Sustained Bronchodilation with Isoproterenol Poly(Glycolide-co-Lactide) Microspheres

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An animal study was carried out to evaluate the *in vivo* bronchodilator action of isoproterenol (Iso) from poly(glycolide-co-lactide) (PGL) microspheres. Microspheres with a mean diameter of 4.5 μm and a drug load of 7% were administered intratracheally to Long-Evans rats. The microspheres released about 70% of the incorporated drug in the instillation medium before administration, which provided immediate action, and the remaining 30% was available for sustained release. A total of 120 animals was anesthetized, paralyzed, artificially ventilated, and divided into 15 groups (n = 8): 3 groups each for saline, blank microspheres, free Iso, blank microspheres with free Iso, and microencapsulated Iso. All instillations were made in a volume of 1 ml/kg and the dose of all Iso preparations was 0.1 mg/kg. At 3, 6, or 12 hr after the intratracheal instillation, a serotonin challenge (40 μg/rat) was administered intravenously to constrict the airways. Airway function tests were performed at each time interval on one group of animals by a maximal expiratory flow-volume maneuver. The heart rate in animals receiving Iso formulations was similar to that in the saline control group, indicating minimal systemic effect of the dose administered. The systemic serum levels were below 2 ng/ml in all the groups. Animals receiving encapsulated Iso resisted the serotonin challenge for at least 12 hr after intratracheal instillation, indicating that the drug was still present over this period of time. On the other hand, the serotonin-induced airway constriction observed in the animals receiving blank microspheres, free Iso, or free Iso with blank microspheres was similar to that in saline controls at all time points. The results clearly show that only a small fraction of the free dose is required in sustained-release form for a prolonged pharmacological effect, resulting in a 50- to 100-fold reduction in the total dose administered.

KEY WORDS: bronchodilation; prolonged action; isoproterenol; poly(glycolide-co-lactide) microspheres; rats.

INTRODUCTION

The treatment of asthma by inhalation aerosol therapy using bronchodilators is an excellent example of targeted drug delivery (1). This route of administration provides adequate therapeutic levels of potent bronchodilators in the respiratory tract and provides a better clinical response than other forms of delivery while minimizing systemic concentrations (2–6). Most of the currently available inhalation bronchodilators have a relatively short half-life and require up to six doses per day (7). The development of longer-acting bronchodilator formulations is desirable to improve the treatment of asthma and other chronic pulmonary disorders. However, to date, sustained-release formulations of bronchodilators have been restricted to orally administered dosage forms which do not provide targeted delivery to the pulmonary region. Although the beneficial effects of divided inhalation doses of isoproterenol (Iso) (8) and metaproterenol (9) over a single large dose are recognized, the development of sustained-release forms of these drugs for inhalation has not been documented. A method to sustain the action of inhaled bronchodilator would be to localize the drug in the airways in a biodegradable carrier device. Liposomes containing bronchodilators have been evaluated with little success (10), probably due to their interaction with the lung surfactant. The objective of this study was to develop microspheres of a potent model bronchodilator, Iso, in a size suitable for administration via inhalation aerosol. The microspheres were evaluated by intratracheal administration in an animal model which had been developed to determine the pharmacological and pharmacokinetic behavior of such a sustained-release dosage form (11). Airway function tests were utilized to assess the resistance to a serotonin challenge in a rat animal model which had received Iso in both the free and the encapsulated forms.

METHODS

Preparation of Microspheres

Iso microspheres were prepared using poly(glycolide-co-lactide) (PGL; 50:50, 34,000 Da, Polysciences, Warrington, PA) by a technique developed earlier (12–14). In brief, Iso, stearic acid (Sigma Chemical Company, St. Louis, MO), and PGL were dissolved in warm acetonitrile (Fisher Scientific) and dispersed into heavy mineral oil (Fisher) containing 0.1% sorbitan sesquioleate (Sigma) with a high-speed dispersator at 3000 rpm for 5 min. The dispersion was then stirred at a slower speed (2000 rpm) for 1 hr with a nitrogen purge to evaporate acetonitrile. The microspheres were recovered by filtration and washed several times to remove adhering mineral oil and surfactant. The resulting PGL-Iso microspheres were dried under vacuum for 24 hr and stored desiccated at room temperature.

Characterization of Microspheres

Total drug content was determined by dissolving a known amount of microspheres in methylene chloride followed by precipitation of polymer and extraction of Iso with 0.1 M phosphate buffer, pH 7.4. The extract was then analyzed for Iso content by UV spectrophotometry at 274 nm.

