The fourth International Standard for Human Urinary FSH and LH: specificities of LH seminal vesicle weight gain assays in the collaborative study differ between laboratories

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Abstract

The fourth International Standard for Human Urinary FSH and LH (IS; in ampoules coded 98/704) was compared with the third International Standard for Urinary FSH and LH (IS 71/264) by 10 laboratories in nine countries, using FSH and LH *in vivo* bioassays.

Estimates of the FSH content of the IS by augmented ovarian weight gain assays were homogeneous within each laboratory and over all laboratories. The combined weighted geometric mean estimate of FSH content of the IS (with 95% fiducial limits) in terms of IS 71/264 was 71.9 (69.0–74.9) IU/ampoule.

Although estimates by seminal vesicle weight gain (SVW) assays of the relative LH activities of the IS and IS 71/264 were homogeneous within laboratories, estimates were heterogeneous between laboratories. This indicated differences between the spectrum of LH isoforms in the IS and IS 71/264, which were obtained from different manufacturers, and differences between the specificities of SVW assays performed in different laboratories. The differences between the specificities of SVW assays appeared to be related to interactions among mean laboratory seminal vesicle weights, age and genetic strain

of rat. The finding of inter-laboratory differences in the specificities of SVW assays is of some significance, as this assay method has been generally adopted by Pharmacopoeias for the control of the LH content of therapeutic products. The combined unweighted geometric mean estimate of LH content of the IS (with 95% fiducial limits) in terms of IS 71/264 by SVW and ovarian ascorbate depletion assays was 70.2 (61.7–80.0) IU/ampoule.

Estimates of the FSH and LH content of ampoules of the IS kept at increased temperatures suggested that the IS would be adequately stable under normal storage conditions.

On the basis of these results, the World Health Organization Expert Committee on Biological Standardization established the preparation in ampoules coded 98/704 as the fourth International Standard for Human Urinary FSH and LH, and assigned to the contents of each ampoule an activity of 72 International Units of urinary FSH and an activity of 70 International Units of urinary LH.

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Introduction

Menotrophin and urinary and recombinant DNA-derived follicle-stimulating hormone (FSH) are widely used in the treatment of infertility in women, and sometimes also in men. The FSH and luteinizing hormone (LH) contents of these therapeutic products are controlled by *in vivo* bioassays. This is because the intrinsic heterogeneity of these gonadotrophins, and the degree of purity of the urinary-derived products, has until now precluded their control by physicochemical methods or by *in vitro* assays. Thus highly purified preparations of FSH and LH have been shown to differ in their isoform compositions (Zaidi *et al.* 1982a, b) and in their biological and immunological properties (Storrington *et al.* 1981, 1982). Bioassays used to control these

gonadotrophin therapeutic products have been calibrated in terms of the third International Standard for Urinary FSH and LH (in ampoules coded 71/264; IS 71/264), which was established by the World Health Organization (WHO) in 1993 (WHO Expert Committee on Biological Standardization (ECBS) 1994). The FSH content of menotrophin and of urinary FSH have also been estimated in terms of the first International Standard for Urofollitropin, which had been calibrated in terms of IS 71/264 and established in 1995 (Rose & Gaines Das 1998, WHO ECBS 1998).

When stocks of IS 71/264 became depleted, the WHO ECBS requested the National Institute for Biological Standards and Control (NIBSC) to obtain and evaluate a replacement international standard (WHO ECBS 2000).

A suitable preparation of menotrophin was therefore obtained and distributed into ampoules to become the fourth International Standard for Human Urinary FSH and LH (IS).

This paper describes the IS and its comparison with the IS 71/264 in an international collaborative study by FSH and LH *in vivo* bioassays. The study provided evidence that the IS was suitable to replace the IS 71/264 as a standard for the bioassay of human urinary FSH and LH, and provided the calibration data used to assign the activities of the IS. The results of the study also showed unexpected between-laboratory differences in the specificities of seminal vesicle weight gain (SVW) assays, which are used to control the LH content of therapeutic products.

Participants

The ten laboratories from nine countries, which took part in the study, are listed below. Throughout this report each participant has been identified by a number between 1 and 10, but these numbers do not relate to the order of listing which is alphabetical by country:

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Materials

The second International Standard for Urinary FSH and LH (IS 71/223) and the third International Standard for Urinary FSH and LH (IS 71/264)

IS 71/223 and IS 71/264 consist of batches of ampoules coded 71/223 and 71/264, respectively, which contain an

extract from the urine of post-menopausal women. Both of these standards were prepared from the same batch of master ampoules, using the same procedure as for the first International Standard for Human Urinary FSH and LH for Bioassay (in ampoules coded 70/45; IS 70/45), and both were included in the collaborative study of that standard (Storrington *et al.* 1976). Subsequent supplementary studies confirmed the activity and stability of IS 71/223 (WHO ECBS 1989) and of IS 71/264 (WHO ECBS 1994).

