Characterisation, calibration and comparison by international collaborative study of international standards for the calibration of therapeutic preparations of FSH

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Abstract

Therapeutic preparations of FSH, used primarily for treatment of infertility, are calibrated by *in vivo* bioassay against international standards (IS) derived from different sources deemed appropriate to their use according to pharmacopoeial monographs. Menotrophins, which have been used for several decades to treat infertility, have been calibrated against the IS for urinary FSH and LH (ISU) but are now being replaced by highly purified urinary FSH or rDNA-derived FSH (rFSH).

The aim of this study was to evaluate two preparations of human rFSH and one preparation of highly purified urinary FSH as candidate WHO IS for bioassay in an international collaborative study by 27 laboratories in 12 countries, and to characterise them in a range of *in vitro* bioassays and immunoassays.

The biological activity of the three candidate standards was confirmed by all laboratories using all assays contributed to the study. Dose-response relationships by *in vivo* bioassay for any of the candidate standards did not differ significantly from that for the ISU. Dose-response relationships obtained in *in vitro* bioassays and immunoassays were also broadly similar among these preparations although dose-response lines for some preparations

appeared to be non-parallel in some immunoassays. For each of the three candidate IS, estimates of the relative potency in terms of ISU by *in vivo* bioassay did not differ significantly between laboratories. In contrast estimates by immunoassays and *in vitro* bioassays showed significant differences between laboratories. Estimates of relative potency of the highly purified candidate IS materials in terms of one another exhibited less inter-laboratory variability than estimates in terms of ISU. Each of the candidate standards showed adequate stability to serve as an IS.

On the basis of the results of this study rFSH (code 92/642) was established as the first IS for FSH, human, recombinant for bioassay with an assigned unitage of 138 IU per ampoule and urinary FSH (code 92/512) was established as the first IS for FSH, human, urinary (urofollitropin) for bioassay with an assigned unitage of 121 IU per ampoule, based on their respective calibration by *in vivo* bioassay in terms of ISU. These assignments of unitage maintain continuity of unitage for preparations in therapeutic use and also appear to be consistent with one another.

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Introduction

Follicle-stimulating hormone (FSH) is a heterodimeric, heterogeneous glycoprotein hormone which has been identified in most vertebrate species. It has a central role in reproduction and hence is widely used in human (and animal) reproductive medicine both as a therapeutic agent and as a diagnostic analyte. The potency of human urinary gonadotrophins (menotrophins), used for several decades as therapeutic products in reproductive medicine, is defined by their activity in *in vivo* bioassay expressed as international units (IU) in terms of the international standard (IS) for urinary FSH and luteinising hormone (LH) (ISU) (WHO Expert Committee on Biological Standardization

1964, 1975, Storrington *et al.* 1976). The relative impurity (Giudice *et al.* 1994) of these materials necessitates the use of bioassays for determining the potency of their dosage forms (Storrington 1988).

Advances in biotechnology have enabled the development of highly purified preparations of urinary-derived and rDNA-derived FSH (rFSH) (De Boer & Mannaerts 1990, Van Wezenbeek *et al.* 1990, Hugues 1994) which have been used successfully in the treatment of infertility (Devroey *et al.* 1992, Howles *et al.* 1994). However, the origin, purity and structural features (Hard *et al.* 1990, Amoresano *et al.* 1996, Harris *et al.* 1996) of rFSH differ from those of naturally derived preparations.

Table 1 Ampouled preparations distributed for analysis

Ampoule code	Material	Nominal content
92/510	Candidate standard for human rFSH	10 µg human rFSH 2 mg human plasma albumin 10 mg mannitol 15.4 µmol NaCl
92/642	Candidate standard for human rFSH	10 µg human rFSH 2 mg human plasma albumin 10 mg mannitol 15.4 µmol NaCl
92/512	Candidate standard for highly purified urinary FSH	10 µg immunoaffinity purified urinary FSH 2 mg human plasma albumin 10 mg mannitol 15.4 µmol NaCl
71/223	2 nd ISU	See reference (Storring <i>et al.</i> 1976)
71/264	3 rd ISU	As 71/223
Accelerated thermal degradation samples	92/510 stored at +20 °C, +37 °C, +45 °C for 717 or 877 days	As 92/510
Accelerated thermal degradation samples	92/642 stored at +20 °C, +37 °C, +45 °C for 570 or 730 days	As 92/642
Accelerated thermal degradation samples	92/512 stored at +20 °C, +37 °C, +45 °C for 392 or 552 days	As 92/512

We describe in this paper the results of an international collaborative study carried out by 27 laboratories in 12 countries to compare candidate standards of highly purified human urinary FSH and of rFSH with the ISU.

The aims of the study were to: (1) calibrate urofollitropin in terms of the ISU; (2) assess the need for a standard for rFSH; (3) calibrate rFSH in terms of the ISU; and (4) assess the stability of candidate IS preparations.

Materials and Methods

Materials

Materials distributed for this study are listed in Table 1 and were identified to participants in the study by a letter code only, and except for *in vivo* bioassays were assumed to have a common nominal potency so that they were all treated as nearly as possible identically. Where assay capacity permitted, two ampoules of the same material but with different codes (coded duplicates) were included to provide an independent assessment of intra-assay variability. For *in vivo* assays different codes were used, and nominal potencies were given based on the best available information so that *in vivo* assays using the minimum number of animals were carried out.

Materials evaluated as candidate standards were generously donated by the Ares-Serono Group, Geneva, Switzerland and Organon International B.V., Oss, The Netherlands. The bulk materials arrived at National Institute for Biological Standards and Control (NIBSC) either as an aqueous solution containing 0.83 mg protein per ml (by the Lowry method using human serum albumin as the standard), or as a lyophilised powder containing 27 µg protein (by amino acid analysis) per mg of excipient (1.54% sucrose, 0.46% citrate) which was redissolved in 10 ml water immediately prior to ampouling. These materials were stored at −20 °C and +4 °C respectively prior to ampouling.

