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The Concentration of Morphine in Serum of Rats Made Dependent Using a Drug-Admixed Food Method

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VAN DER LAAN, J. W., J. G. LOEBER, G. DE GROOT AND V. M. SEKHUIS. *The concentration of morphine in serum of rats made dependent using a drug-admixed food method.* PHARMACOL BIOCHEM BEHAV 31(1) 123–128, 1988.—The present study reports on the induction of physical dependence in rats using morphine-admixed food and addresses the question of the resulting concentration of morphine in serum. The stability of morphine in food is good, since no decrease in concentration could be observed. The concentration of morphine in serum during the experiment was measured using a radioimmunoassay technique. A correlation was found between the food intake during a 7-hour period and the concentration of morphine in the serum at the end of that period, both for a 1 g/kg and a 2 g/kg batch of morphine-admixed food. The concentration of morphine in serum was also found to be dose-related during a period of 6–23 days when the rats were fed for a prolonged period. After long-term administration of 1 g/kg morphine in food a steady-state level of about 0.5 mg/l serum was obtained. Similarly with 2 g/kg morphine in food a steady-state level of 0.8–1.1 mg/l serum was reached. After withdrawal of morphine the serum concentration of morphine dropped to 0.1 mg/l within 24 hours and to below the detection limit within 48 hours. During the withdrawal period sharp drops were noted in body weight (20%) and food intake (50%) after one day.

Morphine dependence Morphine-admixed food Rats Concentration of morphine in serum

FOR the study of morphine dependence in animals a regular administration of morphine for a long period is necessary in order to induce a significant withdrawal syndrome after abrupt abstinence without the use of antagonists. For this purpose a variety of techniques can be used including daily injection of morphine [4], continuous intraperitoneal infusion [8], implantation of pellets [9], a subcutaneous reservoir of morphine solution [2] or dissolution of morphine in the drinking water [1].

The morphine can also be admixed in the food [10] and this method has the advantage of avoiding the stress of surgery or injection during the induction of dependence. However, it is well-known that morphine has a poor bioavailability after oral administration and it appears to be important to confirm that using the food method similar serum concentrations can be obtained as found using the methods mentioned above.

The food method may also be advantageous in providing a natural exposure rhythm, divided over at least twelve hours of the day during the most active period of the animals. A number of authors have studied the degree of dependence produced by the drug-admixed food method [7,10]. However, data concerning the concentration of morphine in the serum or the stability of morphine in the food was not included.

In the present paper data is given regarding the stability of morphine in the food, the relationship between the food intake and the resulting concentration of morphine in serum, and the course of the concentration of morphine in serum during a long period of administration. To check whether the administration of morphine by the food method induces dependence we have measured the body weight loss and food intake during the abstinence phase as these are characteristic symptoms for most kinds of sedative-drug withdrawal [3,10].

METHOD

Animals

Male albino Wistar rats (CPB-TNO, Zeist, The Netherlands), were used in all experiments. The animals were housed individually in wire mesh cages and could obtain food and water ad lib unless otherwise indicated. The food

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consisted of semisynthetic diet (SSP-Tox, Trouw Ltd., Putten, The Netherlands) unless otherwise indicated. During the induction of dependence the food consumption was measured by weighing the food tins on Monday, Wednesday and Friday every week. A light/dark schedule of 12 hours (7.00 a.m. lights on) was maintained. The temperature was controlled at 21–23°C.

Morphine in Food

Morphine was admixed into SSP-Tox food using standard operating procedures, in quantities of 10–15 kg food. The concentration of morphine (base) in the food was 1 or 2 g/kg (w/w). In a preliminary study the stability of morphine was tested in two kinds of food namely:

(a) Hope-farm powdered natural diet and

(b) the semisynthetic protein diet (SSP-Tox).

Samples were taken at day 1, 2, 3, 7, 10, 20 and 30 days after the preparation of the admixed-food in a concentration of 1 g morphine/kg food. During the experiments when the food was offered to the animals 4 random samples of 1 g were taken from the food at weekly intervals. In some cases samples were taken several months after the end of the experiment. On the sampling day morphine was extracted from these samples using ethanol after addition of codeine as an internal standard. The morphine concentration in the ethanol extract was assayed using gas-liquid chromatography and flame-ionization detection after trimethylsilyl derivation of both morphine and codeine.