In 1988, when stocks of IS 70/45 were becoming depleted, the WHO ECBS established the material in ampoules coded 71/223 as the second International Standard for Urinary FSH and LH, and assigned to the contents of each ampoule the same activities as had been assigned to each ampoule of IS 70/45, namely an activity of 54 International Units of urinary FSH and 46 International Units of urinary LH (WHO ECBS 1989).

In 1993, when stocks of the second International Standard were becoming depleted, the WHO ECBS established the material in ampoules coded 71/264 as the third International Standard for Urinary FSH and LH, and assigned to the contents of each ampoule an activity of 54 International Units of urinary FSH and 46 International Units of urinary LH (WHO ECBS 1994).

The IS 71/223, which had been discontinued in 1993 with the establishment of IS 71/264, was included in the study to supplement the available stocks of IS 71/264 for the calibration of the IS.

The fourth International Standard for Human Urinary FSH and LH (IS)

This consists of a batch of ampoules (coded 98/704) containing an extract from the urine of post-menopausal women.

Bulk FSH/LH This consisted of approximately 5.8 g of menotrophin (Batch No. 61662697), extracted from the urine of post-menopausal women, and generously donated to WHO by the Instituto Massone, Buenos Aires, Argentina. It was stated to have an FSH specific activity of 86.2 IU/mg by augmented ovarian weight gain assay and an LH specific activity of 82.7 IU/mg by SVW assay.

Distribution into ampoules The IS was prepared at NIBSC in November 1998. Some 5.8 g of the bulk menotrophin was dissolved in 6000 ml of 0.5% (w/v) lactose. The solution was passed through a 0.45 µm membrane filter (Sartobran P, Sartorius), and distributed into ampoules as approximately 1.0 ml aliquots. The solution of menotrophin was kept at 4 °C throughout. The ampoule contents were freeze-dried, secondarily desiccated and sealed under nitrogen (Campbell 1974, WHO ECBS 1978). The IS consisted of 5672 ampoules. The mean weight of filling solution in 113 weighed

ampoules was found to be 1.006 g, with a coefficient of variation of 0.13% and a range as a percentage of the mean of 1.09. Each ampoule of the IS contains about 0.967 mg of the extract of human menopausal urine and 5 mg of lactose.

Activity of ampoule contents Estimates of the FSH and LH activities of the IS and of the bulk menotrophin by FSH and LH *in vivo* bioassays at NIBSC suggested that there had been no significant loss of FSH or LH activity during the distribution of the bulk menotrophin into ampoules.

Accelerated thermal degradation samples of the IS

Ampoules of the IS which had been kept at +20 °C, +37 °C or +45 °C for 320 days were coded, and were also included in the study.

Methods

Design of the study

Participants were asked to contribute *in vivo* bioassays which were, as far as possible, specific for FSH and LH. In particular they were asked, if possible, to contribute FSH estimates by the augmented ovarian weight gain (AOW) method (Steelman & Pohley 1953), and LH estimates by the seminal vesicle weight gain (SVW) method (Van Hell *et al.* 1964) and the ovarian ascorbate depletion (OAA) method (Parlow 1961). Participants were asked to assume an FSH potency of 70 IU/ampoule and an LH potency of 60 IU/ampoule for assays of the IS and its accelerated thermal degradation samples. Participants were asked to provide full results of their assays, including all raw data in addition to their own calculated estimates of potency.

Statistical analysis

For each assay for which the final body weights were reported, these variables were assessed by analysis of variance for any significant differences among treatment groups. As far as possible for each assay, four response metameters – namely organ weight, log of organ weight, organ weight relative to final body weight and log of organ weight relative to final body weight – were calculated and assessed for heterogeneity of variances and statistically significant outliers (using an in house program, SCAN, Gaines Das & Rice 1985). Data for all assays using each of the available response metameters were also analysed as multiple parallel-line assays (see, for example, Finney 1978). The response metameter used for the reported analysis is that which, for the contributed assays, gave the more homogeneous variances and greatest precision (assessed as λ = the ratio of the slope of the log

dose–response metameter line to the pooled standard deviation of the responses). Estimates of potency for calibration of any preparation in terms of another have been calculated from direct analysis as a parallel-line assay of data for the preparation calibrated and the standard in terms of which it is calibrated, except where two thermally accelerated degradation samples were included with the IS in the same assay, when they were analysed as a multiple parallel-line assay. Estimates from assays for which the deviations from linearity or parallelism were significant ($P < 0.01$ or $P < 0.05$) have been separately considered, and geometric mean potencies have been obtained both including and excluding these estimates. For each potency estimate, a statistical weight has been determined as the reciprocal of the variance of the $\log_{10}$ of the estimate.

