BW and 25 mg of HCB/kg of BW (S+HCB), and of Pair-Fed Rats Taking 47 g of Normal Food/Day per Four Animals during 5 Days (PF)[a] (± SEM)

	C	S	HCB	PF	S+HCB
initial weight, g	150.75 ± 1.98	149.05 ± 1.32	160.25 ± 1.64	165.5 ± 1.98	157.5 ± 1.43
final weight, g	173.43 ± 2.31	154.5 ± 2.73	183 ± 2.23	166 ± 1.70	163.87 ± 1.92
difference, g	+22.68 ± 0.65	+5.45 ± 2.04[b]	+22.75 ± 0.79	−0.5[1] ± 1.14[b]	+6.37 ± 2.51[b]
food intake, g/day per 4 rats	64.75 ± 0.82	46.87 ± 2.31[b]	68.26 ± 1.39	47[b]	53.12 ± 2.46
seminal vesicle, % of BW	0.091 ± 0.006	0.051 ± 0.004[b]	0.094 ± 0.006	0.065 ± 0.004[b]	0.056 ± 0.004[b]
left testis, % of BW	0.604 ± 0.015	0.637 ± 0.024	0.592 ± 0.017	0.671 ± 0.014	0.614 ± 0.021
left kidney, % of BW	0.438 ± 0.07	0.478 ± 0.012	0.439 ± 0.056	0.442 ± 0.009	0.497 ± 0.032
liver, % of BW	4.647 ± 0.093	4.831 ± 0.130	4.895 ± 0.123	3.513 ± 0.020[b]	5.386 ± 0.091

[a] Eight animals in each group; ANOVA followed by t-test. [b] $p < 0.01$ compared with C.

illary column (25 m × 0.32 mm i.d.); CP Sil-19 CB; column temperature, 120 °C; detector, NPD; limit of detection, 50 ng/mL of blood for the given experimental conditions (Angerer et al., 1988).

In a second experiment eight rats were injected subcutaneously with a daily dose of 25 mg of hydroxocobalamin acetate (HCB)/kg of BW in 0.5 mL of acetate buffer solution, pH 5, for 5 days (HCB); HCB was a gift of Continental Pharma Belgium. Eight rats received 250 mg of simmondsin/kg of BW by gastric intubation for 5 days; at the same time they received 25 mg of HCB/kg of BW (subcutaneous injection) (S+HCB). After the experimental period, blood was taken by heart puncture after asphyxiation with CO_2. The concentrations of cyanide and thiocyanate were measured in the blood, whereas the internal organs were weighed, fixed in formalin, and examined anatomopathologically, as described above.

RESULTS AND DISCUSSION

Experiment 1. A daily dose of 250 mg of pure simmondsin/kg of BW causes a reduction in food intake of about 27% in young male rats of about 150 g. This provokes a reduced weight gain compared to the controls (Table I). Rats, pair-fed with the simmondsin-treated animals, behave as simmondsin-treated animals as far as BW is concerned. This confirms our results of an earlier study (Cokelaere et al., 1992). Food intake and weight gain of HCB-treated rats did not differ from the controls. The animals receiving HCB and simmondsin simultaneously reacted exactly like the animals taking only simmondsin. Their food intake inhibition was about 18% but was not statistically different from the food intake inhibition of animals receiving only simmondsin (Table I).

These experiments demonstrate the food intake inhibition effect of simmondsin after gastral intubation. The food intake reduction of about 27% after 250 mg/kg of BW simmondsin is significantly less than the inhibition of 60–65% after 120 mg/kg of BW we described elsewhere (Cokelaere et al., 1992). This could possibly be explained if we take into account that the animals of this experiment are very young (about 150 g compared with 350–450 g in the earlier experiments), actively growing and with a high metabolism per kilogram of body weight. Therefore, the breakdown and excretion of simmondsin may be much faster than in older animals. Furthermore, we are currently conducting long-term studies on the influence of simmondsin on growing rats, where an increase in sensitivity to simmondsin from week 4 to week 13 was observed (Cokelaere et al., unpublished results).

The stomach of the PF animals was empty, whereas the stomach of the other animals contained food. Weights of seminal vesicles, left testis, left kidney, and liver are shown in Table I. This table contains also data of animals treated with HCB and with simmondsin and HCB, expressed as percent of BW. Seminal vesicles of S, PF, and S+HCB animals weighted less than those of the control group.

Table II. Biochemical Blood Values of Young Rats (±150 g) after Normal Food Intake (C) (n = 8), after 5 Days of Simmondsin Administration (250 mg/kg of BW) (S) (n = 8), and after 5 Days of Pair-Feeding (11.75 g/Day per Animal) (PF) (n = 4) (±SEM)[a]

	C	PF	S
glucose, mg/dL	106.5 ± 2.53	46.2 ± 4.63[b,c]	94.88 ± 5.78
uric acid, mg/dL	2.8 ± 0.20	1.93 ± 0.42	2.17 ± 0.42
urea, mg/dL	44.33 ± 2.4	32.25 ± 2.65	43.5 ± 2.78
creatinin, mg/dL	0.89 ± 0.01	0.83 ± 0.03	0.80 ± 0.04
bilirubin, mg/dL	0.58 ± 0.14	0.30 ± 0.02[d]	0.54 ± 0.06
total protein, g/dL	6.37 ± 0.17[e]	7.81 ± 0.03	6.53 ± 0.37[e]
lipase, units/L	24 ± 14.04	21 ± 11.7	40 ± 18.9
GOT, units/L	96 ± 37.89	151 ± 12.95[d]	102.75 ± 9.49
GPT, units/L	55.66 ± 21.66	75 ± 24.59	31.75 ± 2.59
alc phosph, units/L	344 ± 49.67	404 ± 46.58	510 ± 76.10
Na+, mmol/L	144.66 ± 0.66	146 ± 0	146 ± 0.57
K+, mmol/L	8.10 ± 0.18	8.13 ± 0.06	8.15 ± 0.38
Cl−, mmol/L	95.33 ± 1.2	94.75 ± 0.62	94 ± 0

[a] ANOVA followed by t-test. [b] $p < 0.001$ compared with C. [c] $p < 0.001$ compared with S. [d] $p < 0.05$ compared with C. [e] $p < 0.05$ compared with PF.