Candidate IS for rFSH and highly purified human urinary FSH These consisted of two batches of ampoules, coded 92/510 and 92/642, containing highly purified human rFSH produced in Chinese hamster ovary (CHO) cells and a batch of ampoules, coded 92/512, containing a preparation of immunoaffinity-purified human urinary FSH. Samples of ampoules of rFSH and highly purified urinary FSH which had been stored at elevated temperatures to provide an assessment of stability were also included in the study. Reports describing the production and characterisation of highly purified FSH

Table 2 List of participants

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Table 3 Summary of immunoassays used in the study

Laboratory number	Immunoassay type (reported as)
1	Two-site IRMA
2	A: Amerlex M RIA B: Amerlite CLA (luminescent 2-site)
3	Solid phase double antibody RIA (in house) (two variants)
5	DELFA (IFMA)
6	A: Double antibody solid phase RIA (DPC) B: FSH IRMA (DPC) C:Two-site CEIA
7	MAIA-Clone (2-site IRMA) (Serono)
8	RIA
9	DELFA
15	Omnia FSH IRMA (IDS)
17	In-house double-antibody RIA
19	Double antibody RIA (DPC)
20	MYRIA
21	Coated tube IRMA (FSHCTK-2 Sorin)
22	WHO matched reagent programme (1993) RIA
24	Two-site coated tube IRMA (EuroDiagnostica)
27	FEIA (Auraflex)
28	A: CLA B: DELFIA
29	DELFIA
30	In house two-site ELISA (two formats)

RIA=radioimmunoassay; IRMA=immunoradiometric assay;
FEIA=fluorescent enzyme immunoassay; CLA=chemiluminescent assay;
ELISA=enzyme-linked immunoassay, CEIA=chemiluminescent enzyme immunoassay; IFMA=immunofluorometric assay.
Amerlex M, Amerlite, DELFIA, MAIA-Clone, Omnia, MYRIA, FSHCTK-2 and Auraflex are proprietary names for assays used.
DPC (Diagnostic Products Corporation) (Los Angeles, CA, USA);
Serono-Baker Diagnostics Inc. (Allentown, PA, USA); IDS (Immunodiagnostic Systems Ltd) (Bolderon, UK); Sorin Biomedica (Saluggia, Italy);
EuroDiagnostica (Apeldoorn, The Netherlands); WHO matched reagent programme (see Sufi *et al.* 1991).

preparations have been published (De Boer & Mannaerts 1990, Hard *et al.* 1990, Van Weezenbeek *et al.* 1990, Amoresano *et al.* 1996, Harris *et al.* 1996).

The bulk solutions were diluted in an appropriate buffer containing excipients and were distributed into ampoules as 1·0 ml aliquots according to WHO guidelines. Ampoule contents for each preparation are summarised on Table 1.

The second and third IS for urinary FSH and LH for bioassay (ISU) Ampoules (coded 71/223 and 71/264) contained a preparation of urinary FSH and LH with a defined content of 54 IU FSH and 46 IU LH per ampoule (Storring *et al.* 1976, WHO Expert Committee on Biological Standardization 1989, 1994). During the period of this study the stocks of the second ISU were effectively exhausted and the third ISU was established. These two materials appear to be indistinguishable, as previously indicated, and were used interchangeably throughout this study except where they have been directly compared with each other.

Participants and assays contributed to the study

The participants in this study are listed in Table 2. Throughout this report each laboratory is identified by a number between 1 and 30 which does not relate to the order of listing. (NB Not all numbers have been used.)

In vivo bioassays

Ten laboratories contributed *in vivo* bioassays based on the gonadotrophin augmentation of ovarian weight gain. These assays are essentially similar and conform to relevant pharmacopoeial monographs: laboratories 25 and 30, European Pharmacopoeia (1986); laboratories 11, 18 and 23, British Pharmacopoeia (1988); laboratories 7, 16, 17, 22 and 29 (Steelman & Pohley 1953). Each participant was requested to carry out at least two independent assays for each preparation tested preferably using at least three dose levels of each preparation so that linearity and parallelism of dose–response lines could be examined. For the purposes of this study an independent assay was considered to be one in which a freshly made up ampoule of test preparation was tested on a separate occasion using fresh batches of reagents where feasible. Participants were also asked to contribute full results of their assays including all raw data for analysis at NIBSC.

In vitro bioassays and radioreceptor assays

Nine laboratories contributed *in vitro* bioassays by six methods and three laboratories contributed radioreceptor assays by four methods. Laboratories 9, 10, 26, 28, 29 and 30 used Sertoli cells from immature rats (Guillou *et al.* 1986, Padmanabhan *et al.* 1987) and determined various end points including aromatase activity (laboratory 9), plasminogen activator (laboratory 26) and cAMP production. Four laboratories used human or rat rFSH receptor expressed in various cell types (Tilly *et al.* 1992, Gudermann *et al.* 1994): laboratory 7, mouse Y1 adrenal cells; laboratory 9, L cells; laboratory 12, human foetal kidney 293 cells; and laboratory 29 CHO cells. End points measured included progesterone and cAMP. Laboratory 8 used aromatase activity in rat granulosa cells (Miro *et al.* 1991). For the radioreceptor assays laboratories 9, 10 and 29 used calf testis as a source of FSH receptor (Cheng 1975) and laboratory 29 additionally used human rFSH receptor expressed in CHO cells. Participants were asked to examine preparations using assay designs and contributing results as described for *in vivo* bioassays.

Immunologically based assays

Nineteen laboratories using 24 assay systems contributed immunologically based assays to the study (Table 3). Seven laboratories contributed RIA (defined as one-site assays for the purposes of analysis in this study), seven laboratories

Table 4 Laboratory geometric mean estimates with 95% fiducial limits obtained by *in vivo* bioassay for the FSH content of the IS for human urinary FSH (urofollitropin), in ampoules coded 92/512, of the IS for human rFSH, in ampoules coded 92/642, and of a preparation of rFSH in ampoules coded 92/510, expressed as IU of the ISU per ampoule. The number of independent estimates contributing to each mean estimate is shown in parentheses. Estimates are weighted geometric means of homogeneous estimates, except as marked* when estimates are the unweighted geometric means of heterogeneous estimates. NA denotes that the preparation was not assayed. The estimates in braces result from deletion of the single estimate with the largest contribution to the heterogeneity chi-square value for all estimates