Estimates of potency have been assessed for homogeneity using the χ^2 test. Homogeneous estimates have been combined as weighted geometric means. Estimates that differed significantly from homogeneity ($P < 0.05$) have been combined as unweighted geometric means, and the variance of these estimates has been calculated using the variance of the log estimates combined.

For the accelerated thermal degradation studies, predicted losses of activity at -20 °C were calculated from degradation rates estimated assuming a linear relationship between log of degradation rate and reciprocal of absolute temperature (Jerne & Perry 1956, Kirkwood 1977).

Results

Assays contributed

The 10 participants contributed results from a total of 56 assays: 29 AOW assays, 25 SVW assays and two OAA assays. Eight of the 10 participants contributed final weights of animals together with organ weights (ovary for AOW assays or seminal vesicle for SVW assays), although laboratory 9 contributed body weights that could not be related unambiguously to the organ weights. The remaining two laboratories contributed organ weights only. As far as could be determined, assay designs were completely randomized, except in laboratory 3, where a block design was used. The data from laboratory 3 have been analysed as if a completely randomized design had been used, as the relatively small effect attributable to blocks did not substantially affect the precision of estimates.

Assessment of body weights and selection of response metameters

In the majority of assays for which data were available (five participants), initial and final weights were significantly

Table 1 Overall laboratory means of final weights of animals used for augmented ovarian weight gain assays, together with the coefficient of variation (CV) expressed as per cent of these. The response metameter used for the reported analysis is also given

	Rat strain (age in days)	Body weight (g)	CV of body weight (%)	Organ weight (mg)	CV of organ weight (%)	Response metameter
Laboratory						
1	CH-BB Thom (20–23)	76	5	121	32	Log ratio†
2	Wistar (24–30)	103	7	133	30	Log organ weight
3	Lewis (21–28)	NA	—	111	32	Log organ weight
4	Sprague– Dawley (21–23)	51	8	93	39	Log ratio†
5	Lewis (20–21)	NA	—	146	27	Log organ weight
6	Wistar (23–26)	63	12	95	45	Log ratio†
7	Wistar (19–22)	49	9	124	27	Log ratio†
8	Sprague– Dawley (24)	74	8	118	20	Log ratio†
9	Sprague– Dawley CrI:CD (21–22)	67	6	96	32	Log organ weight
10	Wistar (20–22)	54	9	90	35	Log ratio†

NA, Not available.
†Denotes log of the ratio of organ weight to body weight.

correlated ($P>0.01$, correlation coefficient generally about 0.8–0.9), and the change in weight over the course of the assay showed no significant relations to the treatment (preparation or dose). The majority of participants who used body weights in their own analysis used the final weight, and further analysis has been carried out using final weights.

Body weights within each assay were homogeneous ($P>0.05$) except in two assays. In one AOW assay of laboratory 1, apparent heterogeneity resulted from a single outlying weight in one group, which was omitted from all further analysis. In one AOW assay of laboratory 2, variances differed significantly between different groups; there was no consistent pattern, and it has been assumed that this was an artefact of assignment of animals to groups.

Final body weights within each assay did not generally show any significant difference between treatment groups (analysis of variance, $P>0.1$). Nevertheless, slopes of log dose–final body weight lines were calculated for each preparation in each assay in the study. The individual slopes were not significantly different from 0. Considered over all SVW assays, the values of the slopes for IS 71/223,

IS 71/264 or the IS did not differ significantly ($P>0.3$) from 0. Considered over all AOW assays, the values of the slopes for IS 71/223 and IS 71/264 also did not differ significantly from 0. However, there was a slight overall tendency for the values of slopes for the IS to be negative (14/21 negative and 7/21 positive; Student’s t -test, $P\sim 0.16$). Whether this small difference would be reproducible and, if so, whether it reflects some characteristic of the IS or artefacts of assay design cannot be determined from these data.

