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Drug-Containing Gelatin Treats as an Alternative to Gavage for Long-Term Oral Administration in Rhesus Monkeys (*Macaca mulatta*)

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Long-term oral administration of immunosuppressive agents to transplanted rhesus monkeys (*Macaca mulatta*) is one of the major challenges in such studies. To avoid the drawbacks of gavage, we tested an alternative method for oral dosing of sirolimus in rhesus monkeys by adding sirolimus, a commonly used immunosuppressant, to gelatin to create drug-containing gelatin 'treats' that our macaques would accept voluntarily. We evaluated the oral bioequivalence of the oral solution and drug-containing gelatin and assayed the whole-blood levels of sirolimus after long-term drug delivery. We found that time to peak concentration but not peak concentration itself or the area under the time–concentration curve differed between the 2 groups. Although the maximal concentration data did not fit the condition of bioequivalence, those for the time–concentration curves from 0 to 24 h and from 0 h to infinity did; therefore the extent of sirolimus absorption did not differ significantly between the 2 formulations. The sirolimus levels for long-term drug delivery were equivalent at 2.97 ± 1.91 ng/mL in the gelatin group and 3.13 ± 2.03 ng/mL in the solution group. The gelatin dosing technique we describe here is convenient and effective for oral administration of sirolimus in rhesus monkeys and likely can be adapted for other drugs.

Abbreviation: AUC, area under the concentration–time curve.

Rhesus monkeys (*Macaca mulatta*) are often used as recipients in preclinical transplantation studies[7,14] but, in our experience, achieving the necessary long-term administration of oral immunosuppressive agents to transplanted rhesus monkeys often is challenging.

Sirolimus, a commonly used immunosuppressive agent consisting of macrocyclic lactone compounds extracted from absorbent chain enzyme fermentation products, is used mainly to prevent and treat transplantation rejection of renal, hepatic, and other grafts.[19] Available oral formulations of sirolimus include solution, tablets, and soft capsules. Because sirolimus displays low water solubility, poor palatability, and rapid degradation, there are many drawbacks to its use in laboratory rhesus monkeys. Although oral gavage can overcome poor palatability and facilitate accurate dosage, it can lead to respiratory interference, damage to the stomach or esophagus, and granulomatous inflammation after repeated dosing.[3] Furthermore, accurate administration of drugs by gavage requires manual restraint, which can be stressful to subjects, particularly nonhuman primates,[3,4,11] which can be difficult to handle and restrain when conscious. Handling-associated stress has significant physiologic effects and may alter experimental outcomes.[8] Gavage of large animals usually is accomplished through the use of flexible cannulas to minimize potential esophageal damage, but this modification does not eliminate handing- or restraint-associated stress. Anesthesia of subjects with agents such as ketamine prior to gavage may prevent handling associated stress but is known to cause unidentified toxicity and adverse side effects such as dizziness, sedation, loss of appetite, nausea, and vomiting.[6,12]

Many alternative dosing methods for rodents have been described recently, including premixed drug-containing chocolate pellets for rats.[10] However, chocolate is a complex mix of compounds, some of which may affect the pharmacokinetics of sirolimus, and the theobromine and caffeine in chocolate show moderate toxicity.[18] Another alternative oral dosing method involved teaching rats to drink a mixture of fruit juice, syrup, and drug solution from a syringe, thereby allowing accurate drug dosing and reducing stress to the animals.[2] This method was used in rhesus monkeys but to be effective required the presence of a caretaker with whom they were familiar.[5] To our knowledge, an effective alternative oral dosing method for rhesus monkeys has not previously been developed. We here describe an effective and reproducible method for voluntary oral drug administration to rhesus monkeys. Specifically, we incorporated sirolimus into fruit-flavored gelatin. After a short training period, the macaques readily consumed the drug-containing gelatin 'treats.' The aim of the current study was to evaluate the uniformity of the gelatin formulation and the bioequivalence of sirolimus between the gelatin and solution dosage forms.

