

Bioassay of Salmeterol in Children Using Methacholine Challenge With Impulse Oscillometry

Pritish Mondal, MD,¹ Sandra Baumstein, PharmD,² Sreekala Prabhakaran, MD,³
Mutasim Abu-Hasan, MD,³ Yaohui Zeng, MS,⁴ Sachinkumar Singh, PhD,⁵ Kai Wang, PhD,⁴
Richard C. Ahrens, MD,⁵ and Leslie Hendeles, PharmD^{6*}

Summary. Background: Bronchoprovocation with methacholine (MC) is the most sensitive method of determining bioequivalence of inhaled bronchodilators. FEV₁ is used to determine the endpoint, but many children cannot perform spirometry reproducibly. The purpose of this study was to determine whether MC, using impulse oscillometry (IOS) as the endpoint, can differentiate between two doses of salmeterol (SM). Methods: This was a single-blind, randomized study of 10 subjects with mild stable asthma, ages 4–11 years. None were taking a long-acting β -agonist but most were on low-dose inhaled corticosteroid. On one study day, MC was performed 1 hr after one inhalation from each of two separate Advair 100/50 Diskus (100 μ g salmeterol treatment). On a second day, MC was performed after one inhalation from Advair Diskus and one inhalation from Flovent Diskus 100 (50 μ g salmeterol treatment). The provocative concentration of methacholine causing a 40% increase in total airway resistance (PC₄₀R₅) was calculated. Results: The reduction in R₅ (bronchodilator effect) was 15.5% and 18.4% for 50 and 100 μ g, respectively (NS). After MC (bronchoprotective effect), the geometric mean (95%CI) PC₄₀R₅ (mg/ml) was 2.4 (1.3–4.4) during screening, 22.9 (8.5–61.6) after 50 μ g SM and 47.0 (25.2–87.8) after 100 μ g SM ($P = 0.051$ for 50 vs. 100 using a linear mixed effects model). No adverse effects were observed. Conclusions: MC with IOS endpoint will be a useful method for determining bioequivalence of a generic inhaler in children. Seventy-two subjects will be required to achieve 80% power to assess bioequivalence of SM. **Pediatr Pulmonol.** © 2015 Wiley Periodicals, Inc.

Key words: bronchoprovocation; β -agonists; bioequivalence; inhaled therapy.

Funding source: Teva Pharmaceuticals.

¹Department of Pediatrics, College of Medicine, Penn State University, State College, Pennsylvania.

²Department of Pharmacotherapy and Translational Research, College of Pharmacy, University of Florida, Gainesville, Florida.

³Department of Pediatrics, College of Medicine, University of Florida, Gainesville, Florida.

⁴Department of Biostatistics, College of Public Health, University of Iowa, Iowa City, Iowa.

⁵Stead Family Department of Pediatrics, Carver College of Medicine, University of Iowa, Iowa City, Iowa.

⁶Department of Pharmacotherapy and Translational Research, College of Pharmacy and Department of Pediatrics, University of Florida, Gainesville, FL.

Conflict of interest: This study was supported by an investigator-initiated grant from Teva. Dr. Hendeles has served on an advisory board and gave a lecture for Teva. As this is a methodology paper, none these represent a conflict. None of the other authors have a conflict to report.

*Correspondence to: Leslie Hendeles, PharmD, University of Florida, 1600 SW Archer Rd, Rm PG-05, Gainesville, FL 32610-0486.
E-mail: hendeles@cop.ufl.edu

Received 26 June 2015; Revised 8 October 2015; Accepted 27 October 2015.

DOI 10.1002/ppul.23345
Published online in Wiley Online Library
(wileyonlinelibrary.com).

INTRODUCTION

Patents are expiring for many of the currently available inhaled medications for asthma and COPD and manufacturers are developing generic products. They require regulatory agency approval. However, there are geographical differences in the requirements for approval. For example, in the United States a generic inhaled drug can be approved based upon the “weight of evidence” of in vitro studies, a pharmacokinetic study in healthy adults and a pharmacodynamic study in subjects with asthma.¹ Studies in children are not required. In contrast, the European Medicines Agency does not allow extrapolation of studies in adults and requires bioequivalence testing in children.² A pharmacokinetic study can satisfy the requirement but a pharmacodynamic study would be required if a pharmacokinetic study does not demonstrate bioequivalence or cannot be performed, that is, the drug cannot be measured in blood.

If a pharmacodynamic study is required for children, the question is what study design and endpoint should be used? In adults, a bronchoprovocation study with methacholine is the most sensitive method of determining

bioequivalence of β_2 agonists such as albuterol using FEV₁ as the endpoint.³ However, it is unclear whether this method can be used for the salmeterol component of a combination product with fluticasone (i.e., Advair[®]). In children, Simons et al.⁴ was unable to distinguish between a single dose of 25 and 50 μ g salmeterol delivered by MDI using methacholine challenge with FEV₁ as the endpoint in 20 children between 6 and 12 years (mean 10.3 years). Similarly, Palmqvist et al.⁵ showed that in adults the dose-response curve for salmeterol was relatively flat when methacholine challenge was used with FEV₁ as the endpoint. These studies suggest that methacholine challenge with FEV₁ is not sufficiently sensitive to detect a difference between generic and reference products for this long-acting β agonist. Therefore, to distinguish between the response to two adjacent strengths of salmeterol a different endpoint is required.