Morphine in Serum

Blood samples (750 μl) were taken from the retro-orbital sinus under light ether anaesthesia. After clotting serum was prepared. The concentration of morphine in serum was determined using the radioimmunoassay kit Coat-a-Count (Diagnostic Products Corporation, Los Angeles). This assay had a very low cross-reactivity (0.5%) with morphine-3-glucuronide.

Design of the Animal Experiments

Experiment 1. Two groups with 10 rats in each were deprived of food during the first and second night of the experiment. During the daytime from 9.00 a.m. to 4.00 p.m. following these nights the rats had access to morphine-admixed food containing either 1 or 2 g/kg morphine.

At the end of food presentation blood was obtained and serum was prepared for the determination of the concentration of morphine.

Also at the start of the second period blood was taken to obtain a basal value of the concentration of morphine on that day. The amount of food taken over those 7-hour periods was weighed.

After the second day the animals received the morphine-admixed food ad lib. On day 9, 16, 23 and 26 at 9.00 a.m. blood samples were taken, serum was prepared and the concentration of morphine was determined. On day 26 the withdrawal phase was started by changing from morphine-admixed food to control food. (At 3.00 p.m. and on the next day at 9.00 a.m. blood samples were taken.) Blood samples were taken at 3.00 p.m. on that day and at 9.00 a.m. on the following day.

Experiment 2. Two groups each with 26 rats were used. Group I received control food and Group II received morphine-admixed food with a concentration of morphine of 2 g/kg. At weekly intervals starting at day 7 blood was obtained and serum was prepared to determine the concentration of morphine.

On day 38 the morphine-admixed food was changed to control food. However, two injections of morphine HCl (50 mg/kg SC) were given on day 38 and on the morning of day 39 as a substitution for the morphine given earlier in the food. The real withdrawal phase, therefore, started on day 39. During the first three days (day 39–41) after the withdrawal of the morphine food blood was again obtained for determination of the concentration of morphine.

Statistical Analysis

The tests for homogeneity and stability of morphine in the food were carried out by ANOVA. Batch homogeneity was tested using an F-test, while for a test on log-linear decay of food concentration Student's *t*-test was used.

RESULTS

Initially, two kinds of food, a natural and semisynthetic one, were compared with respect to the stability of morphine. In the natural food (Hope-farm) a rapid decrease of the concentration of morphine was found mainly during the first few days after preparation. The concentration stabilized at 60% after 10 days. On the contrary in the semisynthetic SSP-Tox-food the morphine remained stable at 95% of the initial concentration during the whole period of 30 days (Fig. 1).

In the first experiment in the rats the mean body weight ($\pm$SEM) of both groups was 286$\pm$7 and 282$\pm$7 g, respectively. For the first and second day food was available for 7 hours. The concentration of morphine in the serum was related to the food consumption (Fig. 2).

For the 1 g/kg group (Group I) on the first day a regression line was calculated through the origin with a slope B_1 of 0.0182$\pm$0.0062 (95% confidence limits). For the 2 g/kg group (Group II) the slope B_2 was 0.0227$\pm$0.0065. The confidence interval for the difference between the slopes was calculated as $B_2 - 2B_1 = 0.0137 \pm 0.0140$ including zero within the interval. The difference in concentrations of morphine between both groups can therefore be related to the difference in concentration of morphine in the food.

For the second day, the B_1 was 0.0157$\pm$0.0040 and the B_2 was 0.0224$\pm$0.0043. The difference B_2–$2B_1$ was 0.00900$\pm$ 0.00902 and again the hypothesis that in the 2 g/kg group the concentration in the serum was two-fold higher than in the 1 g/kg group, was confirmed.

After these two days, the animals had access to morphine-admixed food ad lib for 24 days. During the whole experiment the absolute morphine intake remained constant since neither the amount of consumed food nor the concentration of morphine in the food changed. The concentration of morphine for the two groups is given in Table 1.

