Pharmaceutical Research, Vol. 13, No. 10, 1996

Commentary

Design of Biological Equivalence Programs for Therapeutic Biotechnology Products in Clinical Development: A Perspective

Joyce Mordenti,[1,3] Joy A. Cavagnaro,[2] and James D. Green[1]

Received May 31, 1996; accepted July 25, 1996

The determination of biological equivalence requires that studies are conducted to establish that two molecules, two formulations, or two dosing regimens, for example, are indistinguishable with respect to safety and efficacy profiles that have been previously established. The criteria that are used to establish biological equivalence will depend on the nature of the change (e.g., molecular, process, formulation), the stage of the development program, the duration of treatment, and the intended clinical indications. Key components of an equivalence program include chemical characterization, *in vitro* and *in vivo* bioactivity against reference material, pharmacokinetics, and safety. Special considerations for patient populations, endogenous concentrations, environmental factors, immunogenicity, assay methodology, biochemical identity, pharmacodynamic equivalence, and statistical methodology are discussed. In addition, the role of preclinical *in vivo* assessments is addressed. Specific case studies provide insight into the varied nature of approaches that are currently employed.

KEY WORDS: bioequivalence; biotechnology products; recombinant proteins; pharmacokinetics; pharmacodynamics; efficacy; immunogenicity; safety.

INTRODUCTION

A biopharmaceutical product that is approved for market by regulatory agencies is subject to rigorous clinical evaluation in studies that are designed to prove safety and efficacy. Ideally, the dosage form and manufacturing process that are intended to be marketed are identical to those used in the pivotal clinical trials. In most cases, however, the "to be marketed" dosage form and manufacturing process are not sufficiently developed at the time when Phase I/II studies are conducted. The dosage form, formulation components, and dose regimen often change during the course of early clinical trials due to attempts to optimize delivery, dose, and product quality. Concurrent with changes in the dosage form, changes occur in the manufacturing process as attempts are made to improve process control, quality, and yield.

To confidently extrapolate data from early clinical studies that employed a dosage form or test material that may be considered "significantly different" from those employed in a pivotal Phase III trial, a prospective "equivalence" program should be developed to support product development. Data from a well-designed, prospective equivalence program supports the conclusion that the test material used throughout the development program was "representative" or "comparable" to the Phase III and/or "to be marketed" product. A database that supports this conclusion would allow the introduction of test material into the clinical program in a manner that does not compromise patient safety or the intended use of the data from these studies to support marketing applications. A similar approach would apply to post-marketing changes.

Currently, there is no "harmonized" regulatory guidance available to the biotechnology industry regarding this issue; however, it represents one of the most important areas in product development. A discussion of this topic occurred at the Third International Conference on Harmonization in Yokohama (1), and the FDA has released for comment a draft guidance document on this subject (Federal Register Docket No. 96D-0132, Vol. 61, No. 82, pp. 18612–13, April 26, 1996). There has been an attempt to relax bioequivalence requirements and speak in terms of 'comparability' for development programs (1–3, Federal Register Docket No. 96D-0132, Vol. 61, No. 82, pp. 18612–13, April 26, 1996). Regardless of the terminology (i.e., 'bioequivalence' *vs* 'comparability' *vs* 'representative' or some other terminology), investigators must decide what *criteria* they are going to use to establish that the new test material (or formulation for example) is "similar" to the current test material (i.e., similar enough to proceed with the development program). Accordingly, the key equivalence program components must be selected such that these *criteria* can be accurately assessed. Even when one only seeks 'comparability,' the *criteria for comparison* should be stated prospectively, prior to testing, and

[1] Experimental Therapeutics, Genentech, Incorporated, 460 Point San Bruno Boulevard, South San Francisco, California.

[2] United States Food and Drug Administration, Center for Biologics Evaluation and Research, HFM-5, 1401 Rockville Pike, Rockville, Maryland.

[3] To whom correspondence should be addressed.

0724-8741/96/1000-1427$09.50/0

then evaluated in a meaningful way to show that the *criteria* have been met and the materials are, indeed, comparable. This review discusses issues, data, and principles that have been developed on this topic for biotechnology-derived pharmaceuticals.[4]

FUNDAMENTAL ISSUES

Pharmaceutical industry and regulatory scientists have made significant progress towards standardizing the design and analysis of clinical equivalence studies. Routine study design issues, which are relevant to both small molecules and protein therapeutics, will not be repeated in this review (see ref. 4 and 5 for a review of these topics). Experience has shown, however, that a subset of study design elements should be considered when evaluating biotechnology-derived pharmaceuticals; namely, subject selection, endogenous concentrations, environmental factors, immunogenicity, assay methodology, biochemical identity, and pharmacodynamic equivalence. These issues are reviewed in detail below.

The statistical methodology for equivalence testing has been and continues to be an area of significant debate. This paper is not intended to address statistical methodology; rather, the reader is referred to the guidance published by the FDA (Biopharmaceutical guidance: Statistical procedures for bioequivalence studies using a standard two-treatment crossover design. FDA Division of Bioequivalence, Office of Generic Drugs, July 1, 1992)[5] and other published sources (see, for example, ref. 6–8). Statistical software packages, such as SAS (SAS Institute, Inc., Cary, NC), BIOPAK (SCI Software, Apex, NC), and other commercial programs, are available to perform the necessary statistical comparisons for equivalence testing, and these topics are not covered in this paper. Suffice it to say, in some instances, a pharmacokinetic comparison, in lieu of a rigorous statistical evaluation, may be sufficient, depending on the nature of the change, the stage of development, the duration of treatment (acute *vs* chronic administration), and the clinical indication (patient population, dosing route/regimen, efficacy endpoints, margin of safety, and so on).

Subject Selection

The issue of subject selection (special populations *versus* normal volunteers) is important whether you are developing a small molecule or a protein therapeutic. There are three major concerns regarding the use of normal volunteers: (a) the potential risks of drug exposure; (b) altered absorption capacity and metabolism compared to special populations (i.e., adult volunteers *vs* children or patients); and (c) endogenous concentrations.

With respect to the first point, if there is no benefit to be derived in normal subjects and exposure represents a definitive risk (e.g., oncolytic agents), then you should conduct your equivalence study in patients. For therapeutic proteins, physicians may be more inclined to use patients because of the concern of potential complications after exposing healthy volunteers to supraphysiologic doses of a recombinant human protien, although with enough clinical experience, it may be possible to use existing safety data to reasonably estimate potential risk. Certainly, if Phase I studies were conducted without incidence in healthy volunteers, then a similar subject population would be acceptable for bioequivalence testing.

The issue of altered absorption capacity and metabolism in special populations was discussed at a Bioequivalence Hearing conducted by the FDA (6). In a summary document (7), the following question was put forth to the Bioequivalence Task Force, *"Does the use of normal volunteers adequately account for the potentially altered absorption capacity and metabolism of special populations (i.e., children or patients)?"* The Task Force offered the following recommendations:

> "The important question is not whether patients are different from volunteers, but whether, and when, these differences could cause two products that seem bioequivalent in normals to be bioinequivalent in a clinical setting. A search of the literature to identify these factors in patients revealed very few relevant publications.
>
> The Task Force believes that it is preferable to subject healthy people, rather than patients, to the rigors of blood sampling and other discomforts of bioequivalence testing. Moreover, use of patients may invariably increase intersubject variability and possibly intrasubject variability as well. Thus far, there have been few, if any, documented examples of problems associated with the use of normals to predict bioequivalence, although there have been relatively few rigorous attempts to document problems. The Task Force believes that at this time, it remains appropriate to determine bioequivalence based on testing in healthy volunteers. The agency recognizes the possibility that some condition could affect bioavailability and is prepared to modify its position regarding the use of normal subjects if such a situation is adequately documented for a given drug."

