Establishing International Units (IU) and the challenges of moving to the SI

Adrian Bristow
National Institute for Biological Standards and Control
UK

NIBSC: Major international centre for producing and distributing biological reference materials. WHO standardization laboratory

- Centre for Biological Reference Materials
  - >95% of WHO International Standards/Reference Reagents
  - >550 Catalogue items
  - 60,000 ampoules/vials distributed p.a. to >60 countries
  - New “state of the art” facility
    - Category 3 containment
    - Freeze drying development capability

- AIDS Reagent Repository: 1500 stock items
- CJD Resource Centre
- UK Stem Cell Bank

In the 20th century:

In the context of Biological Standardization, WHO has defined a biological substance as "a substance which cannot be completely characterized by physico-chemical means alone, and which therefore requires the use of some form of a bioassay". The underlying principle of such assays is that they depend on the comparison of the response of the test substance with that of a reference material, and since the 1920's the International Standards, currently supplied by WHO, have, in many cases served as the international biological reference materials for such procedures.

WHO Biological reference materials Established by NIBSC Number > 400, including:	**Analytical methods supported Include:**
Clotting factors Thrombolytics Hormones Cytokines and growth factors Enzymes Vaccines Micobiological antigens Toxins Antisera and immunoglobulins Genomic DNA, cDNA and RNA	In vivo bioassay In vitro bioassay Enzyme assays Receptor binding assays Function assays Microbiological assays Gene amplification methods

A great many WHO standards have diagnostic application, either by adoption or design

-Hormones
-Clotting factors
-Microbiological antigens
-Reference sera
-Nucleic acids
-Etc etc

WHO consultation on International Biological Standards
For *in vitro* diagnostic procedures

Geneva, Switzerland
14-15 September 2000

A collision of two worlds!

The clinical chemistry community (IFCC, ISO, national metrology Institutes, IRMM)	The WHO biological standardization crowd
ISO standards (34, 15194, 17511) Reference methods	Paradigm based on inability to characterise analytes
The “reference system”	No codified principles
Hierarchy of calibration Uncertainty	Lack of reference methods, and lack of belief in the principle
Traceability	No role for uncertainty, traceability or commutability
Commutability	
SI units	Distrust of SI units

The clinical chemistry community (IFCC, ISO, national metrology Institutes, IRMM)

The WHO biological standardization crowd

Went away thinking…..

“these guys need to learn some science”

The clinical chemistry community (IFCC, ISO, national metrology Institutes, IRMM)

Went away thinking…..

“these guys need to learn some science”

The WHO biological standardization crowd

Went away thinking…..

“these guys need to get out more”

However, over the next 4 years we realised:

1 Metrologists are not necessarily the anti-christ

2 We needed to put our house in order, and base the WHO approach on codified principles rather than established practices

3 Principles such as traceability, uncertainty and commutability cannot be discounted, and may bring added value

WORLD HEALTH ORGANIZATION
ORGANISATION MONDIALE DE LA SANTE

WHO/BS/04.1995

ENGLISH ONLY

RECOMMENDATIONS FOR THE PREPARATION, CHARACTERIZATION AND ESTABLISHMENT OF INTERNATIONAL AND OTHER BIOLOGICAL REFERENCE MATERIALS

(Revised 2004)

The report of the collaborative study should include……*(Inter alia)*

- A formal statement of the traceability path of the International Unit established by the proposed standard

- A consideration of the relationship of the Unit established by the proposed standard with previous units for the same material, including evaluation of the extent to which continuity of the IU has been maintained

- A formal consideration of uncertainty, including a statement of the uncertainty of content derived from the variance of the fill, and an evaluation of the requirements of uncertainty statements in the context of the traceability path

- An evaluation of the extent to which commutability has been demonstrated in the collaborative study.

In the establishment of the standard a variety of methods is usually used and the value assignment to the standard, and therefore the definition of the unit, is not necessarily dependant on a specific method of determination .

Generally, WHO reference standards are established for analytes where no reference measurement procedure ("reference method") has been agreed or established. In these cases the principle set out above will apply. Where a reference method has been defined and agreed, then establishment of the standard and value assignment may be specifically based on that method.

The definition of a medicinal substance, used in treatment, prevention or diagnosis, as a "biological" has been variously based on criteria related to its source, its amenability to characterization by physico-chemical means alone, the requirement for biological assays, or on arbitrary systems of classification applied by regulatory authorities. For the purposes of WHO, including the present document, the list of substances considered biologicals is derived from their earlier definition as "substances which cannot be fully characterized by physico-chemical means alone, and which therefore require the use of some form of bioassay". However, developments in the utility and applicability of physico-chemical analytical methods, improved quality control of biological and biotechnology-based production methods, and an increased applicability of chemical synthesis to larger molecules, have made it effectively impossible to base a definition of a biological on any single criterion related to methods of analysis, source or method of production. Establishment of WHO measurement standards for any substance or class of substances is therefore based on an evaluation of current analytical methodologies, and where biological, immunological or enzymological methods are employed, an evaluation of the need for global measurement standards for calibration of these methods

For example, many small proteins, such as cytokines and hormones, classed as "well-characterized", are now considered to be completely defined by physico-chemical methods. Nonetheless, the need for biological measurement standards may be dictated by the need to define the specific activity of new products, or by the on-going requirement to demonstrate specific activity of production batches. In the diagnostics field. the requirement for global measurement standards for otherwise well characterised proteins and other macromolecules is driven by the routine use of comparative assay procedures such as immunoassays and nucleic acid amplification tests, and by the absence of reference methods for the definition of the analyte in absolute terms in reference materials.

What's left to do?

1. **Buy-in rather than lip service**

2. **Establishing International Units (IU) and the challenges of moving to the SI**

Case 1: Follicle stimulating hormone

The International Standard for Pituitary FSH: collaborative study of the Standard and of four other purified human FSH preparations of differing molecular composition by bioassays, receptor assays and different immunoassay systems.

Storring PL, Gaines Das RE.