The *in vitro* release of Iso from the microspheres was performed by suspending a known amount of microspheres in 0.1 M phosphate buffer, pH 7.4, containing 1 mg/ml sodium metabisulfite and 0.05 mg/ml EDTA. Iso was found to be stable in this buffer medium at 37°C for at least 24 hr. At predetermined intervals the samples were centrifuged and

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supernatant collected for analysis of released Iso by UV spectrophotometry.

The size distribution of the microspheres was measured using a Malvern laser diffraction particle sizer. The microspheres were suspended in 0.05% (w/v) Tween 80 solution and loaded into the counting chamber. Particle size distribution was obtained and analyzed by computer to produce volume and number distribution curves.

Dose–Response Relationship of Iso

To select a suitable dose of Iso for protection against serotonin-induced bronchoconstriction, a time based dose–response profile was generated. Various doses of Iso in saline were administered to sodium pentobarbital anesthetized Long-Evans rats weighing about 330 g via intratracheal instillation at time 0. Evaluation was performed on animals at predetermined time points between 15 min and 2 hr.

Sustained Pharmacological Effects

Three Iso formulations, equivalent to 0.1 mg/kg, and two control formulations were evaluated for sustained bronchodilatory effects. The formulations were administered via intratracheal instillation to ether anesthetized Long-Evans rats weighing about 330 g as solutions or suspensions in a volume equivalent to 1 ml/kg. A total of 120 rats was evenly divided into five treatment groups, which were further divided into three subgroups each for the three time points (Table I). The solution control group was administered a 0.1% (w/v) Tween 80 in normal saline (vehicle), free Iso was administered as a solution in the vehicle, and the microspheres were administered as a suspension in the vehicle. Each subgroup was tested at one of the three time periods after dosing (3, 6, or 12 hr; $n = 8$ for each subgroup). The animals were prepared for evaluation 30 min before the time point.

Pharmacological Evaluation

The animals were evaluated for bronchodilatory effect of Iso by challenging them with a bronchoconstrictor, serotonin, followed by measurement of airway changes using airway function tests.

Animal Preparation. Rats were anesthetized with an intraperitoneal injection of sodium pentobarbital (35–50 mg/kg) and their trachea and jugular vein were cannulated. The animals were placed inside a 2.8-liter whole-body plethysmograph and artificially ventilated with a Harvard rodent respirator. The animals were then paralyzed with gallamine triethiodide (10 mg/kg; Sigma) and the tidal volume and respiratory frequency of the respirator were set to be the same as those observed in the same animal during spontaneous breathing before anesthesia and paralysis. In addition, platinum electrodes (Model E2B, Grass Instruments, Quincy, MA) were implanted subcutaneously in two limbs to record cardiac electrical activity, which was used to determine heart rate.

Table I. Experimental Design

Groups[a]	Formulation administered
I_3, I_6, I_{12}	Saline solution
II_3, II_6, II_{12}	Blank PGL microspheres
III_3, III_6, III_{12}	Free Iso
VI_3, VI_6, VI_{12}	PGL-Iso microspheres
V_3, V_6, V_{12}	Free Iso with blank PGL microspheres

[a] Subscripts represent the time (hours) of assessment after dosing. $n = 8$ for all groups; total, 120 animals.

Airway Function Tests. At a designated time, airway function tests were performed before and after serotonin challenge in anesthetized-paralyzed rats. In order to examine the bronchodilator effect of isoproterenol, serotonin (40 μg/rat) was injected intravenously to constrict airway smooth muscle of rats. The maximal expiratory flow-volume (MEFV) maneuver was performed within 20 sec after the serotonin administration.

The MEFV curve was obtained according to a previously reported method (11). Briefly, the animal was inflated to total lung capacity (the lung volume at airway opening pressures of 30 cm H_2O) three times; the third inflation was through a solenoid valve. At peak volume during the third inflation, the inflation valve was shut off, and immediately another solenoid valve for deflation was automatically opened. The deflation valve was connected to a 4-liter container, which maintained a subatmospheric pressure of -40 cm H_2O. This negative pressure produced maximal expiratory flow ($\dot{V}_{max}$). The MEFV curve was printed on a teletypewriter of a Buxco Pulmonary Mechanics Analyzer (Model 6) and also stored on a cathode-ray storage oscilloscope (Hitachi V-134). The basic principle of this functional test is that the $\dot{V}_{max}$ is a function of airway dimension in the presence of a fixed pressure gradient from alveoli to the airway opening. In other words, a high $\dot{V}_{max}$ can be generated with a large airway dimension (bronchial relaxation), or only a low $\dot{V}_{max}$ can be generated during the condition of bronchial constriction. Following each flow-volume maneuver, the functional residual capacity was determined by a neon dilution method (11). Blood samples (0.5 ml) were collected at each time point at the end of physiological measurements for analysis of serum Iso concentration by HPLC.