	92/512	92/642	92/510
Laboratory number			
7	97 (1) 70–143	133* (2) 119–148	97* (2) 20–467
11	112 (5) 98–127	139 (5) 120–161	91 (5) 78–108
16	147 (2) 119–181	138 (4) 118–161	100 (3) 86–115
17	NA	124 (2) 101–152	90 (2) 76–106
22	129 (1) 104–160	144 (1) 116–182	73 (1) 58–97
23	151 (2) 129–177 [146 (1) 120–179]	160 (2) 130–195	98 (2) 84–114
25	120 (2) 102–140	145 (2) 119–176	101 (2) 83–124
29	125 (2) 104–150	156 (2) 128–189	102 (2) 86–121
30	102 (3) 85–123	132 (5) 117–148	105 (4) 67–165
All estimates	126* (18) 110–142 [121 (17) 113–128]	138 (25) 131–145	95 (23) 90–100

contributed IRMA and eight laboratories contributed other assay types including ELISA and similar assays with different detection modes including chemiluminescence and fluorescence (defined as two-site assays for the purposes of analysis in this study). The majority of these assays were commercially available assays in kit form which included a kit standard calibrated against the International Reference Preparation for pituitary FSH (Code 78/549). Other assays were in-house assays with standards derived from various sources including the National Institutes for Diabetic, Digestive and Kidney Diseases (NIDDK, Bethesda, MD, USA). Participants were asked to carry out at least two independent assays, each to include each of the preparations supplied at as many dilutions as practicable (at least three and preferably six or more) to provide information about the dose–response curves and to return full results of their assays for analysis at NIBSC.

Statistical analysis

***In vivo* bioassays** The majority of participants contributed data for both body weight and organ weight for each response. These data were examined using both organ weight only and organ weight relative to body weight, both as given and following log transformation as the assay response. Within each laboratory the response used for subsequent analysis was that which best satisfied the criteria of statistically valid assays with homogeneous groups of responses and precise estimates of relative potency. Doses with mean responses which contributed

significantly to non-linearity and which appeared to be at the extremes of the response range were omitted from analysis.

Log potency estimates for the rFSH and purified urinary FSH in terms of the ISU were obtained as the displacement of parallel log dose–(log) response lines from analysis as paired or multiple parallel-line assays. Statistical weights for the paired analysis were computed as the reciprocal of the variance of the log potency. Assays described as statistically valid showed no significant ($P>0.05$) deviations from linearity or parallelism.

***In vitro* bioassays, receptor assays and immunoassays**

For each assay response data (as far as possible the directly measured values) were plotted against log dose and assessed both graphically and statistically for outliers, consistency of increase (or decrease) of dose–response relationship and homogeneity of variances. Groups of responses with an excessively large variance relative to the variances of other groups in the same assay were examined further. If such groups consisted of only two replicates, both were deleted; individual outlying responses in groups of three or more replicates were deleted. Response–error relationships were examined and where variances showed a significant increase with increasing response, the relationship of variance to mean response raised to a positive power, α , was fitted using a maximum likelihood method (Raab 1981). This relationship, with α taken as the average value of α for assays of the same type from the same laboratory, was used in subsequent analysis. Dose–response relationships were assessed for conformity to a four parameter

Figure 1 Individual estimates of FSH content for ampoules of 92/512, expressed as IU of the ISU in ampoules coded 71/223 plotted on a log scale. Each square denotes an individual estimate. The three character code in the square indicates the assay type (B denotes *in vivo* assay; R receptor assay, I *in vitro* assay; i two-site immunoassay and r one-site immunoassay) followed by the laboratory number. Shading has been added to emphasise assay type: open squares are *in vitro* bioassays or receptor assays, solid squares are *in vivo* assays, hatched squares are one-site assays and dotted squares are two-site assays. Double squares with a single code indicate two estimates which were obtained in the same assay using differently labelled ampoules. The arrowed number shows the weighted geometric mean of the estimates by bioassay.

logistic function, maximum and minimum responses for each assay were determined using this model, and data for each assay were analysed as parallel-line assays using a logit transformation of the responses (and the program WRANL (Gaines-Das & Tydeman 1982)). Log dose–logit response lines were assessed for linearity and parallelism and log potency estimates were obtained as the displacement of parallel log dose–logit response lines. In a few cases either the response range was so limited that a four parameter logistic model could not be assessed or conformity to this model was not satisfactory and the responses from the linear part of the response range have been analysed as described for *in vivo* bioassays. Slopes of log dose–logit response lines were examined for any consistent trends. Differences between slopes of coded duplicate preparations were used to provide a direct measure of intra-assay slope variability.

Combination of potency estimates Estimates of log potency from analysis of *in vivo* bioassays for specified pairs of preparations were examined for heterogeneity using a chi-squared test and combined as weighted geometric means where estimates were homogeneous, or unweighted geometric means where estimates were heterogeneous. Estimates of log potency from analysis of *in vitro* bioassays, receptor assays and immunoassays were combined as unweighted geometric means. Fiducial limits for unweighted means were determined from the variance of the logarithms of the combined estimates. Comparisons among groups of estimates were made using unweighted analysis of variance of the log potency estimates of the groups compared.

Inclusion, where possible, of a coded duplicate of one of the FSH preparations provided a direct measure of the intra-assay variation of potency estimates calculated as the

Figure 2 Individual estimates of the FSH content for ampoules coded 92/642, expressed as IU of the ISU per ampoule. Codes and shading in squares as for Fig. 1. The arrowed number shows the weighted geometric mean of the estimates by bioassay.

deviation of the log relative potency estimate of the duplicate in terms of the identical material from its expected value of 0. This is taken to provide a measure of the minimum inherent variability in these systems, and is taken as the base against which comparisons among other estimates are considered.

Results

Comparison of the second and third ISU

Over the period of this study the second ISU (in ampoules coded 71/223) was replaced by the third ISU (in ampoules 71/264). These two preparations were derived from the same bulk material and were ampouled under identical conditions. Several of the laboratories received ampoules of both preparations. Pairwise comparison of these two preparations confirmed the continuity of this unitage. Nine independent *in vivo* bioassays in six laboratories were statistically valid and gave estimates which were homogeneous with a weighted geometric mean (95% fiducial

limits) of 0.99 (0.91–1.08) IU of the second ISU equivalent in activity to 1 IU of the third ISU (data not shown). These two preparations were also compared in 39 assays in 14 immunoassay systems. Slopes of log dose–logit response lines for these two preparations were similar and estimates of the relative potency of 71/264 in terms of 71/223 (IU/IU) ranged from 0.80 to 1.20 with an unweighted geometric mean (95% limits) of 1.02 (0.99 to 1.05) IU/IU. For subsequent analysis these two preparations have been treated as identical and referred to as a single preparation (ISU). Where one (but not both) of the second or the third ISU, was included in an assay, it has been recorded as the ISU. Where both preparations were included in the same assay, responses have been combined and treated as if given by a single preparation so that maximum precision is obtained for calibrations in terms of the ISU.