For both groups the F-test revealed a significant difference [for Group I, $F(3,20)=46.3$, $p<0.001$ and for Group II, $F(3,20)=11.12$, $p<0.001$], indicative of either batch-inhomogeneity or a day-dependent difference caused by the assay. There was no significant log-linear decay of morphine in the food.

The concentration of morphine in serum on day 9, 16, 23 and 26 was given in Fig. 3A. The difference in the concentration of morphine between the two groups is evident. After 1 week a steady-state concentration has been reached. Statistical testing was performed by calculating the average con-

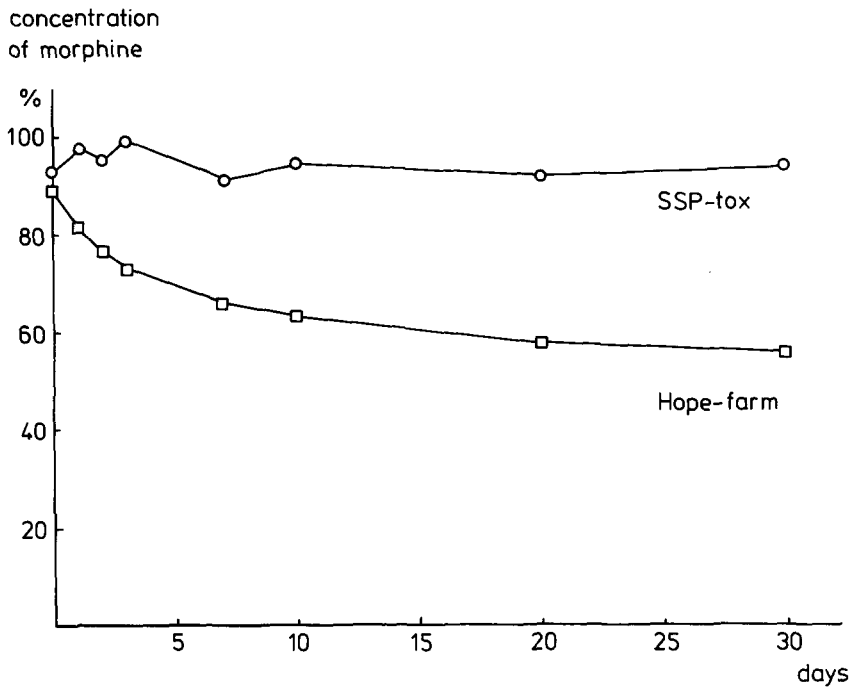

FIG. 1. Stability of morphine in two different kinds of food given as a percentage of the declared concentration. From batches of 1.5 kg of morphine-admixed food (1 g morphine/kg food) samples were taken at various intervals after preparation of the food (day 0) (○) SSP-Tox food; (□) Hope-farm food. The SSP-Tox food contained 20 mg/kg Vitamin E (tocopherol) and 90 mg/kg butylhydroxy-toluene (BHT). The Hope-farm food contained 96 mg/kg Vitamin E and no BHT.

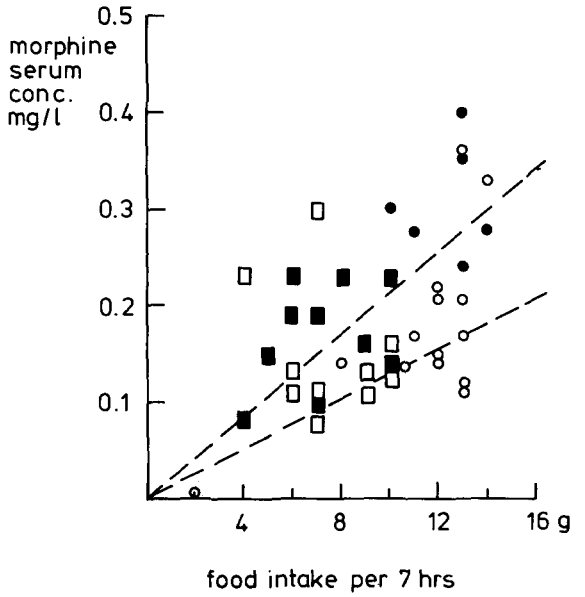

FIG. 2. Relationship between the amount of food intake and the resulting concentration of morphine in the serum. Rats were deprived of food for 24 hours. Thereafter they had access to morphine admixed food (1 or 2 g/kg) of 7 hours of the day for 2 days. At the end of both periods on each day and at the start of the period on the second day blood samples were taken and the concentration of morphine was assayed. The concentration of morphine at the end of the second day was corrected for the concentration of morphine at the start of that period. □: 1st day; ○: 2nd day—morphine 1 g/kg; ■: 1st day; ●: 2nd day—morphine 2 g/kg.