Although these discussions focused on small molecule chemical entities and did not specifically address protein therapeutics, they do provide appropriate guidance for subject selection, as well as extravascular absorption and metabolic issues for biotechnology-derived products. Ultimately, each case must be decided on its own merit, and differences between patients and normals, such as alterations in the binding proteins, irregularities in endogenous concentrations, diminished peripheral blood/lymph flow (for extravascular dosing), presence of antibodies (from prior treatment), etc., should be considered.

The last point, endogenous concentrations, is discussed below.

Endogenous Concentrations

One must understand the biology of the system to adequately address study design issues related to endogenous concentrations (Table 1). The normal pattern of endogenous production can vary substantially; for example, cytokines (such as interferon-γ) are released in response to a specific event, while growth hormone (GH) or insulin-like growth factor I

---

[4] This is a summary report based on the following presentations: J. Mordenti, *Criteria for the Establishment of Bioequivalence for Recombinant Protein*, VI International Congress of Toxicology, June 28–July 3, 1992, Rome, Italy; J. Mordenti, *Bioequivalence of Protein Pharmaceuticals: Fundamental Issues*, AAPS/ACCP Joint Symposium: Preclinical and Clinical Pharmacology Issues with Proteins and Peptides, February 6–9, 1995, San Diego, CA.

[5] Copies of FDA guidances may be obtained from Drug Information Branch, CDER, FDA, 5600 Fishers Lane (HFD-210), Rockville, MD 20857.

Table 1. Endogenous Concentrations

Issues to consider

What is known about production and inhibition?
- pulsatile *vs.* continuous pattern
- autoregulation and feedback loops

Will the test material perturb the normal physiology?
- displacement from binding proteins and subsequent changes in disposition
- effect of supraphysiologic concentrations

What factors influence endogenous concentrations?
- external factors (stress, diet, trauma, exercise, etc.)
- patient factors (genetics, disease state, concomitant medications, etc.)

Is it appropriate to subtract "baseline" endogenous concentrations?

Do you expect tremendous inter- and intra-subject and inter- and intra-day variability in endogenous concentrations?

What does your assay measure?

Methods of control, data collection, and analysis

Volunteer/patient selection
- ethical and logistical concerns for special populations

Intentional suppression
- potential PK/PD interactions

Study design
- schedule administration (time of day, month, season, and so on) to coincide with lowest endogenous concentrations
- manipulate external and patient factors to minimize influence
- include a placebo treatment to measure endogenous concentrations, if appropriate

(IGF-I) are produced continuously but with very distinct profiles, which can be either continuous (IGF-I) or pulsatile (GH) and extremely sensitive to patient/environmental factors.

With therapeutic proteins, isolation of a recombinant protein from the identical endogenous protein is not possible with standard techniques. The use of a radiolabeled protein to measure exogenous material and to establish equivalence is not recommended. Radiolabeled proteins have stability limitations, especially external labels such as ^{125}I. Proteins, which are removed from the circulation by the liver, kidney, and other organs, are not necessarily excreted. There can be significant metabolism, followed by reabsorption and incorporation of radiolabeled amino acids into other proteins, creating pharmacokinetic artifacts. For these reasons, urinary excretion measurements are usually meaningless. Finally, deposition/incorporation of the radiolabel into thyroid, gut, bone, muscle, and other tissues has short- and long-term risks, depending on the nature of the radionuclide.

Described below are approaches to consider for evaluating a therapeutic protein in the presence of endogenous concentrations:

Method 1) Measure the endogenous concentration in a predose sample (sometimes referred to as the 'baseline' concentration) and subtract this concentration from all subsequent samples. This approach works well for proteins that are produced continuously and that create rather stable (steady-state) endogenous concentrations, but it is not appropriate for episodic production or when one expects alterations in production, secretion, or clearance due to feedback mechanisms.

Method 2) Measure endogenous concentrations during a placebo phase. The sample collection times can be the same as the collection times in the bioequivalence study or samples can be collected at a fixed, repetitive interval to accurately characterize endogenous concentrations over the period of interest (i.e., every 5 min, every 30 min, . . . every 2 h . . . , whatever is appropriate for the particular protein). There is not universal agreement on when to monitor endogenous concentrations nor on how to evaluate these data. Emotional and physical stress tend to affect endogenous concentrations such that the information collected during a placebo-phase may not even apply the very next day due to day-to-day variability.

Method 3) Recruit a patient population with negligible endogenous concentrations. For example, one might consider volunteers who have negligible endogenous growth hormone concentrations (e.g., adults or growth hormone-deficient children) for growth hormone bioequivalence studies. In some instances, a special patient population, which may meet your criteria, may show greater variability both in the rate of input after extravascular administration (i.e., ka), in the fraction absorbed (i.e., F), and in the disposition (i.e., CL and Vd).

Method 4) Administer a compound that suppresses the endogenous production and/or secretion. For example, one might pretreat volunteers with somatostatin to suppress endogenous growth hormone secretion for growth hormone bioequivalence studies. This approach introduces the additional uncertainty of the effects of treatment on the pharmacokinetics of your test article.

Method 5) Do nothing and assume that the endogenous concentrations will affect both phases of the study similarly. In this approach, you are evaluating the equivalence of the total concentrations (i.e., endogenous and exogenous) and not solely the equivalence of your injected material.

When you collect information about endogenous concentrations, you need a sound scientific rationale for subsequent data analyses. FDA Biopharmaceutic Guidances for oral products that have measurable endogenous concentrations and/or inducible binding proteins (e.g., potassium chloride and norethindrone/ethinyl estradiol) are helpful to review when you are designing a study because they provide insight on baseline monitoring periods, dosing schedules, 'washout' intervals, analytical specifications, data analysis, etc (Guidance for *in vivo* bioequivalence study for slow-release potassium chloride tablets/capsules. FDA Division of Bioequivalence, Office of Generic Drugs, May 15, 1987; and Guidance for *in vivo* bioequivalence study and *in vitro* dissolution testing on norethindrone and ethinyl estradiol tablets. FDA Division of Bioequivalence, Office of Generic Drugs, March 18, 1988). If you subtract the baseline concentrations from your treatment phase and perform statistical comparisons on the 'uncorrected' and 'baseline-corrected' data sets, theoretically you could get bioequivalence through one comparison and not the other. Usually, bioequivalence is established on baseline-corrected data (Dr. Rabi Patnaik, FDA, personal communication).

Environmental Factors

Environmental factors such as the site, depth, concentration, and volume of the injection; temperature and local perfusion of injection site; and physical activity of patient can influence the absorption profiles of therapeutic proteins that

are administered extravascularly. It goes without saying that it is important to control these factors in a bioequivalence study.

The intramuscular (IM) and subcutaneous (SC) routes, the most common extravascular routes of administration for therapeutic proteins, exhibit dissimilar absorption profiles (Figure 1) due to the differences in the physiology of the absorption sites. Detailed studies of SC absorption have shown that molecules with a molecular weight greater than 16,000 are absorbed mainly by the lymphatics that drain the application site (9,10). Typically, peak concentrations are lower and occur later following SC injection compared to IM (11–13), although these differences rarely affect the biological response.

When proteins are administered by the same route but at different anatomical sites, differences in absorption have been identified. For instance, a SC injection of growth hormone is absorbed better from an abdominal site than from the thigh in both normal volunteers (14) and growth hormone-deficient patients (15), and leuteinizing hormone releasing hormone is better absorbed SC from upper arm than from abdominal site (16). To minimize the influence of injection (17,18), one should consider using the same nurse or physician to inject the subjects in all phases of the study.

The effect of physical activity on absorption profiles is best illustrated with an example. Recombinant human growth hormone (0.1 mg/kg) was administered SC in the left buttock of healthy male volunteers in a standard two-treatment crossover design. The absorption was erratic, and the 'terminal' phase difficult to evaluate (Figure 2, upper panel). The multiple spikes and peaks in the concentration-time profiles were probably due to volunteer activity (sitting, standing, etc.), which had a direct effect on absorption from the SC injection. When recombinant human growth hormone was administered SC in the triceps of the non-dominant arm and the arm immobilized in a sling, there was less inter- and intra-subject variability, diminished observation of 'abrupt' peaks, and the terminal phase was amenable to analysis (Figure 2, lower panel).