Of the four response metameters, log transformation of either organ weight or ratio of organ weight to body weight gave markedly less heterogeneous groups than the untransformed variables in the majority of assays. For any one assay, values of λ were generally not very different for the four response metameters (most values of λ being within 10% of one another).

The overall laboratory means of final body weight and organ weight for the AOW and SVW assays, together with the response metameters used for the reported analysis are shown in Tables 1 and 2. The mean organ weights are broadly comparable between laboratories, because the

Table 2 Overall laboratory means of final weights of animals used for seminal vesicle weight gain assays and of organ (seminal vesicle) weights, together with the coefficient of variation (CV) expressed as per cent of these. The response metameter used for the reported analysis is also given

	Rat strain (age in days)	Body weight (g)	CV of body weight (%)	Organ weight (mg)	CV of organ weight (%)	Response metameter
Laboratory						
1	CH-BB Thom (20–23)	83	8	35	26	Log ratio†
2	Wistar (24–30)	122	5	49	36	Log organ weight
3	Lewis (21–28)	NA	—	46	26	Log organ weight
4	Sprague– Dawley (26–28)	81	4	50	36	Organ weight
5	Sprague– Dawley (20–21)	NA	—	53	26	Log organ weight
6	Wistar (21–24)	55	13	14	32	Log ratio†
7	Wistar (19–22)	48	10	20	25	Ratio
8	Sprague– Dawley (23)	87	8	47	31	Log ratio†
9	Sprague– Dawley CrI:CD (21–22)	76	7	31	26	Log organ weight
10	Wistar (20–22)	63	9	16	33	Log ratio†

NA, Not available.

†Denotes log of the ratio of organ weight to body weight.

majority of assays comprised three doses of each preparation, with broadly similar ratios between largest and smallest doses and equal numbers of animals at each dose. For the FSH assays, the correlation of laboratory mean ovary weight and laboratory mean body weight was marginal ($r=0.6$, $P \sim 0.1$) and there was no significant correlation of ovary weight and age; there was significant correlation ($r=0.8$, $P \sim 0.02$) of laboratory mean body weights and age. The rats used for these FSH assays did not show any significant difference between strains for body or organ weight after allowance for age differences. For the LH assays, the correlation of laboratory mean seminal vesicle weight and laboratory mean body weight was significant ($r=0.8$, $P \sim 0.01$), and the correlation of laboratory mean seminal vesicle weight and age was marginal ($r=0.6$, $P \sim 0.1$); laboratory mean body weight and age were correlated ($r=0.7$, $P \sim 0.05$). Among the animals used for these LH assays, the Sprague–Dawley rats were significantly heavier than the Wistar rats, with the difference more marked for the older rats, and the seminal vesicle weights of those groups of rats with an average age

of less than 24 days were significantly ($P < 0.01$) larger for Sprague–Dawley than for Wistar rats.

Data for all assays were assessed using the selected response metameter. Responses were homogeneous ($P > 0.05$) for all except eight of the 56 assays. These eight assays were further examined. In three of these assays there were either one or two markedly anomalous responses, which when deleted gave homogeneous groups of responses and were included as such for further analysis. In the remaining five assays there was no apparent reason for the observed heterogeneity. However, there was insufficient reason to reject these data, because these five assays neither contained any anomalous individual responses or groups of responses, nor gave any other evidence of invalidity. Such otherwise unexplained heterogeneity of responses can be an artefact of assay design.

Comparison of IS 71/223 and IS 71/264

FSH activity Comparisons of the FSH activities of these two preparations by AOW assays in two earlier

Table 3 Estimates of the LH content of the second International Standard for Urinary FSH and LH (IS 71/223) in terms of the third International Standard for Urinary FSH and LH (IS 71/264). The number of estimates is given in parentheses

Assay method	Laboratory	LH content (as IU of IS 71/264 per IU of IS 71/223)	
		Geometric mean	95% fiducial limits
Seminal vesicle weight gain	2	0.94 (1)	0.64–1.35
	3	1.08 (1)	0.96–1.22
	4	1.12 (1)	0.83–1.52
	6	0.99 (1)	0.71–1.39
	7	1.03 (1)	0.85–1.26
		1.06 (5)	0.97–1.15
		1.03 (5)	0.94–1.12
Ovarian ascorbate depletion	3	0.76 (1*)	0.44–1.21
Both		1.04 (6)	0.96–1.14

*This estimate is from an assay with significant ($P \sim 0.02$) deviation from parallelism.

collaborative studies had shown that their FSH potencies per ampoule, and hence per assigned IU, were equivalent (Storring *et al.* 1976, Rose & Gaines Das 1998). In order to conserve stocks of IS 71/264, calibration of the IS by AOW assays was therefore carried out in terms of IS 71/223 and expressed in terms of IS 71/264.