Materials and Methods

Animals. Rhesus monkeys (5 female and 5 male; age, 3 to 7 y; weight, 5.0 to 8.0 kg) were obtained from the Chengdu Ping'an Experimental Animal Reproduction Center (Chengdu, Sichuan, China). Procedures involving animal care and use were conducted at the National Center for Safety Evaluation of Traditional Chinese Medicine (Chengdu, Sichuan, China), under

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the guidance of the institutional Animal Laboratory Protocol and as approved by its IACUC. The macaques were kept in an air-conditioned room (20 to 25 °C) with lights on from 0800 to 1800. All of the macaques were shown to be healthy based on medical history, physical examination, electrocardiogram, and routine laboratory tests (blood chemistry, hematology, and urine analysis). The macaques did not receive any other medication starting 2 wk before the study and continuing throughout the study.

Preparation of sirolimus-containing gelatin and uniformity test. For preparation of 100 mL gelatin stock, 5 g gelatin powder (Jidianmingjiao, Chengdu, Sichuan, China), 5 g sucrose (Kelong Chemical, Chengdu, Sichuan, China), and 5 g imitation orange flavoring (Guanlidabishi Food, Shenzhen, Guangdong, China) were combined in a 200-mL glass bottle; water was added to a volume of 86 mL; and the mixture was heated and stirred until the gelatin dissolved completely. The gelatin stock was cooled to 45 °C, supplemented with 14 mL sirolimus oral solution (1 mg/mL, Zhongmei Huadong Pharmaceutical, Hangzhou, Zhejiang, China) or 14 mL water, mixed well, and immediately poured into a mold (12 mL per well). A key point is that the gelatin stock should be only as warm as needed to remain clear and fluid, to ensure optimal mixing with the sirolimus solution. In addition, the volume of drug-containing gelatin solution can be adjusted to yield the correct dose per treat. The gelatin treats were stored at 4 °C.

To evaluate the uniformity of the sirolimus–gelatin treats, we individually weighed 10 treats and transferred each to a 100-mL volumetric flask containing 95% methanol. The samples were sonicated for 10 min followed by mechanical shaking for 45 min and filtered through a 0.45-µm membrane. Each sample was diluted with 95% methanol and analyzed by HPLC.

Sirolimus administration and training of rhesus monkeys. For the macaques in the oral solution group, sirolimus was administered by gavage. For this procedure, the macaques were manually restrained against the steel-bar grating in the cage, and the drug was delivered into the stomach via a flexible cannula inserted into the esophagus. Macaques that displayed poor compliance subsequently were anesthetized by using ketamine prior to gavage.

Macaques in the sirolimus–gelatin group were trained to eat treats without drug prior to administration of sirolimus-containing treats. Caretakers familiar to the macaques gave them the nontreated gelatin treats daily, rewarding them with apples when they ate the treats. This training continued until the macaques readily voluntarily accepted the treats; many macaques became excited when handed a gelatin treat and ate it within 1 min. Others even took the treat directly from the caretaker's hand. The macaques were trained to eat the sirolimus-containing gelatin treats in the same way as for the drug-free treats.

Pharmacokinetics assays. ***Bioequivalence assay of sirolimus-containing gelatin and oral solution.*** The bioequivalence study was performed by using a 2-phase crossover design with a 4-wk washout period between phases. In each phase, after overnight fasting, 4 macaques received a single dose of 0.6 mg/kg (the minimal human dose suggested in the insert provided with the sirolimus oral solution) of either sirolimus–gelatin treats or oral solution. Macaques received and readily consumed the gelatin treats in their home cages in the morning prior to feeding; sirolimus oral solution was administered by gavage. Blood samples (1 mL each) were collected from the great saphenous vein before (0 min) and at 0.5, 1, 1.5, 2, 3, 4, 6, 8, 12, 18, and 24 h after drug administration. All samples were stored at −20 °C until sample preparation for HPLC.

Sirolimus levels after long-term administration. We randomly assigned 10 macaques each to receive either sirolimus–gelatin treats or sirolimus oral solution to 0.2 mg/kg daily (the dose suggested in the package insert for long-term administration to human patients) for 1 mo. Blood samples were collected 24 h after the last dose, and sirolimus levels were determined by HPLC.

