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Normocalcemic Effect of Gallium Nitrate in a Hypercalcemic Rat Model[1]

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ABSTRACT

An established rat hypercalcemia model was used to study the effects of gallium nitrate on elevated serum calcium levels. Gallium nitrate was administered by i.v. or i.p. injection at daily doses of 0.07–0.45 mmol/kg for 5 days to the hypercalcemic rats beginning 1 day following surgery. A dose-correlated normocalcemic response was observed. Gallium nitrate administered late after the induction of the hypercalcemic state was also effective in reducing serum calcium levels. The p.o. administrations, however, even at doses as high as 0.45 mmol/kg, did not reduce serum calcium to normal levels. The values of area under the concentration *versus* time curve (0–24 h) of gallium in normal rats were comparable after i.v. [49.2 (μg/ml)h] or i.p. [57.0 (μg/ml)h] injections. In contrast, the p.o. route achieved only 15% bioavailability, which may explain the ineffectiveness of p.o. administered gallium nitrate at that dose level. This study suggests that daily i.v. bolus injections of gallium nitrate for managing hypercalcemia may be potentially as effective as the current regimen of continuous i.v. infusion.

INTRODUCTION

Hypercalcemia in cancer patients not only causes dehydration, nausea, stupor, and coma but also can become a potentially fatal disorder (1). Gallium nitrate, one of several calcium-lowering agents, which also includes calcitonin, mithramycin, and bisphosphonates, is currently used for managing cancer-related hypercalcemia (2–5). Gallium nitrate has been reported to inhibit bone resorption *in vitro* and *in vivo* by directly affecting osteoclast function (6–12). It does not inhibit the activity of osteoblasts or interfere with mineralization in bone formation, but it does increase the number of osteoclasts (13). One of the causes of cancer-related hypercalcemia has been demonstrated to be the PTH[5]-related protein (14–19). This protein binds to PTH receptors in bone and kidney, increases the level of renal adenylate cyclase, and causes hypercalcemia in rodents and in patients with cancer (18, 19). Unlike other antihypercalcemic drugs, gallium nitrate causes no cytotoxic reactions in bone (10, 12, 13). Gallium nitrate is effective in treating patients with refractory hypercalcemia caused by metastatic parathyroid carcinoma (20). Conventional antihypercalcemia treatment in cancer patients uses continuous i.v. infusions of gallium nitrate. To investigate the effect of the delivery route of gallium nitrate on hypercalcemia, we used an animal model of hypercalcemia originally implemented to investigate hyperparathyroidism (21, 22).

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[5] The abbreviations used are: PTH, parathyroid hormone; rPTH, rat parathyroid hormone (peptides 1–34); PTX, parathyroidectomy; AUC, area under the concentration × time curve.

MATERIALS AND METHODS

Alzet osmotic minipumps (Model 2001) were purchased from Alza Corp. (Palo Alto, CA). L-Cysteine·HCl and rPTH (peptides 1–34) were obtained from Sigma Chemical Co. (St. Louis, MO). Gallium nitrate solution (each ml contains 25 mg gallium nitrate and 28.75 mg sodium citrate dihydrate) was supplied by Fujisawa USA, Inc. (Deerfield, IL). Metofane was purchased from Pitman-Moore, Inc. (Mundelein, IL). Male Sprague-Dawley rats weighing 125–150 g were purchased from Harlan Sprague-Dawley, Inc. (Indianapolis, IN). Normal rat serum obtained from Pel-Freez Biologicals, Inc. (Rogers, AR), was used for preparing standards and for diluting samples. A calcium analyzer (NOVA 7; NOVA Biochemical Corp., Waltham, MA) was used for determining serum total calcium concentrations. Gallium concentrations of the metal in plasma were determined on a Model AA-1475/GTA atomic absorption spectrophotometer (Varian Instrument Division, Palo Alto, CA). Because the metal has low volatility, gallium levels were determined by adapting the methodology that uses platinum as a modifier to allow higher temperatures to be used for efficient ashing and charring of samples, thereby improving sensitivity of detection (22).