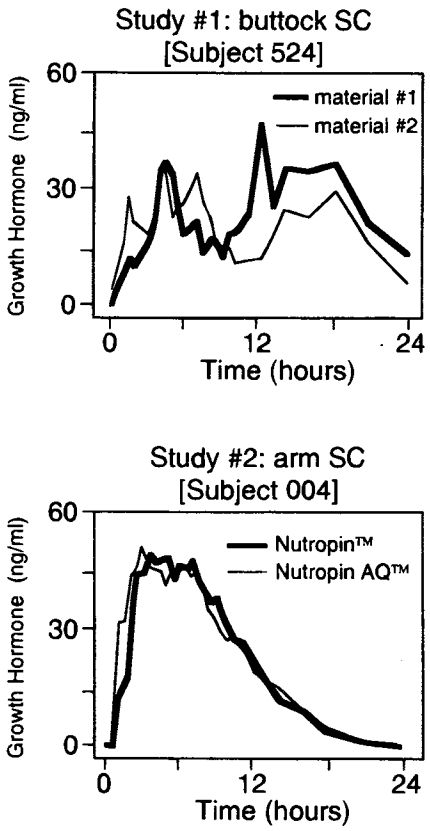

Fig. 2. Comparison of sites of administration. Two bioequivalence studies were conducted. In both studies, healthy adult men received 0.1 mg/kg recombinant human GH subcutaneously in a standard two-treatment crossover design. In bioequivalence study #1, the test materials were administered in the left buttock (upper panel); in bioequivalence study #2, the test materials were administered in the triceps of non-dominant arm (lower panel). Differences in the pharmacokinetics of human GH (study #1 *vs* study #2) are attributed to the effects of volunteer activity on absorption from the SC injection sites (Genentech, Inc., data on file).

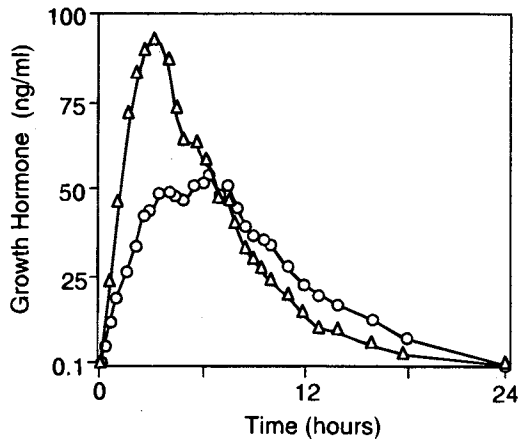

Fig. 1. Comparison of routes of administration. Twenty healthy adult men received 0.1 mg/kg recombinant human GH (IM or SC) in a standard two-treatment crossover design [△ denotes mean data for IM route; ○ denotes mean data for SC route]. Differences in the pharmacokinetics of human GH (IM *vs* SC) are attributed to the differences in blood supply, absorption pathways, body fat, skin temperature, etc. (Genentech, Inc., data on file).

Immunogenicity

The experience in the clinic with respect to the immunogenicity of therapeutic proteins has been variable and not necessarily predictable based on preclinical studies. With most compounds, a small number of patients develop antibodies to the test material (19–21); with murine monoclonal antibodies, the percentage of patients who develop an immune response can approach 100% (22). The immune response is quite variable both within and across species, although the magnitude of the response and the time of appearance often correlate quite well to dose (i.e., the higher the dose or the more frequent the administration, then the more rapid and stronger the immune response) and route (i.e., subcutaneous administration is often more immunogenic than intravenous administration). The impact of an immune response on the pharmacokinetics of a therapeutic protein (in this case, a murine monoclonal antibody in non-human primates) is illustrated in Figure 3. The pharmacokinetics in the low dose group (upper panel) exhibited a relatively smooth exponential profile; whereas, the pharmacokinetics in the high dose group (lower panel) showed a precipitous

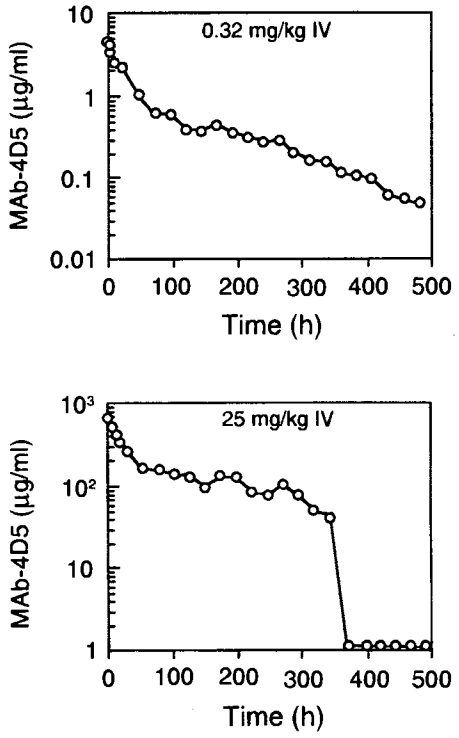

Fig. 3. Adult female cynomolgus monkeys received murine monoclonal antibody 4D5 (MAb-4D5) as a single IV bolus dose of 0.32 or 25 mg/kg (n = 3/group). Serum samples were collected over a three-week period and evaluated for MAb-4D5 and antibodies to MAb-4D5. Antibodies to the test material were not observed in the low dose group (upper panel), while the high dose group (lower panel) developed an immune response approximately two weeks after dosing at which time the serum concentrations of MAb-4D5 diminished rapidly (Genentech, Inc., data on file).

drop in circulating concentrations of test material due to the formation of antibodies that promoted rapid clearance.

Sometimes the immune potential of a therapeutic protein is influenced by the expression system in which it is produced through the introduction of modified sialic acids. Sialic acids are often the terminal carbohydrate moieties on cell surface glycoproteins and glycolipids (23). The sialic acid on adult human glycoproteins is N-acetylneuraminic acid (NANA); N-glycolylneuraminic acid (NGNA) is found in rodents, but it is not found in adult humans[6] (23). If a normal adult human is exposed to glycoconjugates containing NGNA, an immunogenic response occurs (23). Antibodies against NGNA are found in patients who have received injections of animal antisera; it is associated with 'serum sickness' (23,24). NGNA is found, to various degrees, on recombinant proteins that are produced in Chinese Hamster Ovary (CHO) cells; these therapeutic agents have an increased potential to be immunogenic.

When antibodies are detected, they are characterized as neutralizing or non-neutralizing, depending on their ability to abrogate the therapeutic effect. It is not necessary to make this distinction for purposes of conducting a bioequivalence study; however, one should prescreen volunteers or patients to insure that they do not have antibodies (or cross-reacting substances) in their serum, which might interfere with the pharmacokinetics (through complex formation and changes in clearance) or the analytical methods (discussed in next section). Also, serum samples should be collected immediately prior to dosing and at the end of each treatment phase for antibody analysis.

When designing preclinical and clinical bioequivalence programs, considerations for immunogenicity can be incorporated in the study designs. For preclinical studies, crossover designs in animals may be problematic due to antibody formation; a parallel design (with a larger n) may be warranted in this situation. In humans, crossover designs are recommended, except when the material is expected to immunogenic or when the half-life is extremely long (on the order of days to weeks), such as a monoclonal antibody or an immunoadhesin; in these cases, a parallel design should suffice. When conducting crossover studies (both preclinical and clinical), a period effect could represent antibody formation.

Assay Methodology

Validation of the analytical techniques that are used for the quantitation of therapeutic proteins in the biological matrix of interest follows the general recommendations set forth for bioavailability, bioequivalence, and pharmacokinetic studies (25). The parameters essential to ensure the acceptability of the performance of the analytical method are stability of the drug in the matrix under study storage conditions, accuracy, precision, sensitivity, specificity (selectivity), response function, and reproducibility (25).