Dose–response relationships for rFSH, purified urinary FSH and ISU

***In vivo* bioassays** Seven of the ten participants provided data for both body weights and organ weights. In general

Figure 3 Individual estimates of the FSH content for ampoules coded 92/510, expressed as IU of the ISU per ampoule. Codes and shading in squares as for Fig. 1. The arrowed number shows the weighted geometric mean of the estimates by bioassay.

the response used for analysis was log transformed organ weight relative to body weight. This response was also used for consistency in the few cases where there was no clear advantage of one response transformation, and for the one participant who supplied this response without separate data for body weights. Two participants supplied organ weight responses only; in one case log transformed response, and in the other untransformed response, gave more precise estimates of relative potency. Analysis of pairwise comparisons was statistically valid for the majority of assays with fewer than 5% exceptions.

In vitro bioassays and receptor assays Log dose–logit response lines for the four preparations in these assays did not show consistent significant deviations from linearity or parallelism; except in the receptor assays using CHO cells there was a tendency for 92/642 to give steeper dose–response lines than the ISU and 92/512 (92/510 was not included in these assays). However, only marked instances of non-parallelism would have been detected for many of these assays because of the limited number of doses tested

in a number of assays and because of the variability of the measured responses in many assays.

Immunoassays For 16 of the 24 assay systems in this study log dose–logit response lines did not differ significantly and consistently from linearity or parallelism, although all four preparations were not included in five of those assay systems. In laboratory 24, these four preparations were tested at a single dose and results from this assay system have been excluded from this analysis. In the remaining assay systems there was a tendency for the dose–response lines for the ISU or 92/642, or rarely both, to differ consistently from those of other preparations. Although these data indicate that some differences in sensitivity or selectivity exist between different assay systems, which is to be expected, the magnitude of the differences are not sufficient to preclude use of any preparation as an IS.

Variability of estimates

A measure of the within-assay accuracy and precision of the assays contributed was enabled by inclusion within

Table 5 Unweighted laboratory geometric mean estimates (gms), with 95% fiducial limits based on the variance of the logarithms of the means combined, for the FSH content of the IS for human urinary FSH (urofollitropin), in ampoules coded 92/512, of the IS for human rFSH, in ampoules coded 92/642, and of a preparation of rFSH in ampoules coded 92/510 obtained by *in vitro* bioassays and receptor assays, expressed as IU of the ISU per ampoule. The number of independent estimates contributing to each mean estimate is shown in parentheses. NA denotes that the preparation was not assayed

	92/512	92/642	92/510
Laboratory number			
<i>In vitro</i> bioassays – Sertoli cells			
9	84 (4) 33–213	182 (4) 121–274	145 (4) 93–226
10	44 (3) 15–130	103 (3) 15–708	220 (3) 36–1345
26	43 (1)	77 (1)	NA
28	48 (4) 12–194	124 (4) 43–352	102 (4) 58–179
29	96 (2) 70–132	170 (2) 16–1760	NA
<i>In vitro</i> bioassays – various cells			
7 (Y1)	89 (2) 10–790	173 (2) 28–1070	123 (2) 16–926
8 (Granulosa)	46 (2) 26–82	77 (2) 73–80	62 (2) 29–134
9 (L)	64 (2) 14–293	135 (2) 18–1030	120 (2) 10–1480
29 (CHO)	96 (2) 6–1570	203 (2) 14–3040	NA
Geometric means of laboratory gms	64 (9) 49–84	131 (9) 98–173	120 (6) 78–186
Receptor assays – calf			
9	83 (3) 53–131	154 (3) 105–224	120 (3) 60–239
10	83 (2) 46–149	166 (2) 135–205	123 (2) 94–160
29	75 (2) 55–104	168 (2) 146–193	NA
Receptor assays – CHO			
29	79 (2) 52–118	180 (2) 145–223	NA
Geometric mean of laboratory gms	80 (4) 74–86	167 (4) 150–185	121 (2) 107–138

some *in vitro* and receptor assays and within most immunoassays of coded duplicates (ampoules of the same material coded with different letters for assay) and by the need to confirm the continuity of unitage between the second and third ISU. It is expected that the relative potency of coded duplicates to one another should be 1, and similarly of the third and second ISU assuming these preparations to be identical. Deviations of the relative potency from the value of 1 reflect variability within the assay. Consistent differences within a laboratory from the value of 1 and consequent differences between laboratories may result if there is a consistent bias in the assay system.

Comparison by *in vivo* bioassay and by immunoassay of the third ISU with the second ISU gave estimates of relative potency which did not differ significantly from 1 and which showed no greater variability between laboratories and assays than within laboratories and assays. Comparison by immunoassay gave geometric coefficients of variation (gcv) of about 10% both within and between assays and laboratories.

Coded duplicates of one of the rFSH preparations or of the purified urinary FSH were included in most immunoassays and a number of *in vitro* or receptor assays. For each pair of duplicates and each type of assay, estimates showed within- and between-assay variabilities and between-laboratory variabilities which were broadly similar. All estimates for duplicates were therefore used to give pooled

variances for each assay type. The gcv for immunoassays was about 10% or less both within and between assays and was similar or marginally larger between laboratories. For *in vitro* bioassays the within- and between-assay gcvs were each about 50% while laboratory geometric means had a gcv of about 30%. For receptor assays, variances within and between assays and between laboratories were similar, with gcvs generally between 10 and 15%.

Variability between assays within laboratories was examined directly for all comparisons by each type of assay, including *in vivo* bioassays. For immunoassays the intra-laboratory variability was smallest with gcv less than 10% for virtually all comparisons. For receptor assays the intra-laboratory gcv was generally between 10 and 15%. For *in vivo* bioassays the intra-laboratory gcv, determined directly using unweighted estimates of potency, was generally about 25%. *In vitro* bioassays gave the largest intra-laboratory variability with gcvs from 40 to 70%.