Rat Hypercalcemia Model. We used a hypercalcemia model described previously (21, 22). Rats, under metofane anesthesia, underwent PTX, followed immediately by rPTH (2.1 units/h) infusion through a s.c. implanted minipump in the dorsal area between the scapulae. rPTH was freshly prepared for each experiment in 150 mM NaCl containing 2% cysteine·HCl, pH 2.1 (23). For initial experiments, minipumps were weighed before and after being filled with rPTH to obtain the exact volume of the solution within each filled pump.

Treatment of Hypercalcemic Rats with Gallium Nitrate. Gallium nitrate diluted in 0.9% NaCl was administered i.v., i.p., or p.o. to rats 1 day following PTX and rPTH infusion for 5 consecutive days. The p.o. administration was performed using an 18-gauge, ball-tipped feeding needle. One-half-inch-long 32-gauge needles were used for all other injections. Unless otherwise stated, i.v. injections were made through a tail vein after the tail had been warmed under a desk lamp and rubbed with alcohol swabs. The dose volume was no more than 0.25 ml for i.v., stomach, and intestinal injections and was less than 0.5 ml for i.p. or p.o. administration. Preliminary studies showed that the extent of hypercalcemia in vehicle control rats varied from experiment to experiment. Therefore, a group of control rats was always included in each experiment. When various routes of administration were studied in the same experiment, in order to keep a manageable number of animals only one group of vehicle control rats could be included. The control rats received i.p. saline after undergoing the same PTX and rPTH (2.1 units/h) infusion regimen as did the gallium-treated animals. Preliminary studies showed that the vehicle solution 0.9% NaCl with or without citrate buffer gave similar results. For convenience, 0.9% NaCl without citrate buffer was used for diluting gallium nitrate throughout the study. Approximately 0.8 ml of blood was collected on the days stated in each experiment from metofane-anesthetized rats by cardiopuncture, and plasma was separated by centrifugation. A portion of the plasma was stored at −20°C until assayed for gallium concentration; serum calcium levels were determined with fresh plasma within 1 h of cardiopuncture. Unless otherwise stated, P values were determined using Student's t test.

Effect of Delayed Gallium Nitrate Treatment on Calcium Levels. Three groups of rats underwent PTX and rPTH (2.1 units/h) infusion. Group 1 (9 rats) began gallium nitrate (0.3 mmol/kg) treatment 2 days following surgery and rPTH infusion (day 3) for a total of 5 doses. Group 2 (4 rats) started the treatment on day 4 for a total of 4 doses, and group 3 (4 rats) began on day 5 for a total of 3 doses. All of the animals received the drug by daily i.p. injections up to day 7. Serum total calcium levels were measured just before the beginning of the gallium nitrate treatment and then every second day.

Comparison of Bioavailability after p.o., Stomach, and Intestinal Injections. A midabdominal incision was made to expose the intestine or stomach of normal rats (no PTX or rPTH infusion) anesthetized with metofane. For

intestinal injections, one ligation using 3-0 silk thread (Ethicon, San Angelo, TX) was made at the pylorus, and the injection was made about 2 cm below the ligation, with the needle pointed away from the pylorus. For stomach injections, an additional ligation was made 1 cm below the first one to ensure that the drug would not leak into the intestine from stomach injections. The injections were made in the middle of the stomach. For comparison, some rats with no ligations were also included. The incision was stapled with metal clips following the injection. After 0.07, 0.39, and/or 0.67 mmol/kg gallium nitrate were given by i.v., p.o., stomach, or intestinal injections, blood was collected in tubes with heparin at 0.17, 0.50, 0.75, 1, 2, 3, and 4 h. Using the trapezoidal rule method (24), plasma gallium $AUC_{0-4\ h}$ values were calculated following administration by different routes. The AUC values after p.o., stomach, or intestinal injection were compared with those after i.v. of the same dosage to obtain percentage bioavailability (24).

