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Safety aspects in the quality control of recombinant products from mammalian cell culture*

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Abstract: The recent approval of the recombinant DNA-derived biological, human tissue-type plasminogen activator (rt-PA), obtained from large scale mammalian cell culture, addressed a number of issues concerning the safety of recombinant products. The assurance of the safety of such highly complex proteins requires that the following topics be investigated: the characterisation of the recombinant production organism; control of large scale cell culture production conditions; design of the purification process and consistency of manufacture; the purity, safety, and stability testing of the final product; and clinical studies.

Each of the topics described above is discussed with respect to those key quality control issues that ensure the safety of the product. The use of analytical techniques such as peptide mapping and multiantigen ELISA assays to guarantee both the genetic stability and the purity of the final product is also discussed.

Keywords: *Recombinant product safety; biotechnology quality control; mammalian cell culture safety.*

Introduction

The quality control of recombinant DNA products offers the unique opportunity to blend the traditional techniques developed by the serum and tissue-derived pharmaceutical industry with modern developments in analytical protein chemistry and molecular biology. The recent approval by worldwide regulatory agencies of recombinant human tissue-type plasminogen activator (rt-PA) for the treatment of acute myocardial infarction required that a number of significant questions be addressed concerning the safety of such products manufactured in large scale mammalian cell culture. It is important to recognise that the safety of these products does not rest on the results of any one particular test, but depends on a carefully designed manufacturing, quality control and clinical evaluation system, which in its totality ensures the safety and consistency of the final product.

The design of such a system is based on five basic principles: (1) the characterisation of the recombinant cell substrate used for production; (2) the control of large scale cell

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culture production conditions; (3) the design of the purification process and consistency of manufacture; (4) the purity, potency, safety and stability of the final product; and (5) the results of supporting clinical studies in humans. These topics involve specific areas of safety and are discussed in detail in the following sections.

The characterisation of the recombinant cell substrate used for production

Safety concerns about the use of continuous cell lines as substrates for the production of biologicals have been examined in great detail with the conclusion that continuous cell lines may be acceptable even if they exhibit abnormal karyology, infinite life, or other manifestations associated with tumorigenicity [1, 2]. Proof of the safety of products derived from such cell substrates resides with the rigorous testing of the final products for evidence of detectable antigenic impurities, adventitious agents and their products (i.e. endotoxin), and residual DNA. These cell substrates, however, must be thoroughly characterised by the manufacturer with attention to the following: (1) the origin and history of the plasmid and its transfection into the host cell; (2) methods and materials used by the manufacturer in the maintenance, propagation, and storage of the cell lines; (3) growth rate and morphological features during cultivation by the manufacturer; (4) determination of cell markers relevant to final product purity and useful for identification of the cell line; (5) demonstration that the manufacturing working cell bank is free of adventitious agents and other products; (6) karyology; and (7) results of tumorigenicity studies. Data obtained from all these studies are required by regulatory agencies to demonstrate that a given cell bank is acceptable for producing a biological product [1].

A major concern in the production of biologicals from cell culture is the presence of endogenous viruses, principally retroviruses, which are encoded by all vertebrate genomes [3]. These viruses may, in some cases, be expressed spontaneously as observable virions with detectable biological and biochemical properties. In other cases, nothing is observable that is consistent with a functional retrovirus. For example, Chinese Hamster Ovary (CHO) cells are known to contain retrovirus-like particles, although no retroviral activities have been detected in these cells despite extensive study, thus indicating either a defective virus or a non-viral phenomenon [3].

Concerns over the tumorigenic attributes of continuous cell lines revolve about the potential for the cell substrate to confer such a tumorigenic potential to the final product [2]. At the present time, there are four hypothetical mechanisms by which tumorigenicity could be transferred from abnormal cells: (1) the presence of live substrate cells; (2) residual cellular protein; (3) residual cellular DNA; and (4) endogenous retroviruses. The manufacturing process used for the production of rt-PA from CHO cells, for example, was specifically designed to address each of these issues in a readily validatable manner and is discussed in a later section.