Calibration of the IS for FSH, human urinary (urofollitropin) in ampoules coded 92/512, the IS for human rFSH in ampoules coded 92/642 and a preparation of rFSH in ampoules coded 92/510 in terms of the ISU

Calibration by *in vivo* bioassays Laboratory geometric mean estimates are given in Table 4 and individual estimates are shown in Figs 1–3.

Table 6 Unweighted laboratory geometric mean estimates (gms) for the FSH content of the IS for purified urinary FSH in ampoules coded 92/512, of the IS for rFSH in ampoules coded 92/642, and of a preparation of rFSH in ampoules coded 92/510 obtained by immunoassay, expressed as IU of the ISU per ampoule. The number of independent estimates contributing to each mean estimate is shown in parentheses beside the laboratory code where there were the same number of estimates for each preparation, and otherwise beside the mean estimate. The 95% fiducial limits for the unweighted geometric mean of laboratory mean estimates is based on the variance of the logarithms of the estimates combined. NA denotes not assayed

	92/512	92/642	92/510
Laboratory number			
Two-site immunoassays			
1 (2)	86	134	88
2 (2)	75	110	72
5 (3)	65	93	61
6A (2)	74	122	65
6B (2)	75	108	65
7 (4)	70	107	65
9 (2)	77	103	NA
20 (2)	NA	122	NA
21 (2)	78	126	73
27 (2)	77	110	65
28A (3)	78	111	65
28B (3)	76	115	67
29A (2)	72	101	66
29B (2)	74	116	78
30A (2)	131*	NA	NA
30B	85 (5)*	97 (6)*	73 (2)*
Geometric mean of laboratory gms	78 (15) 72–86	111 (15) 105–117	69 (13) 65–73
Omitting estimates marked*	75 (13) 72–78	112 (14) 106–119	69 (12) 65–73
One-site immunoassays			
2 (2)	73	105	74
3 (2)	67	91	60
6 (2)	72	95	55
8 (1)	144*	120*	66*
19 (2)	78	101	61
22 (4)	63	85	52
28 (3)	72	96	65
Geometric mean of laboratory gms	78 (7) 60–101	98 (7) 89–109	62 (7) 55–69
Omitting estimates marked*	71 (6) 65–77	95 (6) 88–103	61 (6) 53–69
One-site and two-site immunoassays			
Geometric mean of laboratory gms	78 (22) 72–86	107 (22) 101–113	66 (20) 63–70
Omitting estimates marked*	74 (19) 71–76	107 (20) 101–113	66 (18) 62–70

Comparison of the IS for human urinary FSH (uro-follitropin), with the ISU was carried out in 21 independent assays in eight laboratories. After deletion of estimates from two statistically invalid assays and of one estimate which contributed significantly to the heterogeneity chi-squared value, the remaining estimates were broadly similar ($P \sim 0.03$ for chi-squared value for heterogeneity) with an unweighted geometric mean of 126 IU of ISU per ampoule. Deletion of an additional estimate which contributed most to the heterogeneity chi-squared value gave a group of 17 estimates which did not differ significantly from homogeneity and which had a weighted geometric mean (95% fiducial limits) of 121 (113–128) IU of the ISU per ampoule of 92/512.

Comparison of the IS for human rFSH with the ISU was carried out in 26 independent assays in nine laboratories. The weighted geometric mean (95% fiducial limit) of 25 homogeneous estimates (after deletion of one estimate from a statistically invalid assay) was 138 (131–145) IU of the ISU per ampoule of 92/642. Comparison of the rFSH preparation in ampoules coded 92/510 with the ISU was carried out in 24 independent assays in nine laboratories. All assays were statistically valid, but one estimate contributed significantly to the heterogeneity chi-squared value both within the laboratory in which it was obtained, and over all estimates, and has been deleted. The remaining 23 estimates did not differ significantly from homogeneity and gave a weighted geometric

Table 7 Laboratory geometric mean estimates with 95% fiducial limits for the FSH content of the IS for rFSH in ampoules coded 92/642, and of a preparation of rFSH in ampoules coded 92/510 obtained by *in vivo* bioassay, expressed as IU of the IS for purified urinary FSH per ampoule, and for the FSH content of a preparation of rFSH in ampoules coded 92/510 obtained by *in vivo* bioassay, expressed as IU of the IS rFSH per ampoule. The number of independent estimates contributing to each mean estimate is shown in parentheses. Estimates are weighted geometric means of homogeneous estimates, except as marked* when estimates are the unweighted geometric means of heterogeneous estimates

	92/642 (as IU of 92/512)	92/510 (as IU of 92/512)	92/510 (as IU of 92/642)
Laboratory number			
7	157 (2) 134–184	NP	104 (2) 92–116
11	148 (3) 125–177	104 (2) 83–132	86 (3) 71–105
16	113 (3) 97–133	70* (2) 2–3000	92* (2) 4–2000
17	NA	93 (2) 75–115	100 (2) 82–122
18	NA	NA	89 (2) 75–105
22	137 (1) 111–170	NP	74 (1) 58–97
23	122 (2) 99–149	79 (2) 89–1400	88 (2) 72–107
25	145 (1) 111–192	77 (1) 56–103	NP
29	155 (2) 128–189	101 (2) 85–120	93 (2) 78–111
30	NA	70 (1) 54–89	102 (4) 86–120
All estimates	138 (14) 129–148	85 (12) 79–92	95 (20) 89–100

NP, Log dose-response lines were non-parallel.

mean (95% fiducial limits) of 94·8 (90·2–99·6) IU of ISU per ampoule of 92/510.

Calibration by *in vitro* bioassays and receptor assays

Laboratory geometric mean estimates are given in Table 5 and individual estimates are shown in Figs 1–3.

Estimates by receptor assays for 92/512, with an unweighted geometric mean of laboratory means (95% limits) of 80 (74 to 86) IU per ampoule, were markedly smaller than those obtained by *in vivo* bioassay. For both of the rFSH preparations 92/642 and 92/510, estimates by receptor assays were significantly larger than those obtained by *in vivo* bioassays, namely 167 (150–185) IU per ampoule for 92/642 and 121 (107 to 138) IU per ampoule for 92/510. Estimates by *in vitro* bioassays varied widely with overall means similar to those obtained by receptor assays. Because of the large intra-laboratory variability, the extent to which the estimates by *in vitro* bioassay in the different laboratories reflect inter-laboratory differences is not clear. For each calibration, the inter-laboratory variability for estimates by *in vitro* bioassay tends to be larger than the pooled intra-laboratory variability, with the largest for the calibration of rFSH preparation coded 92/510.

