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Pharmacokinetics analysis of sustained release hGH biodegradable implantable tablets using a mouse model of human ovarian cancer

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ABSTRACT

This paper presents the pharmacokinetic of human growth hormone (hGH) implantable tablets tested on a human ovarian cancer mouse model. In order to obtain a sustained release device which permits to administer a high dose of the hormone that keeps its integrity and stability, three different formulations of hGH–poly (D,L-lactic-co-glycolic acid) (PLGA) were elaborated by direct compression method varying hormone load, PLGA content and compactation time. *In vitro* studies showed that drug release was mainly controlled by hormone load. Pharmacokinetic studies were conducted by using immunodeficient female mice. Four days before the insertion of hGH implantable tablets in the peritoneal cavity, every mouse received 5×10^6 human ovarian cancer cells (SKOV3.ip1). Hormone serum levels were monitored through bleeding from eye orbital vessels. The population pharmacokinetic model used was based on the in series tank model and model parameters were estimated using the maximum likelihood method. The null hypothesis test about differences between formulations leads us to the conclusion that the three formulations showed the same kinetic behavior except for the hGH load. The hormone release was extended all over 2 weeks but no increase or decrease in survival time was observed. These results suggest that hGH serum levels do not facilitate tumoral cells proliferation, an expected effect of hGH and this could explain why survival times of mice treated with implantable tablets are not shorter than those treated with the control ones.

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1. Introduction

Human growth hormone (hGH) is a monomeric protein secreted by the somatotrophs of the anterior pituitary gland and it is necessary to confer normal postnatal growth and development. hGH secretion is stimulated by growth hormone releasing hormone (GHRH) and ghrelin while it is negatively regulated by somatostatin (SS). The hepatic insulin-like growth factor-1 (IGF-1) secretion is stimulated by hGH although the hormone has many additional IGF-1 independent effects on growth (Perry et al., 2006). hGH replacement therapy requires daily injections which negatively affect patient compliance and comfort. Biodegradable polyesters, such as poly (D,L-lactide-co-glycolide) (PLGA) are widely used as components of marketed biodegradable parenteral drug delivery systems, in form of microparticles, matrices, fibres and films, intended to be injected, implanted or inserted for local or systemic treatments (Blasi et al., 2005; Wang et al., 2007; Rouse et al., 2007). The availability of these types of systems which deliver hGH with a controlled rate via subcutaneous route has been a significant improvement of the current daily treatment (Vlugt-Wensink et al., 2007; Santoveña et al., 2007; Chung et al., 2008; Kwak et al., 2009). The most sustained release formulations of proteins and peptides are administered via parenteral route (Johnson, 2000), due to the rapid and reproducible response and localized therapy and controlled release for nonstable drugs in gastrointestinal tract (McLennan et al., 2005) as comparison with other routes which have plasmatic clearance mechanisms, absorption barriers and enzymatic activity.

In recent years, a great interest has been generated on studying the potential role of the IGF-1 in ovarian cancer (Perry et al., 2006; Spentzos et al., 2007). High circulating levels of this peptide has been associated to an increasing risk before age-55 (Lukanova et al., 2002) and its role through *in vitro* increased cell growth has been tested (Khandwala et al., 2000). In contrast, other studies suggest that hGH based gene therapy may offer new therapeutics prospects in cancer therapy for the treatment of chemoresistant tumors (Cherbonnier et al., 2002; Haeffner et al., 1997). The use of replicative adenovirus to potentiate cytopathic effects in tumor cells represents an exciting recent approach for solid tumor therapy (Barnes et al., 2002). The use of replication adenovirus encoding human growth hormone (AdXGH) or del32-71 growth

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Table 1
Formulations' characteristics.