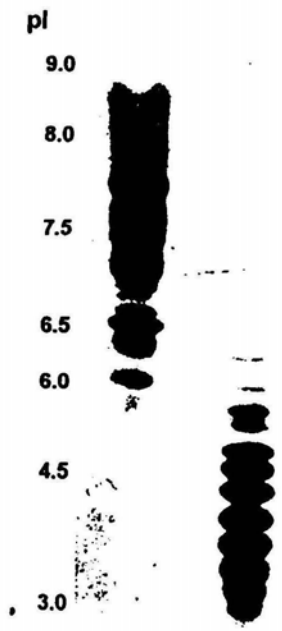
J Endocrinol. 1989 , Nov;123(2):275-93

National Institute for Biological Standards and Control, WHO International Laboratory for Biological Standards, South Mimms, Potters Bar, Hertfordshire

Follicle stimulating hormone

FSH is a two subunit glycoprotein, of approximately 30kD

It exhibits marked heterogeneity at the biochemical level, usually illustrated as isoelectric point variants due to differences in charged groups at the termini of the carbohydrate moieties. Different isoelectric point isoforms also exhibit microheterogeneity



Purified urinary, pituitary and rDNA preparations are available These preparations differ in their isoform distribution, and in the case of the rDNA material, in the nature of the carbohydrates

Carbohydrate structure is known to affect biological activity, and may affect immunological activity

There is evidence that the isoform distribution of the circulating hormone may differ with different physiological states

There is no real evidence of any diagnostic potential in being able to anaylse isoform distribution by immunoassay

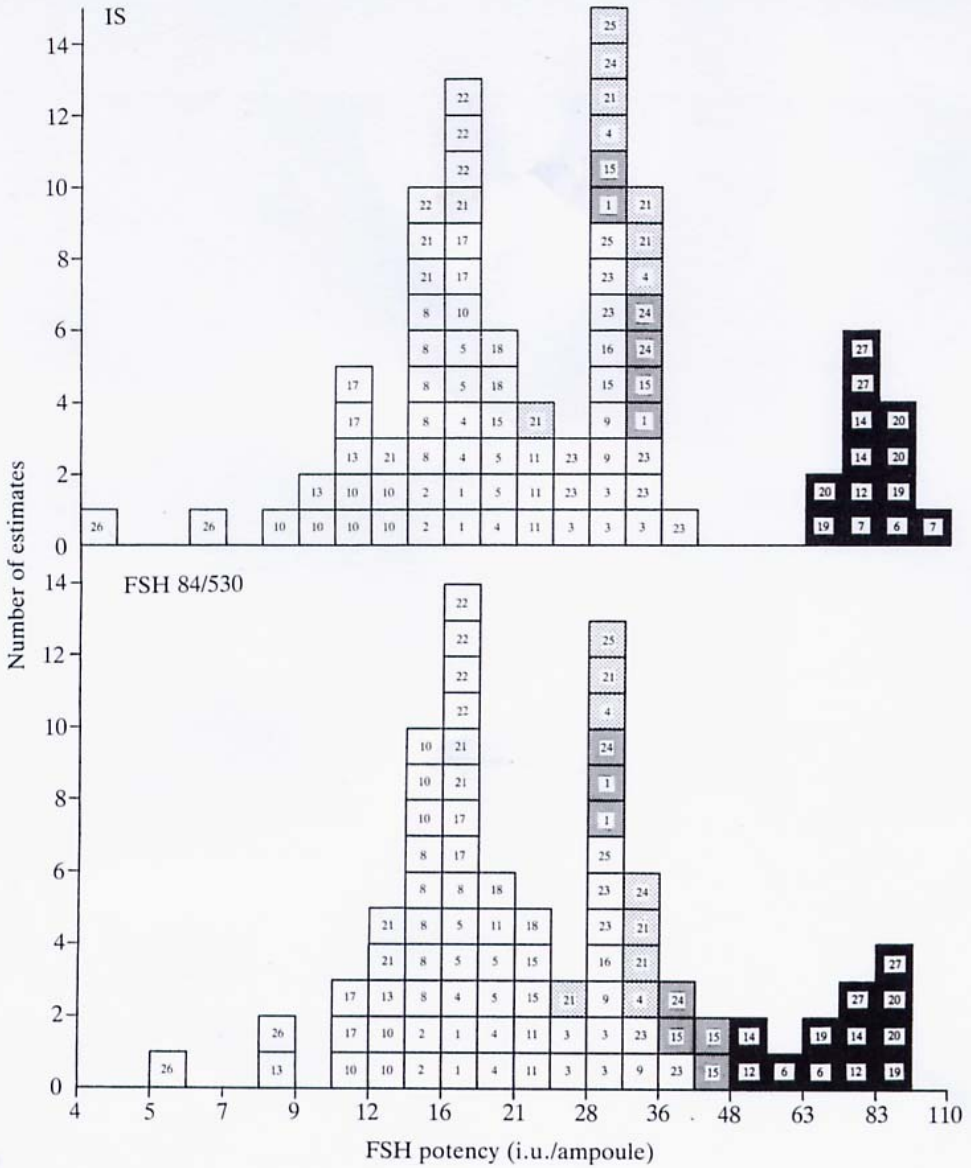


FIGURE 1. Frequency distribution of log potencies of the International Standard for Pituitary FSH (IS) and of FSH 84/530 in terms of IRP 78/549 by in-vivo bioassays (solid squares), in-vitro bioassays (heavily stippled squares), receptor assays (lightly stippled squares) and immunoassays (open squares) in different laboratories (identified by numbers).