The sensitivity and specificity of your analytical methodology will determine the nature and extent of potential interferences. For immunoassays, which are commonly employed for quantitating therapeutic proteins, potential interferences include heterophilic anti-IgG antibodies, autoantibodies against the analyte, metabolites and/or clipped inactive protein, and, of course, endogenous concentrations (26,27). The following two examples illustrate these issues.

The immunoassay for interferon-γ is a double-antibody sandwich ELISA, which uses a rabbit anti-interferon-γ antibody for capture and a horseradish peroxidase conjugated mouse monoclonal antibody to interferon-γ for detection. Some of the variability of extravascular bioavailability of recombinant human interferon-γ in humans (reported to be as high as 575% based on AUC) has been attributed to catabolism at the site of administration, absorption of metabolites, and subsequent analytical interferences (13). It was shown that antibodies in ELISA formats differ in their abilities to detect proteolytically (trypsin, *in vitro*) processed forms of recombinant human interferon-γ (28). When the assay format and antibodies were changed, the calculated human bioavailability decreased dramatically. Further, a study in monkeys with radiolabeled material showed that the production of lower molecular weight fragments was route-dependent (13).

The bioassay for interferon-γ detects antiviral activity against vesicular stomatitis virus in A549 cells. This assay measures the extent to which the cytopathic effect of the challenging virus is inhibited. As part of the preclinical assessment of recombinant human interferon-γ, monkeys received interferon-γ (0.1 mg/kg) by the IV, IM, and SC routes in a randomized three-way crossover design (28). On the first treatment

[6] In humans, NGNA is an oncofetal antigen (23).

(i.e., naive animals), the ELISA and bioassay results were comparable (Figure 4, panels A and B). Following the first treatment, some of the monkeys developed antibodies, and on subsequent treatment, the pharmacokinetic profiles, as measured by the two assays, were very different. In both animals, the bioassay was able to detect the activity of interferon-γ; however, in one instance (Figure 4, panel C) the activity in the ELISA is diminished, while in the other animal (Figure 4, panel D) no activity is detected by ELISA.

For HPLC, MS, and other specific assays, the potential for interference diminishes. As mentioned, prescreening volunteer/patient serum for interfering substances is an important element of the study design.

Biochemical Identity

Many biotechnology products are more accurately characterized as mixtures, with the composition (i.e., biochemical identity) controlled by the manufacturing specifications. Purified recombinant human factor VIII, for example, is a glycoprotein consisting of multiple polypeptides with relative mobilities (M_r) ranging from 80,000–210,000, which is similar to the pattern of polypeptides observed for plasma-derived factor VIII (29). Tissue plasminogen activator (t-PA) exists as a mixture of 1-chain and 2-chain forms; the ratio of these forms changes depending on the production method, i.e., roller bottle versus suspension culture (30). Human monoclonal antibody (MAb) 10058 (an IgM MAb specific for gram-negative bacteria and endotoxin) produced in ascites is cleared from plasma more slowly than tissue culture material in the rat (Figure 5) (31). The nature of the molecular structures on MAb 10058 responsible for the differences in the systemic clearance were not reported by the authors.

Glycoproteins that carry terminal mannose, galactose, N-acetylglucosamine, or fucose residues are rapidly removed from the circulation by carbohydrate-specific recognition systems in hepatic and reticuloendothelial tissues, such as the galactose-specific receptor in hepatic parenchymal cells (e.g., the hepatic asialglycoprotein receptor) and mannose-specific receptor on macrophages (32–34). Sialic acids shield the carbohydrates from these clearance receptors; removal of sialic acid exposes the carbohydrates, resulting in increased systemic clearance and decreased *in vivo* circulating half-life. Changes in the manufacturing process that affect glycosylation and sialic acid content have the potential to change the kinetics and/or pharmacodynamics of a protein, as demonstrated by erythropoietin (35), follicle-stimulating hormone (36), interferon-γ (37), prourokinase (38), and monoclonal antibodies (39).

Physical and/or chemical degradation of proteins may occur during various stages of production, purification, formulation, and storage, resulting in a biologically inactive moiety or changes in the pharmacokinetic profile that may ultimately alter the pharmacodynamics. Physical instability, such as aggregation, gelation, and precipitation, refers to changes in the higher order structures; chemical instability involves modification of covalent bonds in proteins, such as hydrolysis, deamidation, oxidation, acetylation, racemization, beta-elimination, and disulfide exchange (40–42.). For example, nerve growth factor (NGF), a homodimer of 120 residues, undergoes hydrolysis ('clipping') under certain storage conditions: C-terminal clipping of the last 3 residues (i.e., residues 118–120) does not

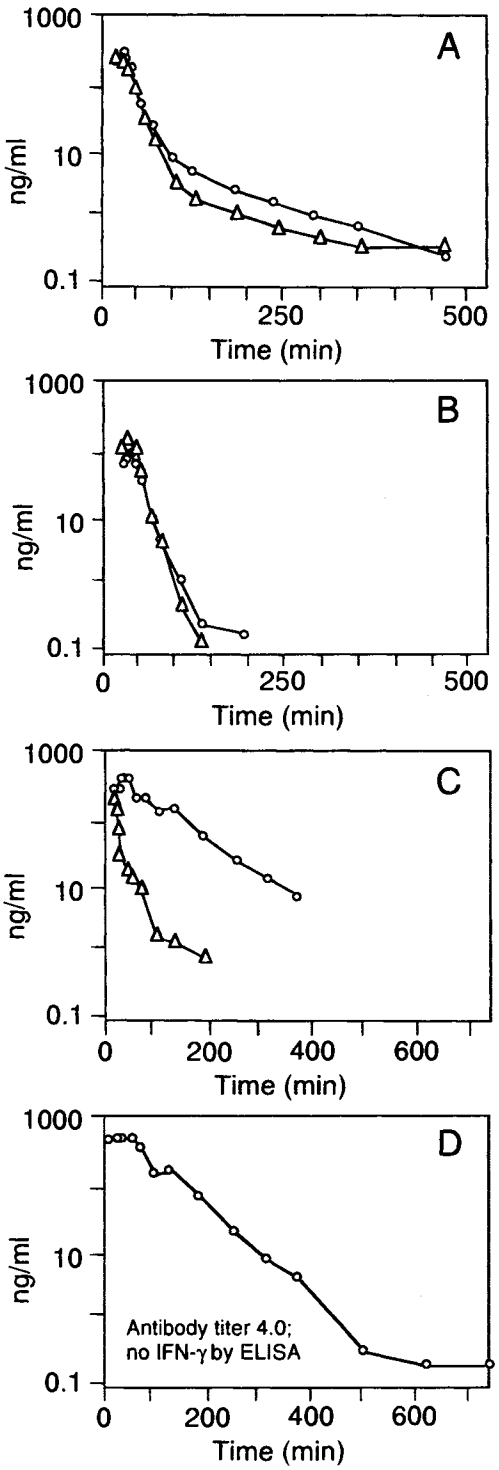

Fig. 4. Healthy monkeys received recombinant human interferon-γ (0.1 mg/kg) by the IV, IM, and SC routes in a randomized three-treatment crossover design (28). On the first treatment (upper panels A and B), the ELISA (△) and bioassay (○) results were comparable (IV data shown). The monkeys developed antibodies to the test material after the first exposure. In subsequent treatments (lower panels C and D), the ELISA and bioassay results were very different (IV data shown). Apparently, the circulating antibodies were able to mask the reactivity in the ELISA, but they had a lesser affect on the bioassay. Redrawn from ref. 28 by permission of the publishers Harvey Whitney Books.

