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A linear mixed-effects statistical model for in-vivo evaluation of recombinant human growth hormone implants in hypophysectomized rats

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Abstract

We have used a linear mixed-effects statistical model to evaluate previously published results of body weight evolution in hypophysectomized (Hpx) rats after administration of two different controlled-release formulations of recombinant human growth hormone (rhGH). Using the linear mixed-effects model, it was possible to distinguish between maximal pharmacological response with time in different subjects and relate it to the structure of the different formulations, the release of the hormone from them and the time necessary to obtain a quantitative result as a consequence of the hormone activity, contrary to the multivariate variance analysis model (MANOVA) used in our earlier work. These results confirmed that the maximum body weight gain obtained with the controlled-release implants is similar to that with subcutaneous rhGH, but with the advantage that laminar biodegradable implants need to be administered only once every 2 weeks.

Introduction

The evaluation of biodegradable recombinant human growth hormone (rhGH) devices using experimental animals is developed practice (García et al 2002; Takada et al 2003a, b; Kim et al 2005). However, because of the difficulties of extrapolating these results to humans, the usefulness of these studies depends on having a relevant pharmacological objective and a proper statistical model to interpret the different experimental variables. A number of issues need to be resolved for a useful model to be developed.

The first issue relates to rhGH. Body weight gain and tibial growth in hypophysectomized (Hpx) rats have been used as indexes for rhGH pharmacological activity in animal models (Van Dyke & Wallen-Lawrence 1930; Evans et al 1943; Clark et al 1996; Carrillo et al 2000).

Secondly, a suitable statistical model must be able to interpret not only the evolution of the pharmacological objective being studied, but also differences between subjects. A variety of statistical treatments of body weight evolution have been reported (Clark et al 1996; Carrillo et al 2000; Takada et al 2003a; Rising et al 2005), some of which have proved the usefulness of rhGH treatment in different animals whereas others have not. Some authors have studied the pharmacological response to rhGH in-vivo over time using a variety of statistical methods, but variability between animals has not been taken into account in such statistical models.

In a previous report, we evaluated in-vivo results obtained with rhGH formulations after implantation in Hpx rats using multivariate variance analysis (MANOVA) and multiple comparison analysis, based on the Bonferroni test or the Scheffé S-method (García et al 2002). In this new research, we have developed a linear mixed-effect statistical model suitable for the evaluation of previously reported results. The aims were to calculate body weight evolution of Hpx rats with time, taking into account variability between animals, and to estimate improvement achieved with rhGH implants as an alternative to subcutaneous injection of rhGH. These implants offer the possibility of a constant release rate, requiring administration only once every 2 weeks.

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Materials and Methods

The materials and methods used for the synthesis and characterization of copoly (D,L-lactic) glycolic acid (PLGA), preparation of rhGH laminar implants by solvent casting, measurement of drug content and in-vitro and in-vivo release studies have been described previously (García et al 2002). Two different laminar implant rhGH formulations (F1 and F2), implanted into the back of the neck, were compared with a similar subcutaneous dose of rhGH (Norditropin; NR) and placebo in Hpx rats. For more details see García et al 2002.

Statistical analysis

The previously used linear mixed-effects model was:

$$y_{ij} = (\beta_1 + \beta_2 z_{2i} + \beta_3 z_{3i} + \beta_4 z_{4i} + b_{0i}) + (\beta_5 + \beta_6 z_{2i} + \beta_7 z_{3i} + \beta_8 z_{4i} + b_{1i}) t_{ij} + (\beta_9 + \beta_{10} z_{2i} + \beta_{11} z_{3i} + \beta_{12} z_{4i} + b_{2i}) t_{ij}^2 + \varepsilon_{ij} \quad (1)$$

where y_{ij} is the weight of i-nth rat observed at time t_{ij} and z_{ij} is a dummy variable to introduce the formulation effect into the model. The vector $\beta = (\beta_1, \beta_2, \ldots \beta_{12})^T$ (T is the transpose of vector β) is the vector of fixed effects, which accounts not only for the formulation effect but also for fixed time effect. The random effect is accepted to be distributed as:

$$b = \sim N(0, \psi)$$

$$\varepsilon_{ij} \sim N(0, \sigma^2)$$

where vector b is the random variation between rats in independent and first- and second-degree time effect, ψ is its covariance matrix and ε is the vector of residual random effect. Pinheiro and Bates (2000) give a detailed description of mixed-effects models.