1. In vivo bioassays (solid squares) fall into one group
2. In vitro/receptor assays (stippled squares) fall into another group
3. Immunoassays (open squares) fall into another group
4. Distribution within the immunoassay group is highly method dependent

FSH (1988) FSH standard (all assays)

- Heterogeneous estimates (between assay types and within immunoassay group)

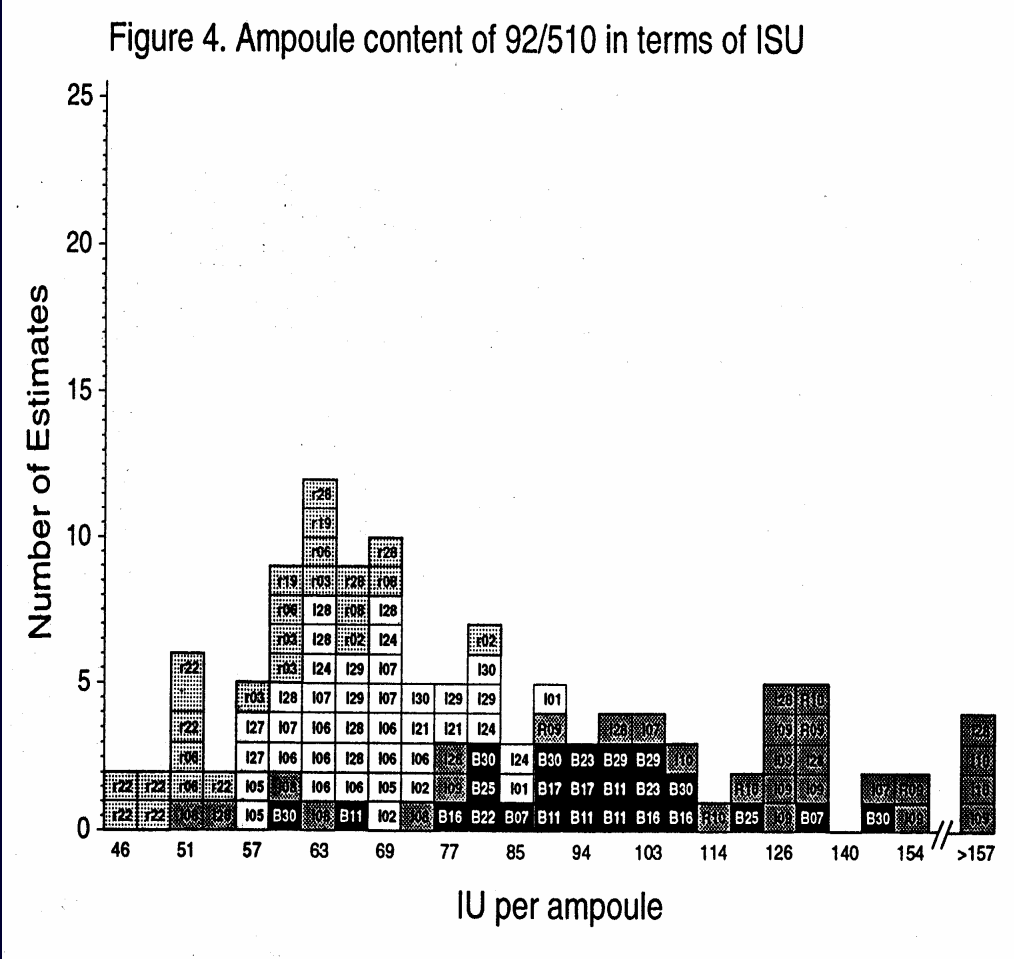
- Decision to define FSH in terms of one assay method (in vivo bioassay anticipates reference method concept

- Potency assigned on basis of in vivo bioassays

FSH (1988)

- Heterogeneous estimates (between assay types and within immunoassay group)
- Decision to define FSH in terms of one assay method (in vivo bioassay anticipates reference method concept
- Potency assigned on basis of in vivo bioassays
- Outcome: chaos

FSH 1997 (immunoassay standard)



Separate immunoassay and bioassay standards

-Short term solution (immunoassayists) and bioassayists went away happy

-Perpetuated some unfortunate myths:

-Immunoassays and bioassays must, on conceptual grounds be considered heterogeneous

-Immunoassays can, on conceptual grounds be considered homogeneous

-Recombinant material, facilitating possible replacement with a similar material

FSH (97) represents the pragmatic approach taken by WHO to complex Heterogeneous analytes

- IU (rather than SI)
- Arbitrary assignment (without formal metrological traceability to previous standards
- No uncertainty assigned
- No defining methodology identified

How, in a situation such as this, can we aspire to formal assignment in SI?

ISO 17511

1 Cases with primary reference measurement procedure and primary calibrator(s) giving traceability to SI units

2 Cases with International conventional reference measurement procedure (which is not primary) and international conventional calibrators without traceability to SI

3 Cases with International conventional reference measurement procedure (which is not primary) but no international conventional calibrators and without traceability to SI

4 Cases with International conventional calibrator (which is not primary) but no international conventional reference measurement procedure and without traceability to SI

5 Cases with manufacturer's selected measurement procedure but neither International conventional reference measurement procedure nor international conventional calibrator and without traceability to SI

What is a "primary reference measurement procedure" ?

ISO 17511

…having the highest metrological qualities, whose operation can be completely described and understood, for which a Complete uncertainty statement can be written down in Terms of SI units, and where results are therefore accepted Without reference to a measurement standard of the quantity Being examined