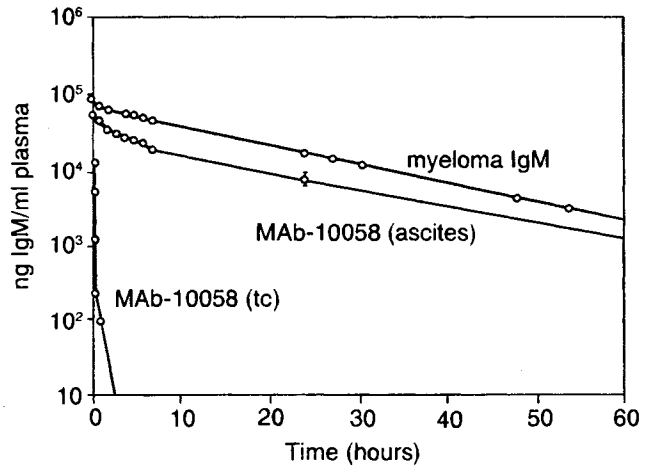

Fig. 5. Pharmacokinetic profiles of purified human MAb-10058 produced in tissue culture (tc) or mouse ascites in comparison with that of a human myeloma IgM protein in the rat following 3 mg/kg IV bolus dose. Redrawn from ref. 31 by permission of the publishers Forefront Publishing.

affect biological activity, while N-terminal clipping (i.e., loss of the first 5 or 9 residues) diminishes the biological activity (43–45).

Pharmacodynamic Equivalence

The preferred (and often easiest) method of demonstrating biological equivalence for a protein product is by comparing drug concentrations in an accessible biological fluid, usually serum/plasma, following a standard dose. There are circumstances when your drug may not achieve measurable concentrations, for instance, the assay may not be sensitive enough (for the administered dose) or the route of administration may not produce adequate systemic concentrations (i.e., drug is poorly absorbed or acts locally, such as topical medication or inhalation products). In the absence of pharmacokinetic data, alternative ways to test bioequivalence (in descending order of FDA preference) include a pharmacodynamic effects study, clinical efficacy trials, *in vivo* animal studies, and *in vitro* studies (Code of Federal Regulations, 21 CFR 320.24). The design and evaluation of a pharmacodynamic effects trial have been described (see, for example, Guidance for topical dermatologic cortico steroids: *in vivo* bioequivalence. FDA Division of Bioequivalence, Office of Generic Drugs, June 2, 1995; and Interim guidance for documentation of *in vivo* bioequivalence of albuterol inhalation aerosols (Metered Dose Inhalers). FDA Division of Bioequivalence, Office of Generic Drugs, January 27, 1994). For pharmacodynamic equivalence, you need acceptable (surrogate) endpoints and appropriate validation; in fact, development and validation of a pharmacodynamic effect bioassay is the same as for any assay of drug or metabolite (see ref. 25).

DESIGN OF BIOLOGICAL EQUIVALENCE PROGRAMS

In order to support the introduction of "new" test material into an ongoing clinical development program, "bridging" studies are necessary. These studies usually include a variety of biochemical characterization measurements, an evaluation of the disposition profile in an animal model (or humans) to confirm relative dosimetry, and some consideration regarding the overall predicted safety profile of the test material. Collectively, the data from these studies comprise the equivalence program and are intended to demonstrate that the test material produced by the "current" process is biologically equivalent to test material produced by the "new" process. Bridging studies should focus attention on the most critical elements for defining safety and efficacy of the biopharmaceutical that is produced by the "new" process. Each product and process are usually very different, so it is generally expected that these studies will be product and process specific (Federal Register Docket No. 96D-0132, Vol. 61, No. 82, pp. 18612–13, April 26, 1996). Ideally, comparative data should be obtained on at least two to three lots of test material produced by the "new" process.

Typically, biochemical characterization may include techniques such as SDS PAGE (reduced and nonreduced), HPLC (reverse phase, size exclusion, ion exchange), amino acid analysis, peptide mapping, NH_2 and COOH terminal sequences, isoelectric focusing, circular dicroism, mass spectrometry, carbohydrate analysis, biological activity, host cell proteins, nucleic acid, LAL, bioburden determination, sterility, and so on. Assessment of glycoforms, sialic acid content, extent of proteolysis, deamidation, oxidation, and aggregation are important and should be evaluated. These changes may have important effects on bioactivity, stability, and/or potential immunogenicity and safety. Furthermore, to ensure the integrity of your program, use test material for preclinical equivalence studies that has been thoroughly characterized, avoiding material that is from early/intermediate manufacturing procedures or from small research scale (bench top) purification (as this material may not be representative of the final vialed product for clinical use). Also, it is important to include specific handling, preparation, and administration procedures in the preclinical and clinical protocols to ensure the material is not inadvertently exposed to denaturing diluents, adsorbent surfaces, or procedures that could affect the ability to administer an accurate, biologically-active dose.

Chemical characterization measurements include an assessment of potency, identity, and purity. Universal specifications or tolerances are difficult to define; however, some attempt to provide acceptable limits should be made. For example, potency could be required to be within 20% of a reference standard as determined by a defined assay. Identity (as reported by primary, secondary, and tertiary structure) would be identical in ideal circumstances. Purity would be required to be within some percentage of a reference standard (e.g., $\pm 10\%$); however, this criteria depends upon the nature of impurity profile (e.g., endotoxin *vs.* variant, potential effects on stability, etc.).

For a biopharmaceutical, the assay performance criteria are often highly variable; therefore, strict statistical criteria that attempt to rigorously establish traditional *in vivo* bioequivalence may not always be appropriate. In some cases, an assessment of rate and extent of absorption as indicated by the maximum concentration (C_{max}), time of maximum concentration (T_{max}), and area-under-the-curve (AUC) may be needed; in other cases, complicating factors related to binding proteins, endogenous concentrations, and unusual concentration-time profiles may need to be considered.

It should be noted to that in some cases, preclinical pharmacokinetic studies may not be necessary prior to human testing. This would usually be true when the overall biochemical characterization profile is judged to be "comparable," and there are no particular safety concerns associated with the molecule. Conversely, there may be a decision to conduct preclinical pharmacokinetic studies if significant differences in biochemical properties are detected (Federal Register Docket No. 96D-0132, Vol. 61, No. 82, pp. 18612–13, April 26, 1996). If this is the case, then determination of the pharmacokinetics or disposition profile in a single species is generally sufficient. The dose range should be similar to that used for the reference material. Ideally, the dose(s) administered to animals should produce serum concentrations that match the range of concentrations observed in humans. There are several situations, however, where this may not be feasible; for instance: (a) the compound may elicit an exaggerated pharmacologic response in animals at equivalent serum concentrations, so the dose must be lowered to avoid untoward pharmacokinetic alterations; (b) there are qualitative and quantitative differences in blood/serum/tissue binding elements that must be considered when selecting an appropriate concentration range; or (c) the assay sensitivity (i.e., the lower limit of quantitation) may require a dose that produces higher serum concentrations in animals to permit full pharmacokinetic characterization for bioequivalence comparisons. Parallel or crossover design may be acceptable; however, complications arising from an immunogenic response to the heterologous protein may make crossover designs in animals inappropriate (1). Also, antibodies to the heterologous protein may compromise your ability to adequately characterize the disposition of test articles that are cleared slowly, such as monoclonal antibodies or immunoadhesins.

Toxicology studies may be warranted in certain cases. The extent of toxicological assessment is contingent upon the previous safety profile, the projected therapeutic ratio, and the clinical indication. For example, if one considers the situation of a monoclonal antibody that has no preclinical or clinical safety issues (i.e., high therapeutic index), minimal *in vivo* toxicology testing would be expected (see Points to consider in the manufacture and testing of monoclonal antibody products for human use. FDA Office of Biologics Research and Review, 1994). In contrast, for a biopharmaceutical that has been previously shown to have a low therapeutic index or indications of cumulative toxicity or other concerns regarding target organ toxicity, more extensive toxicology studies may be indicated. Consider a hemostatic agent that could induce a life-threatening condition, such as disseminated intravascular coagulation, in a narrow concentration range; for this compound, the level of concern regarding any detected differences in the overall "characterization" profile would be high compared to a relatively benign monoclonal antibody.

