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Relative Bioavailability of Two Drug Products of Somatropin Obtained from Either the Milk of Transgenic Cows or Bacterial Culture

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Key Words
Somatropin · Relative bioavailability · Transgenic cow · Pharmacodynamics · Insulin-like growth factors · Human growth hormone treatment

Abstract
Background: Our objective was to assess the relative bioavailability of the first somatropin produced in transgenic cloned cows that carry the human growth hormone (GH) gene (Biohormon®) and somatropin produced in *Escherichia coli* culture (HHT®), the procedure most frequently used for the commercial production of the hormone. ***Methods:*** Upon approval by an independent ethics committee and the National Regulatory Agency of Argentina, we compared the time-concentration profiles of somatropin in 24 healthy volunteers, in a randomized, 2-period, 2-sequence crossover design after inhibition of endogenous GH secretion with lanreotide, a long-acting somastostatin analogue. After the subcutaneous administration of 1.33 mg of each formulation, serum somatropin was analyzed by chemiluminescent immunoassay and IGF-I by immunoradiometric assay. Safety was assessed by clinical and laboratory parameters. Pharmacokinetic parameters were calculated with Win Nonlin® 5.2 using a non-compartmental model and bioequivalence was assessed. ***Results:*** The test/reference ratios of AUC, AUC_{last} and C_{max} were 106.4 (90% CI = 100.2–112.9), 105.3 (90% CI = 99.1–111.8) and 105.49 (90% CI = 92.6–120.1), respectively. No serious adverse events were reported and no GH antibodies were detected. ***Conclusion:*** This study demonstrates that a single dose of Biohormon, the first product with somatropin obtained from milk of transgenic mammals, is bioequivalent to the reference product HHT according to standard criteria.

Introduction

Growth hormone (GH) therapy with exogenous human growth hormone (hGH) has been used for many decades to treat children with GH deficiency (GHD) [1, 2]. In the early times, the supply could not meet the demand to treat all patients with GHD. Since 1985, hGH produced using recombinant DNA technology has been approved and has been assigned the international non-proprietary name somatropin, with the Anatomical Therapeutic Chemical

M.E.C. and R.A.D. are staff of Bio Sidus S.A.; J.F. and I.B. are paid advisors to Bio Sidus S.A.; FP Clinical and CDM were contracted for the execution of this study.

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code H01AC01. Thereafter, GH has been produced by several manufacturers and used worldwide. This has resulted in improved treatment designs allowing to optimize the dose and frequency of subcutaneous injections for children with GHD and also to treat growth failure other than classic GHD, such as Turner syndrome, chronic renal failure and children born small for gestational age [3–8].

More recently, the new indication of GH treatment for children with idiopathic short stature in the USA [9] may increase the demand for drug supply. Although the introduction of DNA technology appeared to offer an unlimited commercial source of somatropin, the price is still high, which is why many underprivileged children mainly from developing countries still cannot access this treatment.

The expression of proteins with potential therapeutic applications in the milk of livestock species seems to be one of the most attractive commercial applications of transgenic animals [10]. Recently, the availability of somatropin in the milk of a cloned transgenic cow has suggested that transgenic cattle could be used as a cost-effective alternative for a large-scale production of this hormone [11].

The main objective of this research was to assess the relative bioavailability of the first somatropin produced in transgenic cloned cows that carry the hGH gene and recombinant somatropin produced in *Escherichia coli* culture, the procedure most frequently used for the commercial production of the hormone.

Materials and Methods

Twenty-four out of 38 healthy male and female volunteers were screened for eligibility and recruited into the study. To be included, the subjects were required to fulfil the following inclusion criteria: age 25–55 years, body mass index in the range of 18–30 and vital signs within the normal range. All volunteers agreed to use barrier contraception during the study and for 1 month following its completion. In addition, women within reproductive age had a negative pregnancy test at the first visit to the clinic and were neither breast feeding nor using any hormonal contraception method. All the subjects were healthy and they did not report to suffer cardiac, gastrointestinal, hepatic, pulmonary, renal, neurological, haematological or endocrine diseases. All the participants had normal haemoglobin, leukocyte, platelet, and erythrocyte sedimentation rates, liver function, creatinine, glucose, blood urea nitrogen, total proteins and coagulation tests.

Subjects were not included in the study if they had a personal history of allergies to milk or drugs and evidence of any surgical, medical or psychiatric condition that might have interfered with the pharmacokinetics of the investigational medicinal products. In addition, patients were excluded when elevated IgE or positive RAST to cow milk proteins were detected.