The quest to assign values in SI is, essentially, a quest for reference methods

Reference methods means different things to different people

ISO 17511

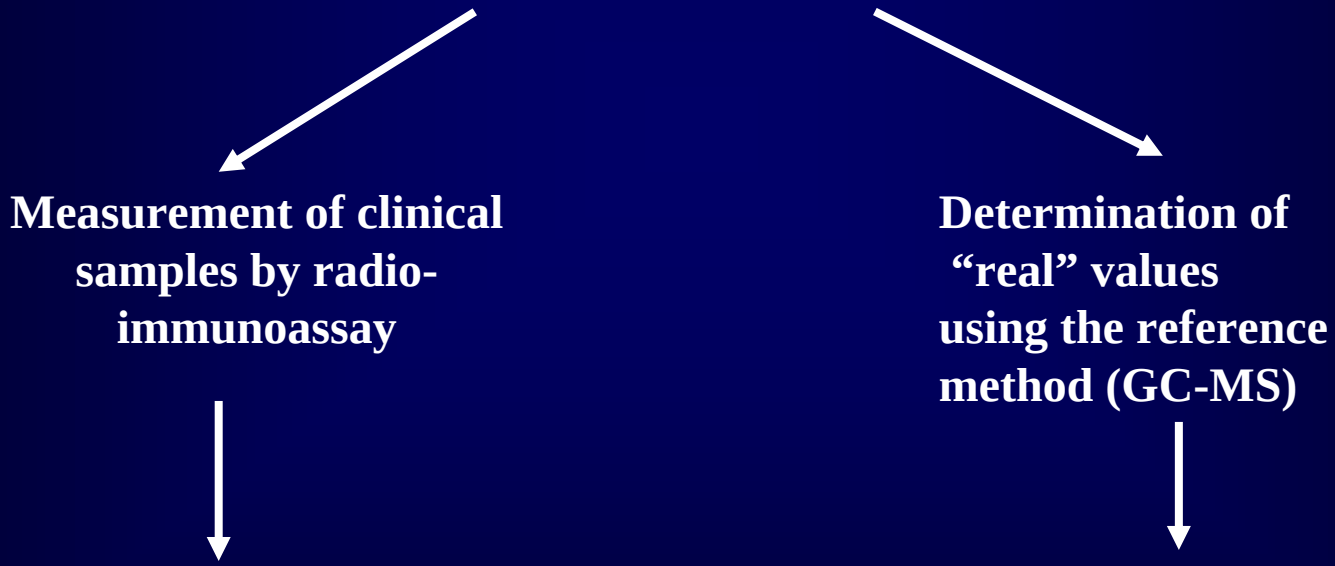
…having the highest metrological qualities, whose operation can be completely described and understood, for which a Complete uncertainty statement can be written down in Terms of SI units, and where results are therefore accepted Without reference to a measurement standard of the quantity Being examined

Clinical Chemistry

A reference method is generally accepted as being One where the analyte can be defined and measured In the sample to be tested (eg plasma). This gives you the Highest level of commutability

To what extent is either applicable in the field of biologicals?

The reference method concept in practice: Urinary free cortisol



1 Can we approach this in practice for biologicals

2 Even if we could, would it help?

Targeted proteomics of low-level proteins in human plasma by LC/MS: using human growth hormone as a model system.

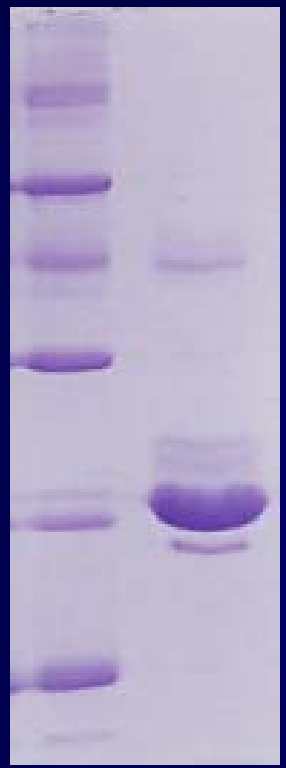
Wu SL, Amato H, Biringer R, Choudhary G, Shieh P, Hancock WS.
J Proteomics Res. 2002 (1) 459-465

Proteomics, ThermoFinnigan, San Jose, California 95134, USA.

This paper describes the profiling of human growth hormone (hGH) in human plasma in order to assess the dynamic range of the ion-trap mass spectrometer for proteomic studies of complex biological samples. Human growth hormone is an example of a low-level plasma protein in vivo, present at sub-femtomole levels. This study was performed on a plasma sample in which hGH has been spiked at 10-fold above the natural level, that is approximately 16 pg/microL of plasma. Initially, the measurement was carried out without any sample enrichment and consisted of the following steps: the full set of plasma proteins were reduced, alkylated, and digested with trypsin,and the resulting peptides were separated on a capillary C-18 column and then detected by ion-trap mass spectrometry (1D LC/MS). In addition, this study provided a global view of the serum proteome with over 200 plasma proteins being preliminarily identified. In the MS/MS analysis, hGH was detected by characterization of the first tryptic peptide (T1). The initial identification was confirmed by alternative approaches, which also allowed the evaluation of different sample purification protocols. First, the plasma sample containing hGH was fractionated on a reversed-phase HPLC column and digested, and hGH could now be identified by MS/MS measurements of two tryptic peptides (T1 and T4) by the same 1D LC/MS protocol. In addition, the assignment of peptide identity was made with higher certainty (as measured by an algorithm score). The plasma sample was also fractionated by 1D and 2D gel electrophoresis, the selected bands were digested and analyzed again by the 1D LC/MS protocol. In both cases using the gel prepurifications, hGH was identified with additional peptides. Finally, the plasma sample was analyzed by 2D chromatography (ion exchange and reversed phase) on a new instrumental platform (ProteomeX), and hGH was identified by the observation of five tryptic peptides. In conclusion, these experiments were able to detect growth hormone in the low femtomole level with a dynamic range of 1 in 40 000 by several independent approaches. ***The amount of growth hormone, while 10-fold above normal in vivo levels, represents concentrations that may be present in disease states (such as acromegaly) and also in doping control measurements.*** These studies have demonstrated that shotgun sequencing approaches (LC/MS/MS) not only can profile high-abundance proteins in complex biological fluids but also have the potential to identify and quantitate low-level proteins present in such complex mixtures without extensive pre-purification protocols. A key to such studies, however, is to use targeted approaches that reduce the complexity of the solute mixture that is presented to the mass spectrometer at a given time point. The various sample preparation protocols described here all improved the quality of the hGH measurement, although in this study the 2D chromatographic approach gave the greatest sequence coverage.

Technology is approaching the level at which the clinical chemists idea of a reference method Can be achieved. Will it help? Probably not.