F[a]	Theoretical dose (mg)	PLGA content (mg)	Time (s)	Drug declared content (%)	Weight (mg)	Thickness (mm)	Hardness (N/mm^2)
A	0.8	20	30	75 ± 1	20.1 ± 0.8	0.8 ± 0.03	4.92 ± 0.11
B	1.6	80	30	75 ± 2	83.7 ± 6.9	3.2 ± 0.3	4.45 ± 0.75
C	0.8	80	60	112 ± 1	73.4 ± 3.0	3.1 ± 0.06	8.97 ± 0.10

[a] *F*: formulation.

hormone, a protein that cannot fold properly (AdXdel32-71GH), to test whether toxicity occurs when infected human ovarian cancer cell lines do not prevent the growth of these cell lines in the case of AdXGH, but prevents it in case of expression of the abnormal folded protein (Graves et al., 2001). Because of this, the use of Adxdel32-71GH to force ovarian cancer cells to express the unfolded protein could be used to prevent tumor growth. Unlike these results, the AdXdel32-71GH treatment had no effect in human ovarian cancer mouse model (Yu et al., 1993). But the expression of wild-type growth hormone using AdXGH prolonged survival and reduced tumor burden in a mouse model of human ovarian cancer, an effect not seen with AdXdel32-71GH and repeated injection of human growth hormone or implanting hGH tablets (Zhu et al., submitted for publication). The aim of this work is to study in depth the pharmacokinetic analysis of the hGH implantable tablets administered to human ovarian cancer mouse model in the process of testing possible therapies as AdXGH. The hGH tablets were *in vitro* characterized before *in vivo* implantation.

2. Materials and methods

2.1. Materials

QA423L Biosynthetic 2-Cistron Human Growth Hormone Cysteine ex. dDAP (hGH), batch ID 274881 (date of manufacture 20/01/2007), with a HPSEC purity of 99.8% and water content of 4.8% (w/w) was provided by Lilly (Madrid, Spain).

Poly (D,L-lactic-co-glycolic acid) (PLGA) (Resomer® RG 504) with a molecular ratio of 50:50 was purchased from Boehringer Ingelheim (Ingelheim, KG, Germany). All other reagents were those of analytical grade.

2.2. PLGA characterization

The particle size distribution of the polymer was measured using a LS™ 100Q laser diffraction particle size analyzer (Beckman Coulter, Fullerton, USA). The mean volume–length diameter of PLGA particles was 35.9 ± 17 μm.

The weight-average molecular weight (Mw) and number-average molecular weight (Mn) of the raw polymer and tablets were determined by gel permeation chromatography (GPC) (Waters, Milford, MA, USA) using Waters Ultrastyragel columns previously calibrated with 2800–700,000 Da polystyrene standards (Tokyo Soda Ltd., Tokyo, Japan), with a refractive index detector Waters 2414 (Milford, MA, USA). Tetrahydrofuran (THF) (Sigma–Aldrich, St. Louis, USA) was used as the mobile phase, at a flow rate of 0.9 ml/min.

The copolymer composition was determined by nuclear magnetic resonance of proton (^{1}H NMR) with a Bruker AMX-400 spectrometer using deutered chloroform ($CDCl_3$) (Sigma-Aldrich, Madrid, Spain) as a solvent. The relative proportions of lactic–glycolic (LA–GA) and glycolic–glycolic (GA–GA) acid bonds were assessed by nuclear magnetic resonance of carbon 13 (13C NMR) at 100.61 MHz using dimethyl-sulphoxide-d6 (DMSO-d6) (Sigma–Aldrich, Madrid, Spain) as a solvent (Dorta et al., 1993).

The effective Mn, Mw and the polydispersivity of PLGA were found to be 31 ± 1 kDa, 54 ± 3 kDa and 1.74 ± 0.03 kDa respectively. The ratio of LA/GA was 53/47 and of LA–GA/GA–GA was 2/1.

Both *in vitro* and *in vivo* polymer degradation was carried out by using the degradation index (DI) (Glynn et al., 1996) defined as the ratio of the number of polymer chain bonds cleaved per chain to the number of chain bonds per chain at zero time (i.e., DI = 1 means that on average, every chain initially present has been broken once). DI is computed from number-average molecular weight by using the equation:

$$\mathrm{DI} = \frac{M_n^0}{M_n^{(t)}} - 1 \qquad (1)$$

being M_n^0 and $M_n^{(t)}$ the number-average molecular weight a time zero and after time '*t*' elapsed.