Microscopic investigation revealed no visible difference in histological structure compared with controls, but the glands contained less fluid. The livers of PF animals weighed less than the livers of controls or of animals treated with simmondsin and showed a lower loading with glycogen. This may be explained by the daily fasting period after the fast intake of the reduced ration at the beginning of each feeding period. The other organs investigated anatomopathologically were normal.

The results of the biochemical investigation of the blood are shown in Table II. There was no difference between controls and animals treated with simmondsin for all parameters examined. The animals of the pair-fed group, however, showed significantly lower glucose and bilirubin concentrations and higher GOT and total protein concentrations than controls and the simmondsin group. The low glucose concentration after pair-feeding can be explained as a normal result of a fasting period (empty stomach) in pair-fed animals who ate their reduced daily rations in the first hours of food presentation. Both control animals and those treated with simmondsin had food in their stomachs. Both groups had the same, normal glucose concentration. The values obtained in our experiments are about the normal ranges published for normal rats (Baker, 1980; Benirschke, 1978; Harkness et al., 1989). Therefore, it is concluded that there are no signs of toxicity in liver, kidney, pancreas, stomach, intestine, seminal vesicle, or testis after a period of 5 days of simmondsin administration at 250 mg/kg of BW in young rats. This confirms the results of other authors, who investigated indirectly the toxicological influences of simmondsin and did not find any toxic effects at the anatomopathological level (Manos et al., 1986; Williams, 1980). However, further

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Table III. Concentration of SCN⁻ (mg/100 mL of Blood) and CN⁻ (ng/mL of Whole Blood) in the Blood of Young Male Rats (±150 g of BW) (± SEM)[a]

	C	S	HCB	S+HCB
SCN⁻	0.72 ± 0.068	0.70 ± 0.073	0.66 ± 0.063	0.79 ± 0.083
CN⁻	<50	<50	<50	<50

[a] C, in control animals (n = 8); S, in animals treated with 250 mg of simmondsin/kg of BW (n = 8); HCB, in animals treated with 25 mg of HCB/kg of BW (n = 8); S+HCB, in animals treated with 250 mg of simmondsin/kg of BW and 25 mg of HCB/kg of BW (n = 8).

long-term investigations have to be conducted to study possible toxicity after chronic administration of simmondsin.

Experiment 2. Table III gives the results of the CN⁻ and SCN⁻ analyses of control animals and of animals treated with simmondsin (S), with HCB (HCB), and with simmondsin and HCB (S+HCB). These analyses do not show any increase in the concentrations of CN⁻ or SCN⁻ in animals treated with simmondsin. HCB has no visible effect on these concentrations either, although the methods we used were very sensitive and reliable. This is in contradiction with the results of Williams (1980) in mice, who observed increased CN⁻ and SCN⁻ but used a much higher dose of simmondsin (up to 750 mg/kg of BW instead of 250 mg/kg of BW in the present experiments). However, the concentrations of CN⁻ and SCN⁻ he published were variable, with highest values obtained in one experiment being lower than the control values in another experiment. We doubt that these values can be interpreted as an indication of the liberation of HCN during the metabolism of simmondsin. That is also what can be concluded from our experiments. Elliger et al. (1973) did not find free HCN following the treatment of simmondsin with acid or base.

In the present study no increase of plasma HCN or SCN⁻ in the simmondsin-treated animals was found. If HCN is liberated, it should be sequestered at least partially by the HCB injected in our rats, since every molecule is capable of binding 3 molecules of CN⁻, and converted to cyanocobalamin (Baumeister et al., 1975). In view of the MW of simmondsin (±376 g), HCB (±1347 g), and HCN (±27 g), one can calculate that, if all of the simmondsin was absorbed and totally metabolized, 37.5 mg of simmondsin requires 44.9 mg of HCB to sequester all of the HCN. We injected only 3.75 mg of HCB a day. However, not all of the simmondsin is absorbed (Booth et al., 1974; Williams, 1980). Moreover, simmondsin is likely to be excreted relatively quickly, because of its hydrophilic nature. Therefore, a substantial part of the possible liberated HCN should be sequestered by the injected HCB. Because no differences in the concentrations of CN⁻ or SCN⁻ were observed between the S- or S+HCB-treated and control animals, it is concluded that there are no indications for liberation of HCN during the metabolism of simmondsin in rats. Moreover, no reduction in food intake inhibition after injection of HCB in simmondsin-treated animals was found, and the weight reduction was the same after S+HCB as after S alone. It is concluded that the food intake inhibition induced by simmondsin and the associated weight loss are not caused by HCN.

Further studies on the working mechanism and possible toxic influences of simmondsin have to be conducted to elucidate the mechanism causing the food intake inhibition in animals after simmondsin administration.

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