Pituitary growth hormone



Dimers

Large loop cleaved forms

22K monomer

20K splice variant

Additional chemical heterogeneity (deamidation, oxidation) and also plasma-based heterogeneity (complexing to binding proteins) also exists. As an analyte, GH cannot be defined as a single chemical entity. It is even worse for glycoproteins

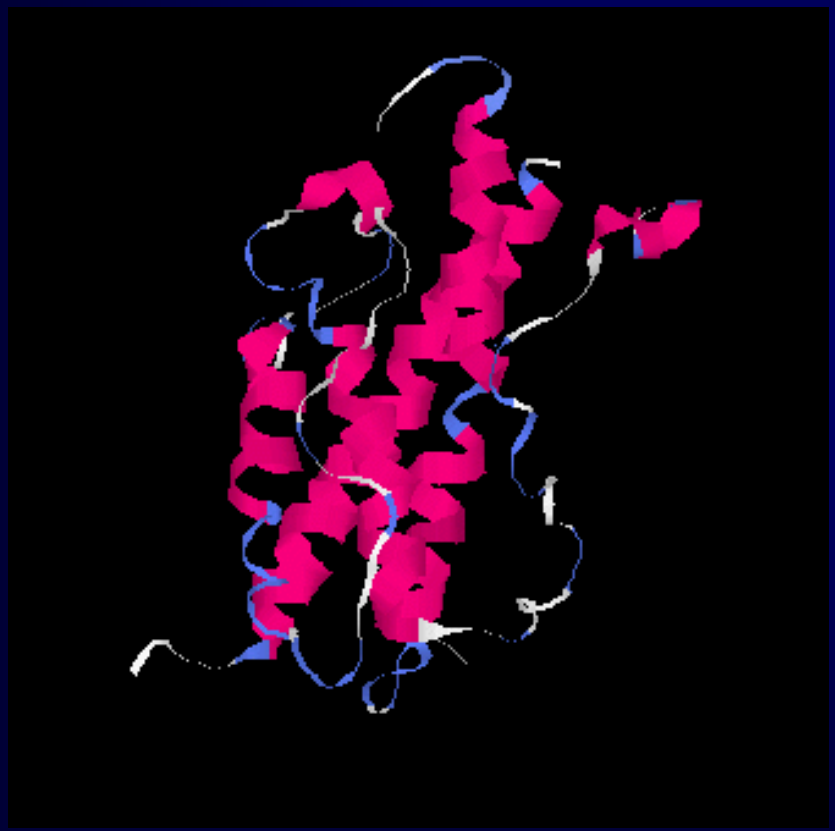
In general terms, the concept of a “a unique, homogeneous chemical entity” does not apply to proteins and other macromolecules

What about the ISO 17511 definition of a reference method?

ISO 17511
…having the highest metrological qualities, whose operation can be completely described and understood, for which a Complete uncertainty statement can be written down in Terms of SI units, and where results are therefore accepted Without reference to a measurement standard of the quantity Being examined

Case 2: growth hormone

(recombinant GH = somatropin)



Molecular weight 22,000
191 amino-acids
3087 atoms

The second IS for somatropin (recombinant growth hormone) : assignment of ampoule content in terms of the 1st

1st IS: assignment of content by amino-acid analysis

2nd IS: assignment in terms of 1st by HPLC

Specified HPLC method

Laboratory	Laboratory mean (mg/ampoule)
1	1.86
2	1.94
3	1.91
4	1.96
5	1.93
6	2.02
7	1.96
8	1.96
9	1.82
10	1.95
11	1.91
12	1.94
13	2.00
14	1.93
15	1.97
16	1.85

Overall mean 1.933mg/amp
RSD 2.69%

1 The physico-chemical assay does not stand alone. It is only valid in the context of a specification which includes:
RP,SE, IEX HPLC, Peptide mapping electrophoretic properties
LC-MS, Bioassay

2 The primary method (amino-acid analysis) seems to satisfy ISO17511 requirements for a reference method

3 The secondary method (HPLC) does not (requires a reference material)

Case 3: the establishment of the WHO Reference Reagents for Human Chorionic Gonadotrophin

IFCC
Ulf-Haake Stenman
Cathie Sturgeon
Steven Birkin

A two-sub-unit heterodimeric glycoprotein

30kD

7-8 major charge variants associated with sialylation and sulphation

Measured by immunoassays for diagnosis of pregnancy, and in oncology, and by bioassay In research and therapeutic product development

A number of variants of the analyte exist, which may be distinguished by Different assays, and which may be of clinical significance (subunits, nicked forms, nicked subunits and degraded forms)

The starting point is a partial structural definition of the measurand. Immunoassay specificity is largely determined by the peptide backbone, and the analytical variants under study are variants in protein, not carbohydrate

Establishment of the RM's for the 6 analytes (hCG, hCG-ß, hCG-α, hCG-n, hCG-ßn, hCG-ßcf), is a three stage process:

1 Exhaustive biochemical characterisation of the preparation in terms of the measurand definition

2 Assignment of content to the bulk preparations is carried out using an absolute method reporting in SI (amino-acid analysis)