Equation (1) takes the following form for group 1 (placebo formulation):

$$y_1(t) = \beta_{1,0} + \beta_{1,1} * t + \beta_{1,2} * t^2 \quad (2)$$

Taking this function of placebo treatment as the normal evolution of body weight in Hpx rats, the values of the constant, linear and quadratic terms for the other experimental groups change as shown:

$$y_2(t) = (\beta_{1,0} + \beta_{2,0}) + (\beta_{1,1} + \beta_{2,1}) * t + (\beta_{1,2} + \beta_{2,2}) * t^2 \quad (3)$$

$$y_3(t) = (\beta_{1,0} + \beta_{3,0}) + (\beta_{1,1} + \beta_{3,1}) * t + (\beta_{1,2} + \beta_{3,2}) * t^2 \quad (4)$$

$$y_4(t) = (\beta_{1,0} + \beta_{4,0}) + (\beta_{1,1} + \beta_{4,1}) * t + (\beta_{1,2} + \beta_{4,2}) * t^2 \quad (5)$$

Because the above equations are used with the average response of body weight gain of Hpx rats in each group, the effect on each rat in each treatment is observed for group 2 as:

$$y_{i(2)}(t) = (\beta_{1,0} + \beta_{2,0} + b_{0,i}) + (\beta_{1,1} + \beta_{2,1} + b_{1,i}) * t + (\beta_{1,2} + \beta_{2,2} + b_{2,i}) * t^2 \quad (6)$$

Model coefficients are estimated using a maximum-likelihood method, allowing the use of a different number of individuals in each group. Software R version 2.1.1 (Statistics Department of Auckland University, Auckland, USA) was used to develop the statistical evaluation of the results using the linear mixed-effects function.

Results and Discussion

Statistical treatment of in-vivo results

Body weight gain in Hpx rats is the response most widely used to test the pharmacological activity of rhGH. The maximum-likelihood estimation shows that the model with weighted power function of errors is preferable to the homoscedastic one. Figure 1 shows the individual weight evolution for each rat studied, together with the predicted values for individual rats and each group studied. The Hpx rats were initially randomized into four groups of seven rats. Rats 1–7 were not treated; rats 8–14 received a total GH dose of 105 μg in seven subcutaneous injections of NR on alternate days; rats 15–21 received placebo implants; rats 22–27 and 34 received a total dose of rhGH of 85 μg in the F_1 formulation; rats 28–33 and 35 received F_2 formulation, total hormone dose 85 μg; however, as rat 35 appeared weaker than the rest and we thought it would not survive, another rat (number 36) was included in the experiment.

Figure 1 shows that the rats in the placebo group lost weight, described by a polynomial function, whereas the evolution of the response is linear for the NR formulation. For F_1 and F_2 formulations, the weight gain evolution shows curves with different maximal values. Estimated values for the mixed-effects statistical model are shown in Table 1. Three treatment coefficients were different from the control group: the values of the constant, linear and quadratic terms for the placebo group show the following estimated function: $y = 83.0 + (-0.47) * t + (+0.017) * t^2$. These placebo values were modified according to the addition of each estimated value to each term of the function for the other groups. Then, the observation function for the group that received the NR treatment is $y = (83.0 - 1.52) + (-0.47) + 1.04) * t + (+0.017 - 0.02) * t^2$. We can similarly obtain the coefficients for the F_1 and F_2 formulations.

Statistical analysis of the results obtained (Table 1) revealed significant differences in the linear and quadratic terms between the formulations administered and placebo. The control group showed a quasi-linear weight loss, being 0.098 the *P* value for the quadratic term of the null hypothesis, although we cannot discount the fact that the Hpx rats weight tended to stabilize in a short time period (see Figure 2A). The NR group showed a linear weight gain because the null hypothesis on the quadratic coefficient was acceptable (0.108). This behaviour depends on the length of the assay: if it is made longer, the rats' weights tend to stabilize. The total weight gain was 8.37 g, with an average rate of 0.492 g per day (Table 2). This result supports the dosage regimen given by Thakkar et al (1998). The group that received the F_1 formulation showed a quadratic weight gain. The estimated plot for this group showed a maximum at 12.8 administering days, with a weight gain at that moment of 7.18 g. The estimated weight gain was 6.42 g at the end of the assay. The group that