Other exclusion criteria were: previous somatropin administration, requirement of chronic treatments with any drug, alcohol or drug abuse within the past 2 years, administration of any other investigational drug, participation in a clinical research trial within the past 6 months, donation of blood or plasma to a blood bank within the past 30 days as well as any condition which in the investigator's opinion might render the subject unable to complete the study or which might pose significant risk to the participant. Subjects who were (themselves or their relatives) employed by Bio Sidus S.A., CDM, or FP Clinical Pharma were also excluded.

Treatments

Two drug products (HHT® and Biohormon®) containing somatropin were used, both manufactured by the same company (Bio Sidus S.A., Buenos Aires, Argentina), under the same conditions and complying with current good manufacturing practices.

HHT 4 IU was dispensed in a vial containing 1.33 mg of somatropin (purified from transfected *E. coli* culture), as lyophilized powder for reconstitution in 1 ml of water for injection. HHT was licensed by the Administración Nacional de Medicamentos, Alimentos y Tecnología Médica, Argentina's national drug regulatory agency, in 1997 and is currently marketed in 6 additional countries of South America, in Central America and in Pakistan. Up to 2008, more than 6 million units had been sold worldwide.

Biohormon is an investigational product that contains somatropin synthesized in cloned cows that have been modified by the transfection of the gene of hGH and express hGH in the milk [11]. After preclinical testing [12] according to the guideline of the European Medicines Agency [13], the product was scheduled for human testing. Biohormon 4 IU is a sterile white lyophilized powder for reconstitution. For both products, after reconstitution the solution has a concentration of 1.33 mg/ml. Both products comply with the European pharmacopoeia specifications for 'somatropin bulk solution' [14] and their specific activity is similar to the somatropin WHO standard (1 mg of somatropin = 3 IU).

The protocol was approved by the Institutional Review Board, by a local independent ethics committee as well as by the Administración Nacional de Medicamentos, Alimentos y Tecnología Médica and conducted in accordance with the Declaration of Helsinki and good clinical practice. All the subjects gave written informed consent to participate in the study. The design was a randomized, open-label, 2-period, 2-sequence crossover study, in which Biohormon (batch No. L04E0J) was analyzed as the test product and HHT (batch No. C047159J) as the reference product.

The subjects were allocated a randomization number in sequential order in accordance with the randomization list. To minimize endogenous GH secretion sufficiently to enable accurate assessment of serum GH concentration-time profiles, all the volunteers received a somatostatin analogue (90 mg of lanreotide; Somatuline Autogel®, Ipsen, France), by intramuscular route 14 days prior to the first dose of somatropin.

The subjects were randomly assigned to 1 of 2 treatment sequences: Biohormon-HHT or HHT-Biohormon. Each study period lasted 1 day, with a wash-out period of at least 7 days between each somatropin administration.

The subjects received both lyophilized somatropin (1.33 mg) products reconstituted in 1 ml of an aqueous vehicle, and subcutaneously injected with standard needle and syringe in the con-

tralateral buttock to the lanreotide injection. Blood samples for hGH determinations were drawn through an indwelling catheter immediately prior to somatropin injection (time 0) and subsequently 0.5, 1, 1.5, 2, 3, 3.5, 4, 4.5, 5, 7, 9, 12, 14, 18 and 24 h administration. IGF-I was measured at 0, 4, 12 and 24 h in both treatment periods. On the study day, after somatropin administration, all subjects received a diet with similar proportions of carbohydrates (50%), fat (20%) and proteins (30%).

GH and IGF-I Assay

Serum GH was determined by an immunochemiluminescent assay (Immulite®; EURO/DPC Ltd., Gwynedd, UK), with a detection limit of 0.05 ng/ml.

Serum IGF-I was measured with an immunoradiometric assay (DSL-5600® Active Insulin-Like Growth Factor I; immunoradiometric assay, Diagnostic Systems Laboratories, Webster, Tex., USA), with previous extraction, the intra-assay coefficient of variation was 1.5–3.4%, whereas the inter-assay coefficient was 3.7–8.2%.

Safety Data

Safety clinical and laboratory evaluations were performed during hospitalization (0 day) and on the 14th and 28th (final visit) days. All clinical chemistry data outside the normal range were identified. Demographic and baseline characteristics of the subjects, as well as their vital signs and clinical chemistry and blood parameters were recorded and tabulated; these data are presented with descriptive statistics.