3 Recovery in ampouled preparations is estimated by immunoassay in terms of bulk

Figure 1: schematic representation of study design

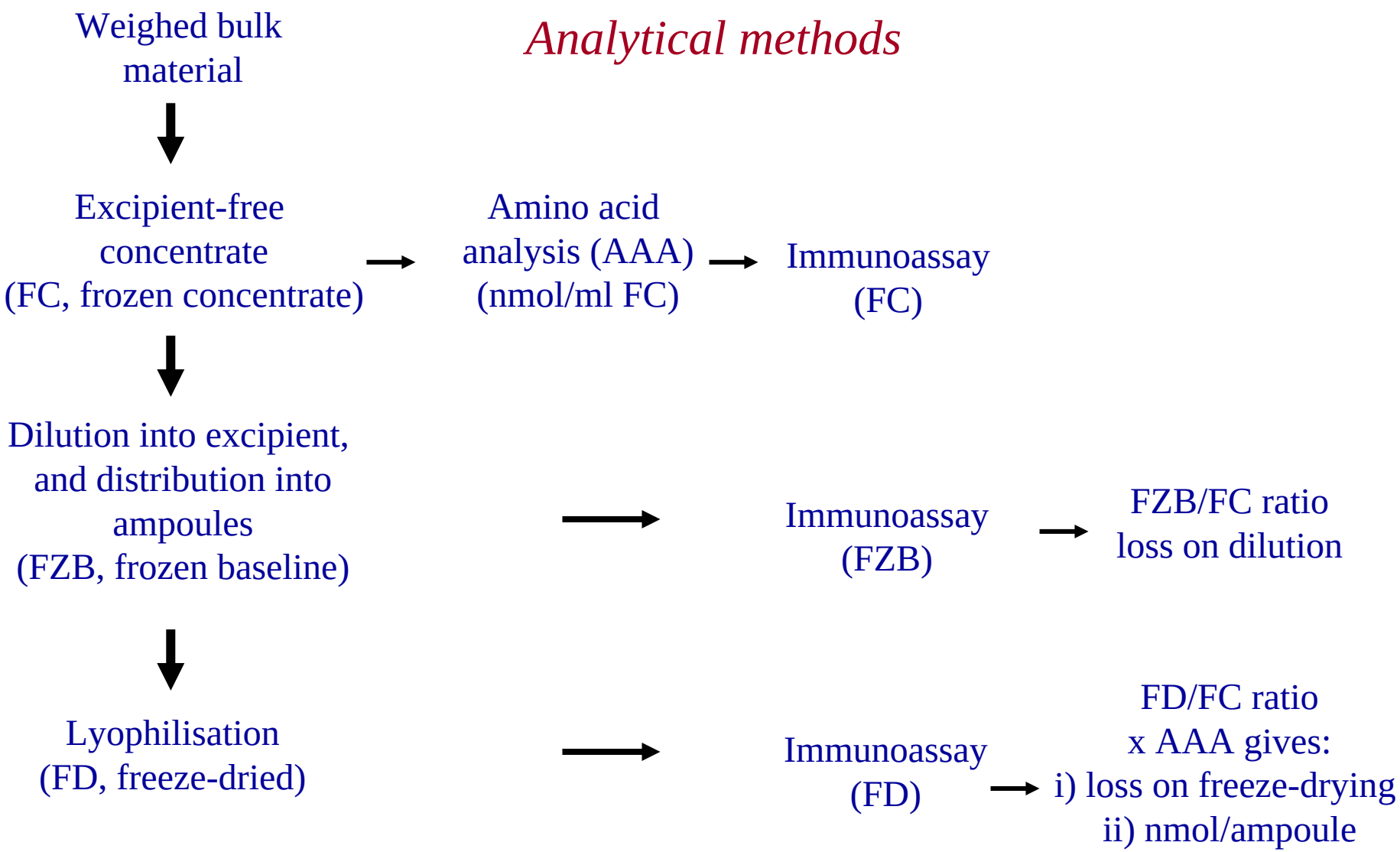


Table 9 Estimates of ampoule content: mean estimates of ampoule recovery, and corrected amino-acid analysis estimates

Preparation	Concentrate (nmol/ml) AAA	lyophilisation recovery (%) (means, tables 3-8)	Lyophilised ampoules: nominal value (nmol)	Lyophilised ampoules: calculated value (nmol)
99/688 hCG	42.78	93.2	2.0	1.88
99/642 hCGn	51	87.2	1.0	0.78
99/650 hCG-β	105.54	88.9	1.0	0.88
99/720 hCG-α	52.86	78.9	1.0	0.84
99/708 hCG-βcf	122.2	104.1	1.0	1.02
99/692 hCG-βn	94.32	85.8	0.5	0.33

Legend: Values for nmol/ml (amino-acid analysis) are derived from Table 2. Estimates of %recovery on lyophilisation are derived from Tables 3-8. In each case, estimates from assays of appropriate specificity, or showing significant cross reactivity have been included, as indicated in Tables 3-8.

How do cases 1 and 2 this differ from the traditional WHO type study (FSH)

-The measurand is defined

-What is done is a consequence of that definition

-The method used to assign the content (amino-acid analysis) is not the method that the RM will be used to support (immunoassays)

-The immunoassay bias/specificity is eliminated from the collaborative study and from the value assignment protocol.

-The interdependence of variable RM and variable assays is broken

- Value assignment is through a specified method, reporting in SI.

Case 4: Enzyme Activities (Colin Longstaff, Division of Haematology, NIBSC)

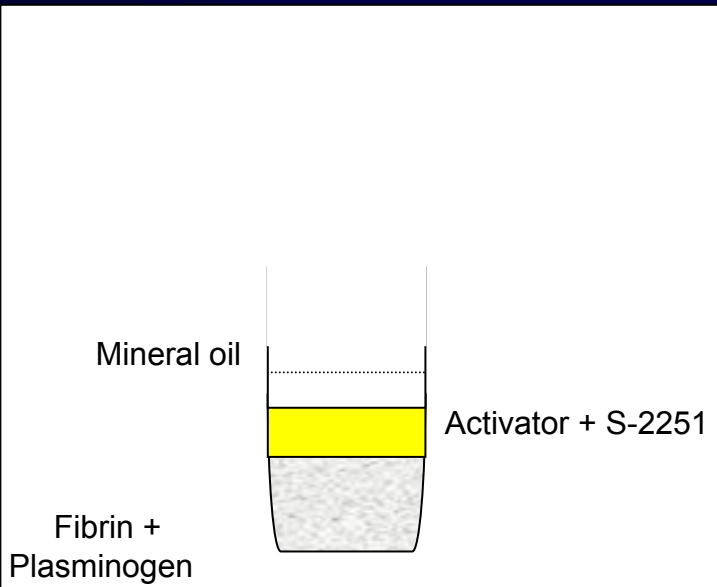
- Units (1µmol in 1 min; katal)
  - Measure product or substrate
  - Conditions carefully defined