Sirolimus quantification by HPLC. ***Sample preparation and processing.*** A C18 SPE column (Agilent Technologies, Polo Alto, CA) was activated by using 3 mL acetonitrile (Sigma Chemical, St Louis, MO), 3 mL methanol (Sigma Chemical), and 3 mL deionized water. The whole-blood samples (1 mL each) were mixed with 1.5 mL deionized water, 500 µL acetonitrile, and 4 mL $ZnSO_4$ solution in a vortex mixer for 2 min, and centrifuged at 2000 × *g* for 3 min. The $ZnSO_4$ solution was prepared by dissolving 3.75 g $ZnSO_4$ (Kelong Chemical) in a mixed solvent of 50 mL methanol, 150 mL acetonitrile, and 200 mL deionized water. After centrifugation, the supernatant was added to the activated column, the column was washed with 3 mL 50% methanol, and 1 mL acetonitrile was used to elute the analytes. After evaporation, the samples were reconstituted in 300 µL 83% methanol, and 75-µL aliquots were injected into an analytical column.[1,9,13]

Rapamycin (Sigma Chemical) was used as the standard. Stock (1 mg/mL) and working solutions of sirolimus were prepared by dilution with methanol. Calibration standards (1, 2, 4, 6, 10, 20, and 30 ng/mL) were prepared from whole-blood samples and working solutions of sirolimus. Quality-control samples with concentrations of 2, 10, and 30 ng/mL were prepared in the same way.

HPLC analysis. The concentration of sirolimus in whole-blood samples was quantified via HPLC–UV (LC-20AD; PDA, 190 to 400 nm; Shimadzu, Kyoto, Japan) by using a reversed-phase Kromasil C18 column (15 mm × 4.6 mm, 5 µm, Eka Chemicals, Bohus, Sweden), 50 °C and a mobile phase (methanol–water 83%) with a flow rate of 1 mL/min. The retention time was 5.9 s. The lower limit of quantification for sirolimus was 1 ng/mL, and the assay range used was 1 to 30 ng/mL. The correlation coefficient for the sirolimus working curve was 0.994. The intraday and interday coefficients of variation for the low- and high-quality control samples were less than 15%.

Data analysis. The area under the whole-blood concentration–time curve (AUC_{0-24} and $AUC_{infinity}$) for sirolimus were obtained by using Excel (Microsoft, Redmond, WA); these data were calculated by using the linear trapezoidal method and further extrapolated to infinity by dividing the last experimental concentration by the terminal slope. The terminal elimination rate constant was determined by log–linear regression. The peak whole-blood concentration and time to peak concentration were obtained by visual inspection of the concentration–time curve.

Statistical analyses were performed by using SPSS software (SPSS 18.0, Chicago, IL). Values for time to peak concentration were compared between the 2 groups by using the nonparametric Wilcoxon signed-rank test. Differences with *P* values of less than 0.05 were considered statistically significant. The bioequivalence of the 2 sirolimus formulations was assessed by calculating individual AUC_{0-24}, $AUC_{0-infinity,}$ and peak concentration values, which were log-transformed, and the means of individual ratios (gelatin:solution) and 90% confidence intervals calculated. The 2 formulations were considered to be bioequivalent when the 90% confidence intervals for $AUC_{0\ to\ 24}$