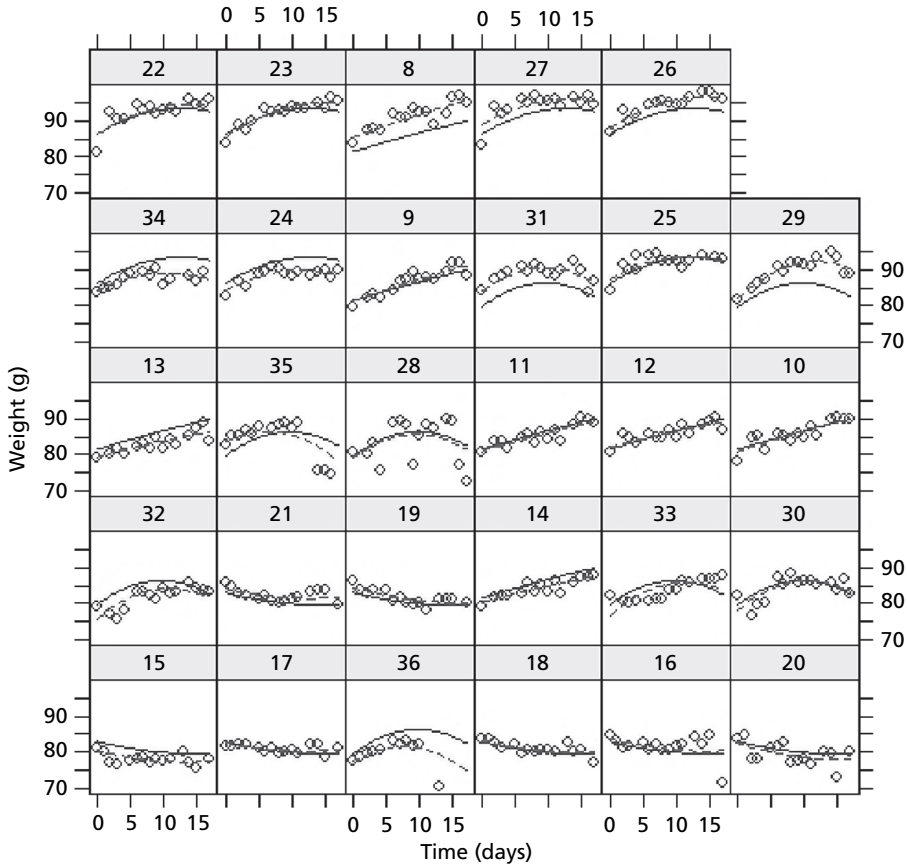

Figure 1 Body weight evolution of each Hpx rat studied over 2 weeks (dots), predicted values for each (discontinuous line) and for each group studied (continuous line).

Table 1 Estimated values of fixed parameters of mixed-effects statistical model. Intercept, days, and days^2 are the constant, linear and quadratic coefficients of the function, respectively. Each term is modified according to the addition of the estimated values of NR, F_1 and F_2. NR: Norditropin subcutaneous injection; F_1 and F_2 are different formulations of rhGH

	Value	s.e.	Degrees of freedom	*T* value	*P* value
Intercept	83.01	1.088	392	76.32	0.000
NR	–1.516	1.567	25	–0.967	0.343
F_1	3.269	1.514	25	2.160	0.041
F_2	–3.363	1.521	25	–2.210	0.037
days	–0.473	0.184	392	–2.569	0.011
days: NR	1.037	0.252	392	4.117	0.000
days: F_1	1.595	0.234	392	6.806	0.000
days: F_2	1.855	0.250	392	7.408	0.000
days^2	0.017	0.010	392	1.659	0.099
days^2: NR	–0.021	0.013	392	–1.612	0.108
days^2: F_1	–0.060	0.012	392	–4.997	0.000
days^2: F_2	–0.088	0.013	392	–6.650	0.000

received the F_2 formulation also showed a quadratic weight gain, with a maximum estimated weight at 9.7 days of treatment and a predicted weight gain of 6.69 g. However, the weight gain was only 2.88 g by the end of the assay. Thus, with the F_1 formulation the maximum weight gain is obtained later than with the F_2 formulation, but with the latter, the weight gain is lower at the end of the assay. This reflects the different preparation procedures and polymeric film degradation (Santoveña et al 2006). In formulation F_1, a lyophilized rhGH cake is inside a polymer film, so the rate of hormone release depends on variation in the molecular weight of the polymer and its diffusion from the core depends on degradation of the laminar implant. Absorption, distribution and pharmacological activity of GH are expected to be later with the F_1 formulation than with F_2 formulation. In the latter the rhGH is dispersed into the polymeric solution used to prepare the film and is therefore distributed evenly over the surface and inside the laminar matrix (Santoveña et al 2006), permitting maximum weight gain earlier than with the F_1 formulation.