Comparison of Gallium Pharmacokinetics after p.o., i.p., and i.v. Administration. An extended 24-h bioavailability study of total gallium was carried out in normal rats to compare the absorption of gallium nitrate after p.o. and i.p. administration to that after i.v. administration. The i.v. injections were made through the saphenous veins. For total gallium determinations, 12 plasma samples (~0.4 ml/sample) were obtained from 3 groups of 5 rats each. Four blood samples were drawn from each rat at different times within 24 h of i.v., i.p., or p.o. administration: from group 1 (5 rats) at 5, 15, and 30 min and 6 h; from group 2 (5 rats) at 1, 2, 4, and 8 h; and from group 3 (5 rats) at 12, 16, 19, and 24 h. Cp_{max} was defined as the highest plasma gallium concentration actually achieved. For i.v. administration, the Cp_{max} was obtained by extrapolating to the time the injection ended. The values of $AUC_{0-24\ h}$ were calculated and total clearance (Cl_T) was then estimated (24). The disappearance curve was analyzed with RSTRIP software (25). The difference, if any, between the i.v. and i.p. elimination phase was analyzed using multivariate ANOVA (26).

RESULTS

Hypercalcemia Model in Rats. For initial experiments, minipumps were weighed before and after being filled with freshly prepared rPTH solutions. The results showed that the volume filled in 34 pumps in 5 separate experiments was 217 ± 8 μl (SD) (coefficient of variance, 0.4%). This volume was reproducible and sufficient to deliver rPTH continuously at a rate of 1 μl/h. Rats became hypercalcemic 24 h after PTX and rPTH infusion (2.1 units/h); the serum total calcium levels increased from normal (10.7 ± 0.7 mg/dl; range, 9.0–11.7 mg/dl; 25 samples from 5 rats) to a hypercalcemic state (14.6 ± 1.9 mg/dl; range, 12.9–18.5 mg/dl; 22 samples from 5 rats). If rPTH was not infused, in less than 24 h the rats became hypocalcemic (calcium levels decreased to 6.8 ± 1.1 mg/dl; range, 5.1–8.1 mg/dl; 5 samples from 5 rats). Histopathological studies of randomly selected trachea samples from which the parathyroid glands had been removed demonstrated that no residual parathyroid gland was present. In fact, in all cases thyroid glands were also partially removed.

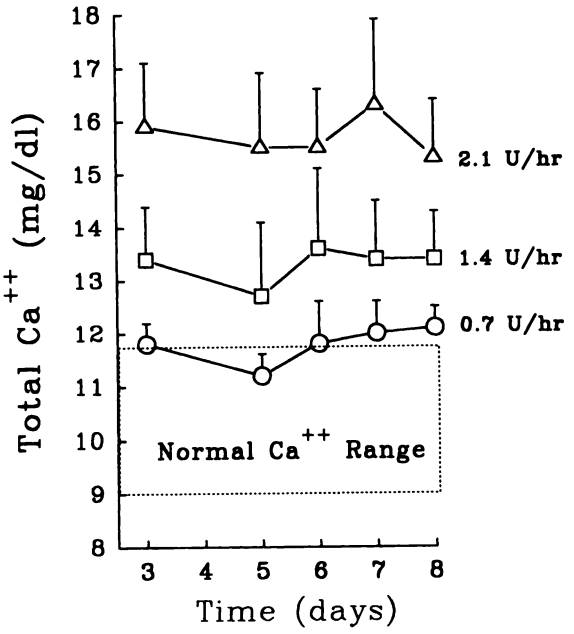

Fig. 1. Effect of different rPTH infusion rates on serum total calcium levels in rats. *Points*, mean; *bars*, SD (n = 5); *U*, units.