Impulse oscillometry (IOS) is a viable alternative as it has been used to effectively measure airway resistance in young children.⁶ It is a safe, objective, effort-independent, and sensitive method that can be used to detect changes in airway resistance with increasing concentrations of methacholine. In children 3–6 years who could also perform spirometry, a 45% increase in total airway resistance (R_5) correlated with a 20% reduction in FEV₁ (PC₂₀FEV₁).⁶ In adults, Short et al.⁷ demonstrated that a methacholine PC₂₀FEV₁ was equivalent to a 43.5% increase in total airway resistance.

The results of methacholine challenge with IOS as the endpoint at two different doses of β_2 -agonist has not been reported, in adults or children. It was our hypothesis that this method would be able to detect a difference in adjacent salmeterol doses in children (i.e., the smallest difference in dose that can be administered because of commercial availability).

MATERIALS AND METHODS

Subject Selection

The study protocol was approved by the University of Florida Institutional Review Board, and an informed consent form was signed by the parent or legal guardian before any procedures were begun. A description of the study is listed in ClinicalTrials.com (identifier # NCT01907334). Subjects 4–11 years with mild stable asthma were eligible for study inclusion and gave verbal assent to participate. The protocol called for airway responsiveness to methacholine defined as a provocative concentration of ≤ 8 mg/ml causing an increase of $> 40\%$ in total airway resistance (PC₄₀R₅). Subjects had to have the diagnosis of asthma for a minimum of 6 months. Female subjects were prepubertal. All subjects were required to be able to perform acceptable IOS technique according to the manufacturer's guidelines. Inclusion

criteria included the ability to demonstrate optimum inhalation technique with the study Diskus[®]. They were allowed to continue a low-dose inhaled corticosteroid as long as the dosage was stable for at least 4 weeks and entrance criteria were met.

Subjects were excluded if they had a history of life-threatening asthma, including loss of consciousness, intubation, and/or admission to ICU; if they had a hospitalization for acute asthma within the past year; if they required a short course of systemic corticosteroids for asthma in last 30 days or more than 4 courses in previous 12 months; if they used a short acting β_2 -agonist more than two times/week in the previous month; or if they were unable to withhold drugs that might interfere with the methacholine challenge for the recommended times. Also, subjects were excluded if they had a history of allergic reaction to any of the medications or formulations administered in this study, if they used a long acting β_2 -agonist in the 3 weeks before the first methacholine challenge, or if there was a change in dosage of nasal steroids in the previous 15 days or montelukast in the last 7 days. Also, subjects were excluded if they were unable to perform the outcome measurements correctly, were unable to demonstrate optimal inhalation technique with study medications or if there was an ECG abnormality of clinical relevance.

Study Design

This prospective, single-blind, fluticasone-controlled, two treatment, randomized, crossover pilot study consisted of four visits. The first visit (1a) was a screening visit to determine eligibility including the demonstration of proper inhalation technique with the Diskus. On the second visit (1b) a baseline methacholine challenge was performed after administration of two actuations of fluticasone propionate (Flovent Diskus 100 μ g). Visits 1a and 1b could have been performed on the same day if the patient had not taken a medication that had to be withheld. On visits 2 and 3, a methacholine challenge was performed 1 hr after pretreatment with the investigator-blinded study medication. After the PC₄₀R₅ was reached or after the highest methacholine concentration was administered (64 mg/ml), subjects inhaled 2–4 puffs of albuterol MDI through a valved holding chamber and IOS was repeated until at least 90% of the pre-challenge R₅ was reached to reverse bronchoconstriction. To control for variation in airway resistance, study visits were performed at the same time of day ± 2 hr. Vital signs were performed at each study visit. Albuterol was not used for at least 6 hr, a long-acting β_2 agonist was not used for 3 weeks, a leukotriene modifier was not used for 24 hr, antihistamines for 96 hr and caffeine containing foods or beverages for 4 hr before every challenge. If a patient was taking an inhaled steroid, it was held until the challenge was completed.

Study Drug Administration

Each treatment day consisted of one inhalation from two different dry powder inhalers. The two treatments were one actuation of Advair Diskus 100/50 μg and one actuation Flovent Diskus 100 μg , (the 50 μg salmeterol regimen); or one actuation from one Advair Diskus 100/50 μg and one actuation from a second Advair Diskus 100/50 (the 100 μg salmeterol regimen).

Advair and Flovent Diskuses are different colors. In order to maintain blinding of the salmeterol dose to the IOS technician and investigators, the study drugs were administered by an unblinded person not involved in data collection.

Optimum inhaler technique was taught at screening and reviewed at each visit. Inspiratory flow using the In-Check Dial[®] (Alliance Tech Medical, Granbury, TX) was measured before dosing to confirm uniform inhalation technique.

Methacholine Challenge Test

Methacholine challenges were performed using a modification of the American Thoracic Society Guidelines for Methacholine Testing.⁸

At screening Visit 1b, IOS was performed before and 1 hr after administration of Flovent. The challenge did not proceed if R_5 increased by $\geq 20\%$ after saline. The starting concentration of methacholine was 0.25 mg/ml with quadrupling concentrations given, at 5 min intervals, up to a maximum of 8 mg/ml. At study Visits 2 and 3 the challenge was started 60 (± 5) min post-administration of study drug. The starting concentration of methacholine was one dilution below the final concentration given at Visit 1b (i.e., that which caused $\text{PC}_{40}R_5$) with quadrupling concentrations given, at 5 min intervals, up to a maximum of 64 mg/ml. When the $\text{PC}_{40}R_5$ was reached, the highest methacholine concentration given, or at any time during testing that the subject requested to stop, the challenge was ended and bronchoconstriction was reversed with albuterol given by MDI through an anti-static chamber. IOS was repeated at 15-min intervals until the subject's R_5 had decreased to 90% of the pre-challenge baseline.