The control of large scale cell culture production conditions

Even before production begins, the quality of the raw materials is the first step that must be ensured. Many raw materials are tested according to defined United States Pharmacopeia (USP) or American Chemical Society (ACS) methods. However, the emergence of recombinant DNA technology has led to the development of new assay methods for several biochemical raw materials. Included in these methods is viral and

mycoplasmal screening of serum products as well as laboratory scale performance testing of many raw materials to ensure that product yield and recovery are consistent. The recombinant organism itself is an example of a key raw material.

Following the required use of a master cell bank that has been shown to be free of adventitious agents, the next important safety factor is the large scale production system itself. At the time of harvest, the manufacturer is required to ensure freedom of the cells from contamination by bacteria, fungi, yeasts, mycoplasmas, and exogenous viruses [4]. Fortunately, such contaminations are readily apparent based on their negative effects on typical operating parameters such as growth rate and cell viability. Well established microbiological methods exist to detect even low levels of bacterial or mycoplasmal contamination and are employed on a lot-by-lot basis. Viral screening assays have been developed that are specifically designed to detect the presence of human viruses that propagate in CHO cells. These assays are performed on samples taken just prior to harvest.

Design of the purification process and consistency of manufacture

The safety and efficacy of a biological drug product are demonstrated during clinical trials. Consistency of manufacture helps to ensure the product's continued safety and efficacy in humans and may even be more important than the absolute purity of the product. This reliance on the consistency of manufacturing establishes the dependence of the biological on the process by which it is manufactured. The manufacturing process developed includes defined purification steps targeted at removal of impurities. Some of these impurities are non-product related and possibly derived from the fermentation or cell culture processes, while other impurities may arise from the degradation of the biological itself.

A generalised flowchart of the manufacturing process used for recombinant DNA-derived pharmaceutical products such as rt-PA is presented in Fig. 1. In this figure, key places for in-process testing and process validations are shown.

In the manufacturing schematic presented, the cell culture process is terminated typically after a finite number of passages, the cells are harvested and removed, and the biological drug product is purified and recovered. Generally, chromatographic purification by ion exchange, size/shape separations, hydrophobic interactions, affinity chromatography or monoclonal antibodies are used. The actual purification scheme depends on the chemical properties of the drug substance itself. Assays are performed during the manufacturing process to monitor the effectiveness of the process in removing DNA, and to measure endotoxin levels, protein concentration, and product potency. One of the most common methods used to determine endotoxin in the drug product is the Limulus Amebocyte Lysate (LAL) assay. This extremely sensitive and rapid test ensures that the safety requirements of the final product are met.

It is important to design in-process tests in such a way that they are simple, fast assays that will give accurate measurements of the quality of the process. Methods that monitor the potency of the final product are essential for process control and reproducibility. However, in addition to process monitoring using analytical methods, process validation studies must be performed to validate the removal of key impurities of concern such as whole cells, DNA, or antibiotics. Steps in the process must be added and validated whose purpose is to remove putative exogenous viruses that might contaminate the product.