Examples of production and process changes that may require the establishment of equivalence prior to use of test material in clinical studies are shown in Table 2. Each change has the potential to change the kinetics and/or dynamics of a protein, which ultimately could have an impact on safety, efficacy, and immunogenicity. For example, a cell line change from *E. coli* to CHO cells can affect glycosylation[7] and tertiary structure[8]; changes in nutrients can alter the nature of the carbohydrates; and changes in formulation components can alter conformation or facilitate dimer and aggregate formation. Each situation must be evaluated with respect to its unique properties.

Table 2. Examples of Production/Process Changes that May Require the Establishment of Equivalence Prior to Use of Test Material in Clinical Studies (modified from ref. 1)

Cell Bank, Cell Line, Plasmid
- Changes in cell clone
- Changes in cell substrates
- Production in ascites versus cell culture
- Use of higher methotrexate concentrations during amplification
- Use of a New Master Cell Bank
- Change in promoter gene vs. structural gene
Cell Culture/Fermentation
- Use of new additives
- Use of new amino acids
- pH changes or temperature shifts
- Changing from serum to serum-free conditions
Purification/Recovery Conditions
- Omission of a purification step
- Major design changes
- Removal of a virus inactivation step
- Substitution of cations vs. anion exchanger
Formulation
- Use of new excipient(s)
- Changes in excipient ratios
- Changes in buffer exchange method
- Changes in container closure
Manufacturing Site
- Site change
- Change from pilot scale to full scale

The significance of a process/manufacturing change may be more important if it is made later in the development program, for example Phase III, in contrast to the same change being made during Phase I or Phase II, and may require that clinical bioequivalence studies be performed. Important considerations for the clinical bioequivalence studies have been previously discussed.

The data derived from these studies should allow an objective reviewer to conclude that the new test material is biologically equivalent to the current test material. These data would also support the conclusion that the test material, which was utilized during the preclinical and clinical development programs, was "representative" or "comparable" on all important measures to the test material that is produced for market at time of registration.

CASE STUDIES

The scope of the equivalence program is usually established in relation to the significance of the change that is being evaluated, the stage of development, and the clinical indication. Naturally, this is very subjective and takes into consideration

[7] *E. coli* does not glycosylate proteins.

[8] *E. coli*, but not CHO cells, has trouble with disulfide bonds, resulting in folding problems.

how the change may effect the finished product, as well as the answers to questions such as ··· How much of a 'difference' is acceptable? Is 'inequivalence' an issue at this stage of drug development? Are the pivotal efficacy trials in progress? Does the dosing regimen/route provide measurable systemic concentrations? Do blood concentrations correlate with toxicity and/or efficacy?

In the early stages of development, significant differences in pharmacokinetics may be overcome by simply adjusting the dosage regimen and proceeding with the clinical development program—strict adherance to traditional/statistical definitions of bioequivalence may not be warranted at this stage. For compounds that are administered chronically or that are in latter stages of development, changes in pharmacokinetics could result in under- or overdosing, accumulation, or changes in response (safety, efficacy) with long term exposure to modified material; in this situation, the scope of the equivalence program may become more elaborate and the comparison criteria more rigorous.

The following two case studies were selected to illustrate equivalence programs for proteins undergoing different types of changes at different stages of development, i.e., recombinant human insulin-like growth factor (Phase I process change) and recombinant human growth hormone (Phase II/III formulation change).

Case Study I: Recombinant Human Insulin-Like Growth Factor

Recombinant human insulin-like growth factor (rhIGF-I) is a 7.4 kD peptide that is produced in *E. coli*. It circulates bound to several binding proteins, which differ quantitatively and qualitatively across species (46,47). The unbound form of IGF-I is associated with hypoglycemia; it is one-seventh as potent as insulin. rhIGF-I is in clinical development for a variety of indications that include immune function restoration, acute and chronic renal failure, anabolic properties, Lou Gehrig's disease, and diabetes.

Genentech filed the IND for rhIGF-I using the ZZ-process, which involves the production, secretion and release into the supernatant of a fusion protein carrying a ZZ-leader sequence (e.g., Z domains of Protein A). The fusion protein is cleaved with hydroxylamine, and the rhIGF-I portion is recovered by subsequent purification steps. While in Phase I, the process was changed to a direct secretion or DS-process, wherein rhIGF-I is released directly into the culture supernatant. The new process is more efficient and requires fewer fermentations to produce the same amount of material. The new process required changes to the cell line, plasmid, fermentation, purification, and formulation.

Key components of the equivalence program included chemical equivalence (i.e., peptide mapping, mass spectrometry, HPLC retention time, and immunoreactivity), *in vitro* bioactivity (i.e., cell proliferation and radioreceptor bioassays), preclinical safety (Table 3), and clinical pharmacokinetics and safety (Table 4). The program did not include a preclinical pharmacokinetic assessment due to the binding protein differences across species. Hypoglycemia was the primary safety parameter because it is rapid, it correlates with the unbound IGF-I plasma concentrations, and a significant database existed for the hypoglycemic effect in both rats and humans. The preclinical doses (i.e., 1 and 100 μg/kg IV) were selected because they represented a no-observable adverse effect level (NOAEL) and a moderate adverse effect level, without mortality; the clinical dose (i.e., 50 μg/kg IV) was selected to provide measurable plasma concentrations and slight hypoglycemia, without other adverse effects.

Table 3. Preclinical Bioequivalence of rhIGF-1 in Rats

Species	Crl:CD®BR/VAF/Plus rats (5/sex/group)
Test Materials	DS- and ZZ-process rhIGF-I
Design	Single tail vein injection; serum glucose 15–20 minutes postdose; sacrifice on day 7
Dose/Route	1 or 100 μg/kg IV bolus of either test material; saline control group
Measurements	Glucose, body weight, food consumption

Endogenous free IGF-I concentrations in each volunteer were assessed in the plasma samples collected 5–30 minutes prior to injection; the free IGF-I concentrations in all cases were below the lower limit of quantitation of the radioimmunoassay (i.e., 6.25 ng/ml). Endogenous concentrations were assumed to contribute equally to each treatment over the 24-hour sample collection period.

Free IGF-I concentration *versus* time data were evaluated by non-compartmental methods to determine C_{max}, T_{max}, AUC from 0 to the last measurable concentration (AUC_{0-t}), and the terminal serum half-life. The pharmacokinetic parameters for each IGF-I preparation were evaluated employing an analysis of variance model appropriate for the two-period crossover design. Bioequivalence was evaluated using a 20% criterion applied to the classical t-test 90% confidence intervals for the ratios of C_{max}, T_{max}, AUC_{0-t}, and the terminal serum half-life. If the 90% confidence interval for these ratios fell between 0.8 and 1.2 (which is equivalent to the mean values differing by 20% or less with 90% confidence), bioequivalence was accepted.

Glucose serum concentrations were evaluated as follows: the AUC between 0 and 2 hours (time of first snack), AUC_{0-2}, was calculated using the linear trapezoidal method. Glucose nadir was the lowest observed glucose concentration. The two-period, crossover ANOVA model that was used to analyze the free IGF-I parameters was also used to examine the glucose response to rhIGF-I administration.

Table 4. Clinical Bioequivalence of rhIGF-1 in Humans

Subjects	24 healthy male volunteers (18–34 years)
Test Materials	DS- and ZZ-process rhIGF-I
Design	Two-treatment randomized crossover study; treatments separated by a 3-day washout
Dose/Route	50 μg/kg IV bolus
Measurements[a]	Free IGF-I plasma concentrations over a 24-hour period after each injection; serum glucose; clinical safety parameters

[a] AUC, C_{max}, T_{max} and half-life for free IGF-I and AUC_{0-2h} and C_{min} for glucose were analyzed according to an analysis of variance model appropriate for the study design. The equivalence of DS- and ZZ-process rhIGF-I was assessed by determining if the mean parameters differed by 20% or less with 90% confidence.