2.3. Preparation and characterization of hGH-PLGA tablets

Three different formulations of PLGA tablets containing freeze-dried hGH were prepared by direct compression in an hydraulic press (Carver 4120, Wabash, IN, USA) by means of applying a force of 0.1 T and fitted with a 6 mm test cylinder and a pellet mold under aseptic conditions at room temperature. 20 units of each formulation was prepared in continuous. The composition and compression time of every formulation are indicated in Table 1.

The hGH content of tablets was determined by dissolving each one in THF, where the stability of the hormone was tested during the contact time. Once the polymer was dissolved, the dispersion was centrifuged at 4000 rpm for 10 min (Econospin Sorvall Instruments, Wilmington, DE, USA), the supernatant removed and residue vacuum-dried. This precipitate was dissolved in mobile phase and the hormone quantified measured by high performance liquid chromatography (HPLC). Ten tablets of each formulation were analyzed.

The used HPLC system (Waters, Millford, MA, USA) consists of a pump, a Model 600 E Multisolvent delivery system, a 717 plus Autosampler, a 2487 Dual absorbance detector and a gel filtration column (Protein-Pak 125,300 mm × 7.8 mm) packed with 10 μm particles of 125 Å pore size as stationary phase. The used data acquisition software was Millenium32 (Chromatographic Manager, Waters Corporation). The mobile phase was acetonitrile/water 30/70 (v/v) mixture with 0.05% trifluoroacetic acid (Sigma–Aldrich, Madrid, Spain) at a flow rate of 1.0 ml/min at room temperature and UV detection at 214 nm was used. All chemicals and reagents were HPLC grade. All solvent were filtered with 0.45 μm pore-size filters (Millipore, Billerica, MA, USA). The mobile phase was filtered and degassed.

In order to validate the analytical method, seven hGH standard solutions were prepared at concentrations of 2.5–40 μg/ml. Each sample was analyzed five times. The variance analysis (ANOVA) of the linear regression confirmed the linearity of the method through rejection of the null hypothesis of linearity deviation for a significance level of 0.05 ($\alpha = 0.05$). The mean equation of the regression line was: area = −29849.5 + 36218.7*C; $r = 0.998$ ($n = 35$), with a residual standard error of 22,851. The method precision (as repeatability) was 0.4 ± 0.2%, it was determined by a six times analysis of the same hGH sample (40 μg/ml). System accuracy was expressed as percentage recovery by the assay of a known added amount of hormone (2.5 μg/m, 15 μg/m and 40 μg/ml), the mean value being 104 ± 8% ($n = 9$). The detection and quantitation limits,

based on the standard deviation of the response and slope, were 2.1 μg/m and 6.3 μg/ml respectively.

The weight and thickness of every tablet were determined by a precision balance (AG285 Mettler Toledo, Madrid, Spain) and a digital micrometer (Mitutoyo, IL, USA).

2.4. In vitro release studies

To determine the release kinetics, every characterized tablet was maintained in individual vials containing 1 ml of isotonic phosphate buffer (Sörensen) pH 7.4 in a water bath (Stuart Scientific, Stone, Staffordshire, UK) at 37 °C for 15 days. To prevent the degradation of the hormone, the release medium was periodically removed and replaced by equal volumes of fresh buffer (Graves et al., 2001). The final hGH content of every tablet at the end of the assay was determined by HPLC as the method described above (Section 2.3). The assays were made in triplicate.

The mean dissolution time (*MDT*) was estimated for collecting data by mixing cup readings (Levenspiel, 1979), see Eq. (2):

$$MDT = \frac{\sum_{i=1}^{n} t_i C_i \,\Delta t_i}{\sum_{i=1}^{n} C_i \,\Delta t_i} \tag{2}$$

being t_i is the time at each observation, C_i is the concentration at time i and Δt_i is the time interval.