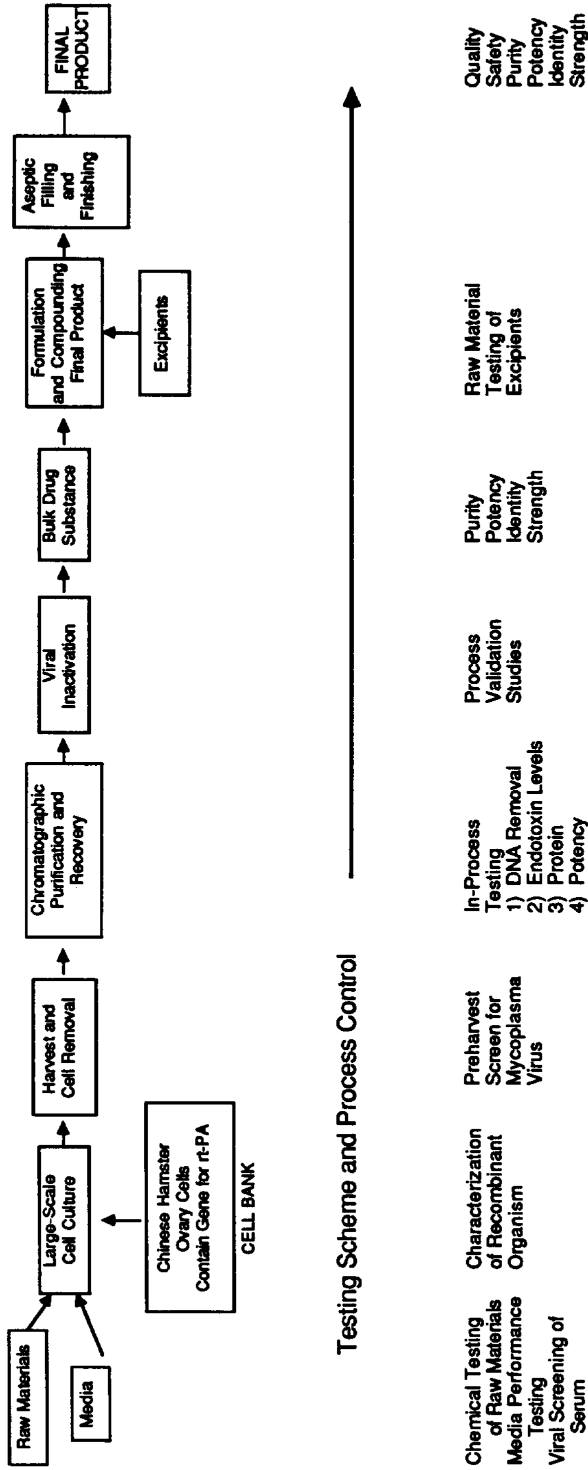


Figure 1
Typical production scheme for the manufacture of recombinant DNA-derived pharmaceutical products.

Purity, potency, safety and stability testing of the final product

Purity

The purity of a biological product is defined as the measurement of the active drug substance in relation to the total substances (not including additives) present in the final product [5]. Impurities are defined as all process related non-adventitious substances present that are not considered to be the active material, additives, or excipients. Examples of common impurities in recombinant DNA-derived biologicals are presented in Table 1 together with a list of the analytical methods typically used for their determination. Contaminants include all adventitious chemical substances or micro-organisms present in raw materials, bulk drugs, or final products. A list of potential contaminants in biologicals is presented in Table 2. Because recombinant DNA products are derived from living organisms, any host cell or host cell component is an impurity. Other substances present during the growth of the cells, associated with the fermentation processes, or used in the purification of the product are also potential impurities. The development of recombinant DNA manufacturing technology in parallel with the development of sophisticated preparatory, analytical, and immunological methods has enabled the detection of impurities and contaminants of concern in these products.

There is no one single method that can address the purity of a biological product. Examples of analytical methods commonly used for detecting the purity of biologicals are presented in Table 3. These various assays are used in combination to give a more complete picture of the purity and consistency of the final product. For example, the use of sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) in tandem with multiantigen ELISAs and high-performance liquid chromatography (HPLC) methods can measure host cell protein impurities at the ppm level. Other impurities may be controlled through process validations (see Fig. 2). Another valuable assay for monitoring product purity and genetic stability is the technique of peptide mapping.

Table 1
Common impurities of rDNA-derived biologicals

Impurities	Detection method
Endotoxin	LAL,* rabbit pyrogen
Host cell proteins	SDS-PAGE,† immunoassays
Other protein impurities (media)	SDS-PAGE, HPLC,‡ immunoassays
DNA	DNA hybridisation, UV spectrophotometry
Protein mutants	HPLC-tryptic mapping
Aggregates	HPLC-SEC,§ light scattering
Oxidised methionines	Amino acid analysis, HPLC-tryptic mapping, Edman degradation analysis
Proteolytic clips	IEF,‖ SDS-PAGE (reduced), HPLC, Edman degradation analysis
Deamidation	IEF (standard comparison), HPLC
Monoclonal antibodies	SDS-PAGE, immunoassays
Amino acid substitutions	Amino acid analysis, HPLC-tryptic mapping
Viruses (endogenous)	CPE,** HAd,†† electron microscopy, reverse transcriptase activity

*Limulus amebocyte lysate.
†Sodium dodecyl sulphate polyacrylamide gel electrophoresis.
‡High-performance liquid chromatography.
§High-performance liquid chromatography–size exclusion chromatography.
‖Isoelectric focusing.
**Cytopathic effect.
††Haemadsorption.