Close monitoring of adverse events was conducted and recorded throughout the study. The occurrence and severity of local reactions (redness, swelling, indurations or bruising) were assessed by inspection at the injection site at pre-scheduled time points of each period. Patient complaints, physical examinations, blood pressure, cardiac and respiratory rate, body temperature, 12-lead ECG and laboratory tests (haemoglobin, haematocrit, leukocytes, erythrocytes, erythrocyte sedimentation rate, blood glucose, lactate, creatinine, total bilirubin, electrolytes, aspartate aminotransferase, alanine aminotransferase and urine analysis) were utilized to evaluate the occurrence of adverse events.

In addition, antibodies against hGH and bovine β-lactoglobulin were determined. For hGH antibodies a radioimmunoprecipitation assay was used, as previously described [15]. Briefly the study was performed on the basis of immunoprecipitation of GH labelled with ^{125}I (using chloramine T as oxidizer and cutting the reaction with sodium metabisulfite). Pure recombinant hGH, batch HCB p-179, provided by Bio Sidus, was used for iodination. In a final volume of 500 μl, the serum was incubated with labelled GH in the presence of sodium borate buffer (pH 8.5), 1% of bovine albumin for 36–48 h, at 4°C. At the end of the incubation period the immune complex was precipitated with 25% polyethyleneglycol (Carbowax 6000; Dow Chemical Co, Midland, Misc., USA, p/v, in saline) and separated by centrifugation (15 min at 3,000 rpm), after discarding supernatant, complexes were counted with Cobra II Auto gamma (Packard, Meriden, Conn., USA). The cutoff was established at 3% binding of labelled GH. Appropriate positive and negative controls were also used.

Antibodies against bovine β-lactoglobulin were detected with a commercial ELISA kit (ENEASYSTEM III®; Bio Genetix, Fiumicino, Rome, Italy) for the in vitro measurement of specific IgG antibodies.

Pharmacokinetic and Statistical Analysis

Serum GH concentrations and kinetics parameters were estimated for each subject by a standard non-compartmental analysis using Win Nonlin professional 5.2 (NCA model; Pharsight Corp., Mountain View, Calif., USA).

The following pharmacokinetic parameters were computed: area under the serum concentration-time curve from the time of dosing (time 0) to the last measurable concentration (AUC_{0-last}), calculated using the linear trapezoidal rule [16]; area under the serum concentration-time curve extrapolated to infinity (AUC_{0-inf}), calculated by extrapolation to infinity using the terminal half-life ($t_{1/2z}$) estimated with log-linear regression ($AUC = AUC_{0-last} + AUC_{last-inf}$); peak serum concentration (C_{max}); time of peak serum concentration (T_{max}); elimination half-life ($t_{1/2}$) and mean residence time (MRT) estimated as the area under the first moment curve (AUMC) divided by AUC. Apparent plasma clearance (CL/F) was defined as the ratio of the dose injected to AUC, and the apparent volume of distribution (Vz/F) was calculated as $(CL/F)/\lambda_z$.

The apparent elimination rate constant (λ_z) estimated by linear regression of the terminal linear phase of the semi-logarithmic plasma concentration-time curve and the apparent elimination half-life were calculated using the equation $t_{1/2} = (\ln 2)/\lambda_z$.

Following logarithmic transformation, an analysis of variance was performed on somatropin parameters (C_{max}, AUC_{0-last}, AUC_{0-inf} and T_{max}) of the whole population under analysis. There were no imputations for missing data. The analysis of variance model consisted of the logarithmically transformed C_{max} as the response variable with factors for sequence, subject nested in sequence, period and somatropin product (treatment). Using an average bioequivalence approach, (Schuirmann and Hauck-Anderson test) a 90% confidence interval (90% CI) for the true ratio test of the Biohormon to reference (HHT) means was calculated and expressed as a percentage. Bioequivalence was assessed according to US [17] and EU [18] criteria, with equivalence acceptance limits for the ratio between 80 and 125%. To obtain a 90% power to demonstrate equivalence within the acceptance limits of 80–125%, assuming that the expected ratio of means was 1.000, the crossover design yielded a sample size of 12 volunteers in each sequence group.

The IGF-I serum concentration in both groups (reference and test) was compared, at baseline and in the 4-, 12- and 24-hour samples with the λ statistic of Wilks, calculated with the software R [19]. A separate analysis of the basal and 24-hour IGF-I values in both groups was performed with the same method. The Mc-Nemar test (with the same software) was utilized to compare adverse events associated with the reference and test products.