Impulse Oscillometry

Impulse oscillometry was performed with the Jaeger MasterScreen IOS (CareFusion, Yorba Linda, CA).

It consists of a loudspeaker on one arm of a y-adaptor, a terminal resistor on the other, a pneumotachograph with mouthpiece on the lower leg, and is connected to a computer. As the subject breathes tidally through the mouthpiece, sound waves are generated by the loudspeaker and superimposed on the subject's breaths, providing a pressure-flow relationship that is used to calculate the various components of airway resistance. The measure of

total airway resistance (R_5) was selected as the primary endpoint. Large airway resistance (R_{20}), peripheral airway resistance ($R_5 - R_{20}$), and area under the reactance curve (AX), a measure of lung compliance, were also collected. The procedure was performed in triplicate, and the average of the three measurements was used.

Data Analysis

Ten subjects were arbitrarily selected. $\text{PC}_{40}R_5$ values were $\log_2$ transformed. If a subject did not experience at least a 40% increase in R_5 after the highest methacholine concentration, 64 mg/ml, a value of 128 mg/ml was assigned as the $\text{PC}_{40}R_5$ on days when salmeterol was administered. A linear mixed-effects model was fitted under a homoscedastic residual covariance structure with subject as random effect and dose as a fixed effect (see online repository for more details). Dose-response was assessed as a priori contrast between 50 and 100 μg . Additionally, the results were fitted to an $E_{\max}$ model.

Monte Carlo simulations were performed to estimate the sample size required to establish bioequivalence of generic and innovator products with 80% and 90% power (online repository).

RESULTS

Of 15 subjects screened, 10 completed the study (Fig. 1). The mean (SD) age was 8.2 (1.6) years (Table 1). All but two of them were prescribed a low dose of inhaled corticosteroid before the study. All of the subjects were able to perform IOS reproducibly. The mean (SD) baseline total airway resistance (R_5) was 6.9 (1.5) cm $\text{H}_2\text{O}/\text{L}/\text{sec}$ and remained constant on each study day (Table 2).

There was a significant bronchodilator response. The decrease in R_5 from pre-dose to 1 hr post-dose, before methacholine, was 15.5% after 50 μg SM ($P = 0.00002$) and 18.4% after 100 μg ($P = 0.002$; Fig. 2). However, there was no significant difference between doses, that is, the dose-response curve was flat. Similarly, there was no trend for a dose-response for bronchodilatation on large airway resistance (R_{20}) or area under the conductance curve (AX; see online repository Figs. S2 and S3).

There was a significant non-linear increase in $\text{PC}_{40}R_5$ with SM dose (Fig. 3). The a priori contrast of 50 and 100 μg SM approached significance ($P = 0.051$). The maximum effect ($E_{\max}$) was 80 mg/ml, the dose of SM producing 50% of the maximum effect (ED_{50}) was 47 μg and the $\text{PC}_{40}R_5$ without SM (E_0) was 2.4 mg/ml. However, variability in $\text{PC}_{40}R_5$ response was observed (Fig. 4).

The geometric mean (95%CI) for the $\text{PC}_{40}R_5$ ratio of 100/50 μg SM was 2 (0.9–5). There was no significant dose-response for the other IOS parameters (see online repository Figs. S4–6).

No adverse effects were observed.

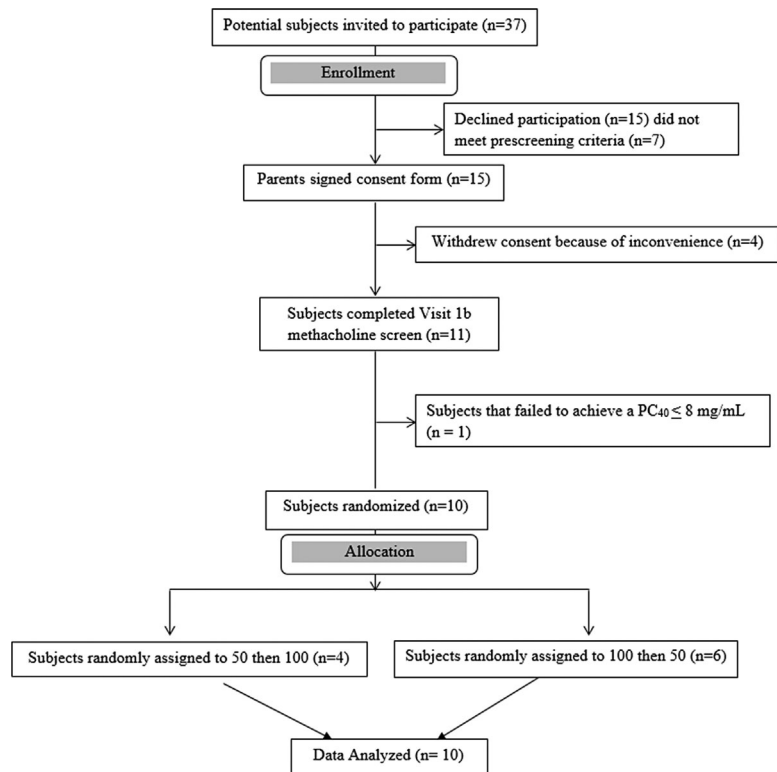


Fig. 1. Consort flow diagram of enrollment, allocation, and analysis.