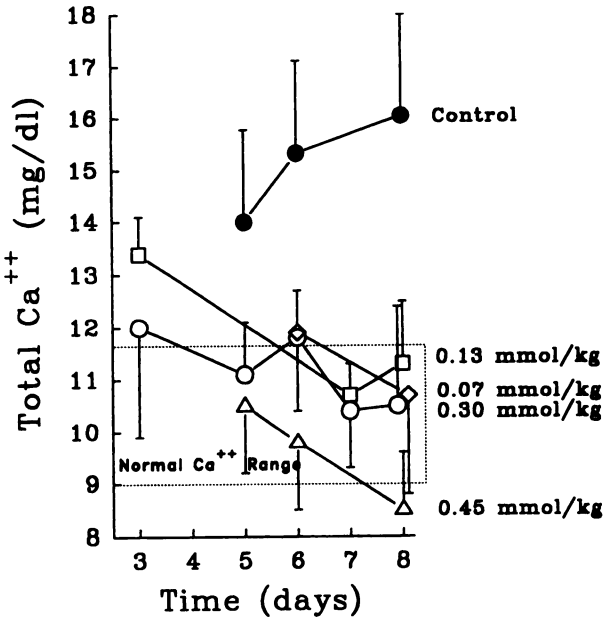

Fig. 2. Effect of various i.p. gallium nitrate doses on serum total calcium levels in hypercalcemic rats. *Points*, mean; *bars*, SD (n = 6–8 rats). All animals received 2.1 units/h rPTH (1–34) post-PTX. Control animals were given i.p. saline.

rPTH Dose Effects on Serum Calcium Levels. A rPTH dose-related response on serum total calcium levels was observed (Fig. 1). The lowest rPTH rate of infusion (0.7 unit/h) did not significantly elevate the serum calcium levels above normocalcemic levels. The next higher rate (1.4 units/h) resulted in the development of hypercalcemia, but the calcium levels were not as high as those after infusion with the highest rPTH infusion rate (2.1 units/h) (Fig. 1). Results from a separate experiment indicated that the serum calcium levels rose within 18 h after rPTH infusion of 2.1 units/h in parathyroidectomized rats (results not shown). An infusion rate of 2.1 units/h was therefore chosen for all subsequent experiments.

Comparison of Serum Total Calcium Levels in Rats after Various Gallium Nitrate Doses by i.p. Injections. Gallium nitrate doses at 0 (control), 0.07, 0.13, 0.3, and 0.45 mmol/kg were administered by i.p. injection to rats. Five daily gallium nitrate treatments began 1 day following PTX and continuous rPTH infusion (2.1 units/h) initiation. Control animals received i.p. saline following the same schedule as that for gallium nitrate. Gallium nitrate produced a dose-related effect on serum calcium levels (Fig. 2). On day 3, rats receiving higher doses of gallium nitrate (0.3 and 0.45 mmol/kg) had normocalcemic levels, whereas rats receiving lower doses (0.07 and 0.13 mmol/kg) remained hypercalcemic. Although administration of lower doses (0.07 and 0.13 mmol/kg) eventually reduced calcium levels to the normal range, it took longer to do so than it did using the higher doses (0.3 and 0.45 mmol/kg) (Fig. 2).

Comparison of Serum Total Calcium Levels in Rats after Different Routes of Administration of Gallium Nitrate. Administration i.p. of gallium nitrate (0.13 mmol/kg) produced a normocalcemic effect similar to those found after i.v. injections (Fig. 3). When

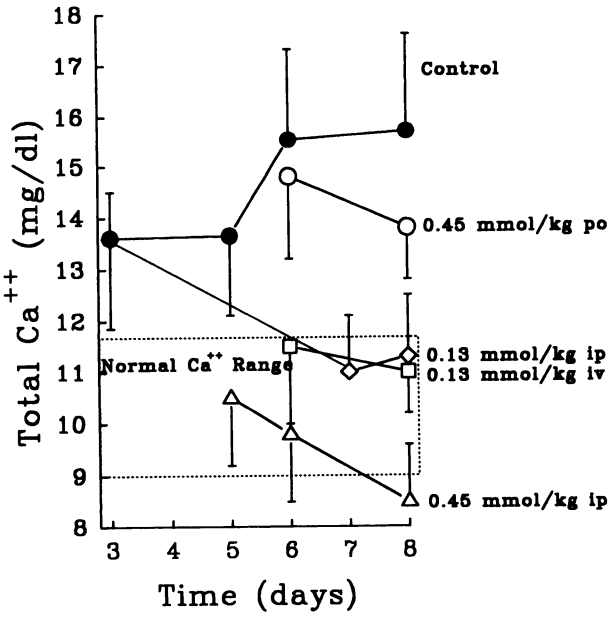

Fig. 3. Comparison of serum total calcium levels in hypercalcemic rats after i.v., i.p., and p.o. administration of gallium nitrate: 6 rats each given 0.13 mmol/kg i.p., 0.45 mmol/kg i.p., or vehicle control; 10 rats given 0.45 mmol/kg p.o.; and 4 rats given 0.13 mmol/kg i.v. Treatment began 1 day after PTX and initiation of 2.1 units/h rPTH (1–34). *Points*, mean; *bars*, SD.