LH activity Comparisons of the LH activities of these two preparations by *in vivo* bioassays in the earlier collaborative study had shown that their LH potencies per ampoule, and hence per assigned IU, were equivalent (Storring *et al.* 1976). However, this comparison was made some years ago, and therefore further direct comparisons of the LH activities of these two preparations have been made in this study in a limited number of assays, as shown in Table 3.

The estimates by SVW assay were homogeneous and gave a weighted geometric mean LH content for all assays (with 95% fiducial limits) of 1.06 (0.97–1.15) IU of IS 71/264 per IU of IS 71/223 and an unweighted geometric mean LH content for all assays (with 95% fiducial limits) of 1.03 (0.94–1.12) IU of IS 71/264 per IU of IS 71/223.

Table 4 Estimates of the FSH content of the fourth International Standard for Human Urinary FSH and LH in terms of the third International Standard for Urinary FSH and LH. The number of estimates is given in parentheses

Laboratory	FSH content (IU/ampoule)	
	Geometric mean	95% fiducial limits
1	77.1 (2)	66.6–89.3
2	68.6 (2)	55.1–85.3
3	73.0 (2)	67.3–79.2
4	65.8 (2)	51.4–84.4
5	73.9 (4) ^a	67.9–80.4
6	59.6 (3) ^{a,b}	48.4–73.3
7	67.7 (2)	58.8–77.9
8	74.2 (2)	61.6–89.4
9	68.7 (2)	58.7–80.4
10	74.2 (2) ^c	64.4–85.6
Weighted geometric mean	71.9 (23)	69.0–74.9
Weighted geometric mean ^d	71.6 (19)	68.5–74.9
Weighted geometric mean ^e	72.3 (25)	69.4–75.2

^aIncluding one estimate from an assay with marginally significant ($0.01 < P < 0.05$) deviations from linearity or parallelism.
^bExcluding two estimates from assays with significant ($P < 0.01$) deviations from parallelism.
^cIncluding two estimates from assays with marginally significant ($0.01 < P < 0.05$) deviations from linearity or parallelism.
^dExcluding estimates from four assays with marginally significant ($0.01 < P < 0.05$) deviations from linearity or parallelism^{a,c}.
^eIncluding the two deleted estimates from assays with significant deviations from parallelism ($P < 0.01$)^b.

These two preparations were also included in a single OAAD assay. In this comparison the OAAD assay showed non-parallelism of dose–response lines ($P \sim 0.02$). The reason for this is not known. However, this assay showed unexplained heterogeneity of responses and apparent outliers. Analysis appropriate to the block design showed significant interaction of blocks and regression. If the apparent statistical invalidity is ignored, the estimate obtained from this assay is not significantly different from 1, and is not heterogeneous in combination with estimates from the SVW assays. Furthermore, if this value were included, the weighted geometric mean estimate of LH content (with 95% fiducial limits) for all LH assays was 1.04 (0.96–1.14) IU of IS 71/264 per IU of IS 71/223, which is very similar to the estimate given by the combination of SVW assays alone.

As the remaining stocks of IS 71/264 were insufficient to carry out the complete calibration of the IS, calibrations in terms of IS 71/223 were used to supplement these data. For all these subsequent analyses, IS 71/223 and IS 71/264 have been treated interchangeably, and the results expressed in terms of IS 71/264. Where the two preparations were included in the same assay, responses have been combined and treated as the responses of a single preparation, to give maximum precision for other

Figure 1 Frequency distribution of estimates of the FSH content of the fourth International Standard for Human Urinary FSH and LH in terms of the third International Standard for Urinary FSH and LH by augmented ovarian weight gain assays in different laboratories. Each box denotes the estimate from an individual assay, and the number in the box identifies the laboratory.

comparisons. All but one laboratory provided at least one SVW assay estimate in terms of IS 71/223. All but two laboratories provided at least one SVW assay estimate in terms of IS 71/264. The IS 71/223 was used alone in 14 SVW assays, the IS 71/264 was used alone in four SVW assays, and both standards were used together in five SVW assays. One OAAD assay used the IS 71/223 alone, and the other OAAD assay used both standards together.