TABLE 1—Demographics of Subjects With Completed Protocol

Subject #	Age (year)	Height (cm)	Weight (kg)	Controller meds
2	9	142	49	QVAR 160 µg/d
3	8	137	44	Flovent 176 µg/d
5	9	137	34	QVAR 160 µg/d
6	8	128	27	QVAR 320 µg/d
8	8	127	22	Flovent 220 µg/d
9	7	125	26	Flovent 176 µg/d
12	6	121	20	None
13	11	147	36	Flovent 176 µg/d
14	10	147	37	QVAR 160 µg/d
15	6	130	28	Singulair 5 mg
Arithmetic mean	8.2	134.1	32.3	
SD	1.6	9.3	9.4	

SD, standard deviation.

DISCUSSION

To the best of our knowledge, this is the first study on the dose-response of SM using methacholine challenge with IOS as the endpoint. The baseline total airway resistance remained relatively constant on each study day for each subject, indicating the reproducibility of IOS. All of the subjects could effectively finish IOS on each visit. Total airway resistance (R₅) was significantly decreased with both doses of SM. However, IOS alone could not detect a significant difference between doses. In contrast,

when methacholine challenge was used as the endpoint, a difference between the bronchoprotective effect of 50 and 100 µg SM was identified ($P = 0.05$ with only 10 subjects). As the concentration of methacholine was titrated against R₅, it was not possible to calculate a PC₄₀ for other endpoints. However, the increase in airway resistance for the small airways (R₅–R₂₀), for example, was not less after 100 µg than after 50 µg, that is, there was no dose-response for bronchoprotection. It may be a function of where methacholine is exerting bronchospasm.

TABLE 2—Baseline R_5 (cm H₂O/L/sec)¹ on Each Study Day

Subject	Screening	Screening MC	Before 50 μ g	Before 100 μ g
2	8.4	7.3	7.1	7.1
3	8.3	7.5	7.6	7.6
5	5.7	5.4	5.5	6
6	7.8	7.4	6.8	8.6
8	6.7	7.3	6.1	6.0
9	7.7	6.0	7.3	7.2
12	8.2	6.8	6.4	6.5
13	3.9	4.2	4.1	4.1
14	5.7	5.6	5.1	4.3
15	7.4	7.3	6.3	6.6
Mean	6.9	6.5	6.2	6.4
SD	1.5	1.1	1.1	1.4

MC, methacholine; SD, standard deviation.

¹SI (kPa $\times$ L/sec) = cmH₂O/L/sec $\times$ 0.098.

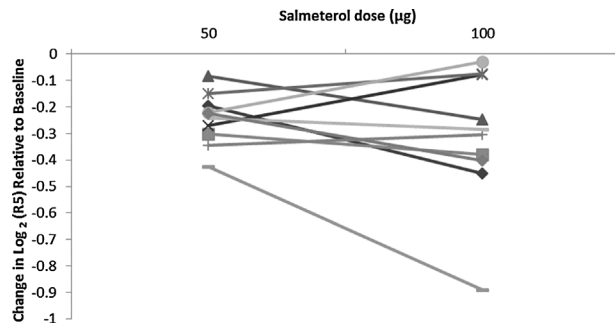


Fig. 2. Bronchodilator response expressed as change in $\log_2$ total airway resistance (R_5) measured before and 1 hr after the salmeterol dose, just before beginning the methacholine challenge. The larger the negative number, the larger the decrease in R_5 . The decrease was significant* ($P=0.00002$ for 50 μ g and $P=0.002$ for 100 μ g) but there was no significant difference between doses.

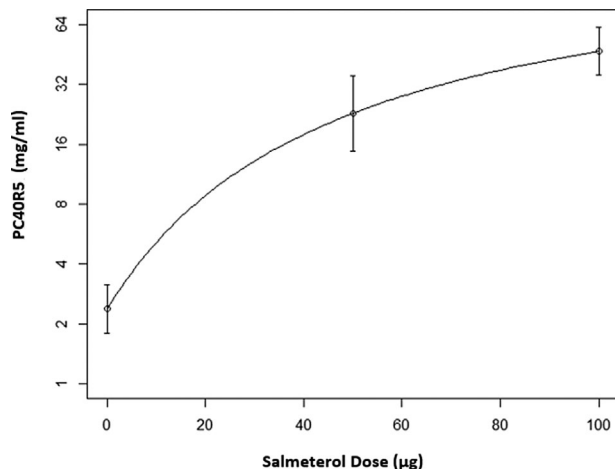


Fig. 3. Geometric mean ($\pm$ geometric SD) provocative concentration of methacholine required to increase total airway resistance by 40% (PC_{40R_5}) fit to an E_{max} curve. The y-axis is $\log_2$ scale but the numbers are the antilog. The difference between the two doses of SM was nearly significant ($P=0.051$). The zero dose PC_{40R_5} (E_0) was 2.4 mg/ml, the maximum effect PC_{40R_5} (E_{max}) was 80 mg/ml and the dose of SM producing 50% of E_{max} (ED_{50}) was 47 μ g.

