Assignment of quantities to biological medicines: an old problem re-discovered

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A distinction exists between ‘chemical’ and ‘biological’ medicines. While, from antiquity, both organic and inorganic substances had been used in therapy, developing chemical sciences were inapplicable to materials extracted from natural sources, and the active principles could be neither identified nor characterized. The distinction between biological medicines or ‘biologics’ grew out of this realization. Such ‘biologics’ in clinical use were, however, variable in efficacy and in safety, and controlling the strength or quality was necessary. Without information on what biological medicines are, it was necessary to quantify what they do, and such medicines were quantified using systems based on biological responses (bioassays) in animals, organs or cells. Bioassays are defined in terms of an external standard rather than in absolute terms, and depend on a number of key assumptions: the need to assay ‘like against like’, the desirability of making the assay principle relevant to the intended clinical effect in man, and the importance of appropriate statistical models of design and analysis. The science of ‘biological standardization’ has kept pace with developments in medicine and continues to allow the use of biological medicines in man to be controlled on the basis of common measurement systems.

Keywords: constants; measurements; metrology

1. Introduction

This paper is the text of a presentation given at the Royal Society during the meeting addressing the subject of the new International System of Units, and the tracing of measurement units to basic physical constants. Although the subject matter of the talk is not related directly to the main topic of the meeting, the intention of the presentation was to draw attention to another domain of metrology in which the quantities and units and the way they are defined and measured are quite different. This paper is intended only as an introduction to the subject for readers whose predominant interest is in the physical sciences. Readers seeking further information on biological medicines should consult a number of publications exploring different aspects of the topic, including Thorpe & Wadhwa [1,2], Hockley et al. [3], Jodar et al. [4], Lin et al. [5] and Bristow et al. [6].

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One contribution of 15 to a Discussion Meeting Issue ‘The new SI based on fundamental constants’.
Many areas of medical science depend critically on the need to measure and report quantities and magnitudes accurately and reproducibly. Chemical substances are measured in two main areas: therapeutic or prophylactic medicinal substances, administered for the treatment or prevention of disease, and diagnostic analytes, measured to diagnose or monitor disease states or treatment efficacies. In these medicinal contexts, measurement of quantities is clearly highly significant: making the difference between lack of efficacy, effective therapy and over-dose in therapy, and the difference between mis-diagnosis and accurate diagnosis in diagnostic medicine. Indeed, the consequences of inaccurate measurement in both areas of medicine can range from minor to fatal.

It is less well recognized, but equally true, that the quantity measured should be relevant. For example, on 13 March 2006, six healthy male volunteers were given low doses of TGN1412, an experimental monoclonal antibody that was under clinical trial as a potential immune-activating agent, through agonistic (stimulatory) interaction with the CD28 T-cell receptor. All developed rapid life-threatening adverse reactions that led to multiple organ dysfunction [7]. This was later confirmed to arise from a toxic ‘cytokine storm’. Owing to rapid intervention, the humans all survived, but at least some will have persistent, irreversible sequelae. The dose (mg kg\(^{-1}\)) used was 500 times lower than the dose that had been safe in primates. In one sense, this was not an adverse drug reaction. TGN1412 is a super-agonist for the CD28 T-cell receptor, with the intended action of stimulating release of T-cell cytokines. It could be argued that, qualitatively, the response was not unexpected. What was unexpected was the magnitude of the response. The clinical trial lacked any reference framework for relating the quantity used to express the dose (mg) to the magnitude of the response in humans. Clearly, in this situation, the mass of the substance administered did not provide a useful indicator of its effect, as the magnitude of the effect in primates was not a useful indicator of the magnitude of the effect in humans.

In therapy, however, most drugs are dosed in units (mg) of the International System of Units (SI). A significant minority, the ‘biologics’, are measured and dosed in arbitrary units. In measurements for diagnostic purposes, the situation is somewhat reversed. Several hundred measurands are measured in the diagnosis of disease, and approximately 20 per cent are measured in units of the SI (mg or mol). The majority are ‘biologics’, and are not traceable to the SI.