An increase or a decrease in the $\dot{V}_{max}$ at 50% total lung capacity ($\dot{V}_{max50}$) was used to indicate dilation or constriction in airways, respectively. In order to eliminate individual differences between animals, percentages of baseline (before serotonin treatment) values were used to analyze any serotonin-induced changes at each time interval. One-way analysis of variance was used to establish differences among groups. Intergroup comparisons were made using Dunnett's test. Differences were regarded as significant if $P < 0.05$.

Heart Rate Measurements

The dominant systemic effect of Iso is an increase in the heart rate caused by a direct sympathomimetic action on the heart. The time lapse needed to increase the heart rate and the extent of increase can be used to track the passage of Iso from pulmonary to systemic regions. Heart rates were measured before the serotonin treatment (baseline) and at specific intervals thereafter. The heart rates were also used to monitor the cardiovascular health of the animals. Pulmonary data were discarded for a particular rat if the heart rate decreased to below 80% of the initial value during the study.

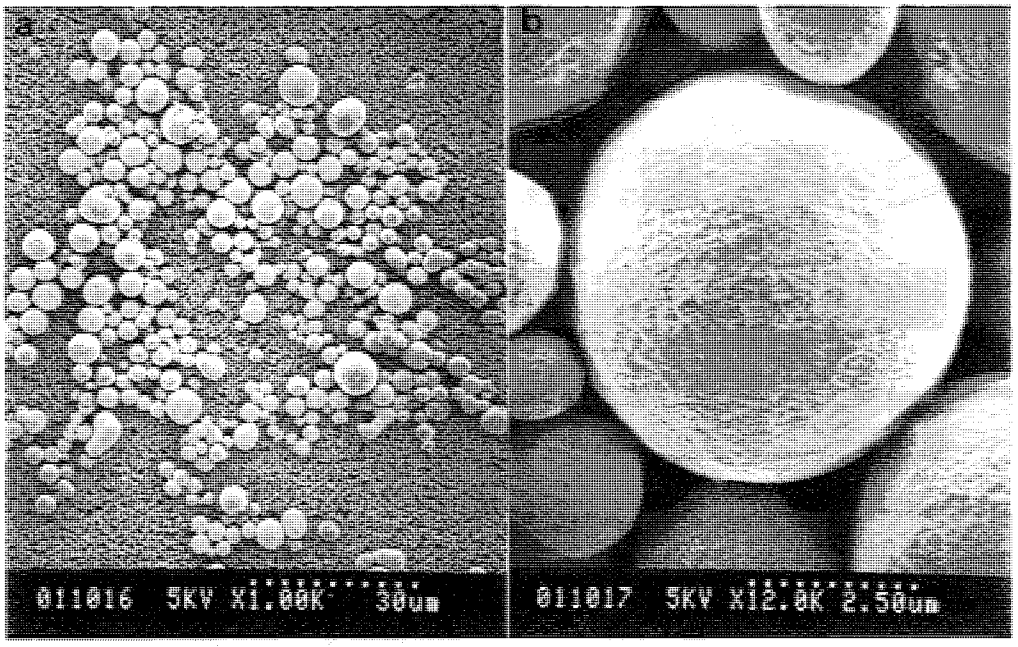

Fig. 1. Scanning electron micrograph of PGL-Iso microspheres.

Isoproterenol Assay

To date, few laboratories have been able to quantify the low levels of Iso found after *in vivo* administration to man or to laboratory animals (15–17). A sensitive HPLC technique employing precolumn derivatization followed by fluorescence detection was established by modification of the reported methods. Arterenol (1.6 μg/tube) was added as an internal standard to a 200-μl plasma sample. An additional 200 μl of 2 *M* ammonia-ammonium chloride buffer containing 8.9 m*M* diphenylborate–ethanolamine complex and 13.4 m*M* EDTA was added and the samples were extracted with *n*-heptane containing 4.6 m*M* tetraoctylammonium bromide and 10 ml/L 1-octanol. The aqueous layer was frozen in acetone–dry ice and discarded. An additional 1 ml 1-octanol was added to the organic layer and was extracted with 200 μl 0.08 *M* acetic acid. The aqueous layer containing Iso was frozen in acetone–dry ice and separated from the organic waste.