Calibration of the IS by FSH assays in terms of IS 71/264

Table 4 and Fig. 1 present the results from this calibration. Two assays from laboratory 6 showed statistically significant deviations from parallelism ($P < 0.01$). A further four assays (one each from laboratories 5 and 6, and both assays from laboratory 10) showed marginal deviations from linearity or parallelism ($P \leq 0.05$). Although there were no significant deviations from parallelism in the majority of assays, there was a tendency for the slope of the log dose–response line for the IS to be larger than that for 71/264 (16 of 23 pairs of slopes, excluding the two assays with significant deviations from parallelism at the 1% level). The overall mean of slope differences was significantly larger than 0 ($P \sim 0.02$, Student's *t*-test).

Estimates of the FSH content of the IS were homogeneous within each laboratory and over all laboratories. The combined weighted geometric mean estimate of FSH content of the IS (with 95% fiducial limits) was 71.9 (69.0–74.9) IU/ampoule (Table 4).

Calibration of the IS by LH assays in terms of IS 71/264

Results are presented in Table 5 and Fig. 2.

SVW assays Three SVW assays (one each from laboratories 1, 3 and 4) showed marginal deviations from linearity or parallelism ($P \leq 0.05$). There was a slight tendency for the slope of the IS to be larger than that of IS 71/264 (15 of 23 pairs of slopes). The overall mean of slope difference was marginally larger than 0 ($P \sim 0.11$, Student's *t*-test).

All estimates by SVW assays were homogeneous within laboratories. However, estimates by SVW assays combined over all laboratories were highly significantly ($P < 0.0001$) heterogeneous. The unweighted geometric mean of laboratory mean estimates of LH content of the IS (with 95% fiducial limits) by SVW assays was 71.8 (62.7–82.2) IU/ampoule (Table 5).

The modest difference between the slopes of the log dose–response lines of the IS and IS 71/264 in the SVW assays, not significant in the majority of assays, was not as great as that observed for the AOW assays, and seemed insufficient to account for the heterogeneity of estimates. However, the inter-laboratory heterogeneity of estimates in the presence of intra-laboratory homogeneity of estimates suggested differences between the specificities of SVW assay methods in different laboratories. In this context, it was noted that the larger estimates of potency appeared to come from assays in which larger overall seminal vesicle weights were observed (Fig. 3). The values

Table 5 Estimates of the LH content of the fourth International Standard for Human Urinary FSH and LH in terms of the third International Standard for Urinary FSH and LH. The numbers in parentheses refer to the number of estimates in the case of laboratory means or the number of laboratory means in the case of means of laboratory means

Assay method	Laboratory	LH content (IU/ampoule)	
		Geometric mean	95% fiducial limits
Seminal vesicle weight gain	1	69.8 (2)*	60.5–80.6
	2	69.0 (3)	59.3–82.4
	3	81.0 (2)*	74.8–87.7
	4	105.1 (2)*	88.7–124.6
	5	68.5 (2)	60.4–77.7
	6	60.5 (4)	52.3–69.9
	7	56.9 (2)	50.1–64.7
	8	89.4 (2)	71.9–112.2
	9	63.6 (2)	56.2–71.9
	10	64.8 (2)	55.5–75.5
Unweighted geometric mean of laboratory mean estimates		71.8 (10)	62.7–82.2
Unweighted geometric mean of laboratory means excluding estimates*		71.9 (10)	62.3–83.1
Ovarian ascorbate depletion	3	56.7 (2)	45.7–70.4
Both			
Unweighted geometric mean of laboratory mean estimates		70.2 (11)	61.7–80.0

*Including one estimate from an assay with marginally significant ($P \leq 0.05$) deviations from linearity or parallelism.

of the potency estimates by SVW assays were found to be significantly correlated with the laboratory mean seminal vesicle weight ($r=0.7$, $P \sim 0.02$), and with age ($r=0.7$, $P \sim 0.03$). It was noted that the two largest estimates of potency came from the two laboratories using Sprague–Dawley rats that were older than 22 days. Lewis rats, which were used in a single laboratory and which had an upper specified age range of 28 days, gave the third largest estimate of potency. These data were consistent with a strain-dependent effect on the specificity of SVW assays for rats older than 22 days. Although assay precision depends on many factors unique to each laboratory, precision of potency estimates also appeared to be greater in assays using Sprague–Dawley and Wistar rats that were younger than 22 days. This effect was not related to differences in the total number of animals used per assay, as shown by calculations of the statistical weight per animal.

The omission of laboratories 3, 4 and 8, which showed three of the four greatest mean laboratory seminal vesicle weights and the largest estimates of potency for the IS, gave log potency estimates for the IS in terms of 71/264 which were homogeneous ($P \sim 0.26$), and which combined to give a weighted geometric mean of laboratory mean estimates of LH content of 64.4 (61.1–67.8) IU/ampoule.