Table 2
Potential contaminants in biologicals derived from cell substrates and/or tissue and serum sources

Source	Type
Tissue or serum	Proteins Live virus and viral proteins Live microbes and microbial proteins
Cell culture	Media components Live virus and viral proteins Antigenic cellular proteins Bioactive cellular proteins Endotoxin Cytokines
Tissue culture media	Heavy metals Endotoxin Live microbes and microbial proteins Small organic compounds
Recovery process	Stabilisers Filter components Detergents Leachable column components Monoclonal antibodies

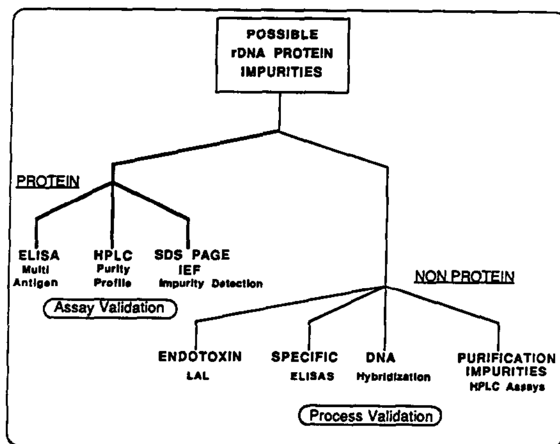


Figure 2
Primary tools for assessing the purity of recombinant proteins.

Because trypsin cleaves proteins selectively at lysine and arginine residues, specific peptidyl fragments of the protein are generated that can be separated by reversed-phase HPLC. Digestion of the protein with trypsin followed by separation of the peptide fragments results in peptide maps, which are one of the most sensitive methods used for monitoring small changes in protein structure. These peptide maps resemble an infrared spectrum of an organic compound and are used in a similar way to "fingerprint" the protein. For example, a single amino acid change at position 275 in rt-PA (see Fig. 3)

Table 3
Advantages and disadvantages of analytical methods used for determining the purity of biologicals

Method	Sensitivity limit	Advantages	Disadvantages
Amino acid analysis	10,000–100,000 ppm (1–10%)	Reliable Valuable for small peptides Amino acid substitutions can be detected	Low sensitivity Provides little information Not very valuable for large proteins Very labour-intensive
HPLC — Purity profile		Fast Reproducible	Low sensitivity at 214 and 280 nm
Reversed-phase Ion-exchange	1000–5000 ppm (0.1–0.5%)	Computer compatible Very high resolution technique	Often not applicable to broad-spectrum purity profiles
SDS-PAGE		Reliable Tremendous amount of information based on molecular weight	Denaturing assay system Resolution limit ~500 DA Very labour-intensive Artifacts in sample preparation
Reduced/non-reduced Coomassie Brilliant Blue stained	5000–10,000 ppm	Quantitative assay	Judgments requiring a high degree of experience necessary
Reduced/non-reduced silver stained	50 ppm	Broad-spectrum technique Can detect single proteins at the 100 to 500 ppm level Semiquantitative	
Isoelectric focusing		Reliable Quantitative	Resolution limit ~0.1 pH units Difficulty in interpretation of results
Coomassie Brilliant Blue stained	~5000–10,000 ppm	Tremendous amount of information Native system	
Silver stained	~50 ppm		
Multiantigen ELISA	0–1 ppm	Reliable Extremely sensitive Reasonable precision (10–20%) Works well in conjunction with HPLC, SDS-PAGE, and IEF	Process specific Critical reagents required: Coat antibodies HRP-conjugates Standard solution Requires revalidation whenever a reagent is changed