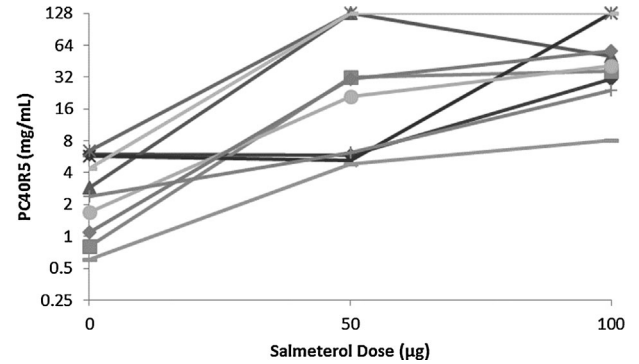


Fig. 4. The concentration of methacholine producing a 40% increase in total airway resistance (PC_{40R_5}) 1 hr after 0, 50, and 100 μ g single doses of inhaled salmeterol. The higher the PC_{40R_5} , the more methacholine it took to achieve the target of 40% increase in R_5 . The difference between the 50 and 100 μ g doses was nearly significant ($P=0.051$).

During each of the three study days, the dose of fluticasone propionate remained constant at 200 μ g. This would not have affected the PC_{40R_5} . It takes about 72 hr of twice daily dosing before airway responsiveness is decreased.⁹ In addition, multiple doses of FP only have a small effect on PC_{20} . In one study of children 6–14 years, 6 weeks of 200 μ g/day only doubled the mean PC_{20} compared to placebo.¹⁰ Moreover, most of the subjects were already on daily inhaled corticosteroids.

The results of the simulations to estimate power and sample size indicate that at a test dose of 50 μ g SM, 72 subjects would be needed to demonstrate bioequivalence at 80% power and 88 subjects at 90% power with a 90%CI of 0.67 and 1.5 (online repository).

In conclusion, methacholine challenge in children using IOS as the endpoint can be used to determine the equivalence of a generic inhaled product containing SM. This method could also be used for determining the equivalence of the same drug delivered by different devices (e.g., MDI vs. dry powder inhaler).

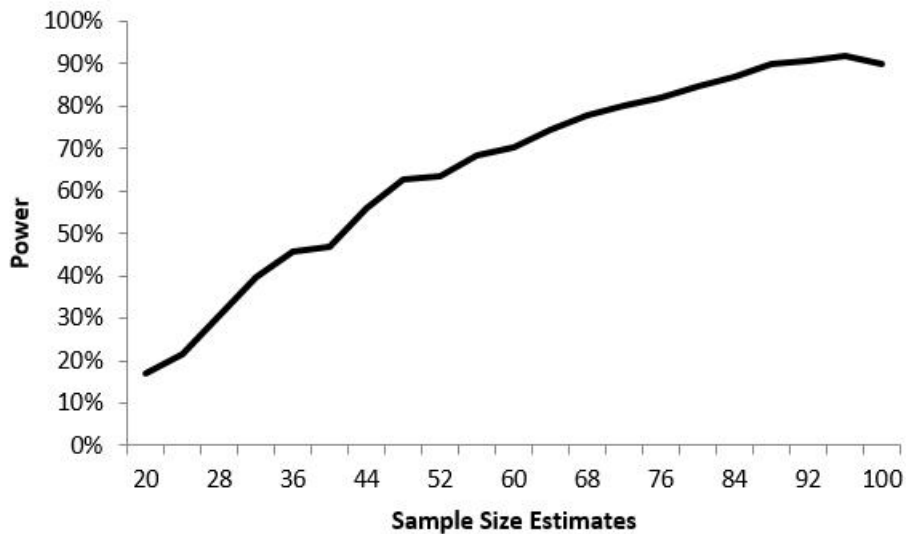
REFERENCES

1. Food and Drug Administration. Draft Guidance on Albuterol Sulfate. Recommended April 2013, Revised June 2013. <http://www.fda.gov/downloads/drugs/guidancecomplianceregulatoryinformation/guidances/ucm346985.pdf>. Accessed 22 May 2015.
2. European Medicines Agency, Committee for Medicinal Products for Human Use (CHMP). Guideline on the requirements for clinical documentation for orally inhaled products (OIP) including the requirements for demonstration of therapeutic equivalence between two inhaled products for use in the treatment of asthma and chronic obstructive pulmonary disease (COPD) in adults and for use in the treatment of asthma in children and adolescents. CPMP/EWP/4151/00 Rev. 1; 22 January 2009. http://www.ema.europa.eu/docs/en_GB/document_library/Scientific_guideline/2009/09/WC500003504.pdf. Accessed 22 May 2015.
3. Ahrens RC, Hendeles L, Clarke WR, Dockhorn RJ, Hill MR, Vaughan LM, Lux C, Han SH. Therapeutic equivalence of Spiros