OAAD assays The estimates from the two OAAD assays, which were carried out in laboratory 3, were consistent with estimates from the SVW assays with smaller mean organ weights, and when combined with all estimates by SVW assay gave an unweighted geometric mean of laboratory mean estimates of LH content (with 95% fiducial limits) of 70.2 (61.7–80.0) IU/ampoule (Table 5). Omitting the SVW estimates from laboratories 3, 4 and 8 gave a combined weighted geometric mean of (homogeneous) laboratory mean estimates of LH content (with 95% fiducial limits) by SVW and OAAD assays of 63.9 (60.8–67.2) IU/ampoule.

Accelerated thermal degradation studies of the IS

Laboratory mean estimates of the FSH and LH content of ampoules of the IS kept for 320 days at 20 °C, 37 °C or 45 °C in terms of ampoules of the IS kept at – 20 °C are shown in Tables 6 and 7, respectively.

It was not possible for all samples to be tested in all laboratories. However, data from the limited number of laboratories testing several of the samples suggests that assays in the different laboratories may differently detect the apparent degradation of the IS.

AOW assays of laboratory 7 suggest that there has been, at most, a very slight loss of detectable FSH activity in any of the samples tested. In contrast, the AOW assays of laboratory 2 show a consistent significant loss of FSH activity with increasing temperature of storage except for an apparent increase in activity for the sample stored at 20 °C. Based on the overall geometric means of laboratory means, the predicted loss of FSH activity at – 20 °C is less than 0.1% per year.

The SVW assays of laboratory 7, as in the case of its AOW assays, suggest that there has been, at most, a very slight loss of detectable LH activity in any of the samples tested. In contrast the SVW assays of laboratory 2, as in the case of its AOW assays, show a consistent significant loss of activity with increasing temperature of storage except for an apparent increase of activity for the sample stored at 20 °C. The geometric means of laboratory means cannot be used to predict loss of LH activity, as there is no apparent change between samples stored at 37 °C and 45 °C. However, if the mean of the sample stored at 37 °C, namely 87% of activity remaining, is used with the ‘most common’ estimate for the sample stored at 45 °C

Figure 2 Frequency distribution of estimates of the LH content of the fourth International Standard for Human Urinary FSH and LH in terms of the third International Standard for Urinary FSH and LH by seminal vesicle weight gain assays (open boxes) and ovarian ascorbate depletion assays (shaded boxes) in different laboratories. Each box denotes the estimate from an individual assay, and the number in the box identifies the laboratory.

(78% of activity remaining) the predicted loss of LH activity at -20°C is also less than 0.1% per year.

Discussion

Comparison of IS and IS 71/264

The spectrum of FSH and LH isoforms present in the IS and in IS 71/264 are likely to show some differences, because these hormone preparations are from different

manufacturers. Such differences in FSH and LH isoform composition are probably the basis for the small, but consistent, differences observed between the slopes of the log dose-response lines for the IS and IS 71/264 in the

Table 6 Estimates of the FSH content of ampoules of the fourth International Standard for Human urinary FSH and LH (IS) kept for 320 days at 20°C , 37°C or 45°C , in terms of ampoules of the IS kept at -20°C . The number of estimates is given in parentheses and the 95% fiducial limits are in square brackets

Geometric mean FSH content (% of that of the IS kept at -20°C)			
Laboratory	IS kept at 20°C (coded C)	IS kept at 37°C (coded A)	IS kept at 45°C (coded B)
1		89 (1)	82 (1)
2	126 (2)	82 (3)	59 (2)
3		77 (1)	67 (1)
4	146 (1)	44 (1)	
6	86 (1)	93 (1)	53 (1)
7	99 (1)	95 (1)	92 (2)
10			60* (2)
Geometric mean of laboratory means	112* [77–163] (4)	80 [74–87] (6)	68* [54–84] (6)
Geometric mean of all individual estimates	110 (5)	80 (8)	68* (8)

*Unweighted geometric mean of heterogeneous estimates.

Figure 3 Comparison of laboratory mean estimates of the LH content of the fourth International Standard for Human Urinary FSH and LH by seminal vesicle weight gain assays in terms of the third International Standard for Urinary FSH and LH (as IU/ampoule) with laboratory mean seminal vesicle weights (as mg). Laboratories are identified by numbers.