Calibration by immunoassays Laboratory mean geometric estimates are given in Table 6 and individual estimates are shown in Figs 1–3. For each calibration, estimates by immunoassay were significantly smaller than estimates by *in vivo* bioassay with overall estimates (95% limits) for 92/512, 92/642 and 92/510 of 74 (71 to 76), 107 (101 to 113) and 66 (62 to 70) IU of ISU per ampoule. The ratio of *in vivo* bioactivity to the overall

mean immunoreactivity was 1·7 for 92/512, 1·29 for 92/642 and 1·44 for 92/510.

For calibration of each of the three ampouled preparations, over all immunoassays or over one- and two-site immunoassays separately, the inter-laboratory variability was larger than the pooled intra-laboratory variability. For two-site immunoassays and for all immunoassays considered together, the inter-laboratory variability for calibration of 92/512 was less than half that for calibration of 92/510 or 92/642. Estimates by one-site assays were less, for each calibration, than estimates by two-site assays with the difference being least for calibration of 92/512. (One-site estimates were 5, 15 and 12% less than estimates from two-site assays for 92/512, 92/642 and 92/510 respectively.)

Comparison of the IS for human rFSH in ampoules coded 92/642 and a preparation of human rFSH in ampoules coded 92/510 with the IS for human urinary FSH (urofollitropin) coded 92/512

Comparisons by *in vivo* bioassay The IS for human rFSH was compared directly with the IS for FSH, human, urinary in 15 *in vivo* bioassays (Table 7 and Fig. 4). One of these estimates contributed significantly to the heterogeneity chi-squared value both within the laboratory in which it was obtained, and over all estimates, and has been deleted. The remaining 14 estimates did not differ significantly from homogeneity and had a weighted geometric mean (95% limits) of 138 (129–148) IU of the IS for FSH, human urinary per ampoule. This value is virtually identical to the value obtained by calibration of the IS for human rFSH in terms of the ISU.

Figure 4 Individual estimates of the FSH content for ampoules coded 92/642 expressed as IU of the IS for urinary FSH (in ampoules coded 92/512) per ampoule of 92/642. Codes and shading are as in Fig. 1. The arrowed number shows the weighted geometric mean of the estimates by bioassay.

The rFSH in ampoules coded 92/510 was compared directly with the IS for human urinary FSH in 16 *in vivo* bioassays (Table 7 and Fig. 5). Three of these assays showed significant deviations from parallelism and were deleted. Further deletion of one estimate, which contributed most greatly to the heterogeneity chi-squared value, gave a group of estimates which did not differ significantly from homogeneity, and which had a weighted geometric mean (95% limits) of 85 (79 to 92) IU of the IS for human urinary FSH per ampoule. This is similar to the value obtained by calibration of this preparation in terms of the ISU, although being based on only half the number of assays, may be less reliable.

Comparisons by *in vitro* bioassays and receptor assays Estimates obtained by direct comparisons of 92/642 and 92/510 with the IS for human urinary FSH are shown in Table 8 and Figs 4 and 5. Overall estimates by both *in vitro* bioassays and receptor assays are some two times larger than those obtained by *in vivo* bioassay using the IS for human urinary FSH as standard. These estimates

are also larger, by a factor of about 1.5, than estimates obtained by calibration of these preparations by *in vitro* bioassays or receptor assays in terms of the ISU. The inter-laboratory variability for comparisons by *in vitro* bioassay of 92/642 with the IS for human urinary FSH is not larger than the intra-laboratory variability. However, comparisons of 92/510 with the IS for human urinary FSH show somewhat less inter-laboratory consistency. There are receptor assays in only two laboratories for the latter comparison. However, comparison of 92/642 with the IS for human urinary FSH gave a larger estimate in receptor assays using CHO cells expressing rFSH receptors than in assays using the classical calf testis membrane assay.

Comparisons by immunoassays Estimates obtained by direct comparisons of 92/642 and 92/510 with the IS for human urinary FSH are shown in Table 9 and Figs 4 and 5. These estimates are significantly larger than those obtained by immunoassay using the ISU as standard and are also larger than estimates obtained by *in vivo* bioassay for these comparisons. Excluding laboratories 30

Figure 5 Individual estimates of the FSH content for ampoules coded 92/510 expressed as IU of the IS for urinary FSH (in ampoules coded 92/512) per ampoule of 92/510. Codes and shading as in Fig. 1. The arrowed numbers show the weighted geometric mean of estimates by *in vivo* bioassay for 92/510 as IU of 92/512 (85) and, for comparison, 92/510 as IU of the ISU (95).

and 8, the inter-laboratory variability for either comparison is slightly more variable than the intra-laboratory variability.

Comparison of a preparation of human rFSH in ampoules coded 92/510 with the IS for human rFSH

Comparisons by *in vivo* bioassay The rFSH preparation in ampoules coded 92/510 was compared directly with the IS for human rFSH in 22 *in vivo* bioassays (Table 7 and Fig. 6). Two of these assays showed significant deviations from parallelism and were deleted. The remaining 20 estimates did not differ significantly from homogeneity and had a weighted geometric mean (95% limits) of 95 (89–100) IU of the IS for human rFSH per ampoule. This is virtually identical to the value obtained by calibration of this preparation in terms of the ISU and similar to the value obtained by comparison of this preparation with the IS for human urinary FSH.

Comparisons by *in vitro* bioassays and receptor assays

Estimates obtained by direct comparisons of 92/510 with the IS for human rFSH are shown in Table 8 and Fig. 6. Overall estimates by both *in vitro* bioassays and receptor assays are larger than those obtained by *in vivo* bioassay using the IS for human rFSH as standard. However, these estimates are similar to estimates obtained by calibration of this preparation by *in vitro* bioassays or receptor assays in terms of the IS for human urinary FSH. The inter-laboratory variability for this comparison was not significantly larger than the intra-laboratory variability.