Fluorescence derivatization was carried out by adding 150 μl acetonitrile, 50 μl 1.75 *M* bicine buffer, pH 7.05, 100 μl 0.1 *M* 1,2-diphenylethylenediamine, and 10 μl 20 m*M* potassium hexacyanoferrate to the extract. The mixture was

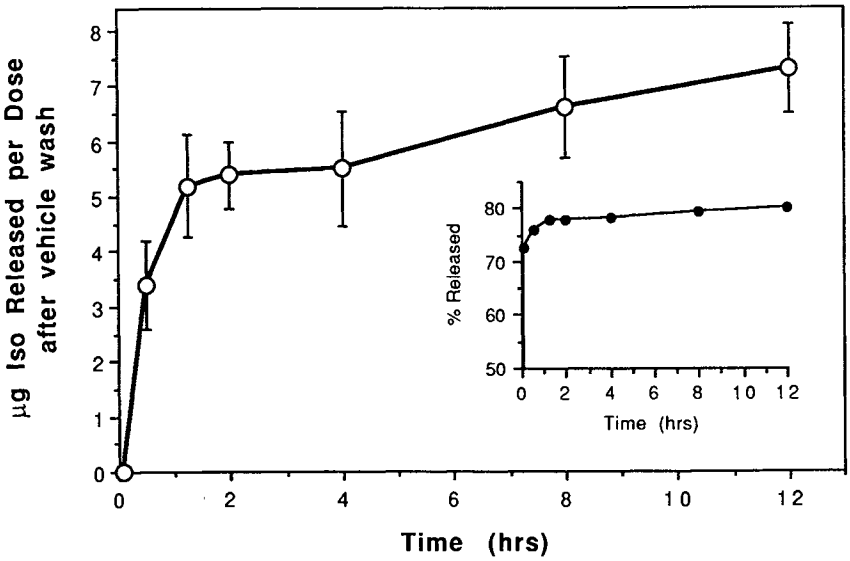

Fig. 2. *In vitro* Iso release from PGL-Iso microspheres. The plot shows the amount of Iso released from a single dose of Iso-PGL microspheres washed in an aqueous medium for 5 min before conducting the release. The inset shows the total drug release profile of PGL-Iso microspheres.

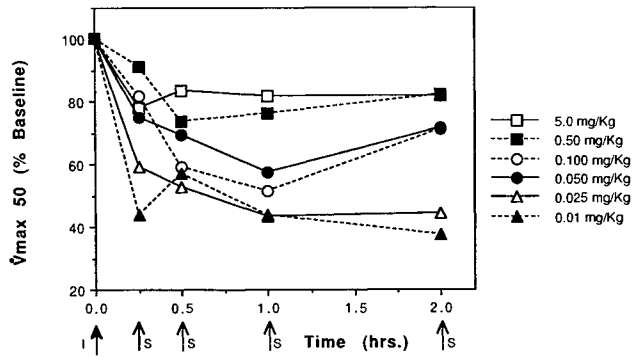

Fig. 3. Kinetic dose–response profiles for protective effect of Iso in solution form. I indicates Iso administration and S indicates serotonin challenge and time of measurement. $n = 2$.

heated for 30 min at 45°C and allowed to cool in a refrigerator for 10 min. A 50-μl aliquot of the reaction mixture was injected onto a 250 × 4-mm Bio-Sil ODS-10 column (Bio-Rad, Richmond, CA) and eluted with a mobile phase of methanol: 50 m*M* Trizma buffer (4:1), pH 7.2, at a flow rate of 1 ml/min. The eluate was measured at an excitation wavelength of 368 nm and an emission wavelength of 470 nm.

RESULTS

Microsphere Preparation and Characterization

PGL-Iso microspheres were spherical in shape, with a smooth, nonporous surface (Fig. 1). The mean diameter of the microspheres was 4.5 μm, with 59% of the microspheres, by weight, under 5 μm in diameter and 98% under 10 μm in diameter. All the microspheres were under 15 μm in diameter. The *in vitro* release profile of PGL-Iso microspheres is shown in Fig. 2. The plot shows release of Iso after removal of surface or freely accessible pore associated drug. As shown in the inset, about 72% of the drug is released in less than 5 min and the drug remaining in the microspheres is released at a slow rate, with an additional 9% release over 12 hr.