Results

Thirty-eight volunteers were screened and 24 complied with the study procedures. Ten subjects did not fulfil the inclusion and exclusion criteria and 4 subjects did not return to visit 2. There were no major protocol deviations among the 24 subjects that completed the study; no subject dropped out. The baseline demographic charac-

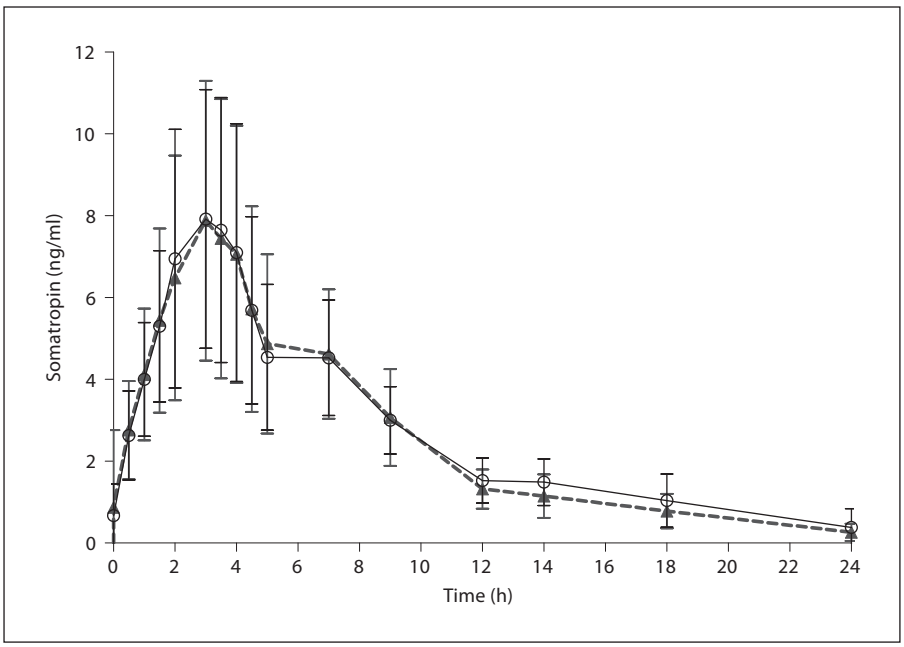

Fig. 1. Serum somatropin concentration × time curves of HHT (reference product, ▲) and Biohormon (test product, ○) after subcutaneous administration of 4 IU to 24 normal subjects.

teristics are shown in table 1. All volunteers received the investigation products between 8 and 8:48 a.m. in both treatment periods.

Figure 1 shows concentration × time profiles obtained after subcutaneous administration of Biohormon and HHT. The concentrations of somatropin for both products illustrate the typical serum pattern of extravascular administration. The pharmacokinetic parameters of somatropin for each product, presented in table 2, were similar (p = non-significant).

The linear model of mixed effects for analysis of variance, designed for defined bioequivalence parameters in crossover studies, using log-transformed AUC_{inf}, AUC_{last} and C_{max}, showed a p value >0.05 for sequence, period and treatment.

Additionally, the linear model for mixed effects in the analysis of pharmacokinetic parameters of crossed studies, using HL λ_z, T_{max}, Vz, CL/F, $AUMC_{0-inf}$ and MRT_{inf} (log transformed), also showed a p value >0.05 for sequence, period and treatment, consistent with the similarity of both treatments.

The intra-subject coefficient of variation was 12% for AUC and 26% for C_{max}, indicating that somatropin shows a low to moderate intra-subject variability.

Table 3 presents the equivalence analysis. All parameters were within the specified acceptance range (80–125%) for average bioequivalence. The 2 unilateral T tests for both end regions showed a $p < 0.05$, thus rejecting the null hypothesis (lack of bioequivalence between formulations) for the 3 parameters analyzed.

Table 1. Baseline demographic characteristics

Variable	Value
Cases	24
Gender	12 female/12 male
Age, years	32.83 ± 5.94
Height, cm	166.77 ± 8.03
Weight, kg	69.83 ± 13.60
BMI	25.62 ± 3.21

Figures are means ± SD.

Pharmacodynamics

Both products increased the serum IGF-I levels, as shown in figure 2. The baseline concentration was significantly increased at 24 h upon injection of both somatropin products ($\lambda = 0.245$, $p < 0.001$). A linear increment was observed up to levels of 250 ng/ml at 12 h after the administration of somatropin, and then the concentrations remained stable for the subsequent 12 h. The mean concentrations of IGF-I at 0, 4, 12 and 24 h were similar after the administration of both products ($\lambda = 0.953$, $p = 0.72$).