TABLE 1

CONCENTRATION OF MORPHINE IN FOOD

Day	Declared Concentration 1 g/kg	2 g/kg
2	0.88 ± 0.02	1.79 ± 0.02
7	0.89 ± 0.01	1.76 ± 0.02
14	0.87 ± 0.01	1.79 ± 0.03
21	0.78 ± 0.02	1.65 ± 0.03
26	0.87 ± 0.01	1.71 ± 0.05

The concentration of morphine in the food is given in g/kg as mean ± SD with n=4.

The declared concentration is morphine. H_2O whereas the concentration determined in food is calculated as morphine itself.

centration over the whole period. The average concentration for Group I was 0.482 mg/l and for Group II 0.796 mg/l with a pooled variance of 0.0146. This difference appeared to be significant with $p<0.001$. There was no decay with time. The amount of food consumed was the same for both groups (Fig. 3B). Initially the body weight in Group II took somewhat longer to increase than that of Group I but after a week the lines were parallel (Fig. 3C).

Figure 3A shows that within 24 hours of the withdrawal of the morphine-admixed food the concentration of morphine fell below the detection limit. Withdrawal symptoms in both groups were a decrease of 10% in the body weight and a

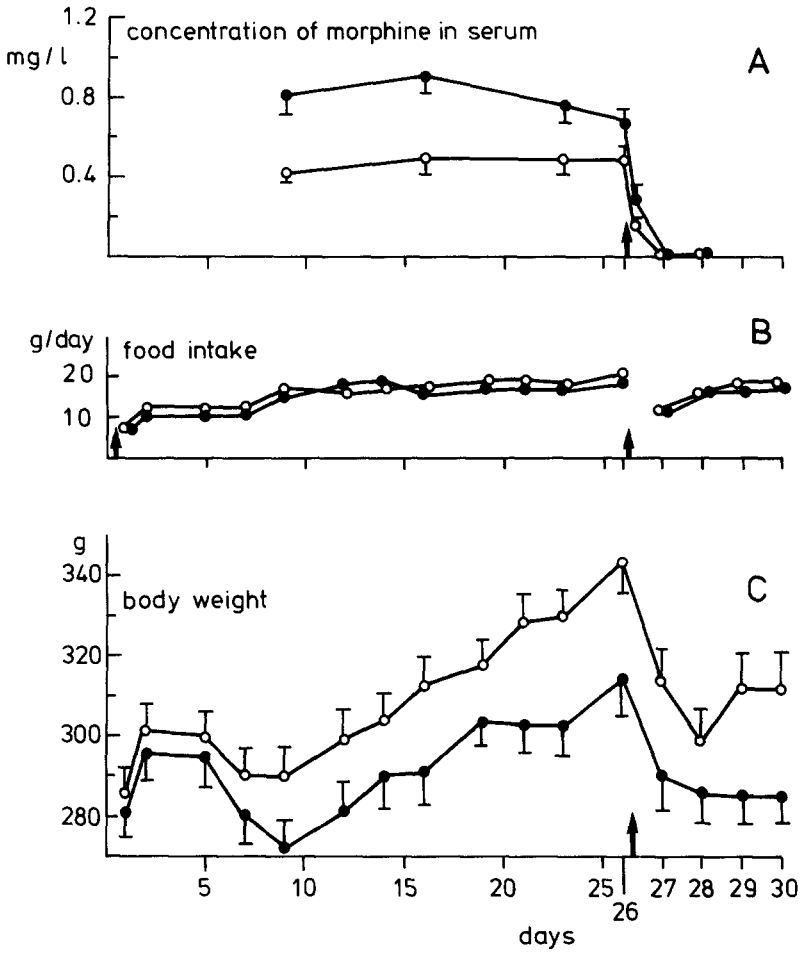

FIG. 3. Morphine dependence: the course of the concentration of morphine in serum (A), the food intake (B) and the body weight (C). Rats were fed with SSP-Tox-food with morphine in two concentrations. The food intake and the body weight were measured every 2 or 3 days. Blood samples were taken on the days indicated. On day 26 (arrow) the morphine admixed food was changed to control food. (○) Morphine in food 1 g/kg; (●) morphine in food 2 g/kg. Each group consisted of 10 rats.