Comparisons by immunoassay

Estimates obtained by direct comparisons of 92/510 with the IS for human rFSH are shown in Table 9 and Fig. 6. These estimates are about 10% smaller than those obtained by *in vivo* bioassay using the IS for human rFSH as standard, and about 30% larger than those obtained by immunoassay using the IS for human urinary FSH as standard. With the previously

Table 8 Unweighted laboratory geometric mean estimates (gms) with 95% fiducial limits obtained by *in vitro* bioassays and receptor assays for the FSH content of the IS for rFSH in ampoules coded 92/642, and of a preparation of rFSH in ampoules coded 92/510, expressed as IU of the IS for purified urinary FSH per ampoule, and for the FSH content of a preparation of rFSH in ampoules coded 92/510, expressed as IU of the IS for human rFSH per ampoule. The number of independent estimates contributing to each mean estimate is shown in parentheses

	92/642 (as IU of 92/512)	92/510 (as IU of 92/512)	92/510 (as IU of 92/642)
Laboratory number			
<i>In vitro</i> bioassays – Sertoli cells			
9	262 (4) 145–476	209 (4) 84–521	110 (4) 78–156
10	283 (3) 100–800	607 (3) 295–1250	208 (6) 91–473
26	217 (1)	NA	NA
28	310 (4) 191–504	256 (4) 85–777	114 (4) 58–223
29	214 (2) 29–1600	NA	NA
<i>In vitro</i> bioassays – various cells			
7 (Y1)	235 (2) 163–338	167 (2) 142–198	98 (2) 81–120
8 (Granulosa)	209 (5) 166–263	163 (2) 134–197	111 (2) 49–251
9 (L)	255 (2) 153–425	226 (2) 4–10 000	122 (2) 2–10 000
12 (Human)	206 (3) 142–299	184 (3) 65–525	123 (3) 62–246
29 (CHO)	256 (2) 234–279	NA	NA
Geometric mean of laboratory gms	243 (10) 220–268	233 (7) 153–354	123 (7) 99–154
Receptor assays – calf			
9	224 (3) 199–251	175 (3) 137–223	108 (3) 79–148
10	243 (2) 166–355	179 (2) 76–422	100 (3) 89–113
29	270 (2) 170–427	NA	NA
Receptor assays – CHO			
29	277 (2) 228–338	NA	NA
Geometric mean of laboratory gms	253 (4) 216–296	177 (2) 151–208	104 (2) 64–169

noted exclusions, the inter-laboratory variability for the comparison by immunoassay of 92/510 and the IS for human rFSH was only marginally more variable than the inter-laboratory variability and similar to that obtained for the comparisons of the IS for human rFSH and the IS for human urinary FSH.

Accelerated thermal degradation studies of the candidate standards

Samples of each of the ampouled purified preparations of FSH which had been stored at elevated temperatures for 1–2 years (ATD samples) were compared within the same assay with ampoules of the same preparation which had been stored continuously at – 20 °C. The ATD samples of 92/642 did not differ significantly from the samples stored at – 20 °C in two *in vivo* bioassays. Estimates of the activity of ATD samples of both 92/510 and 92/512 relative to samples stored continuously at – 20 °C in two to four bioassays showed a loss of about 30% for samples stored at 45 °C and a loss of 10–15% for samples stored at 20 °C. Based on these results and using an Arrhenius equation for prediction, predicted yearly losses of activity at – 20 °C for either of these two preparations would be less than 0.1%.

For each of the three ampouled preparations, estimates by *in vitro* bioassays for the ATD samples and the samples

stored continuously at – 20 °C did not differ significantly from one another relative to the intra-assay and intra-laboratory variability.

Estimates by immunoassays and receptor assays for ATD samples of each of the three ampouled preparations differed significantly between different assay systems, although estimated losses of activity were generally less than those obtained by *in vivo* bioassays except for 92/642, and not detectable by some assay systems. This indicates that these various assay systems show significantly different specificities for the degraded and less biologically active FSH.

On the basis of these results, each of the ampouled preparations appears to be sufficiently stable to serve as a standard.

Discussion

Separate standards for FSH from pituitaries and from post-menopausal urine were established in the 1960s on the basis that gonadotrophins extracted from these two sources differed from one another, although the unitages for each were derived from a common preparation. The ISU is the standard in current use for calibration by bioassay of therapeutic products. However, the development and therapeutic use of high purity and

Table 9 Unweighted laboratory geometric mean estimates (gms) for the FSH content of the IS human rFSH in ampoules coded 92/642, and of a preparation of rFSH in ampoules coded 92/510 obtained by immunoassay, expressed as IU of the IS for human urinary FSH in ampoules coded 92/512 per ampoule and of the preparation of rFSH in ampoules coded 92/510 expressed as IU of the IS for human rFSH per ampoule. The number of independent estimates contributing to each mean estimate is shown in parentheses beside the laboratory code where there were the same number of estimates for each preparation, and otherwise beside the mean estimate. The 95% fiducial limits for the unweighted geometric mean of laboratory mean estimates is based on the variance of the logarithms of the estimates combined

	92/642 (as IU of 92/512)	92/510 (as IU of 92/512)	92/510 (as IU of 92/642)
Laboratory number			
Two-site immunoassays			
1 (2)	189	124	90
2 (2)	177	115	90
5 (3)	173	113	90
6A (2)	199	107	74
6B (2)	174	106	84
7 (4)	185	113	84
9 (2)	161	NA	NA
15 (4)	180	118	90
20			
21 (2)	196	114	80
27 (2)	174	102	81
28A (3)	172	100	81
28B (3)	183	107	80
29A (2)	169	111	91
29B (2)	189	128	93
30A (2)	129*	85*	90*
30B	142* (5)	97* (2)	88* (5)
Geometric mean of laboratory gms	173 (16) 163–184	109 (15) 103–115	85 (15) 83–89
Omitting estimates marked*	180 (14) 174–186	112 (13) 107–117	85 (13) 82–89
One-site immunoassays			
2 (2)	173	123	98
3A (2)	166	108	90
3B (3)	174	109	87
6 (2)	159	92	80
8 (2)	101*	55*	76*
17 (2)	166	105	87
19 (2)	155	94	84
22 (4)	163	100	85
28 (3)	163	111	94
Geometric mean of laboratory gms	156 (9) 137–177	98 (9) 82–117	86 (9) 81–92
Omitting estimates marked*	165 (8) 159–170	105 (8) 97–113	88 (8) 83–93
One-site and two-site immunoassays			
Geometric mean of laboratory gms	167 (25) 157–177	104 (24) 97–112	86 (24) 83–88
Omitting estimates marked*	174 (22) 169–179	109 (21) 105–113	86 (21) 84–89

rDNA-derived preparations of FSH has led to a review of standards for therapeutic FSH preparations. This study has provided comparisons of the ISU with candidate preparations of high purity urinary FSH and of rFSH.