Table 2. Pharmacokinetic parameters of somatropin in HHT and Biohormon after subcutaneous administration of 4 IU in 24 normal volunteers

	HHT	Biohormon	p
C_{max}, ng/ml	8.32 ± 3.42	8.69 ± 3.39	NS
T_{max}, h	3.27 ± 1.08	3.20 ± 1.39	NS
AUC_{last}, ng · h/ml	61.69 ± 17.59	64.34 ± 16.10	NS
HL λ_z, h	4.22 ± 1.59	4.68 ± 1.47	NS
λ_z	0.18 ± 0.05	0.16 ± 0.04	NS
$AUC_{inf-obs}$, ng · h/ml	63.60 ± 17.27	67.39 ± 16.88	NS
Vz_{f-obs}, l/h	139.10 ± 69.40	140.45 ± 52.56	NS
CL_{f-obs}, l	22.40 ± 6.10	20.90 ± 5.01	NS
$AUMC_{inf-obs}$, ng · h^2/ml	480.60 ± 159.90	562.54 ± 218.6	NS
$MRT_{inf-obs}$, h	7.70 ± 2.36	8.33 ± 2.12	NS

C_{max} = Peak serum concentration; T_{max} = occurrence time of C_{max}; AUC_{last} = area under the serum concentration-time curve from time 0 to the last experimental point; HL λ_z = apparent elimination half-life; λ_z = apparent elimination rate constant, AUC_{0-inf} = area under the serum concentration-time curve from time 0 extrapolated to infinity; Vz_{f-obs} = apparent volume of distribution; CL_{f-obs} = apparent plasma clearance; $AUMC_{inf-obs}$ = area under the first moment curve; MRT = mean residence time from time 0 to infinity λ_z.

Safety

No serious or life-threatening adverse events were observed and no subject was withdrawn due to adverse events.

The Mc-Nemar test did not show significant differences in the rate of adverse events when Biohormon and HHT were compared (p = 1). The majority of adverse events were mild and transient. The most frequently recorded were diarrhoea (45%, 11/24), headache (33%, 8/24) abdominal distension (12%, 3/24), abdominal pain (4.1%, 1/24), mild elevation of transaminases (4.1%, 1/24), nodule in the lanreotide application zone (12.5%, 3/24), all of them related to lanreotide rather than to HHT or Biohormon. No local reactions at the injection site of somatropin were observed. No antibodies against human GH or to β-lactoglobulin were detected at the final visit (not shown).

Discussion

The results of this study demonstrate that the concentration-time profile of GH following subcutaneous injection of 1.33 mg of a product containing somatropin obtained from milk of a cloned transgenic cow is similar to the concentration-time profile of the same dose of somatropin obtained by classic expression in *E. coli*. Though the dose in the present study is lower compared with other pharmacokinetics/pharmacodynamics studies of somatropin [20–22], the drug inter-subject variability was consistent with other somatropin pharmacokinetics/pharmacodynamics studies [23–25].

Regulatory guidance for equivalence acceptance stipulates that the 90% CI for the ratio (test to reference) of the areas under the serum concentration versus time curves (AUC ratio) and that of the maximum serum drug concentrations (C_{max} ratio) must fall between 80 and 125% [26]. This study shows that both the rate and extent of exposure of somatropin meet the accepted criteria for bioavailability equivalence. The high statistical power of the tests, between 0.90 and 0.99, showed a type II error of less than 10% (false negative) with a type I error of 5%.

A problem when assessing somatropin pharmacokinetics is the variable influence of endogenous GH over the measured GH concentration. The GH-deficient patient population is of limited size. Therefore, recruiting a relatively homogeneous group of GHD subjects is more difficult compared to healthy subjects, who are the usual participants in bioequivalence studies. To decrease such influence, in previous research a 24-hour somatostatin infusion was used [27, 28]. In our study, we utilized a long-acting somatostatin analogue to avoid an additional venous catheter, thus reducing the possibility of infections and other complications, and providing better conditions to the subjects during the stay at the study site.