another group of rats received gallium nitrate by p.o. administration at the highest i.p. dose tested (0.45 mmol/kg), on day 6 the calcium levels did not differ ($P > 0.07$) from those in control animals. However, on day 8, a limited but significant ($P = 0.01$) decrease in serum calcium levels was observed when compared with those of vehicle control rats; however, the levels were still hypercalcemic (Fig. 3). This mild decrease was negligible when compared with the normocalcemic effect demonstrated by the same dose given by i.p. injection.

Effect of Delayed Gallium Nitrate Treatment on Reversal of Hypercalcemia. Three groups of rats began daily gallium nitrate (0.3 mmol/kg) treatment on day 3, 4, or 5 after PTX and rPTH initiation for 5, 4, or 3 doses, respectively. Their serum total calcium levels were first measured on the day prior to treatment and then every other day before the daily treatment was given (Fig. 4). A 2-day course of gallium nitrate therapy was required to bring serum calcium levels into the normal range regardless of the starting time for drug administration (Fig. 4).

Bioavailability Study. Gallium nitrate injected into the nonligated stomach of normal rats yielded low plasma gallium levels, which were even lower when 2 ligations were made (Fig. 5). On the other hand, gallium nitrate given by p.o. gavage or injected directly into the intestine (with 0 or 1 ligation) produced higher gallium levels (Fig. 5). The gallium levels in rats after gallium nitrate injection into ligated or nonligated intestines were not significantly different ($P > 0.18$). Therefore, the results from both groups were combined for analysis. In comparison with the levels produced by i.v. injections, however, these levels were still low; the 4-h gallium bioavailabilities after p.o., intestinal (0 or 1 ligation), stomach (0 ligation), or stomach (2 ligations) administrations were 11.2, 6.1, 2.3, and 0.7%, respectively.

Disappearance Curve of Gallium in Plasma. Total gallium levels measured in plasma at various time points following i.v., i.p., or p.o. administration of gallium nitrate at a dose of 0.13 mmol/kg are shown in Fig. 6. Following p.o. administration of gallium nitrate, the maximum absorption of gallium did not occur until 2 h. The bioavailability was only 15% at 24 h. Five min after i.p. injection, gallium was already measurable in plasma, and the gallium level reached a peak in 30 min. Initially, these levels were much lower than those obtained by i.v. injection; but once gallium was absorbed, the plasma gallium levels and the $AUC_{0-24\ h}$ values were comparable for both routes of administration. In fact, i.p. injections yielded somewhat higher $AUC_{0-24\ h}$ values [57.0 (μg/ml)h] than did i.v. injections (49.2 (μg/ml)h], although the difference was not statistically significant. The disappearance curves of both routes were biphasic, and the half-lives of the elimination phases were 31 and 26 h for i.v. and i.p. routes, respectively. The pharmacokinetic parameters are summarized in Table 1.