Calibration of candidate IS by in vivo bioassay

Some 25 comparisons by *in vivo* bioassay for each of the candidate preparations with the ISU gave assays which were broadly statistically valid and estimates of relative potency which, for each comparison, were homogeneous, with a maximum of two heterogeneous estimates for any comparison. Thus, for each candidate standard, valid

calibration by *in vivo* bioassay in terms of the ISU was obtained, giving for 92/512 a weighted geometric mean (95% fiducial limits) of 121 (113–128) IU per ampoule, for 92/642 a weighted geometric mean of 138 (131–145) IU per ampoule, and for 92/510 a weighted geometric mean of 95 (90–100) IU per ampoule.

These results are consistent with studies which have found that the *in vivo* biological activity of rFSH (De Boer & Mannaerts 1990, Galway *et al.* 1990), and its pharmacokinetic and pharmacodynamic characteristics (Porchet & le Cotonnec 1994, Weinbauer *et al.* 1994) are similar to those of urinary FSH, and that rFSH gave a dose-response curve in the Steelman–Pohley bioassay which

Figure 6 Individual estimates of the FSH content for ampoules coded 92/510 expressed as IU of the IS for FSH (in ampoules coded 92/642) per ampoule of 92/642. Codes and shading as in Fig. 1. The arrowed number shows the weighted geometric mean of the estimates by bioassay.

was parallel to a menotrophin standard and linear in the same part of the curve. Thus clinical protocols and dosage regimens similar to those for urinary FSH have been proposed for rFSH (Porchet & le Cotonnec 1994).

Since rFSH preparations appear to behave overall in a manner similar to preparations derived from urinary sources and in the interests of maintaining continuity of unitage from one standard to another and hence from one therapeutic product to another the IS for rFSH was calibrated directly in terms of the IS for menotrophin.

Comparisons by *in vitro* bioassay

In the present study estimates of activity by *in vitro* bioassays showed significant differences between laboratories. The variability of estimates obtained from *in vitro* bioassays may reflect the selectivity or specificity of these assays which in general select only one action of

FSH as a measurable end point. A number of studies have compared *in vitro* bioactivity, or structural features of rFSH with preparations derived from natural sources. Thus rFSH has been found to have similar activity to natural FSH in some *in vitro* assay systems (Galway *et al.* 1990, Mannaerts *et al.* 1991, Mason *et al.* 1993) and to be more potent in other assay systems (Flack *et al.* 1994, Lambert *et al.* 1995). Results obtained here using receptor binding assays were more consistent between laboratories than those from *in vitro* bioassays but there were a limited number of systems used. However, it may be that receptor binding and signal transduction are dissociable functions which require separate sites of the FSH molecule (Valove *et al.* 1994). Thus while receptor binding may require properties of FSH which are consistent between different preparations, *in vitro* bioassays may determine properties which are variable between different preparations.

Comparison by immunoassay

Estimates of activity by immunoassay differed consistently and significantly between laboratories indicating that immunoassays may have different molecular specificities. Numerous publications have highlighted the variability between different immunoassay systems (Seth *et al.* 1989, Taylor *et al.* 1994) which may arise from differences in specificity or affinity of different antibodies for standard materials or test samples (Seth *et al.* 1989, Jockenhovel *et al.* 1990), different standard preparations used in different assays and different reference preparations used for calibrating kit standards. Thus even if a highly purified standard were used for calibration of diagnostic immunoassay some variability in estimates of activity in clinical samples could be expected.

The *in vivo* bioassays, which are specified by the Pharmacopoeia for calibration of therapeutic products, did not show consistent inter-laboratory differences between estimates for any of the comparisons considered in this study. However, estimates obtained by *in vitro* bioassay and immunoassay varied depending on the assay system and on the preparation used as standard. Some of these latter assay systems appeared to distinguish between rFSH preparations, for example. The ratio of biological activity to immunological activity for *in vivo* bioassays was about half that for *in vitro* bioassays for some comparisons. Thus assays which do not take account of biological clearance in the same way may give significantly different estimates of biological activity. The inter-laboratory variabilities for both *in vitro* bioassays and immunoassays suggest that the ratio of biological activity to immunological activity is not a stable parameter.

Conclusions

The standards describe here enable calibration of therapeutic products derived from highly purified urinary and from pure rFSH on the basis of the underlying principle of comparing 'like with like'. There is a discontinuity of unitage between the calibration of all the candidate standards by immunoassays and by *in vivo* biological assays. Studies of the calibration of FSH standards for diagnostic immunoassays will be considered separately.

Based on the results of this study the Expert Committee on Biological Standardization of WHO established the preparation in ampoules coded 92/512 as the IS for urinary FSH (urofollitropin) with an assigned potency of 121 IU per ampoule on the basis of its calibration by *in vivo* bioassay in terms of the ISU.

The preparation coded 92/642 was established as the IS for human rFSH and assigned a value of 138 IU per ampoule on the basis of its calibration by *in vivo* bioassay in terms of the ISU. This unitage maintains a continuity of activity with ISU used to calibrate urinary-derived

therapeutic materials. The unitages assigned to 92/512 and 92/642 are also consistent with their direct calibration by *in vivo* bioassay in terms of one another. The ISU was retained as a standard for bioassay of urinary FSH and LH (menotrophin). All of the above standards were deemed suitably stable to act as WHO IS. They have been calibrated by the *in vivo* bioassay and are intended as standards for the calibration of such assays.

These standards are available from the National Institute for Biological Standards and Control (Fax +(44) 1707 646977; Tel +(44) 1707 646399; email standards@nibsc.ac.uk; mail NIBSC, PO Box 1193, Blanche Lane, Potters Bar, Hertfordshire EN6 3QG, UK).

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