2.5. In vivo studies

The procedure was performed under sterile conditions. CB17 severe combined immunodeficiency (SCID) female mice were from Charles River (Willimantic, CT) as indicated and maintained in specific pathogen-free housing. The Yale University Institutional Animal Care and Use Committee approved all protocols in compliance with the US Public Health Policy on Humane Care and Use of Laboratory Animals. All mice received 0.2 ml of human ovarian cancer cells (SKOV3.ip1) in phosphate buffered saline (PBS), 5×10^6 cells, on day 0 in the peritoneal cavity. Mice were injected with carprofen, 5 mg/kg sc, before the operation. The anesthetic was 100 mg/kg ketamine and 10 mg/kg rylazine sc, followed by the use of an isofluorane nose cone if necessary. Every tablet was implanted in the peritoneal cavity 4 days after injections with SKOV3.ip1 cells and the wall of the peritoneal cavity closed with stitches. The skin layer was closed with staples and removed after 2 weeks. Mice 3–4 were the controls (they received a placebo formulation) and mice 1–2, 5–8 and 9–15 received formulations A, B and C respectively. According to the gradual improvement in the surgical tablet implantation procedure, the number of mice included in each assay was increased. Carprofen 5 mg/ml was present in the drinking water for 3 days after the operation.

Serum levels were determined by bleeding from eye orbital vessels, three times a week in each mouse and assaying the serum for hGH by an immunoassay (Immulite Growth Hormone, Siemens Healthcare Diagnostics, Deerfield, IL, USA). The interassay variability and accuracy of the method were 5.7 ± 0.43% and 95 ± 1.1% respectively. The survival estimates were calculated by using the Kaplan–Meier method and log rank tests performed to determine significant differences using PRISM software (GraphPad Software, San Diego, CA).

2.6. Population pharmacokinetic analysis

Serum hormone levels were interpreted using a population pharmacokinetic model based on a tank in series model. Preliminary attempts to build a pharmacokinetic model using standard modeling revealed two pitfalls: first, classical pharmacokinetic models fails because a substantial lack of fit; second, individual parameter distributions deviated considerably from the assumed normal distribution.

Table 2
Estimated values of fixed parameters with a log-normal distribution of nonlinear mixed-effects statistical model.

	Value	s.e.[a]	Degrees of freedom	*t*-Value	*p*-Value
C_0	1.912	1.286	84	1.486	0.141
Ln *a*	−9.27	0.229	84	−40.5	0.000
Ln *k*	−0.965	0.122	84	−7.91	0.000

[a] s.e.: standard error.

We use a form of the in series tank model instead; a popular solution in modeling chemical reactors because of its ability to fit data, suitable to explain individual pharmacokinetic parameters distribution using the log-normal model (Table 2). This is an already used strategy in pharmacokinetic modelling (Yafune and Ishiguro, 1999; Thomson et al., 2003; Gupta and Li, 2006). Serum hormone levels, $c(t)$, were therefore fitted using the model:

$$c(t) = c_0 + \frac{X_0 \exp(a_L) \exp(k_L)^{n-1} t^{n-1}}{(n-1)!} \exp(-t \exp(k_L)) \tag{3}$$

where c_0 is the basal steady state hormone level resulting from the equilibrium between hormone secretion and metabolism: a_L is the (natural) logarithm of the ratio between hormone bioavailability and hormone volume of distribution: k_L is the logarithm of the model rate constant: n is the number of tanks and it is estimated as 3 by trial and error: and finally, X_0 is the hormone dose (hGH implant load). The statistical model was,

$$C_{i,j}(t_k) = f(\theta_i, \beta_j, t_k) + \varepsilon_{ijk}, \quad \varepsilon_{ij} = N(0, \sigma^2) \tag{4}$$

being $c_{i,j}(t_k)$ the hGH serum concentration in k-ieme mouse using the i-eme formulation at time t_k, $\theta_i = (a_{Li}, k_{Li})^T$ the mean (population) parameter vector for formulation i-eme and β_j the contribution of mouse j-eme which is assumed log-normal distributed with mean zero vector and (in our model) diagonal variance-covariance matrix. Parameter estimation was conducted using the nonlinear mixed-effects (nlme) function included in R statistical software (The R Foundation for Statistical Computing, WU, Vienna). Null hypothesis about population parameters was conducted using ANOVA test and the distribution of individual parameter through normal plots (Pinhero and Bates, 2000).