From the initial evaluations (chemical characterization and *in vitro* bioactivity), rhIGF-I made by the DS-process was shown to be similar to the material made by the ZZ-process. Following dosing in rats, there was no evidence of overt clinical signs of toxicity and no effect on body weight gain with either compound compared to controls. As expected, the 1 μg/kg dose had no effect on serum glucose concentrations in rats, while the 100 μg/kg dose produced mild to moderately lower serum glucose concentrations, which was similar in both treatment groups. It was concluded that rhIGF-I produced by the two methods were biologically equivalent in rats with respect to safety and their effect on lowering serum glucose concentrations. In humans, the test materials were considered indistinguishable with respect to free IGF-I plasma concentrations, glucose lowering effects, and clinical safety parameters. Based on the findings of the equivalence program, rhIGF-I produced by the DS-process replaced the rhIGF-I produced by the ZZ-process in ongoing clinical trials.

Case Study II: Recombinant Human Growth Hormone

Recombinant human growth hormone (rhGH) is a 22 kD nonglycosylated protein produced in *E. coli*, which is approved for short stature associated with growth hormone deficiency. A change from a lyophilized powder to a liquid formulation was proposed to provide an improved, more convenient dosage form. At the time that this formulation change was being considered, rhGH was in clinical development for chronic renal insufficiency and Turner Syndrome; the lyophilized form was already approved for marketing.

Key program components of the equivalence program included chemical characterization (i.e., peptide mapping, HPLC retention time, and SDS PAGE and isoelectric focusing gels), a rat weight gain bioassay, and clinical pharmacokinetics and safety (Table 5). Endogenous hGH concentrations in volunteers were assessed in the serum samples collected 5–30 minutes prior to injection; the hGH concentrations in most cases were below the lower limit of quantitation of the immunoradiometric assay (i.e., 0.5 ng/ml). Endogenous concentrations were assumed to contribute equally to each treatment over the 24-hour sample collection period.

Serum hGH concentration versus time data were evaluated by non-compartmental methods to determine C_{max}, T_{max}, AUC from 0–24 hours (AUC_{0-24}), AUC extrapolated to infinity ($AUC_{0-\infty}$), and the terminal serum half-life. All parameters were reported; however, bioequivalence was based on the logarithmically transformed ($\log_{10}$) AUC and $\log_{10}$ C_{max} parameters. T_{max} was not included in the bioequivalence assessment because the concentration-time profile is essentially flat around the peak (see Figure 2, lower panel). This flatness is due to complicated absorption processes; thus, T_{max} is highly variable and not indicative of absorption rate in the classical sense (see Biopharmaceutical guidance: Statistical procedures for bioequivalence studies using a standard two-treatment crossover design. FDA Division of Bioequivalence, Office of Generic Drugs, July 1, 1992).

Table 5. Clinical Bioequivalence of rhGH in Humans

Subjects	36 healthy male volunteers (18–34 years)
Test Materials	Nutropin™ (somatropin [rDNA origin] for injection); Nutropin AQ™ (somatropin [rDNA origin] injection)
Design	Two-treatment randomized crossover study; treatments separated by a 7-day washout period
Dose/Route	0.1 mg/kg SC bolus in triceps of the non-dominant arm
Measurements[a]	hGH serum concentrations over a 24-hour period after each injection; clinical safety parameters

[a] The equivalence of the two formulations was assessed by determining whether the 90% confidence limits for the ratio of geometric means using log-transformed ($\log_{10}$) AUC and $\log_{10}$ C_{max} were within 80–125%

From the initial evaluations (chemical characterization and rat weight gain bioassay), rhGH in the two formulations was considered to be similar. In humans, the two formulations were declared equivalent based on the demonstration with 90% confidence that the ratio of geometric means using $\log_{10}$ AUC and $\log_{10}$ C_{max} were within the 80 to 125% range.

FUTURE DIRECTIONS

This is a very exciting time with respect to bioequivalence issues (see 1–3, 48–59). The FDA is actively reviewing the methodology and seeking input from academic and industrial scientists. We are witnessing a departure from AUC, C_{max}, and T_{max} as the sole criteria for bioequivalence, with more emphasis placed on *in vitro* characterization, therapeutic index, clinical indication/population, and kinetic/dynamic variability for choosing the appropriate bioequivalence metric.

Improvements in analytical techniques have facilitated the design and conduct of equivalence studies, such that the clinical studies for protein pharmaceuticals are now similar in size and scope to those conducted for small molecules. Continued analytical improvements, as well as a better understanding of the physiology of a particular disease state and patient population, should offer insight into the nature of and appropriate modeling techniques for endogenous concentrations.

New human therapies on the horizon (such as gene therapy, antisense oligonucleotides, and tissue-engineered products) will provide challanges for pharmaceutical scientists. For these pharmaceuticals, one cannot simply measure the decline in serum concentration after administration to evaluate equivalence, since the disposition profile in these instances does not represent 'clearance' in the classical sense. Targeted and promiscuous cellular uptake (with unknown site and efficiency of incorporation) occur simultaneously with degradation, thus complicating this analysis. Clearly, serum concentrations are not an appropriate surrogate for tissue concentrations because activities in these two compartments are dissimilar. How will we evaluate the equivalence of test materials in these situations: measure the product of gene insertion? . . . conduct clinical safety/efficacy trials (big, costly, time consuming)? . . . rely on preclinical tissue distribution, safety, and efficacy?

Lastly, it seems appropriate to permit a greater reliance on biochemical characterization, *in vitro/in vivo* bioassays, and pharmacokinetic comparisons (with suitable clinical dosing adjustments) in early stages of development to facilitate process and formulation changes. More intense, statistically-rigid, clinical kinetic/dynamic comparisons should be reserved for Phase III and beyond.

ACKNOWLEDGMENTS

We would like to acknowledge the scientific contributions of the following rhIGF-1, rhGH, and muMAb4D5 project team members: Sharon Chen, Pamela Garzone, Beth Hutchins, Allen Izu, Joyce Kuntze, Daniel Maneval, and Andrew Perlman; and the advice, comments and support of the following individuals: Joffre Baker, Rob Garnick, Bryan Lawlis, Rabi Patnaik, Mary Slikowski, Robert Strickley, Karen Thomsen, Thomas Tozer, Roger Williams, and John Winslow. Also, we thank Charles Hoffman for the illustrations.