Dose–Response Relationship of Iso

The duration of a protective effect after administration of Iso at various doses is illustrated in Fig. 3. An Iso dose above 0.5 mg/kg protected the animal from the serotonin challenge for up to 2 hr. A 0.1 mg/kg Iso dose protected the animal for 15 min following which an increase in the serotonin-induced bronchoconstriction was evident, indicating that the duration of the protective effect of Iso was about 15 min. At doses below 0.025 mg/kg, no protection was seen and the challenge with serotonin produced a severe bronchoconstriction. However, it was found that administration of 0.05 and 1.0 mg/kg Iso in solutions provided a similar magnitude of protection against the serotonin-induced decrease in $\mathring{V}_{max50}$ as did 5.0 mg/kg Iso at 15 min following administration.

Evaluation of the Sustained Pharmacological Response

For evaluation of the sustained response, each animal was used for measurement at only one time point, i.e., each treatment at each time point was studied in a different animal. This was essential to eliminate time-dependent deterioration in the cardiovascular condition of the animals after surgery and paralysis. Body weight, baseline values of respiratory parameters, and heart rate are listed in Table II. There were no significant differences in these values between groups. The baseline heart rate at 12 hr, however, was significantly lower than that at 6 hr in the free Iso group.

The temporal changes in serotonin induced bronchoconstriction for various Iso formulations are shown in Fig. 4. The results are represented as percentages of baseline values. Complete protection against serotonin would result in a $\mathring{V}_{max50}$ of 100%. Serotonin challenge produced a 30–45% decrease in the $\mathring{V}_{max50}$ in the control group (Group I), with no consistent temporal pattern, indicating a mild to moderate bronchoconstriction during the 3- to 12-hr period. In the free Iso, blank PGL, and free Iso with blank PGL groups (Groups II, III, and V), the serotonin challenge produced a bronchoconstriction similar to that in the control group, indicating that the effective period for the intratracheally instilled isoproterenol is less than 3 hr. In Group IV, the PGL-Iso group, serotonin failed to induce any significant bronchoconstric-

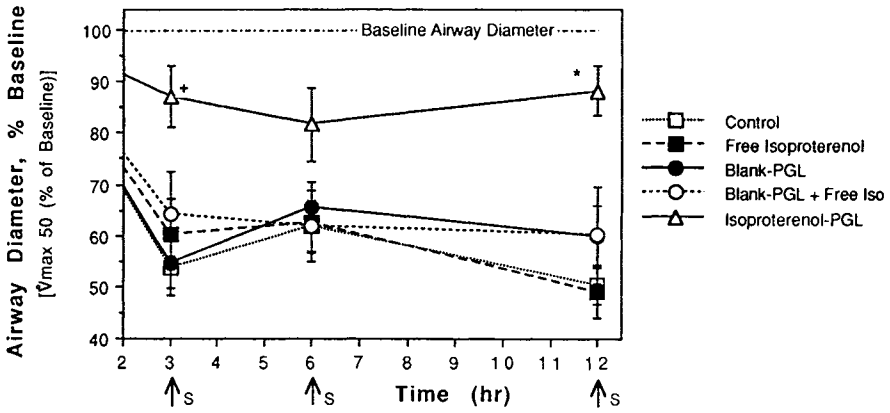

Fig. 4. Changes in maximum expiratory flow at 50% total lung capacity ($\mathring{V}_{max50}$) in five groups ($n = 8$) administered different formulations at 0.1 mg/kg intratracheal Iso dose. S indicates administration of serotonin. Statistical differences ($P < 0.05$) between groups: (+) compared to control, free Iso, and blank microsphere groups; (*) compared to all other groups.