Statistical moments, area under hormone serum levels curve (AUC) and mean residence time (MRT) were derived from Laplace transform of nonsteady state component of Eq. (3) (excluding c_0), resulting:

$$AUC = X_0 \exp \frac{a_L}{k_L} \tag{5}$$

$$MRT = \frac{n}{\exp(k_L)} \tag{6}$$

where X_0 is the hGH dose, a_L is the logarithm of the ratio bioavailability/volume of distribution, k_L is the logarithm of the model rate constant and n is the number of tanks (estimated as 3).

Standard deviations of *AUC* and *MRT* were computed by using delta method.

3. Results and discussion

Three consecutive formulations were elaborated with the aim of reducing the *in vitro* hGH release from the tablets. The declared content, weight, thickness and hardness determined for each formulation were summarized in Table 1. Formulation B was elaborated with the double theoretical value of drug and the quadruple one of polymer content than formulation A, but with the same maximum force in time. In order to reduce the hGH release

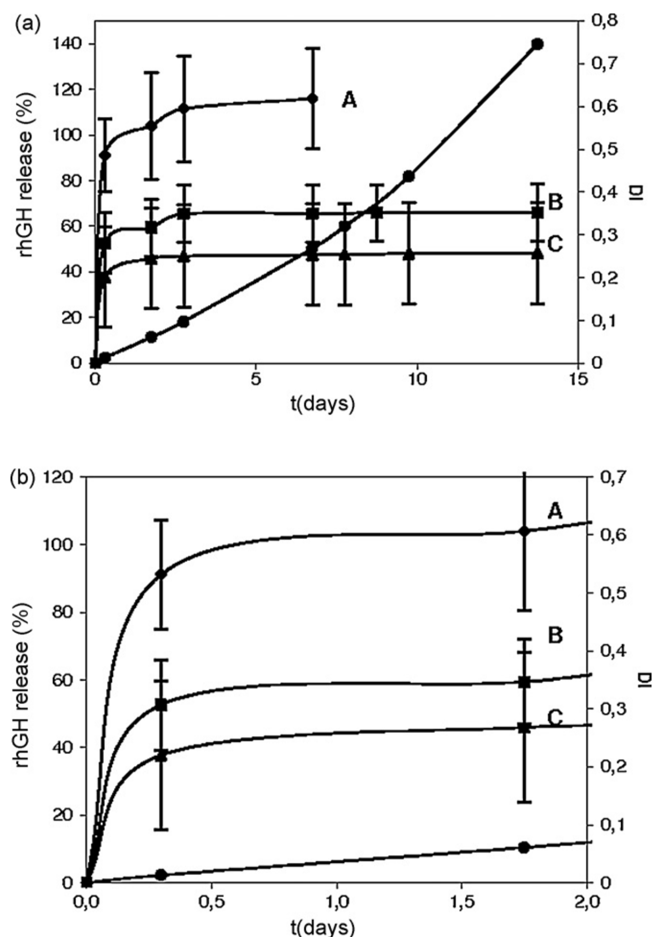

Fig. 1. Formulations A (rombus), B (square) and C (triangle) *in vitro* release profile and DI (circles) in the second axes versus time. Fig. 4B shows in detail the evolution in the first 2 days. Error bars represent the standard deviation.