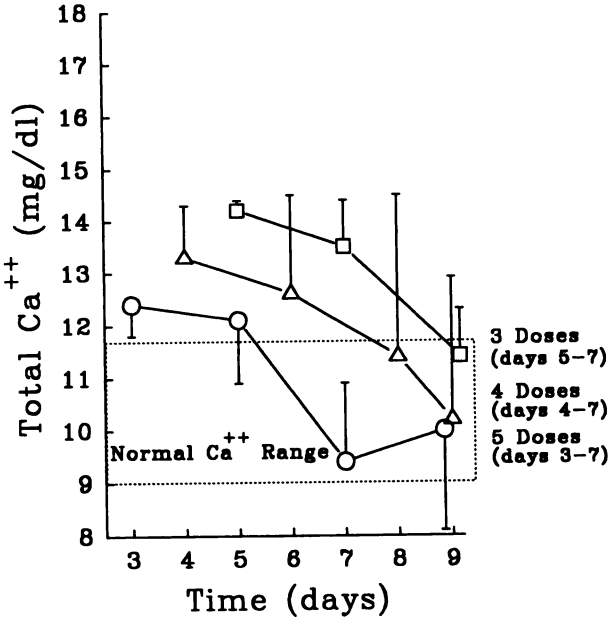

Fig. 4. Effect on serum total calcium levels of delaying start of gallium nitrate (0.3 mmol/kg) treatment until day 3, 4, or 5 after PTX and initiation of 2.1 units/h rPTH (1–34). Each group consisted of 5 rats. *Points*, mean; *bars*, SD.

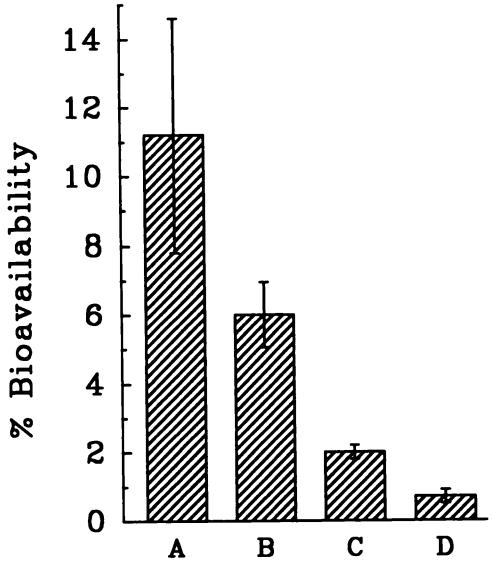

Fig. 5. Four-h bioavailability of total gallium in normal rats following administration of gallium nitrate by various routes. *A*, p.o. (0.07 mmol/kg, 9 rats; 0.39 mmol/kg, 2 rats; and 0.67 mmol/kg, 5 rats); *B*, intestinal route (0.07 mmol/kg, 6 rats with 0 ligations; 0.39 mmol/kg, 1 rat with 0 ligations and 1 rat with 1 ligation); *C*, stomach route (0.39 mmol/kg, 4 rats with 0 ligations); *D*, stomach route (0.39 mmol/kg, 3 rats with 2 ligations). *Columns*, mean; *bars*, SD.

Table 1 *Pharmacokinetic parameters of total gallium in rats following i.v., i.p., or p.o. administration of gallium nitrate (0.13 mmol/kg)*

Route of Administration	T_{max} (h)	Cp_{max} (ng/ml)	$Cp_{24\ h}$ (ng/ml)	$AUC_{0-24\ h}$ (μg/ml)h	Cl_T ml(min·kg)	Bioavailability (%)
i.v.		29,200	1,264[a] ± 354	49.2	3.1	100
i.p.	0.5	7,900[a] ± 1,028	1,636 ± 136	57.0	2.7	116
p.o.	3.0	839 ± 292	82 ± 62	7.6	3.0	15

[a] Data are presented as mean ± SD.

DISCUSSION

Several hypercalcemia animal models are available (12, 21, 22, 27–29). We elected to use a model originally developed in rats to simulate hyperparathyroidism in humans (21, 22) that is excellent for testing antihypercalcemic agents. The surgery (PTX and implantation of the Alzet pump) is easy to perform, and the animal survival rate has been 95% in our laboratory. Gallium nitrate has been shown to inhibit *in vitro* PTH-mediated release of calcium from bone (9) and to be effective in treating rodents (12) and humans with refractory hypercalcemia caused by metastatic parathyroid carcinoma (9, 20). The PTH-related protein has been shown to be responsible for hypercalcemia in patients with cancer (17–19). Preliminary studies have shown that i.p. gallium nitrate (0.45 mmol/kg) does not affect serum calcium levels in normal rats (data not shown). Our studies also show that gallium nitrate is very potent because it is effective even when treatment is started late in the course of the metabolic disorder.