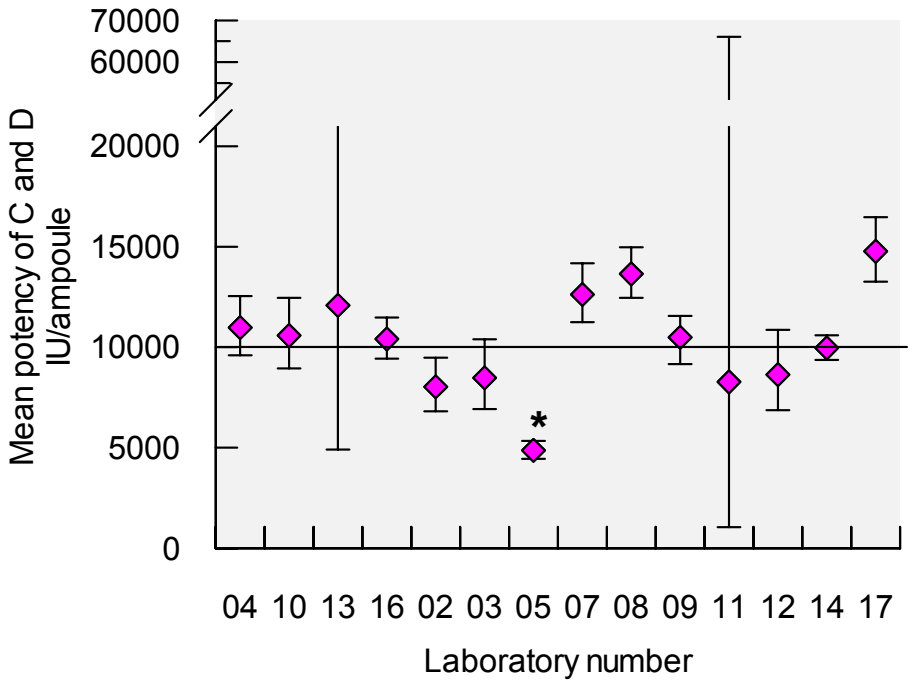
- International Units (IU)
  - Specific for each enzyme
  - Reference preparations
  - Method not defined

Fibrinolysis IS and IU

Standard	***Potency (current Standard)***	***Unit origin (1st Standard)***
Plasmin 3rd IS	5.3 IU	Release 0.1 µeq Tyr from casein
Plasminogen BS	10 U	Plasmin
Streptokinase 3rd IS	1030 IU	NIH unit
uPA HMW 1st IS	4300 IU	Hydrolysis of Arg-lys-gly ME
tPA 3rd IS	10 000 IU	Arbitrary
PAI-1 1st IS	7.0 and 27.5 IU	Vs uPA and tPA
Reteplase	0.0285 U	1 U = 1 mg
Staphylokinse	1 U	1 U = 1 ampoule

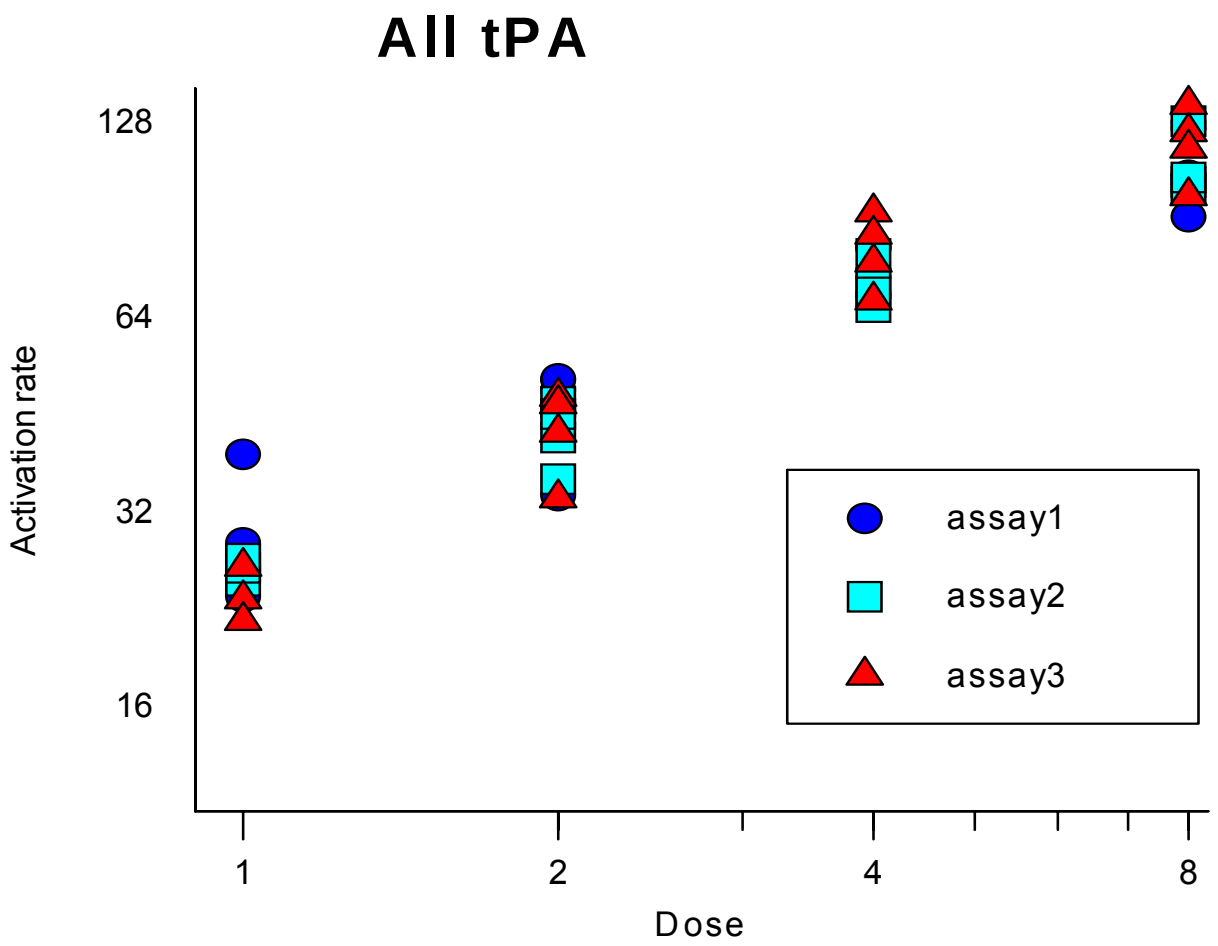
SSC: Can we measure fibrinolysis proteins in absolute units? ([Plasmin]/s in a defined method)