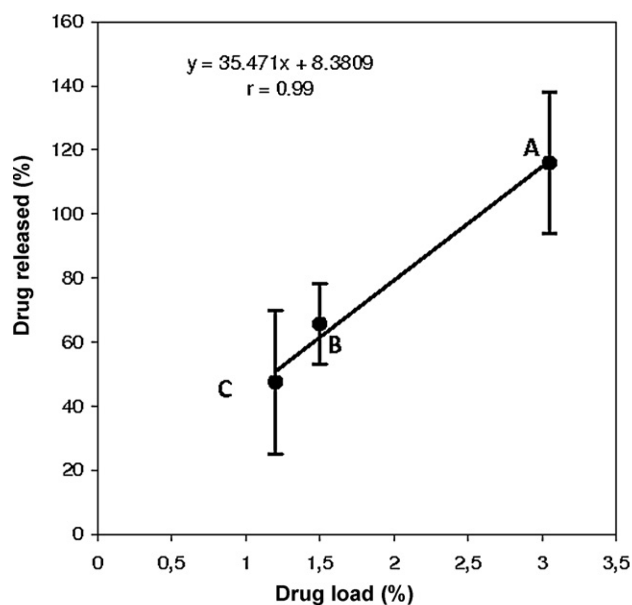

Fig. 2. Percentage of drug release versus drug load for every formulation. Error bars represent the standard deviation.

obtained in formulations A and B, the formulation C was elaborated with the least hormone dose and the highest PLGA content with the same maximum applied force than the other formulations, but within 60 s. Formulation A weight and thickness were lower than formulations B and C, elaborated with more content of PLGA. The Mw was similar than the obtained for the crude polymer (52 ± 1 kDa). No differences in Mw were determined between formulations.

The hGH *in vitro* release profiles from formulations A–C are shown in Fig. 1. As can be seen, the three formulations have the same initial release rate, characterized by a fast delivery in the first days. Formulation A releases all the drug in less than 48 h and the assay was stopped in seventh day. Formulation B releases a 60% of the hormone within 2 days and it is maintained near to this value up to 2 weeks. The formulation C discharges a lower amount of drug than the other formulations within 24 h, approximately 40% and it is maintained near to this value up to 15 days as formulation B. These results show that the drug release from the tablet slows down as more quantity of polymer and lower drug dose are added and more compression time is applied.

In the second *y* axes of Fig. 1, it is represented the polymer DI evolution with time. Since the increase of polymer DI does not coincide with a change in the hormone release profile, the hormone release from the tablet after the first days does not seem to be influenced by the polymer Mw evolution (Santoveña et al., 2009). We have to highlight that formulations B and C at the end of the assay does not release the total drug content. The incomplete release may be caused by the high amount of polymer incorporated to these two formulations or by a nonspecific adsorption mechanism as described in the literature (Kim and Park, 1999; Park et al., 1998). Fig. 2 shows the relationship between the percentage of drug release and the drug load in every device. Formulation A with a lower amount of drug, polymer, weight and thickness permits a faster hormone release through the thin tablet matrix. From formulations B and C, with less drug load, the hormone takes more time in diffusing though more layers of polymer. The remanent hGH inside the tablets maintained its stability at the end of the studies and the mass balance was accomplished.

Fig. 3 summarizes *in vivo* results. Solid lines are computed from population mean parameter whereas dotted one are those fitted to each mouse. Null hypothesis about no difference between formulation parameters was accepted (data not shown) and therefore, the differences between solid lines in Fig. 3 are due solely to the differences in hormone loads. However, when data from mice 3 and 4 (controls) are analyzed using a nonlinear mixed model, this same hypothesis is rejected. We attribute this contradiction to the fact that only two mice were used as controls and therefore we decided to remain c_0 in the model. Except for mice #9 (formulation

Table 3
Comparison with pharmacokinetics parameters published by different authors.

Route	Bioav (%)	Dose (mg)	AUC (mg day/ml)	AUC/Dose	Animal model	Ref.
Intravenous injection	100	0.034	8.82×10^{-6}	2.6×10^{-4}	Rat	Kagatani et al. (1998)
Subcutaneous injection	80.6	0.025	5.22×10^{-6}	2.1×10^{-4}	Rat	Kwak et al. (2009)
Subcutaneous injection	16.5	2.0	8.6×10^{-5}	4.3×10^{-5}	Monkey	Kim et al. (2005)
Intranasal	0.20	20	1.0×10^{-5}	5.0×10^{-7}	Sheep	Cheng et al. (2005)
Subcutaneous implantation (formulation C)	96.0	0.97	2.4×10^{-4}	2.5×10^{-4}	Mouse	This study

Bioav.: bioavailability. Ref.: reference.