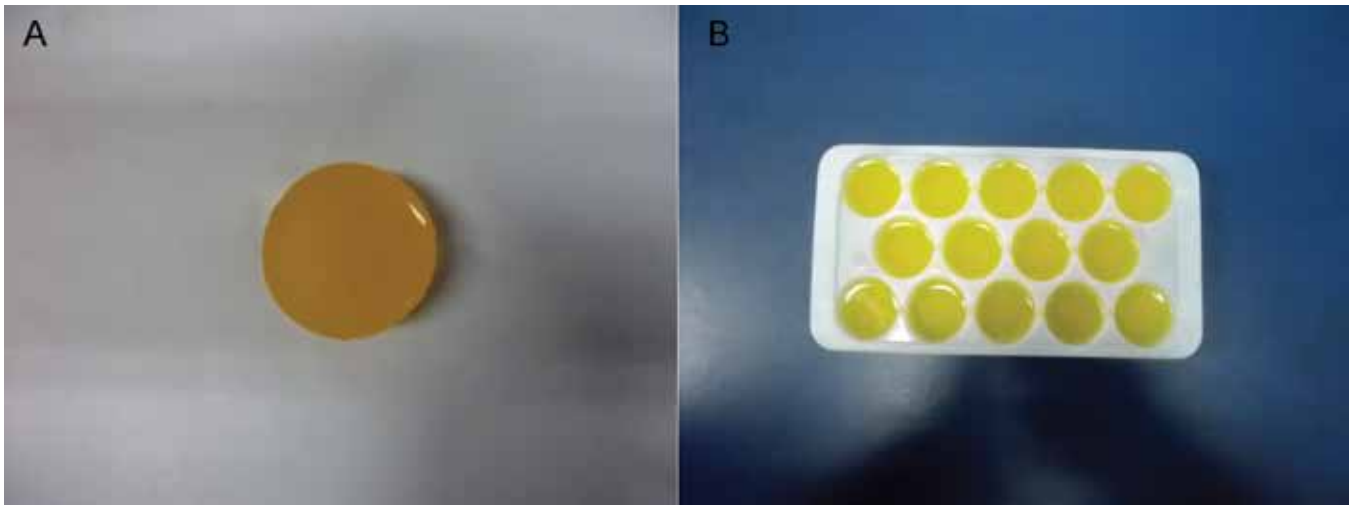

Figure 1. Appearance of sirolimus-containing gelatin treats. The treats were semisolid, nonsticky, and brightly colored.

Figure 2. Voluntary oral administration of sirolimus-containing gelatin treats by rhesus monkeys. A rhesus monkey readily accepts and eats the sirolimus-containing gelatin treat on day 4 of training.

and $AUC_{0\text{-infinity}}$ were within the range of 80% to 125% and that for peak concentration was within 75% to 133%.

The sirolimus levels after long-term drug delivery were compared between the 2 groups by using *t* tests; differences with *P* values that were less than 0.05 were considered statistically significant.

Results

Characteristics of sirolimus–gelatin treats. The sirolimus-containing gelatin treats were brightly colored, semisolid, and nonsticky (Figure 1). They had an average weight of 11.71 ± 0.39 mg and a content uniformity of 100.18% ± 0.91%, indicating appropriate consistency between treats.

Animal behavior. Sirolimus was administered by gavage to macaques in the oral solution group. Before gavage, the macaques had to be restrained to keep their heads still and mouths open, and they showed poor compliance with the procedure. Even after 15 d of training, the macaques remained noncompliant, screaming and hiding in the corners of the cages, without ketamine sedation.

In contrast, all the macaques in the sirolimus–gelatin group learned to voluntarily consume the treats after 4 d of training. On the first day of training, the macaques came forward to inspect the drug-free treat on the cage floor and then sniffed and licked it. They were rewarded with apples after eating the treat. Most (13 of 14) of the macaques readily ate the treat after just 1 d of training. After just 4 d of training, all 14 macaques displayed excitement when they saw the treats and consumed them within 1 min of their placement in the cage. Macaques continued to readily eat the treats when the drug-free treats were replaced with those containing sirolimus (Figure 2). Therefore, no physical restraint was necessary, the macaques adapted readily to this administration procedure, and sirolimus could be administered in the animal's home cage. In addition, the macaques continued to show interest in the gelatin treats even after long periods (as long as 1 y) during which no such treats were presented (data not shown).

Bioequivalence of the 2 dosage forms by single-dose oral delivery. Single doses of sirolimus yielded maximal blood concentrations of 11.4 ± 2.4 ng/mL within 3 to 4 h in the macaques that received the treated gelatin and 16.6 ± 3.8 ng/mL within 1.5 to 2 h in the oral solution group. Blood concentrations declined steadily thereafter and reached values of 1.95 ± 2.8 ng/mL in the gelatin group and 1.85 ± 3.3 ng/mL in the oral solution group by 24 h after treatment (Figure 3).