decrease to 60% of the food intake during the first night of withdrawal. By the third day the food intake was restored to normal.

The second experiment was set up to study the effect of morphine itself on body weight and food intake during the induction of dependence and during withdrawal. At the start of the second experiment the body weight (mean±SEM) of the rats was 110±4 g (n=32). The course of the concentration of morphine in serum is given in Fig. 4A. Statistical analysis did not reveal any decay of the concentration during the five-week period, $F(3,8)=0.94$, n.s. The concentration of morphine in food was not determined in this experiment. The food intake in Group II was somewhat smaller than that of the control Group I (Fig. 4B). After 10 days the growth curves of the body weight appeared to be parallel (Fig. 4C).

During the withdrawal phase the lowest food intake was found on day 40 (Fig. 4B) resulting in a minimum body weight on day 41 (Fig. 4C).

DISCUSSION

The present paper shows the morphine-admixed method to be useful in inducing dependence on morphine in rats.

The initial study on morphine stability in two kinds of food revealed a good stability in the semisynthetic food and a decay of morphine in the natural diet. One important difference between these two kinds of food may be the amount of antioxidants such as tocopherol and butylhydroxytoluene, which is much higher in the semisynthetic diet (Van Leewen, personal communication). The determination of morphine in the food during the animal experiments revealed a stability in the semisynthetic diet for longer than 6 months.

A major problem encountered by investigators using oral administration of morphine is refusal by experimental animals to consume significant amounts of morphine, presumably because of its bitter taste [6]. This may explain the smaller food intake in the 2 g/kg group during the first few

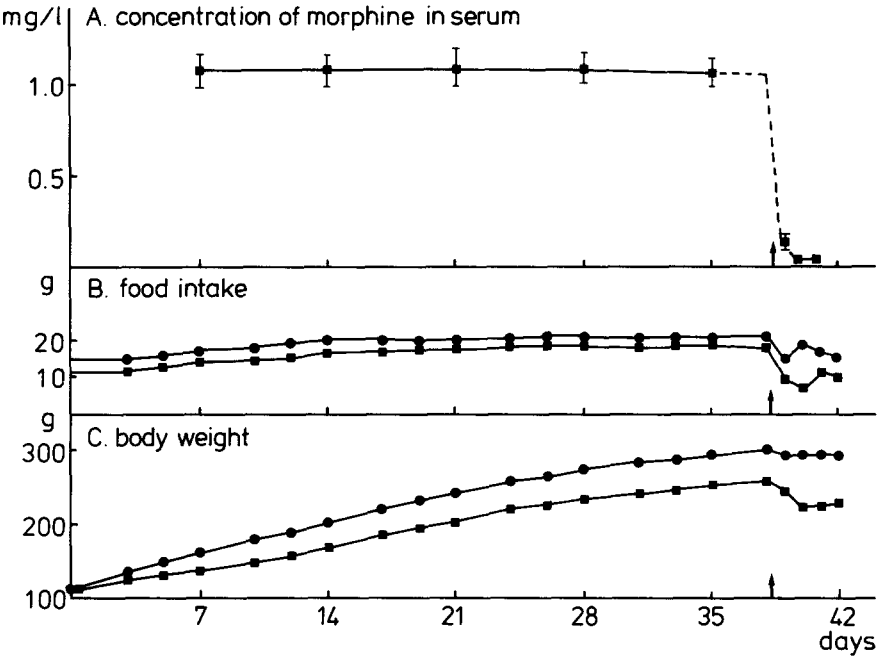

FIG. 4. Induction of morphine dependence comparison with a control-fed group of the course of the concentration of morphine in serum (A), the food intake (B) and the body weight (C). Rats were fed with SSP-Tox-food (●) or SSP-Tox-food with morphine in a concentration of 2 g/kg (■). The food intake and the body weight were measured every two or three days. Once a week blood samples were taken. On day 38 the morphine food in group II was changed to control food. On days 38 and 39 injections with morphine (50 mg/kg SC) were given to group II. Group I received saline injections. For four successive days the animals were weighed daily and their food intake was measured.