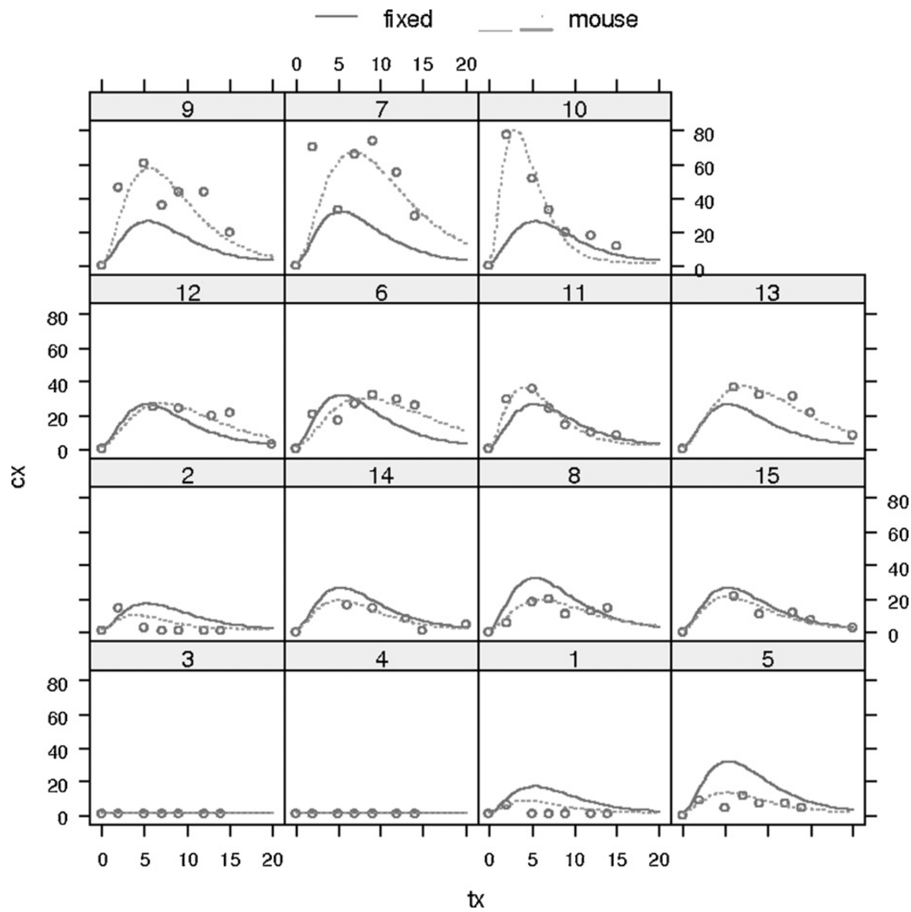

Fig. 3. hGH plasmatic concentrations (ng/ml) of every studied mouse over 15 days (dots), predicted values for each one (discontinuous line) and for each group studied (continuous line).

C) and #7 (formulation B) which show large intra-subject variability, the agreement between observed and predicted serum levels was quite satisfactory. Inter-subject variability also agrees with the log-normal proposed model as can be concluded from Fig. 4.