The maximum weight gain obtained with the F_1 and F_2 formulations (7.18 / 8.37 = 0.85 and 6.69 / 8.37 = 0.80, respectively) is similar to that obtained for the NR group (with the above mentioned difference obtained at different times). In fact, these values are close to the rate for the total quantity of hormone administered in both types of implant and NR subcutaneous injection (85 / 105 = 0.81). Thus, there is an in-vivo relationship between the maximum weight gain obtained for each formulation and the total rhGH dose administered. It can be concluded that the linear mixed-effects statistical model

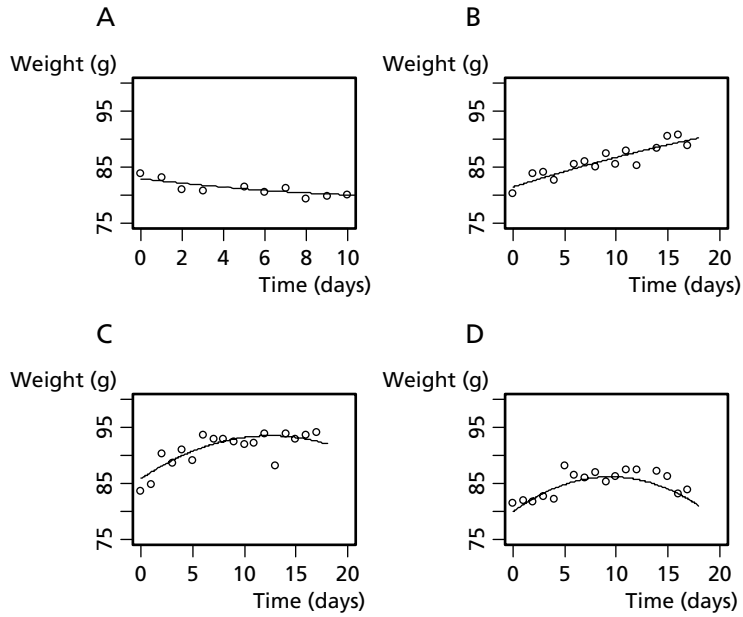

Figure 2 Estimated and experimental evolution of rat body weight over 15 days' treatment in the placebo group (A) and in rats receiving subcutaneous injection of rhGH (NR) (B), formulation F_1 (C) and formulation F_2 (D).

Table 2 Summary of the mixed-effects statistical results

Formulation	Time max (days)	Max weight gain (g)	Average rate (g day^{-1})	Weight at 17th day (g)
NR	17.0	8.37	0.492	8.37
F_1	12.8	7.18	0.561	6.42
F_2	9.7	6.69	0.691	2.88

NR, Norditropin subcutaneous injection; Time max, time at which the maximum value of weighed plots is reached; Max weight gain, predicted weight gain at time max; Average rate, predicted weight gain divided by time max; Weight at the 17th day, predicted weight gain with respect to initial weight.

allows evaluation of in-vivo results obtained with controlled-release formulations when growth curves are employed to study a pharmacological response with time of a certain drug – in this case, rhGH. Unlike the MANOVA statistical method used in our earlier work, the relationship obtained between the two rhGH laminar-implant formulations and the rhGH subcutaneous medication shows nearly the same maximum weight gain, even if we compare it with the relationship between rhGH dosage in both types of treatments. The in-vivo results with the two rhGH controlled-release formulations show that the maximum body weight gain in Hpx rats occurred more quickly than with the subcutaneous rhGH treatment. Using this statistical treatment, we can differentiate not only the variability between subjects but also the time of maximum response obtained after administering different biodegradable formulations, and relate these to the distribution of the drug in the film, the ease of drug release from each device and the period of time necessary to obtain a pharmacological response. It would be interesting to study body weight evolution with this type of laminar implant but with a higher dose of GH, in order to prolong the effect of a single dose beyond 15 days.

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