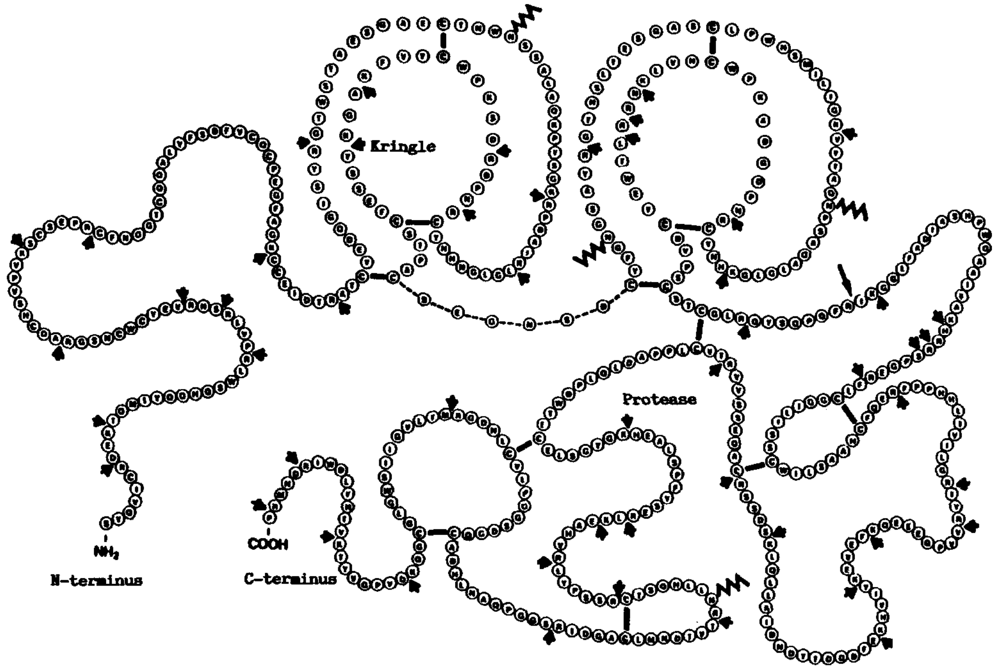


Figure 3
Primary structure of rt-PA. Arrows indicate tryptic cleavage sites.

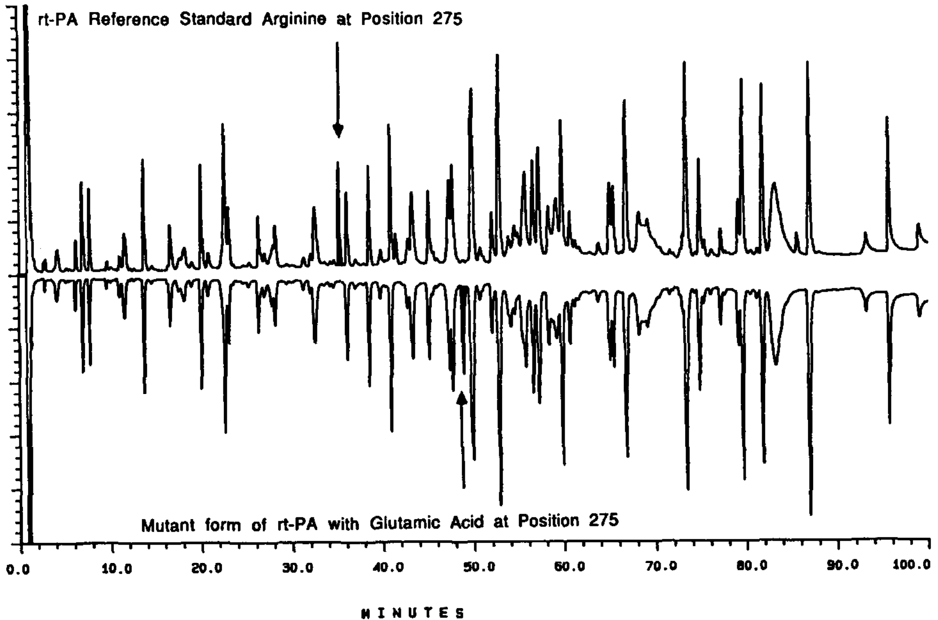


Figure 4
Chromatograms of the tryptic maps of a mutant form of rt-PA with a glutamic acid residue in place of the normal arginine residue at position 275 and the rt-PA reference standard. Arrows illustrate the differences in the two chromatograms caused by the substitution.

results in the chromatogram presented in Fig. 4. This method is invaluable in demonstrating the genetic stability of a biological on a lot-to-lot basis.