2. Biological medicines: their origin and definition

Kambaskovic [8] described two diabetics with remarkably long life span. Hazel Davies, who had lived for 80 years on insulin therapy after being diagnosed in 1921, and Roy Cross, who lived to be greater than 100 after being diagnosed in 1938. The well-established need for a good long-term control of blood glucose levels suggests that insulin was a well-controlled drug. Paradoxically, however, insulin available in the early decades of the twentieth century was extremely impure, and determination of content/potency using modern analytical methods would have been quite impossible. Moreover, its structure was unknown until the 1950s, when Sanger elucidated it in his Nobel prize winning protein structure.
studies. In the absence of any meaningful knowledge of the structure or analytical methods to measure the content, the dose of insulin to be administered was determined by quantitative measurement of its biological activity. The insulin mouse convulsion test illustrates this principle. Injecting fasting mice with insulin will cause some to go into hypoglycaemic convulsions, where the number of convulsing mice is related to the amount of insulin injected.

A typical approach is illustrated in figure 1. To carry out an insulin assay, fasting mice are allocated to four groups of 24 animals. Each group receives either a high or a low dose of a standard, or a test preparation, with the administered doses being estimated to elicit hypoglycaemic convulsions in most of the animals receiving the high doses, and only a few of the animals receiving the low doses. The number of animals convulsing can then be used to calculate potency, as described in figure 1.

Thus, activity estimates approaching the necessary level of precision could be obtained by using the response in a relatively simple biological assay system, combined with the application of appropriate methods of bio-statistical analysis.

Measurement of the biological activity of insulin exemplifies all aspects of a ‘biological’ as the term is used in medicine:

— the medicinal substance was isolated from a biological source (extracts of animal pancreata—bovine, porcine),

— accurate quantification of the active principle was achieved by quantifying its activity in a test measuring function rather than quantity of substance, i.e. what it does, rather than what it is, and

— quantification is dependent on the science of biological standardization.

(i) the unit of insulin can only be described in terms of a reference material, not in terms of an absolute response (e.g. number of convulsions), and

(ii) statistical combination of independent assays can produce precise numbers from an inherently imprecise and irreproducible method.
The science of biological standardization is actually divided into three disciplines, developed in parallel, largely at the Medical Research Council from the 1920s onwards:

— development of quantitative biological assay systems, in animals, organs, cells and sub-cellular fractions,
— the development of statistical methods to analyse such assay systems, and
— the development of procedures to prepare, store and test reference materials, solving problems of scale, retention of activity, stability and homogeneity.

The successful introduction of insulin and other macromolecules into therapeutic use led to a formalization of the status of a biological medicine, which has been defined by the World Health Organization as a medicinal substance ‘...of biological origin, which cannot be characterized adequately by chemical and/or physical means alone...’ [9].

Standardization of biological activity, however, remains a complex field, in which it has been frustratingly difficult to draw up a single, simple set of approaches. Although the need to measure ‘like against like’ has been frequently cited and adopted as the overarching principle, in practice, this is frequently complicated by the nature of biological medicines and their biological test systems. For example, heparin—a glycosaminoglycan (and a true ‘biological’) — has two distinct biological activities: anti-factor Xa activity and anti-factor IIa activity. Different heparins have these activities in different ratios, and will therefore behave differently depending on which activity forms the basis of the assay system. Such complexity is the rule rather than the exception in biologicals, and this paper will later consider examples of biological medicines where in vivo and in vitro biological activity, or activity and receptor binding can be seen to be essentially different activities.

Indeed, comparison of the already cited examples insulin and TGN1412 antibody further illustrates potential weaknesses of the biological activity-based approach. In the insulin case, the structural homologies of insulin and its receptor, and the physiology of the insulin response, are sufficiently similar between the two species, such that the effects of human insulin in mice are sufficiently similar to effects in human to allow accurate quantification of the dose. This is clearly not the case in TGN1412, where the effects of the drug in species as closely related as primates and human are quite different. Although it is rare for such gross species differences to the same drug to be observed, it is also well recognized that measurement of biological activity in animals, while being a useful quantitative analytical tool, cannot be assumed to parallel clinical effectiveness in human.

In summary, notwithstanding, the obvious long-term successes in permitting therapies based on the measurement of biological activity, the approach makes assumptions about the test and the standard, their behaviour in the assay systems being used, and the relationship between biological test systems and clinical effect that have to be understood, which limit the information that can be obtained, and have driven the development of alternative analytical approaches to supplement the biological approach, and where possible, replace it.
Table 1. Biological medicines (therapeutic and diagnostic).