Table 7 Estimates of the LH content of ampoules of the fourth International Standard for Human Urinary FSH and LH (IS) kept for 320 days at 20°C, 37°C or 45°C, in terms of ampoules of the IS kept at – 20°C. The number of estimates is given in parentheses and the 95% fiducial limits are in square brackets

		Geometric mean LH content (% of that of the IS kept at – 20°C)		
		IS kept at 20°C (coded C)	IS kept at 37°C (coded A)	IS kept at 45°C (coded B)
Assay method	Laboratory			
Seminal vesicle weight gain	1	92 (1)	–	78 (1)
	2	116 (2)	83 (2)	79 (1)
	3	–	91 (1)	–
	4	91 (1)	–	–
	6	–	–	77 (1)
	7	100 (2)	–	99 (1)
	10	–	–	78* (2)
wgmlm	–	100 [91–110] (4)	87 [79–97] (2)	86 [76–97] (5)
Ovarian ascorbate depletion	3	–	–	83 (1)
Both wgmlm	–	–	–	85 [76–95] (6)

*Unweighted geometric mean of heterogeneous estimates. wgmlm, weighted geometric mean of laboratory means.

AOW and SVW assays contributed to this collaborative study. Furthermore, differences in LH isoform composition between the IS and IS 71/264 are probably also the basis for the highly significant between-laboratory heterogeneity of estimates by SVW assay for the LH content of the IS in terms of IS 71/264. In contrast, estimates by SVW assay of the relative LH potencies of IS 71/223 and IS 71/264, which originated from the same hormone preparation, were homogeneous between laboratories.

Selectivity of SVW assays

Although estimates by SVW assays of the relative LH activities of the IS and IS 71/264 were homogeneous within laboratories, estimates were heterogeneous between laboratories. This indicated differences between the specificities of SVW assay methods in different laboratories. These inter-laboratory differences in the selectivity of SVW assays in detecting the differences between the spectrum of LH isoforms in the IS and IS 71/264 appeared to be related to the size of the seminal vesicles and age of the assay animals, and hence perhaps the degree of maturity of their LH target cells. Thus there was a significant correlation between the value of the LH potency estimate for the IS in terms of IS 71/264 obtained in a laboratory and both the overall mean seminal vesicle weight and age of the assay animals in that laboratory.

The limited data in this study are consistent with a strain-dependent effect among older rats.

In contrast, for the AOW assays, in which organ weights did not differ greatly between laboratories, the correlation between organ weight and the FSH potency estimate was not significant (correlation coefficient $r=0.38$, $P\sim 0.3$).

We are not aware of any previous report in the literature of such relationships between mean assay seminal vesicle weights, age or rat strain and the selectivity of SVW assays. The assay as originally described by Van Hell *et al.* (1964) used pre-pubertal rats of unspecified strain with ages in the range from 21 to 28 days. (The age range of rats for the SVW assay described in the British Pharmacopoeia (2000) and European Pharmacopoeia (1997) is 19–28 days, and for that described in the United States Pharmacopoeia (2000) is 20–21 days. The strains of rat to be used for the SVW assays described in these three pharmacopoeias are not specified.) The earlier generation of LH assay in which weight gain of male accessory organs was used as response, namely the ventral prostate weight gain assay (Greep *et al.* 1941), used 21–22-day-old hypophysectomized rats of unspecified strain, in which the maturity and selectivity of LH target cells may have been more precisely defined. This finding of inter-laboratory differences in the specificities of SVW assays is of some significance because, as indicated above, this assay method has been generally adopted by

Pharmacopoeias for the control of the LH content of therapeutic products.

Suitability of the IS for the standardization of FSH and LH

The participants in the collaborative study agreed that the IS appears to be suitable to replace the IS 71/264 as the international standard for urinary FSH and urinary LH. Thus the IS was found (a) to contain urinary FSH and urinary LH with specific FSH and LH activities of the same order as those of the IS 71/264, as estimated by *in vivo* bioassay methods which are specific for FSH and LH, and which are specified for the estimation of urinary FSH and LH in pharmacopoeial monographs; and (b) to be adequately stable, on the basis of accelerated thermal degradation studies, when stored under normal conditions, at -20°C in the dark.

Establishment of the fourth International Standard for Human Urinary FSH and LH

On the basis of the results of the collaborative study, and with the agreement of the participants, the WHO ECBS established the preparation in ampoules coded 98/704 as the fourth International Standard for Human Urinary FSH and LH and assigned to the contents of each ampoule an activity of 72 International Units of urinary FSH and an activity of 70 International Units of urinary LH (WHO ECBS, 51st Report).

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