The safety profile of a newly introduced recombinant protein is a potential concern for manufacturers as well as regulatory authorities. This has been thoroughly investigated in previous hGH derivatives from pituitary-derived and recombinant GH products. In this study, Biohormon and HHT were well tolerated in terms of both adverse events and local tolerability. No serious adverse events were reported. The most frequently mentioned adverse events involved the gastrointestinal system (11/24, 45%), and most of them were related to the somatostatin analogue administration. Somatostatin often causes gastrointestinal side effects, and these constituted the majority of the adverse events observed in the present study. The rate of adverse events in our study was similar to the above-mentioned studies [28].

The specific issue of our product relates not only to the potential immunogenicity of GH but also to cow-milk-derived proteins. We did not detect any antibodies against hGH nor bovine β-lactoglobulin with a radioimmunoprecipitation assay and ELISA specifically developed to detect such proteins. However, in future efficacy studies

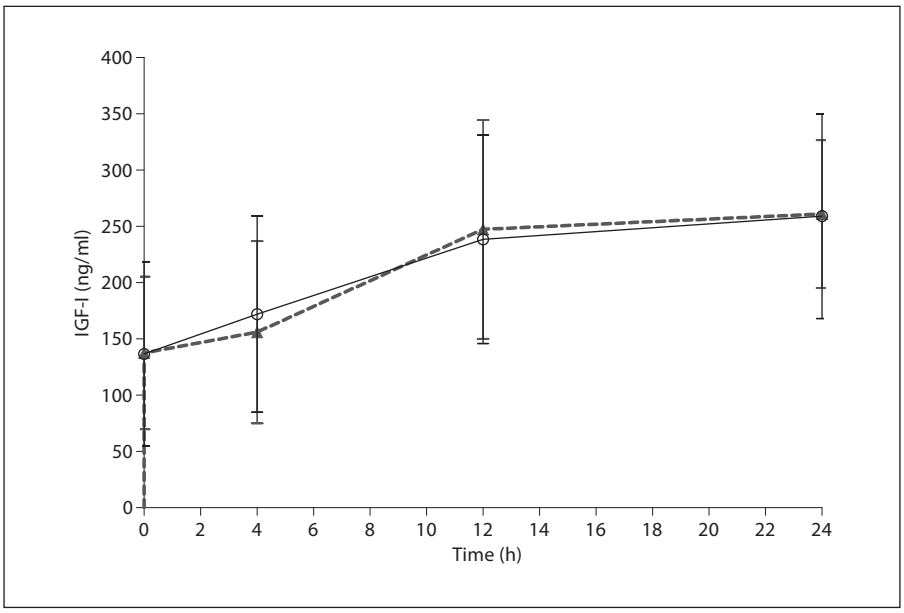

Fig. 2. Effect of HHT (reference product, ▲) and Biohormon (test product, ○) on the mean serum IGF-I concentration of 24 normal subjects after subcutaneous injection of 4 IU of each product.

Table 3. Bioequivalence analysis of somatropin obtained from bacterial cultures (HHT) and somatropin obtained from cloned transgenic cows (Biohormon)

Parameter	Ref. HHT	Test Biohormon	Ratio % ref.	90% CI lower limit	90% CI higher limit
$AUC_{inf-obs}$, h · ng/ml	4.11	4.18	106.43	100.2	112.9
AUC_{last}, h · ng/ml	4.08	4.13	105.30	99.1	111.8
C_{max}, ng/ml	2.03	2.08	105.49	92.6	120.1

with daily injections with this cow-derived somatropin, the assessment of immunogenicity against GH or cow milk proteins will need to be further explored.

For the purpose of establishing bioequivalence, somatropin obtained from transgenic cows (Biohormon) was compared with somatropin from bacterial expression (HHT), which, together with somatropin produced in mammal cells, are considered to be the standard reference of somatropin therapies.

Recombinant DNA-derived proteins expressed in different biological systems can exhibit the same structure and physicochemical properties as the natural protein. Usually, somatropin has been obtained by expression in bacteria or mammal cell cultures, and their safety, as well as pharmacokinetic-pharmacodynamic profiles, are well established. Other therapeutic proteins have been produced in the milk of transgenic animals: factor VIII is being developed for haemophilia and Atryn® (antithrombin-α) has already been authorized for use in patients with congenital antithrombin deficiency [29, 30].

Somatropins obtained from a different biological system such as the yeast *Saccharomyces cerevisiae*, recently approved for clinical use, needed a comparison to an already marketed reference product [31]. The present study was performed as part of the required comparison to a registered reference product.

In summary, we have demonstrated that somatropin obtained from transgenic cows does not present differences in bioavailability with respect to somatropin produced in bacteria, resulting in bioequivalence of products (HHT and Biohormon) containing somatropin of either origin.

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