<table>
<thead>
<tr>
<th>class of medicines</th>
<th>examples</th>
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<tbody>
<tr>
<td>clotting factors</td>
<td>blood coagulation factor VIII</td>
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<tr>
<td>thrombolytics</td>
<td>tissue plasminogen activator</td>
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<tr>
<td>hormones</td>
<td>follicle-stimulating hormone</td>
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<td>cytokines and growth factors</td>
<td>interferon</td>
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<td>enzymes</td>
<td>thrombin</td>
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<td>vaccines</td>
<td>whole cell Pertussis vaccine</td>
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<td>microbiological antigens</td>
<td>hepatitis B antigen</td>
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<tr>
<td>toxins</td>
<td>Botulinum toxin</td>
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<tr>
<td>anti-sera and immunoglobulins</td>
<td>anti-HPV-16 Ab</td>
</tr>
<tr>
<td>genomic DNA, cDNA and RNA</td>
<td>human immunodeficiency virus</td>
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<td>clade 1 genetic reference panel</td>
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3. The current biological portfolio

At the present time, a wide range of medicinal substances based on human blood, biotechnology, cellular or microbiological sciences is considered to be ‘biological’ (table 1).

The unit of measurement remains based on an appropriate biological response. Clotting factors and thrombolytics are measured in terms of their ability to promote or reverse blood coagulation, respectively, interferon by its actions on the growth of cells in culture, vaccines in systems measuring the protection of test animals against microbiological challenge and toxins in whole animal lethality tests.

At the present time, for the purposes of regulation, in most areas of the world, medicines are considered in two distinct categories, chemical drugs, where the dose or clinical effect can be related to quantities that are expressed in SI units, and biological drugs, where the dose or clinical effect can only be related to the response in a complex measurement system, such as an animal or a living cell, and can be related only to the effect produced by a reference material (bioassay).

4. Does the distinction between chemical and biological drugs remain valid?

The definition of a biological set out above is a practical definition, and relates not only to the nature of the substance, but also to the utility of physico-chemical analytical methods being employed. As would be expected, there has been a trend for the cut-off to increase in molecular size and complexity. Antibiotics, for example, from their discovery in the 1960s, would have been regarded as biologicals, and indeed, were dosed on the basis of microbiological inhibition assays. They are now SI-traceable chemical drugs, as indeed are many proteins, up to and including, for example, human growth hormone at molecular mass of 22,000. Despite this trend, the majority of macromolecules used in therapy are still considered to be biologicals, indeed, regulatory authorities are inherently
reluctant to relax the dependence on biological assay, and progress in removing biologicals from the list remains frustratingly slow. More significantly, many of the developments offering promise for significant medical breakthroughs in the near future, such as engineered antibodies, stem cells, gene therapy and targeted drugs would be expected to be classed as biologicals for the foreseeable future. In short, we are gaining biologicals much faster than we are losing them.

Despite the clear advances in physico-chemical analytical technology that is applicable to small- to medium-size proteins, predominantly in the areas of mass spectroscopy and high-performance liquid chromatography, there remain some fundamental difficulties to be overcome in complete characterization of macromolecules.

5. Structural complexity

Although insulin (51 amino acids, molecular mass of 5800) and human growth hormone (191 amino acids, molecular mass of 22000) are now regarded as chemical drugs, dose in milligrams, and approved for use in human without the need for any form of bioassay, these are, in fact, rather small, simple protein structures. Therapeutic proteins can be at least an order of magnitude larger and more complex. Clotting factor VIII, for example, used in the treatment of haemophilia and purified either from blood or recombinant DNA expression systems, has a primary protein backbone of 2361 amino acids with a molecular mass of 268000. It is also further processed by glycosylation and intra-chain cleavage. Conventional approaches to resolving the complexity of protein structure such as tryptic mapping, where the protein is enzymatically cleaved into reproducible and predictable fragments, are rendered inapplicable, simply as a result of the complexity of the mixture. Growth hormone, for example, produces some 20 peptides on tryptic fragmentation, mostly between 3 and 20 amino acids in length. Factor VIII produces nearly 200, a mixture whose complexity is essentially unresolvable in a one-dimensional fractionation. Many of the most therapeutically promising proteins are being developed, for instance, monoclonal antibodies (molecular mass of 150000) are of a similar order of complexity.

6. Heterogeneity

The second seemingly intractable problem associated with the physico-chemical characterization of macromolecules is their tendency to be inherently heterogeneous. There are a number of underlying reasons for this. In many cases, it is simple because they are intended to be heterogeneous, are complex mixtures of microbial antigens intended to mimic infection with the pathogenic organism, and to elicit a broad spectrum and protective immune response. In other cases, the heterogeneity is associated with the nature of biosynthesis.