days in the first experiment. The problem does not appear to be as great as with morphine dissolved in the drinking water since in our study all animals survived the study in contrast to what has been described by Nichols and Davis [5].

After about one week the animals appeared to be habituated to the food and the daily food consumption did not differ significantly from that of control-food groups.

The daily intake of morphine with a concentration in food of 2 g/kg would be 40 mg and in relation to the body weight 120–140 mg/kg. This dose is similar to the maximal dose used in administering morphine via drinking water [1] and to the amount of morphine used with chronic intraperitoneal infusion [8]. Initially a delay in growth was observed. In older, heavier animals (see the first experiment) a decrease of the body weight was found. After a certain time the growth curves are parallel to that of the control group. The animals fed with morphine remained slightly anorexic as they did not regain the original growth curve of the control group (Fig. 4, second experiment). Such a delay in growth appears to be unavoidable but could be minimized by starting at a lower dose, e.g., 0.5 g/kg and by increasing the concentration of morphine at weekly intervals (results yet to be published).

It is remarkable that the concentrations of morphine in serum can be measured after administration via food. The fast metabolism of morphine after oral administration is apparently not an impediment. There is an approximate correlation between food intake and concentration of morphine in serum (Fig. 2). The variation is substantial, however. This may be caused by the long time period (7 hours) during which the animals had access to the food. Animals usually eat for short periods of about 2–3 hours. When such a period happened to coincide with the start of the 7-hour period the morphine has been partly metabolized since the elimination halflife in rats is about 1–5 hours [8]. On the other hand, when an intake period had ended just before the end of the 7-hour period, there would have been insufficient time to absorb the morphine from the gastroinestinal system. Both factors may be responsible for the variation. Furthermore, it is not known whether rats have dose-related elimination kinetics with regard to morphine. Despite the variability, the statistical analysis did not reject a ratio of 2 for the slopes of the regression lines of both groups.

The difference in the concentration of morphine in the food is also evident in the concentration of morphine in serum after long-term treatment, especially after 9–15 days. Administration of morphine in food (1 g/kg) induced a level of 0.4–0.5 mg/l and using the higher concentration (2 g/kg) a level of 0.8 mg/l was found. In the second experiment the concentration in serum was somewhat higher, possibly due to the lower body weight of the animals. These values represent the concentration a few hours after the end of the period and are nearly the maximal concentrations reached during the day. In the first experiment at the end of the afternoon on the first day of withdrawal the concentration of morphine had decreased to 0.2 mg/l (Fig. 3A). It is reasonable to suggest that the concentration of morphine will fluctuate daily between these extremes. The method described here using morphine-admixed food is therefore similar to the in-

traperitoneal infuson technique for which a concentration of morphine in serum of about 0.8 mg/kg has been reported as well [8].

The present data indicates that there is no tolerance due to increased metabolism in rats with respect to morphine although in Fig. 3 the concentration on day 23 in Group II seemed to be decreased. Analysis of the data did not confirm statistical significance for this decrease.

The degree of dependence can be estimated by measureing the body weight loss and the decrease in food intake [7,10]. The body weight loss was found in both experiments and appeared to be irrespective of the dose of morphine. Yanaura *et al.* [10] could not find a dose-related effect on body weight loss either, whereas other people suggest that it may be related to the duration of exposure to morphine [7]. It is possible that in our experiments a maximal effect is present. The food intake showed a decrease during the first few days of the withdrawal phase. It appears to occur for all drugs with a sedative effect on behaviour such as narcotics, benzodiazepines and barbiturates [10].

In conclusion, the morphine-admixed food method will be a useful method to induce dependence in rats.

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