Table II. Baseline Values for Five Groups of Rats[a]

Group	n	Wt (g)	TLC (ml)	FRC (ml)	Peak $\dot{V}_{max}$	$\dot{V}_{max50}$ (ml/sec)	Heart rate[b] 3 hr	6 hr	12 hr
Control	24	283 ± 9	12.8 ± 0.5	1.64 ± 0.05	122 ± 3	55 ± 3	353 ± 9	364 ± 12	338 ± 11
Blank microspheres	24	295 ± 11	12.4 ± 0.6	1.66 ± 0.07	116 ± 5	56 ± 3	360 ± 13	353 ± 9	340 ± 14
Free Iso	24	281 ± 11	13.3 ± 0.4	1.69 ± 0.04	119 ± 2	58 ± 3	360 ± 13	364 ± 12	326* ± 9
Encapsulated Iso	24	287 ± 12	12.9 ± 0.4	1.78 ± 0.07	118 ± 2	54 ± 3	340 ± 16	341 ± 16	318 ± 17
Free Iso + blank microspheres	24	273 ± 10	12.9 ± 0.4	1.69 ± 0.07	114 ± 3	54 ± 3	326 ± 13	330 ± 11	345 ± 21

[a] Values are mean ± SE. TLC, total lung capacity; FRC, functional residual capacity; $\dot{V}_{max50}$ maximum expiratory flow rate ($\dot{V}_{max}$) at 50% baseline TLC.

[b] Heart rate data were obtained from eight animals in each group at a fixed time point.

* $P < 0.05$ compared to the 6-hr values in the same group.

tion for at least 12 hr, indicating that the microsphere encapsulated Iso had a duration of action exceeding 12 hr.

Heart rates following Iso administration in different formulations of PGL microspheres are shown in Table II. Although a large dose of Iso can produce systemic effects such as an increase in heart rate (18), there was no significant increase in heart rate produced by 0.1 mg/kg Iso. Therefore, Iso microspheres produced profound pulmonary effects and insignificant systemic side effects at a dose of 0.1 mg/kg Iso in this study.

Systemic Isoproterenol Analysis

The liquid extraction technique used for sample preparation resulted in a good recovery of Iso and the internal standard, arterenol. Figure 5 shows the standard curves and chromatogram for the assay. The retention time for Iso is 7.1 min and that for arterenol is 3.3 min, with good resolution without any interfering peaks. The standard curves were linear from 2000 to 5 ng/ml, with a minimum detectable concentration of 2 ng/ml. The coefficients of regression were greater than 0.998 for all the standard curves during the validation procedure. Plasma samples obtained from the animals during the course of study did not show any Iso when the dose was 0.1 mg/kg.

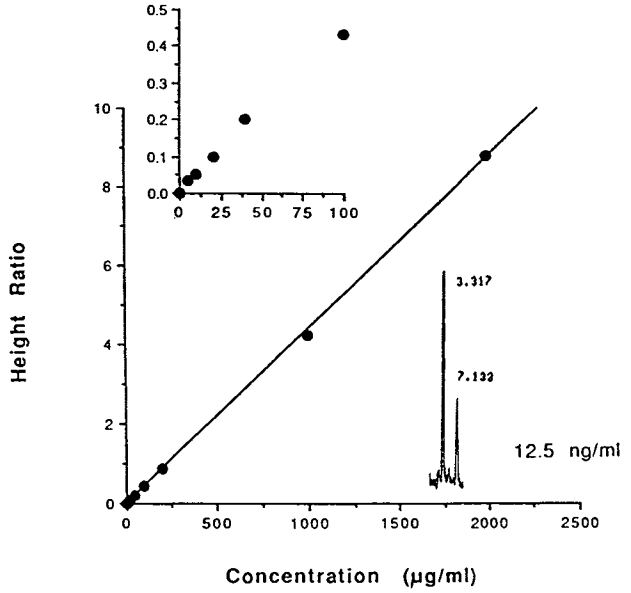

Fig. 5. Standard curve for HPLC assay of Iso. The correlation coefficient is 1.000. The insets show a lower range of concentrations and a typical chromatogram at 12.5 ng/ml. Retention times: Iso, 7.1 min; arterenol, 3.3 min.

In our previous experiments at a much higher intratracheal dose (5 mg/kg), all the formulations exhibited absorption rate-limited elimination ("flip-flop kinetics"), with no differences in systemic bioavailability between the formulations at that dose. However, at 0.1 mg/kg the systemic absorption is negligible, which would be consistent with a lack of a change in heart rate and undetectable systemic Iso concentrations.

DISCUSSION

The microspheres developed are well suited for inhalation delivery of bronchodilators. The 4.5-μm mean diameter is suitable for targeting the conducting airways, without delivering a large amount of drug and microspheres to the alveoli. The major pharmacological advantage of targeting the conducting airways is the proximity of the released drug to the site of action, which in the case of the bronchodilators is the conducting airways. Another possible advantage from a toxicity viewpoint is the ease of removal of microspheres from the lungs by the mucocilliary escalator mechanism (19). This would clear the conducting airways and avoid excessive buildup of exhausted microspheres in the lungs. Microspheres penetrated deep in the alveoli would be cleared by biodegradation.