The analysis of endotoxin has been a critical issue in the manufacture of biological products. Endotoxin is considered to be a marker of bacterial contamination and itself can produce severe febrile responses in humans. As previously described, endotoxin can be assayed by the LAL assay, by the USP Rabbit Pyrogen Test, or by the endogenous pyrogen assay [6]. Another nonprotein impurity is host cell DNA, which is usually analysed by the technique of DNA hybridisation or dot blot analysis [7]. This method can typically detect nanogram to picogram levels of host cell DNA. However, this method is lengthy, labour intensive, and quite difficult to perform. No other techniques with the required sensitivity exist at this time. This is clearly an area where new analytical technology needs to be developed.

Potency

The potency of recombinant DNA-derived products is preferably measured in an assay that mimics the action of the drug in humans. For example, the biochemical assay used for determining the potency of rt-PA is presented in Fig. 5. This assay was designed specifically to mimic the action of rt-PA when administered during a myocardial infarction and is based on the production of a synthetic fibrin clot from the action of thrombin on fibrinogen. The mechanism of this assay is presented in Figs 6 and 7.

Purpose

To develop an *in vitro* assay that mimics as closely as possible the action of rt-PA in the lysis of a blood clot causing a myocardial infarct.

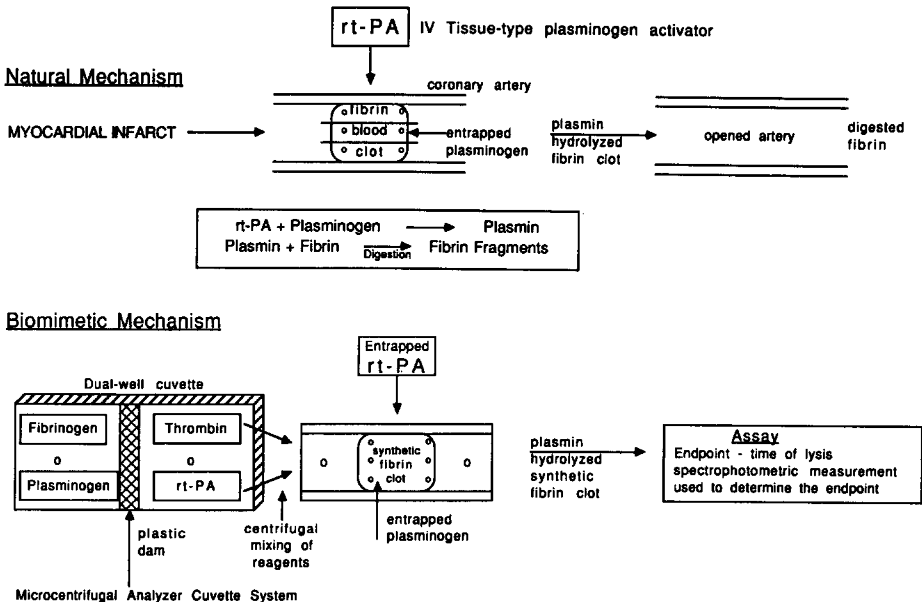
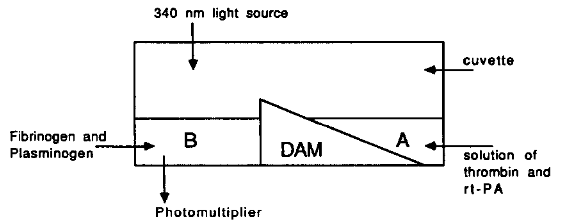


Figure 5
Biomimetic–*in vitro* assay for the activity of rt-PA.