- dry powder inhaler and Ventolin metered dose inhaler: a bioassay using methacholine. *Am J Respir Crit Care Med* 1999;160:1238–1243.
4. Simons FE, Soni NR, Watson WT, Becker AB. Bronchodilator and bronchoprotective effects of salmeterol in young patients with asthma. *J Allergy Clin Immunol* 1992;90:840–846.
 5. Palmqvist M, Ibsen T, Mellén A, Lötvall J. Comparison of the relative efficacy of formoterol and salmeterol in asthmatic patients. *Am J Respir Crit Care Med* 1999;160:244–249.
 6. Schulze J, Smith HJ, Fuchs J, Hermann E, Dressler M, Rose MA, Zielen S. Methacholine challenge in young children as evaluated by spirometry and impulse oscillometry. *Respir Med* 2012;106:627–634.
 7. Short PM, Anderson WJ, Manoharan A, Lipworth BJ. Usefulness of impulse oscillometry for the assessment of airway hyper-responsiveness in mild-to-moderate adult asthma. *Ann Allergy Asthma Immunol* 2015;115:17–20.
 8. Crapo RO, Casaburi R, Coates AL, Enright PL, Hankinson JL, Irvin CG, MacIntyre NR, McKay RT, Wanger JS, Anderson SD, et al. Guidelines for methacholine and exercise challenge testing—1999. This official statement of the American Thoracic Society was adopted by the ATS Board of Directors, July 1999. *Am J Respir Crit Care Med* 2000;161:309–329.
 9. Sovijärvi AR, Haahtela T, Ekroos HJ, Lindqvist A, Saarinen A, Poussa T, Laitinen LA. Sustained reduction in bronchial hyper-responsiveness with inhaled fluticasone propionate within three days in mild asthma: time course after onset and cessation of treatment. *Thorax* 2003;58:500–504.
 10. Hofstra WB, Neijens HJ, Duiverman EJ, Kouwenberg JM, Mulder PGH, Kuethe MC, Sterk PJ. Dose-responses over time to inhaled fluticasone propionate treatment of exercise- and methacholine-induced bronchoconstriction in children with asthma. *Pediatr Pulmonol* 2000;29:415–423.

SUPPORTING INFORMATION

Additional supporting information may be found in the online version of this article at the publisher's web-site.



Salmeterol dose (μg)