Only 15% of p.o. administered gallium nitrate is bioavailable, suggesting that larger doses may be required to achieve effective treatment or that new gallium ligands with improved bioavailability should be formulated. However, our studies demonstrated that daily i.v. bolus injections of gallium nitrate were effective in decreasing blood calcium levels to normal range. The cumbersome continuous i.v. method of administration could thus be replaced by daily bolus injections. Furthermore, daily i.p. injections were as effective as daily i.v. injections, producing equally high plasma gallium concentrations and high AUC values. Warrell *et al.* (1) noted that rodents had excellent bioavailability of gallium in blood and bone after they received s.c. injections of gallium nitrate. It was also demonstrated that gallium nitrate given s.c. significantly decreased serum calcium levels in hypercalcemic nude mice bearing a canine adenocarcinoma, a model of humoral hypercalcemia of malignancy (12). Our studies indicated, furthermore, that rats receiving a gallium nitrate dose of 0.13 mmol/kg by either i.v. or i.p. injection had plasma gallium concentrations above 1000 ng/ml for at least 24 h. The pharmacokinetic studies suggested that a threshold plasma gallium concentration of approximately 1000 ng/ml must be attained to achieve acute normalization of elevated serum calcium levels (1). A gallium nitrate dose of 0.13 mmol/kg/day in rats is equivalent to 0.78 mmol/m^2/day or approximately 200 mg/m^2/day in humans. This dose, given as a continuous infusion, was previously used successfully for treating 21 cancer patients with hypercalcemia (1). Therefore, a daily schedule of bolus i.v., s.c., or i.p. injections of gallium nitrate at a dose of 200 mg/m^2/day is recommended for future clinical trials to investigate its antihypercalcemic effect. The safety of this regimen should be carefully evaluated in clinical trials. While a good model of hypercalcemia, this model does not lend itself to toxicity assessment. In our experiments, both saline control and drug-treated rats underwent multiple cardiopunctures and surgeries (PTX and Alzet pump implantation). Both control and treated animals exhibited piloerection, and daily weighing indicated that both groups had similar weight losses (data not shown). For example, weight loss in gallium nitrate-treated rats on day 6 after 5 days of the highest dose (0.45 mmol/kg; i.v., i.p., or p.o. injection) did not differ significantly from that after a lower dose (0.13 mmol/kg, i.v. injection) ($P = 0.11$) or from that in control rats ($P > 0.3$).

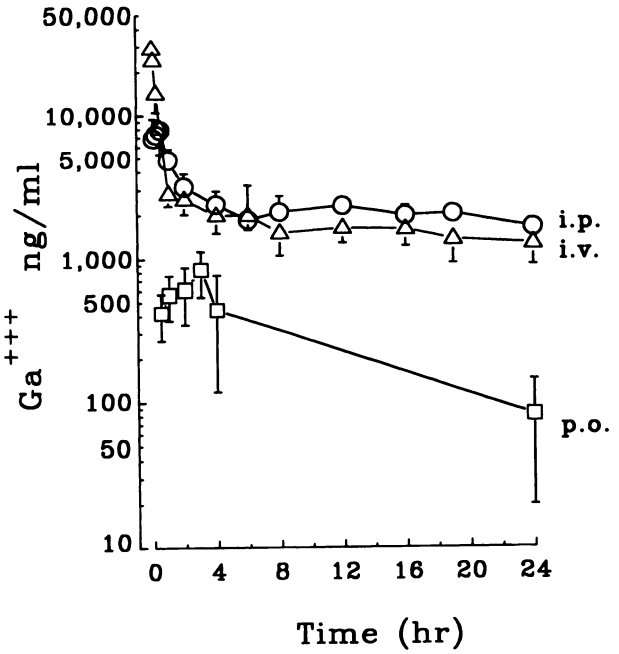

Fig. 6. Plasma total gallium disappearance curve in normal rats following i.v., i.p., or p.o. administration of gallium nitrate (0.13 mmol/kg). *Points*, mean; *bars*, SD.

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