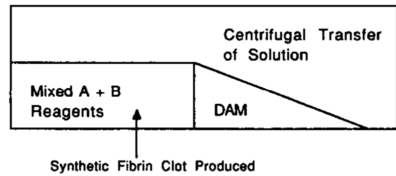
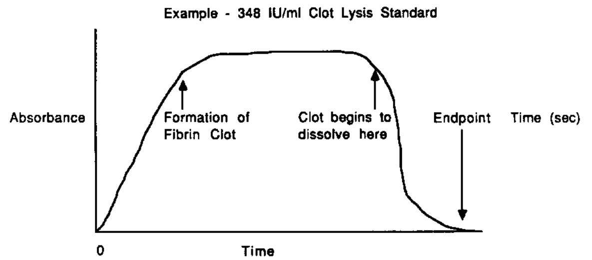


Figure 6
Mechanics of the *in vitro* clot lysis assay.



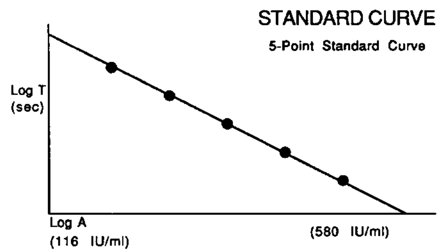


Figure 7
Analysis of the data from the *in vitro* clot lysis assay.

Plasminogen is also mechanically trapped within the synthetic clot just as it would be during an actual myocardial infarct. The addition of rt-PA results in the conversion of plasminogen to plasmin, which rapidly dissolves the synthetic fibrin clot. Automation of this assay using a microcentrifugal analyser and a spectrophotometrically determined endpoint has resulted in a highly accurate and precise method for assessing the potency of rt-PA [8].

Safety

The safety of the final product may be assessed in several ways. As stated earlier, the absence of endotoxin contamination is monitored throughout production using the LAL assay and is verified in the final product using the USP Rabbit Pyrogen Test. General safety testing in mice and guinea pigs is routinely performed per USP requirements prior to product release. Finally, the sterility of the drug is confirmed in the bulk drug substance and in the final product.

Stability

The stability of biologicals traditionally has been determined by the measurement of the potency or activity of the product in a whole animal bioassay. It has been extremely difficult to measure and/or identify the degradation products of most biologicals because of their complexity and relative purity levels. However, the modes of protein degradation are reasonably well understood and include denaturation, proteolysis, aggregation, dimerisation, oxidation, chemical reduction by excipients, deamidation, and photolysis. For a complex biological, a variety of analytical methods must be employed to screen for evidence of these degradation modes. Thus, a number of methods such as SDS-PAGE, Isoelectric Focusing (IEF), peptide sequencing, and potency or activity procedures can be used to evaluate the stability of a product.

Clinical studies in humans

The safety of a recombinant biological must first be assessed via suitable animal toxicity testing, followed by safety and efficacy studies in humans. Results of clinical studies with rt-PA demonstrated efficacy as measured by infarct artery patency in up to 85% of patients studied (N = 85) [9, 10]. In a double-blind randomised trial (N = 138) comparing rt-PA to a placebo, patients experienced improvement in left ventricular function after rt-PA administration [10]. The most common side effect of rt-PA therapy is bleeding. Because rt-PA is highly specific in its lysis of fibrin blood clots, its action can be expected wherever blood clots may be found in the body.

It is estimated that since its approval and introduction for the treatment of myocardial infarction, rt-PA has been used in literally thousands of people. Its efficacy and safety have been thoroughly confirmed and its benefits to humans have only been estimated to date.

Conclusion

The safety of recombinant DNA products manufactured in mammalian cell culture is ensured by a thoroughly characterised and documented manufacturing and control system. State-of-the-art analytical methods are employed wherever necessary to confirm the consistency of manufacture. Each aspect of manufacturing, from incoming raw materials to the final product, relies on a system of checks and balances to ensure that

potential errors are avoided. Process validation studies ensure that the process functions as designed. Quality control analytical testing is performed on a lot-by-lot basis and the stability of the product is monitored. Finally, clinical studies in humans demonstrate the safety and efficacy of the product.

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