The most widely quoted and studied source of molecular heterogeneity in biological medicines is glycosylation. Unlike protein synthesis, glycosylation is a non-template-dependent process, in which the attachment of carbohydrate moieties to specific sites on a protein can produce a range of molecular structures. N-linked glycosylation of erythropoietin, for example, exists in bi-,
tri- and tetra-antennary structures, and up to six main isoforms based on charge heterogeneity resulting from variations in the number of terminal sialic acid residues (figure 2).

When working with products exhibiting a range of molecular structures, value assignment and traceability to the SI are extremely problematic. Any attempt to express the quantity of material in milligrams, even if it were technically possible, is essentially meaningless. The different glycoforms present have different molecular weights, and describing the glycoproteins present in milligrams would not reflect the number of molecules of the substance present. One solution that has been adopted is to measure and describe only the protein backbone, which, being template directed, is essentially homogeneous. Although this approach is technically possible, estimates of glycoprotein content based on measurement of protein backbone frequently do correlate with estimates of potency. Figure 3b, for instance, shows that for a range of erythropoietin products, biological activity in vivo is highly dependent on glycosylation, suggesting that the amount of protein to express the quantity of the drug would be essentially irrelevant.

7. Activity measurement in the International System of Units

There is one particular field, enzymology, in which properties of biological substances have always been successfully expressed in SI units, notably the katal (kat) s$^{-1}$ mol. This difference, however, does not reflect an inconsistency in

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Figure 3. Therapeutic erythropoietin was produced using a purification process designed to selectively enrich specific glycosylation isoforms, producing products with a range of glycosylation properties, represented by the Z number, an analytical parameter reflecting increasing structural complexity of glycosylation. (a) Biological activity in vitro (proliferation of erythropoietin receptor expressing cell). (b) Biological activity in vivo (reticulocyte count in mice). Data from Yuen et al. [10].

The dependence of in vivo activity on glycosylation is widely recognized and usually explained in terms of half-life in vivo. Bioassays based on in vitro systems, such as cells in culture, usually depend on three limiting factors: the amount of substance, receptor-binding affinity and signal transduction to produce a
measurable response in the cell, with the receptor-binding event usually being activity limiting. In contrast, \textit{in vivo} activity depends on additional \textit{in vivo} dynamic factors, particularly the half-life, which is usually the activity-limiting factor \textit{in vivo}.

The dynamics of \textit{in vivo} clearance may, in addition, extend beyond the well-recognized role of glycosylation. For example, insulin analogues with up to 10-fold higher receptor affinity or with similarly reduced receptor affinity have been produced (e.g. B-10 Asp insulin). Although the receptor-binding activity of such analogues is reflected in increased \textit{in vitro} potency, it is not reflected in the \textit{in vivo} potency, which is usually similar to the parent molecule. Similarly, insulin analogues with reduced receptor binding do not show reduced activity \textit{in vivo}. Ribel \textit{et al.} \citep{11} explained this apparently paradoxical observation in terms of plasma clearance rates. The main physiological route of insulin clearance is receptor mediated. While increasing the affinity of insulin for its receptor has the effect of increasing its potency on target cells, it also increases its rate of clearance, thus bringing about a paradoxical reduction in activity \textit{in vivo}. Similarly, reducing the affinity of insulin for its receptor has the paradoxical effect of increasing the plasma half-life, counteracting the loss of activity at the target cell receptor-binding event.

In summary, despite the clear advances in analytical technology available to measure receptor-binding events or other molecular interactions, the recognition that biological actions can seldom be described in terms of simple two-component binding events, and in fact, such descriptions are often highly misleading, has prevented the widespread use of this type of approach in describing the potency of biological medicinal substances.

8. Summary

— There is a need for robust and accurate quantification in all areas of medicine, including biological medicines.
— For biological macromolecules, quantification has traditionally been based on quantitative biological methods, traceable to arbitrary units of biological activity defined by reference materials rather than to the SI.
— Recent decades have seen advances in the utility of physico-chemical analysis in the biologicals field such that many smaller proteins may now be regarded as chemicals and value assigned in SI units, a trend that is likely to continue.
— At the present time, physico-chemical analytical methods cannot completely address the structural complexities, and inherent heterogeneity of some therapeutic macromolecules and the ‘biologicals’ approach to quantification is still considered to be the most appropriate.
— The continuing trend of more powerful analytical methods and the increasing ability to relate biological activities to measurable biochemical events such as receptor binding offers the promise of reduction in dependence on biological systems for quantification.
— It is likely, however, that quantitative determination of biological activity will continue to be considered an essential requirement for many biological medicines, to satisfy both scientific and regulatory requirements.
References