REFERENCES

1. J. D. Green. In P. F. D'Arcy and D. W. G. Harron (eds.) *Proceedings of the Third International Conference on Harmonization, Yokohama, 1995*, Queen's University of Belfast Press, Northern Ireland, 1996, pp. 230–236.
2. R. Williams. (abstract). *Bio-International '92. Conference on Bioavailability, Bioequivalence and Pharmacokinetic studies.*
3. L. Z. Benet. In K. Midha and H. Blume (eds.), *Bio-International. Bioavailability, bioequivalence, and Pharmacokinetics*. Medpharm, Stuttgart, 1993. pp. 27–37.
4. V. Steinijans, D. Hauschke, and H. Jonkman. *Clin. Pharmacokinet.* **22**:247–253 (1992).
5. H. Blume and K. Midha. *Pharm. Res.* **10**:1806–1811 (1993).
6. Proceedings of the FDA bioequivalence hearings, Docket number 86N-0251, Washington, DC., September 29-October 1, 1986.
7. Report by the bioequivalence task force on recommendations from the bioequivalence hearings conducted by the Food and Drug Administration, 86N-0251, RPT00002, pp. 19–21, January 1988.
8. H. Blume and K. Midha (eds.). *Bio-International 2. Bioavailability, Bioequivalence, and Pharmacokinetic Studies*. Medpharm, Stuttgart, 1995.
9. A. Supersaxo, W. Hein, H. Gallati, and H. Steffen. *Pharm. Res.* **5**:472–476 (1988).
10. A. Supersaxo, W. Hein, H. and Steffen. *Pharm. Res.* **7**:167–169 (1990).
11. J. Kahn, J. D. Allan, T. Hodges, L. Kaplan, C. Arri, H. Fitch, A. Izu, J. Mordenti, S. Sherwin, J. Groopman, and P. Volberding. *Annals of Internal Medicine* **112**:254–261 (1990).
12. W. Saal, H-J. Glowania, W. Hengst, and J. Happ. *Fertility and Sterility* **56**:225–229 (1991).
13. J. Mordenti, S. A. Chen, and B. Ferraiolo. In A. H. C. Kung, R. A. Baughman, and J. W. Larrick (eds.), *Protein Therapeutics: Pharmacokinetics and Pharmacodynamics.*, W. H. Freeman and company, New York, 1993, pp. 187–199.
14. S. Beshyah, V. Anyaoku, R. Niththyananthan, P. Sharp, and D. Johnston. *Clin. Endocrinol.* **35**:409–412 (1991).
15. T. Laursen, J. Jorgensen, and J. Christiansen. *Clin. Endocrinol.* **40**:373–378 (1994).
16. S. Blunt, R. Clayton, and W. Butt. *Clinical Endocrinology* **25**:589–596 (1986).
17. P. Hildebrandt, L. Sestoft, and S. Nielsen. *Diabetes Care* **6**:459–462 (1983).
18. J. Thow and P. Home. *Br. Med. Journal* **301**:3–4 (1990).
19. H. Kirchner, A. Korfer, P. Evers, M. M. Szamel, J. Knuver-Hopf, H. Mohr, C. R. Franks, U. Pohl, K. Resch, M. Hadam, H. Poliwoda, and J. Atzpodien. *Cancer* **67**:1862–1864 (1991).
20. K. Lundin, L. Berger, F. Blomberg, and P. Wilton. *Acta Paediatr. Scand. (suppl).* **372**:167–168 (1991).
21. C. M. Zwickl, K. S. Cocke, R. N. Tamura, L. M. Holzhausen, G. T. Brophy, P. H. Bick, and D. Wierda. *Fundamental and Applied Toxicology* **16**:275–287 (1991).
22. R. Dillman. *Antibody Immunoconj. Radiopharm.* **3**:1–15, (1990).
23. E. Muchmore, M. Milewski, A. Varki, and S. Diaz. *J. Biol. Chem.* **264**:20216–20223 (1989).
24. Y. Kozutsumi, T. Kawano, H. Kawasaki, K. Suzuki, T. Yamakawa, and A. Suzuki. *J. Biochem.* **110**:429–435 (1991).
25. V. Shah, K. Midha, S. Dighe, I. McGilveray, J. Skelly, A. Yacobi, T. Layloff, C. Viswanathan, C. E. Cook, R. McDowall, K. Pittman, and S. Spector. *Pharm. Res.* **9**:588–592 (1992).
26. L. Boscato and M. Stuart. Clin. Chem. **34**:27–33 (1988).
27. T. Weber, K. Kapyaho, and P. Tanner. Scand. J. Clin. Lab. Invest. 50, Suppl **201**:77–82 (1990).
28. A. Chen, D. Baker, and B. Ferraiolo. In P. Garzone, W. Colburn, and M. Mokotoff (eds.), *Peptides, Peptoids, and Proteins*. Harvey Whitney Books, Cincinnati, OH, 1991, pp. 54–71.
29. D. Eaton, P. Hass, L. Riddle, J. Mather, M. Wiebe, T. Gregory, and G. Vehar. *J. Bio. Chem.* **262**:3285–90 (1987).
30. R. Baughman, Jr. In B. Sobel, D. Collen, E. Grossbard (eds.) *Tissue Plasminogen Activator in Thrombolytic Therapy*, Marcel Dekker, Inc., New York, 1987, pp. 41–53.
31. S. Gauny, J. Andya, J. Thomson, J. Young, and J. Winkelhake. *Hum. Antibod. Hybridomas* **2**:33–38 (1991).
32. G. Ashwell and A. Morell. In A. Meister (ed.) *Advances in Enzymology and Related Areas of Molecular Biology*, John Wiley and Sons, New York, 1974, pp. 99–128.
33. G. Ashwell and J. Harford. *Ann. Rev. Biochem.* **51**:531–54 (1982).
34. D. Barbeau. Controlled Release Newsletter, Controlled Release Society, Inc., Deerfield, IL, July 1990, pp. 6–8.
35. M. Fukuda, H. Sasaki, L. Lopez, and M. Fukuda. *Blood* **73**:84–89 (1989).
36. W. Blum and D. Gupta. *J. Endocr.* **105**:29–37 (1985).
37. T. Sareneva, K. Cantell, L. Pyhala, J. Pirhonen, and I. Julkunen. *J. Interferon Res.* **13**:267–269 (1993).
38. J. Henkin, D. Dudlak, D. Beebe, and L. Sennello. *Thrombosis Research* **63**:215–225 (1991).
39. M. Mattes. *J. Nat. Cancer Inst.* **79**:855–863 (1987).
40. M. Manning, K. Patel, and R. Borchardt. *Pharm. Res.* **6**:903–917 (1989).
41. J. Cleland, M. Powell, and S. Shire. *Crit. Rev. Therap. Drug Carrier Systems* **10**:3007–377 (1993).
42. S. Li, C. Schoneich, and R. Borchardt. *Biotech. and Bioengineer.* **48**:490–500 (1995b).
43. C. Schmelzer, L. Burton, W.-P. Chan, E. Martin, C. Gorman, E. Canova-Davis, V. Ling, M. Slikowski, G. McCray, J. Briggs, T. Nguyen, and G. Polastri. *J. Neurochem.* **59**:1675–1683, (1992).
44. C. Drinkwater, P. Barker, U. Suter, and E. Shooter. *J. Biol. Chem.* **268**:23202–207 (1993).
45. A. Shih, G. Laramee, C. Schmelzer, L. Burton, and J. Winslow. *J. Biol. Chem.* **269**:27679–686 (1994).
46. R. Baxter and J. Martin. *Biochem. Biophys. Res. Commun.* **147**:408–415 (1987).
47. A. Lord, S. Bastian, L. Read, P. Walton, and F. Ballard. *J. Endocrin.* **140**:475–482 (1994).
48. A. Rescigno. *Pharm. Res.* **9**:925–928 (1992).
49. W. Hauck and S. Anderson. *J. Pharmacokin. Biopharm.* **22**:551–564 (1994).
50. S. Chen, A. Izu, J. Mordenti, and A. Rescigno. *Am. J. Therapeutics* **2**:190–195 (1995).
51. A. L. Gould. *J. Pharmacokin. Biopharm.* **23**:57–86 (1995).
52. L. Endrenyi and P. Al-Shaikh. *Pharm. Res.* **12**:1856–1864 (1995).
53. A. W. Boddy, F. C. Snikeris, R. O. Kringle, G. C. G. Wei, J. A. Oppermann, and K. K. Midha. *Pharm. Res.* **12**:1865–1868 (1995).
54. R. P. Basson, B. J. Cerimele, K. A. DeSante, and D. C. Howey. *Pharm. Res.* **13**:324–328 (1996).
55. T. N. Tozer, F. Y. Bois, W. W. Hauck, M. L. Chen, and R. L. Williams. *Pharm. Res.* **13**:453–456 (1996).
56. R. G. Buice, V. S. Subramanian, K. L. Duchin, and S. Uko-Nne. *Pharm. Res.* **13**: 1109–1115 (1996).
57. H. S. Pentikis, J. D. Henderson, N. L. Tran, and T. M. Ludden. *Pharm. Res.* **13**: 1116–1121 (1996).
58. R. Schall and R. Williams. *J. Pharmacokin. Biopharm.* **24**:133–149 (1996).
59. L. Z. Benet and J. E. Goyan. *Pharmacotherapy* **15**:433–440 (1995).