The microspheres exhibited a large burst, releasing over 70% of the incorporated Iso in a few minutes. The burst also occurred prior to the intratracheal administration in *in vivo* studies during preparation of the suspension in a vial. The microsphere suspension thus administered contained a mixture of 0.07 mg/kg free Iso and 0.03 mg/kg encapsulated Iso. A similar release profile with a slower burst may be expected

after inhalation administration via dry aerosol, as the hydrodynamic effects would be much smaller in lungs as compared to the vial in which the suspension is prepared for instillation.

The dose–response relationship seen in this study is similar to that obtained by others in human subjects (20,21). Those investigators carried out a dose–response study of inhaled Iso and observed that supermaximal doses of isoproterenol cannot produce more bronchodilation, as indicated by the increase in forced expiratory flow at 1.0 sec (FEV_i) or in peak expiratory flow rate. The ineffectiveness of a supermaximal dose was probably related to the removal of Iso from the lungs and subsequent metabolism and/or excretion via the systemic circulation (22). Thus, at a dose of 0.1 mg/kg Iso, sufficient bronchodilatory effect was seen; however, the duration of the effect was relatively short. Accordingly, a dose of 0.1 mg/kg was selected for the delivery of Iso in microspheres to examine the effect of sustained release on the duration of bronchodilatory effect. This estimation of the effective period is comparable to that obtained by Choo-Kang *et al.* in human subjects (6). In this study, free Iso at a dose of 0.1 mg/kg was found to have a duration of action lasting only about 15 min in rats (Fig. 3). The microspheres, on the other hand, contain only 0.03 mg/kg encapsulated Iso after intratracheal instillation. A dose of 0.025 mg/kg free Iso failed to protect the animals, whereas a dose of 0.05 mg/kg free Iso had a duration of action lasting about 15 min. Thus the extended duration of action may be attributed to the release of very small amounts of drug at the site of action and prevention of the metabolism of unreleased drug due to encapsulation in the microspheres.

Although the mechanisms for the sustained bronchodilator effect of the encapsulated isoproterenol are not clear, two possible mechanisms can be speculated: (i) Iso remaining after burst release is entrapped in the microspheres and, therefore, is not available for clearance from the lungs or for intracellular metabolism in the Group IV animals; and (ii) the slow and repeated release of Iso from the entrapped microspheres can produce a type of "enhanced" effect. Riley *et al.* (8) compared the bronchodilator effect of a single 800-μg inhalation dose of Iso to four 200-μg doses given 20 min apart in asthmatic human subjects. Specific conductance (an indicator of bronchodilation) increased significantly in the group given divided doses than the group given a single 800-μg dose, indicating an enhanced bronchodilator response to a fixed amount of Iso by inhaling the drug in divided doses sequentially. Heimer *et al.* (9) also demonstrated the enhanced bronchodilator effect using sequential doses of another bronchodilator, metaproterenol. Either or both of these mechanisms could account for the increased duration of action of Iso microspheres. Most recently, a 10-year study in Saskatchewan involving 12,300 asthmatic patients suggested that the excessive use of β-2-agonist might increase the risk of fatal asthma attacks (23). It appears that a polymeric carrier which released a small dose of the drug gradually and could be administered less frequently would reduce the risk of excessive use.

In summary, Iso administered as 70% free and 30% encapsulated at a dose of 0.1 mg/kg significantly ameliorated serotonin-induced bronchoconstriction for at least 12 hr after intratracheal instillation. Free Iso at the same dose provided protection for less than 30 min and a free Iso dose equivalent to that remaining in the microspheres at the time of administration did not provide any protection. Thus, the Iso dose in the microsphere formulation could be decreased by 50- to 100-fold to provide the same protection as free drug. The results show the advantage of a sustained-release formulation for targeted delivery of a model bronchodilator to the lungs. The animal model was relatively easy to use and provided useful information on the pharmacological effect of bronchodilators. The reported study was carried out by intratracheal administration of Iso formulations to minimize the variability in the dose. An aerosol formulation of the microspheres is undergoing development (24) and will be evaluated for pharmacological effects using the animal model described.

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