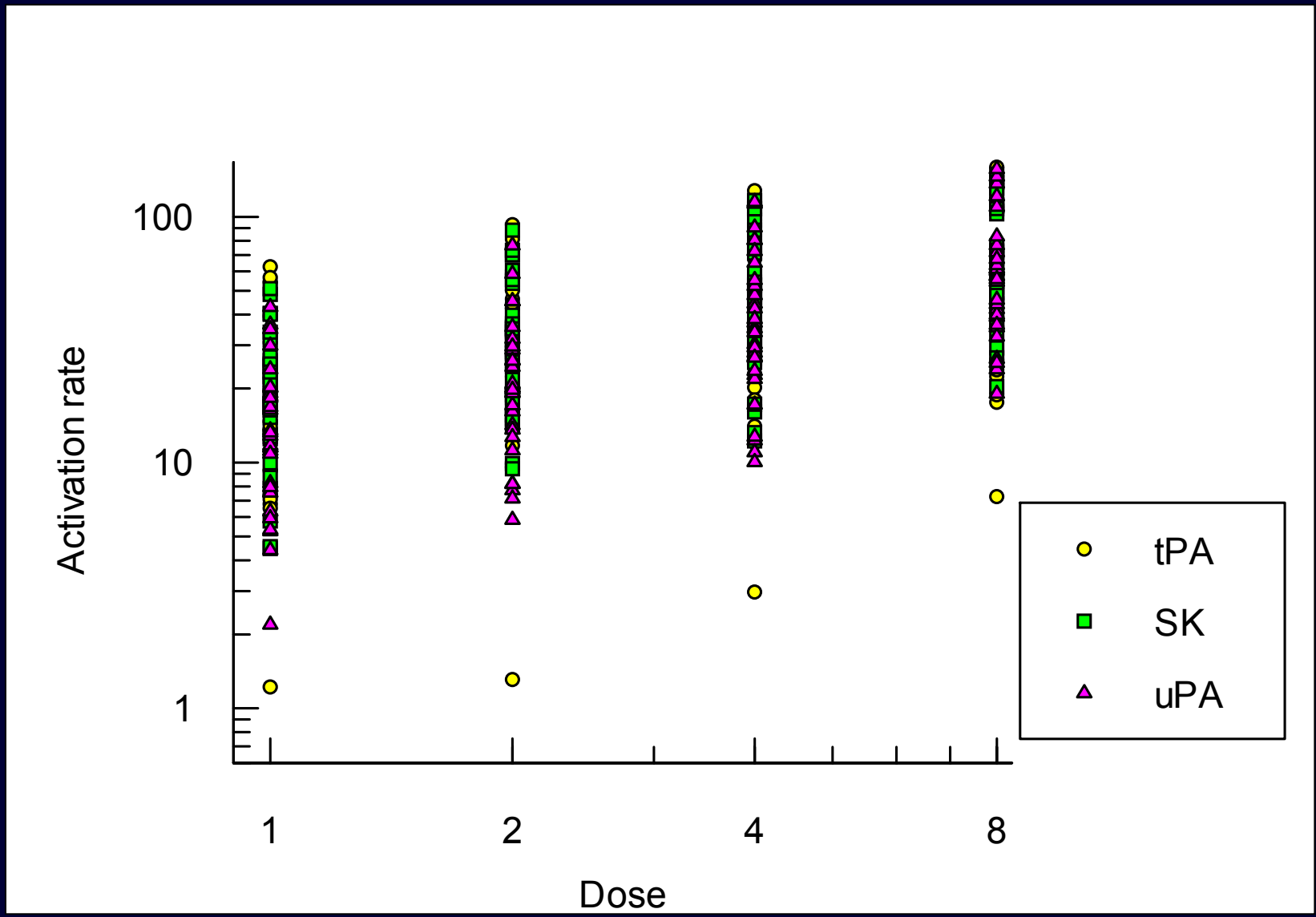


A pad of fibrin + plasminogen is preformed in a microtitre wells. Onto this is added plasminogen activator in chromogenic substrate. A range of plasminogen activator doses may be studied. The plasmin generated is measured by following the change in OD with time. The rate of plasmin generated is calculated from a plot of the transformed data of OD versus time squared.

Between assays: one laboratory



Between laboratories



No major methodological problems reported

tPA, SK and uPA all behaved equally well
Intra-assay agreement good

Intra-laboratory agreement OK

Inter-laboratory agreement poor

Absolute measures in enzyme units are much more difficult than relative measures in IU

Summary and conclusions

1 WHO has derived its approach to standardisation of biologicals from a historic inability to characterize the analytes by physico-chemical methods

2 The current WHO approach recognises developments in physico-chemical methods, and also seeks to incorporate concepts of traceability, uncertainty and commutability

3 Calibration of biological RM's in SI depends on the elaboration of reference methods

4 For well-characterised biologicals, reference methods linked to a specific definition of the measurand can be developed, and can be successfully applied. For other analytes, limitations on physico-chemical characterization remain real.

5 The limitations on the ability to define biological reagents remain real. Proposed reference methods that depend on biolgical reagents are unlikely to be successful

Future activities:

1. Debate and reach an understanding of what kind of measurement procedure can constitute a reference method

2. Recognize that reference measurement procedures can be developed in some areas

3. Seek to promote this development

4. Invoke the help of the proteomics community in developing analytical methods for direct measurement of biological analytes in complex matrices