Summary statistics for the pharmacokinetic parameters of sirolimus are shown in Table 1. Values for ACU_{0-24} and peak concentration did not differ significantly between the 2 groups in regard to formulation, individual animal, medication order, and cycle were not statistically significant. However, time to peak sirolimus concentration differed significantly ($P < 0.05$) between the gelatin and oral solution formulations. The geometric mean (90% confidence interval) was 1.01 (0.84 to 1.17) for AUC_{0-24h}, 1.11 (0.82 to 1.23) for $AUC_{0-infinity}$, and 0.71 (1.04 to 1.98) for peak concentration. Results revealed significant differences in the peak concentration and time to peak concentration between the 2 groups. However, given that values for $AUC_{0-24\,h}$ and $AUC_{0-infinity}$ fit the condition of bioequivalence, absorption of sirolimus from the gelatin formulation was not significantly different from that of the oral solution.

Serum sirolimus levels after long-term drug delivery. HPLC analysis of small volumes (1 mL) of serum gave average sirolimus levels of 3.13 ± 2.03 ng/mL for macaques that received the gelatin formulation daily for 1 mo and 2.97 ± 1.92 ng/mL for the oral solution group. The difference between these 2 values was not statistically significant.

Table 1. Pharmacokinetic parameters of sirolimus in 4 rhesus monkeys

	Sirolimus oral solution		Sirolimus–gelatin treat		
	Arithmetic mean	Geometric mean (coefficient of variation)	Arithmetic mean	Geometric mean (coefficient of variation)	Ratio of gelatin treat to oral solution (90%confidence interval)
AUC_{0-24} (ng × h/mL)	87.86	87.21 (13.9%)	88.29	87.97 (10.0%)	1.01 (0.84, 1.17)
$AUC_{0-imfinity}$ (ng × h/mL)	214.62	212.71 (15.4%)	213.09	211.28 (14.9%)	1.11 (0.82, 1.23)
Peak concentration (ng/mL)	16.63	16.23 (23.8%)	11.40	11.47 (20.8%)	0.71 (1.04, 1.98)
Time to peak concentration (h)	not done	2 (1.5, 2)[a]	not done	3 (3, 4)[a]	not done

[a]Median (minimum, maximum)

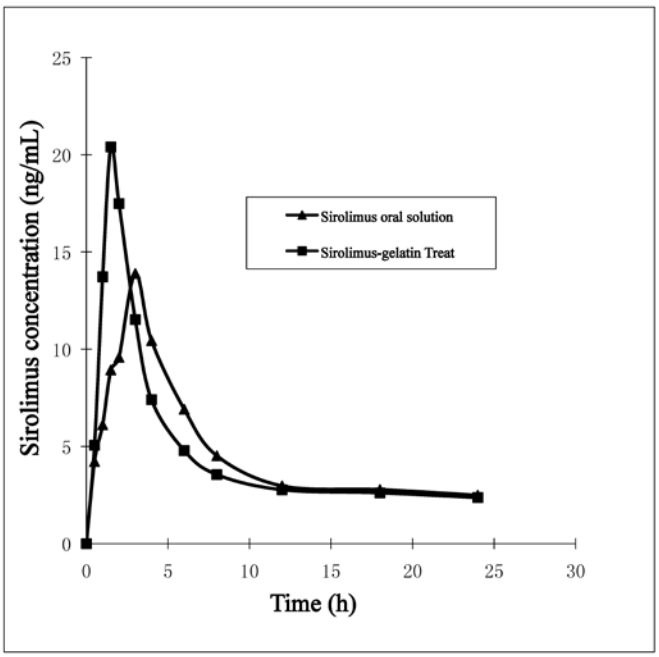

Figure 3. Concentration–time profile after the administration of a single dose of sirolimus in 4 rhesus monkeys. Blood samples were collected over a 24-h period after the administration of a single dose (0.6 mg/kg) formulated as a drug-containing gelatin treat or an oral solution. Data are expressed as means (n = 4).

Discussion

This report is the first to describe the administration of sirolimus to rhesus monkeys by using a drug-containing gelatin dosage form. The gelatin dosing technique appears to provide a useful alternative to gavage because it enhances the compliance of the animals, allows accurate drug dosage, reduces the distress associated with physical restraint,[4,8] and avoids the physical damage caused by the gavage procedure.