50

100

Change in Log_2 (R20) Relative to Baseline

0.20

0.10

0.00

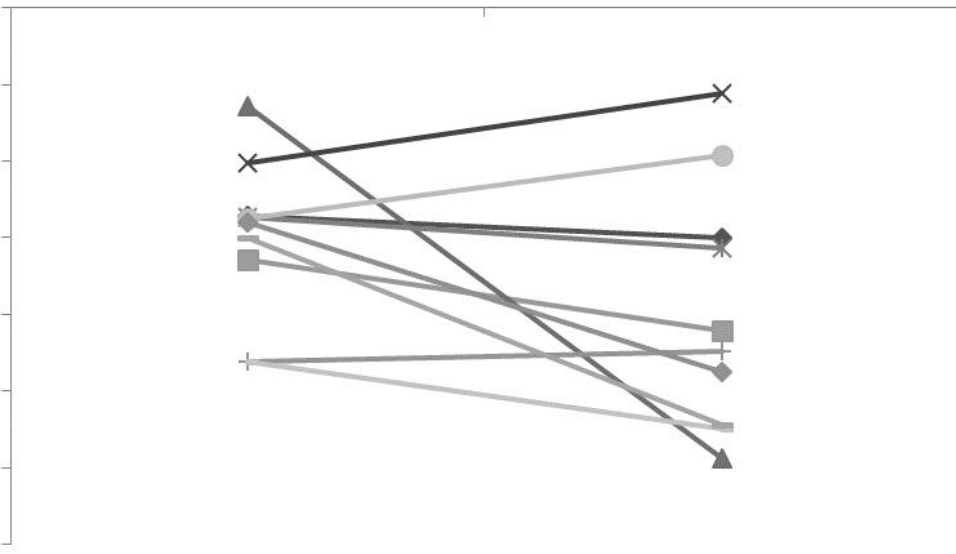
-0.10

-0.20

-0.30

-0.40

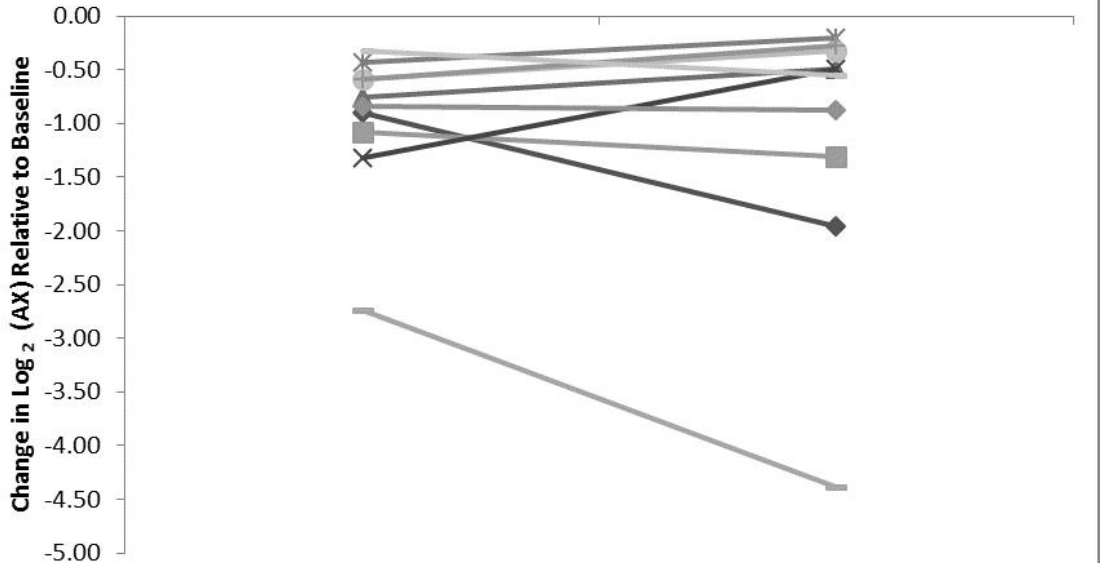
-0.50

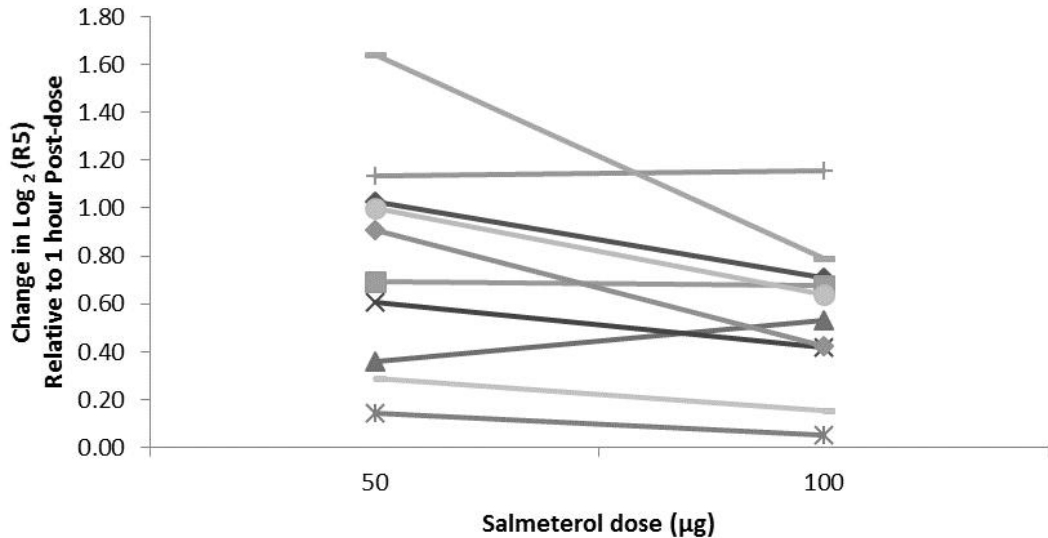


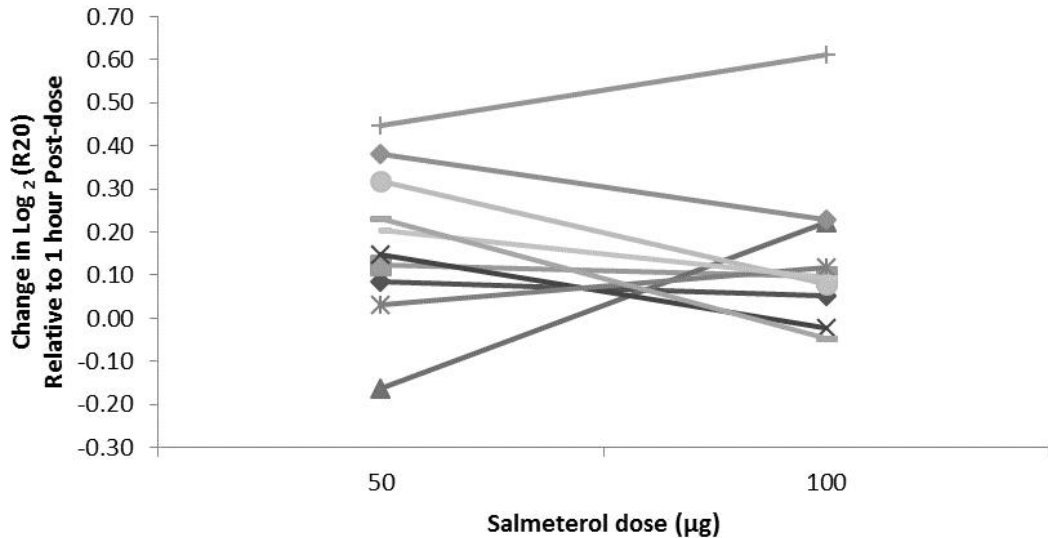
Salmeterol dose (μg)

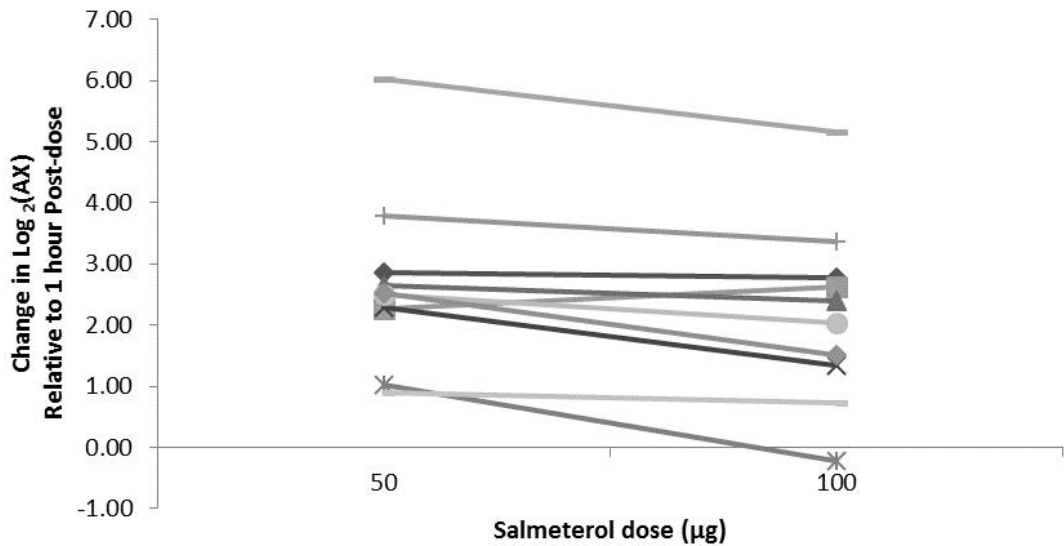
50

100









Online Repository

Statistical Method:

Distribution of the PC_{40R5} values at each of the three doses were assessed separately using the Kolmogorov-Smirnov test for normality. The data approximated a log-normal distribution at each dose level ($p > 0.05$). Linear mixed-effects models were fitted under homoscedastic and heteroscedastic residual covariance structures with subject as a random effect and dose as a fixed effect. These two models were compared using the corrected Akaike Information Criterion (AICC). Dose-response was assessed as an *a priori* contrast between the 50 and 100 μg . Additionally, an E_{max} model was estimated based on the pooled means at the three reference dose levels (0, 50, and 100 μg).

Monte Carlo simulations were conducted to estimate the sample size required to establish bioequivalence (BE) within predefined limits [0.67, 1.5] with 80 and 90% probability of successfully meeting these bioequivalence (BE) criteria (i.e., 80 and 90% power). The dose effects, and between-and within-subject variances required for the simulations were estimated from the linear mixed-effects model using the dataset described in the text.

A 4-way, randomized, crossover experimental design with three reference doses (0, 50 μg , 100 μg) and one test dose (50 μg) was assumed for the simulations. Varying sample sizes (N) were used (ranging from 20 to 100 in increments of 4) and 500 datasets were simulated for each value of N. For each of these simulated datasets, 1000 bootstrap samples were obtained. For each bootstrap sample the data points at reference doses were used to fit an E_{max} model to obtain the

estimates (E_O , $E_{\max}$, and ED_{50}) and the $PC_{40}R_5$ values at the test dose were averaged to estimate the test dose response (E_T). The dose of reference (D_R) required to achieve the response level E_T was obtained by inverting the $E_{\max}$ model:

$$D_R = \frac{(E_T - E_O + ED_{50})}{E_{\max} - E_T + E_O}$$

The potency ratio (PR) for test dose (D_T) = 50 μ g was then computed as:

$$PR = \frac{D_R}{D_T}, \text{ where } D_T = 50 \mu\text{g}$$

For each of the 500 simulated datasets, 1000 PRs computed in this manner were used to obtain a 90% confidence interval (CI) for the PR for that simulated dataset. The proportion of these CIs that fell within the predetermined bioequivalence limits [0.67, 1.5] was defined as the power to test bioequivalence at the given sample size.

Statistical Results:

The dataset contained $PC_{40}R_5$ values for 10 subjects measured at 3 dose levels: 0 μ g; 50 μ g; and 100 μ g. The means ($\pm$ standard deviations) of the log base 2-transformed $PC_{40}R_5$ values at 0 μ g, 50 μ g, and 100 μ g were 1.24 ($\pm$ 1.27), 4.52 ($\pm$ 2.01), and 5.55 ($\pm$ 1.26), respectively. Based on the AICC, the model assuming a homoscedastic covariance structure was selected for further analysis. The estimated fixed effects for the dose from the mixed model at 0 μ g, 50 μ g, and 100 μ g were 1.2465, 4.5145, and 5.5548 ($p = 0.051$ for the *a priori* contrast of 50 μ g and 100 μ g).

The estimated between- and within-subject variances were 1.0211 and 1.3770, respectively. The estimated parameters for an $E_{\max}$ model of these results were: $E_O = 1.24651$ (antilog=2.4 mg/mL); $ED_{50} = 46.7043$ mcg; and $E_{\max} = 6.3204$ (antilog 80 mg/mL).

The results of the simulations to estimate power and sample size suggested that with a test dose of 50 µg, 72 subjects would be needed to demonstrate bioequivalence at 80% power, while 88 subjects would be needed to achieve 90% power (E-Figure 1).

Image Legends:

1. Results of Monte Carlo Simulation to estimate power at varying sample sizes at test dose of 50 μg .
2. Bronchodilator response expressed as change in $\log_2$ large airway resistance (R_{20}) measured before and 1 hour after the salmeterol dose, just before beginning the methacholine challenge. The larger the decrease in post-salmeterol R_5 the greater the bronchodilator effect and the larger the negative value on the y-axis.
3. Effect of salmeterol on lung compliance expressed as change in $\log_2$ area under the reactance curve (AX) measured before and 1 hour after the salmeterol dose, just before beginning the methacholine challenge. The larger the decrease in AX the greater the bronchodilator effect and the larger the negative value on the y axis.
4. Bronchoconstrictor response to methacholine expressed as $\log_2$ change in total airway resistance (R_5) from post salmeterol dose before the challenge to the value after the last dose of methacholine. The larger the positive value the greater the resistance and thus bronchoconstrictor effect.
5. Bronchoconstrictor response to methacholine expressed as $\log_2$ change in large airway resistance (R_{20}) from post salmeterol dose before the challenge to the value after the last dose of methacholine. The larger the positive value on the y-axis the greater the increase on resistance in the large airways.
6. Effect of methacholine on lung compliance expressed as change in $\log_2$ area under the reactance curve (AX) measured 1 hour after the salmeterol dose before the methacholine challenge to the value measured after the last concentration of methacholine. The larger the value on the y-axis the greater the bronchoconstrictor response.