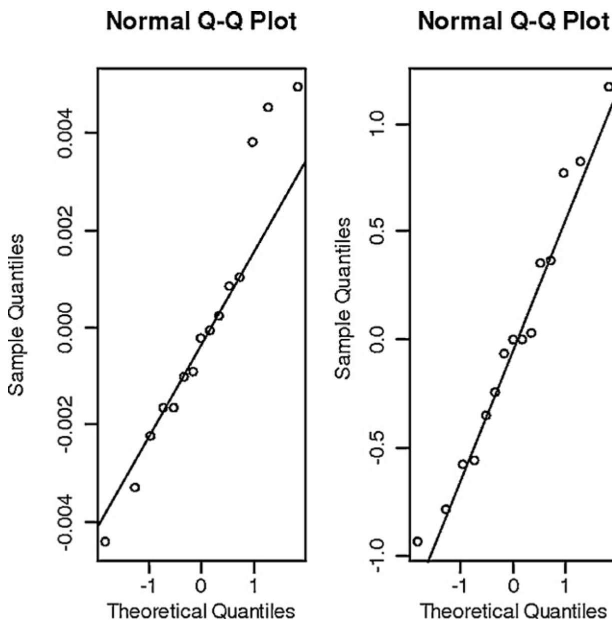

Fig. 4. Q–Q plot of the log-normal distribution of the random effects.

As long as it has been accepted that the three formulations show the same kinetics, the predicted AUCs were 150.2 ± 37 ng day/ml, 295.5 ± 74 ng day/ml and 238.9 ± 60 ng day/ml for formulations A, B and C respectively and the predicted MRT was just the same for all of them: 7.88 ± 0.95 days. This value is quite larger than those observed for MDT (1.75, 1.12 and 1.60 days for formulations A, B and C respectively) and, of course, larger than hGH half-life (0.7 h) (Jorgensen, 1991). This fact leads us to the conclusion that not only is the proposed manufacturing method suitable to get a sustained release hormone, but also the inappropriate of *in vitro* testing to simulate *in vivo* conditions. When a comparison between the DI calculated after *in vitro* (0.24, 0.83 and 0.95) and *in vivo* (0.12, 0.79 and 0.71) assays for formulations A, B and C respectively is made, similar values at difference of MDT and MRT is showed. The polymer degradation shows a similar *in vitro* and *in vivo* evolution during the 15 days of the assays, an interval in which these types of tablets did not notably reduce the glass transition temperature (T_g) and the viscous and elastic moduli but it can significantly condition the hGH release rate as it has been found in previous works (Santoveña et al., 2009). If we compare these pharmacokinetics parameters results to those obtained in the literature for the intravenous route (Kagatani et al., 1998) see Table 3, we can show how the calculated bioavailability for formulation C is higher than the obtained for other routes as subcutaneous injection (Kim et al., 2005; Kwak et al., 2009) or intranasal (Cheng et al., 2005) and similar to that obtained for a hGH daily injection.

The implantation of these hGH formulations in a mouse model of human ovarian cancer had a mean survival of 34 days, not significantly different from control mice and control tablets (31.5 days and 34 days respectively) (Zhu et al., Submitted for publication). The

hormone levels remain high for over 2 weeks as compared with the control mice (Fig. 3), but these levels do not decrease the survival time as other authors suggest (Tworoger et al., 2007; Lukanova et al., 2002).

4. Conclusion

The elaboration procedure of hGH implantable tablets does not compromise the stability of the hormone and permits the incorporation of a high dose of the drug. The hGH *in vitro* release from these implantable tablets shows the same profile than other drugs from these types of devices (Choonara et al., 2006; Takahashi et al., 2004). The amount of *in vitro* drug release from every formulation is directly related to the drug load. The remaining drug content inside the tablets depends on the matrix structural degradation evolution and the device structure (Witt and Kissel, 2001). The software R with the nonlinear mixed-effects statistical model permits to fit all the *in vivo* data to a unique series tank model (O'Hara et al., 2001; Gaynor et al., 2007), with the tablet dose as the main factor that affected the value of the pharmacokinetics parameters independently to the type of implanted tablet. This population pharmacokinetic analysis allows us to establish a statistical model of studying the evolution of hGH plasmatic concentrations produced after the release of the hormone from different types of implantable tablets in every animal and group of animals. The increase in control hormone levels for over 2 weeks after the tablet implantation in a mouse model of human ovarian cancer does not produce a statistically significant enhance or reduction in the survival time.

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