Sirolimus-containing gelatin treats were easy to make and required no specialized equipment or materials. The results of the uniformity test showed that sirolimus was dispersed homogenously among the treats, ensuring accurate administration of drug dosage. The semisolid consistency, nonsticky texture, and high palatability of the brightly colored treats helped the macaques to rapidly (within 3 d) overcome their innate avoidance of new objects. In a previous study, 5 to 10 d were required to train rhesus macaques to drink a mixture of apple juice and syrup,[5] and some animals displayed "syringe-sucking boredom" after several weeks of drug delivery, even when they received the liquid from a caretaker with whom they were familiar. Furthermore, the macaques required 1 to 2 d to adapt to an unfamiliar caretaker before they would consistently ingest all of the liquid.[5] In the present study, we fasted our macaques overnight before the first day of training to maximize the likelihood that they would overcome their innate neophobia of the drug-free gelatin treats. After 4 d of training without food restriction, all of the macaques began to eat the treats as soon as they were offered. In addition, the trained macaques maintained their interest in the sirolimus-containing treats even after they had not been presented for as long as 1 y. Compared with gavage and syringe-feeding methods, our gelatin–sirolimus formulation is less time-consuming to administer, which is particularly important in experiments involving numerous animals.

Adding a drug to drinking water is a common technique that allows oral drug delivery with minimal stress to experimental animals. However, this technique does not ensure accurate drug dosing and cannot be used for drugs that are unstable.[20] Oral gavage allows accurate drug dosing but also is problematic. It is stressful to nonhuman primates,[3,11] typically requires the use of ketamine anesthesia in cases of poor compliance, and can lead to damage of the respiratory system or upper gastrointestinal tract.[3] Our drug-containing gelatin method avoids these drawbacks of gavage and treated drinking water.

We performed pharmacokinetic profiling to compare the blood sirolimus concentrations due to feeding rhesus macaques by using drug-containing gelatin compared with gavage. The single-dose pharmacokinetic time-course study showed significant differences in the time to peak concentration and the data for peak concentration did not demonstrate bioequivalence. However because AUC_{0-24} and $AUC_{0-infinity}$ did fit the conditions for bioequivalence, we conclude that sirolimus absorption did not differ between the gelatin formulation and the oral solution. Although the sirolimus–gelatin formulation showed a delayed time to peak concentration compared with the oral solution (3 h versus 2 h), both formulations achieved similar effective concentrations. In addition, serum levels of sirolimus after long-term administration did not differ between the 2 experimental groups. We therefore think our sirolimus–gelatin treats are an effective alternative to the use of gavage and the oral solution to treat rhesus macaques. Future efforts will address improving the rate at which the gelatin treat dissolves and promoting the absorption rate of sirolimus given by using this method. In addition, in clinical transplantation experiments, it will be important to regulate the dose and frequency of the treats administered to maintain the blood sirolimus concentration within the effective range.

Previous studies indicate that the effective blood concentration of sirolimus to prevent transplant rejection is 4 to 12 ng/mL in humans.[17] Because of the lack of similar data for nonhuman primates, we based the dose we used in the current study on the recommended human dosage of 0.2 mg/kg daily, which achieved a blood sirolimus concentration of only 3 ng/mL in our macaques. Given that the drug dosage conversion factor from human to nonhuman primate is about 3,[16] we need to use a sirolimus dose of 0.6 mg/kg daily in our macaques to maintain blood concentrations in the 4- to 12-ng/mL range targeted for humans.

One limitation of our gelatin treat dosing method is a lack of stability; by day 4, the treats appeared to be dried out. Because the treats should be prepared shortly before use, this method may be more appropriate for long-term studies involving daily doses.[2] Our method can be adapted easily for administration of other drugs in rhesus macaques and likely can be extended to long-term drug studies of other nonhuman primates and rodents.[15,20] The gelatin treat method is unsuitable for the delivery of heat-labile compounds and for rodent experiments that require precise individual drug dosing. However, this limitation regarding rodent studies might be overcome through housing the animals individually and using a smaller-scale gelatin delivery system.[15] Finally, the use of sucrose-containing gelatin is inappropriate for diabetes research. Saccharin or other sweeteners may be useful alternatives to sucrose for masking the bitter taste of various drugs.[2]

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