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A Rapid, Sensitive and Reliable Assay for Inhibin Bioactivity

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Abstract

A rapid 2-day quantitative assay for inhibin bioactivity based on FSH secretion from pituitary cells of immature female rats is described. The bioassay exhibited steeper slopes, improved precision and greater (fourfold) sensitivity compared with a previously established pituitary FSH cell content assay. Whole pituitary glands were used for the preparation of pituitary cells and the method for cell dispersion required a single enzymatic treatment with trypsin. Cells (180 000 viable cells per well) were dispensed into culture media containing inhibin and incubated for 48 h. Media were removed and assayed for FSH by radioimmunoassay. Using a ram rete testis fluid preparation as standard the inhibin dose–response curves of 25 consecutive experiments showed indices of precision of $-0{\cdot}08$(mean)[range $-0{\cdot}04$ to $-0{\cdot}17$] and Finney's G values of $0{\cdot}017$[$0{\cdot}003$–$0{\cdot}06$]. The mean ED_{40} was $0{\cdot}17$ units of inhibin activity per well with interassay variation of $16{\cdot}2\%$ at this point of the dose–response curve. The assay had a practical capacity of 400 wells, permitting the measurement of dose–response curves of at least 40 unknowns with three dose points and triplicate wells per dose. The assay is specific for inhibin-containing preparations from several animal species. Overall, the assay is simple, precise, and sensitive, indicative of its applicability to the measurement of inhibin samples with low inhibin bioactivity and to the screening of large numbers of fractions during inhibin purification.

Introduction

Assays for inhibin activity based on suppression of FSH secretion or cell content of rat anterior pituitary cells in culture are the best characterized, reliable and precise methods available (see reviews by Hudson *et al.* 1979; Baker *et al.* 1983; Channing *et al.* 1985; de Jong and Robertson 1985). Although these assays have been shown to be satisfactory *in vitro* bioassays they require at least 4–6 days of incubation. Furthermore, the preparation of the pituitary cells require a combination of collagenase, trypsin and deoxyribonuclease (DNase) treatment of anterior pituitary glands. To render these assays more attractive and practical the experimental procedures must be simplified and incubation times shortened. In this study we have examined the use of cells prepared from whole pituitary glands of immature female rats in a rapid 2-day assay for inhibin activity based on inhibition of basal secretion of FSH.

The choice of pituitary glands from immature female rats was based on several experimental observations. Firstly, in immature rats or mice injected with pregnant mare's serum gonadotrophin (PMSG), endogenous FSH levels were completely suppressed as circulating inhibin concentrations were increased by the PMSG

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treatment, suggesting that the pituitary glands in these animals were sensitive to feedback inhibition by ovarian inhibin (Lee *et al.* 1981, 1982; Lee and Gibson 1985). Secondly, the decrease in circulating levels of FSH in female rats with age was closely related to increased ovarian and peripheral blood inhibin levels (Lee *et al.* 1984). In other studies where inhibin was administered to castrated rats of different ages, maximum suppression of FSH was observed in 25-day-old recipient animals, indicative of increased feedback inhibition (Hermans *et al.* 1980). Lastly, Denef *et al.* (1978) have demonstrated a more constant FSH : LH ratio in pituitary cells of varying sizes from female rats compared with cells isolated from adult or immature male rats. This observation suggests that a more homogenous and uniform population of pituitary cells coud be prepared from female rats and reduce any possible differences in responsiveness of heterogenous pituitary cell types to inhibin action.

In our laboratory, we have previously described an assay for inhibin based on inhibition of pituitary cellular FSH content (Scott *et al.* 1980). In this assay cells prepared from anterior pituitary glands of adult male rats are used. A comparison of the characteristics of this assay system with a bioassay which is more rapid, sensitive, precise and reproducible using cells from immature female rats is described in this paper.

Materials and Methods

Adult male rats (130–160 days old) and immature female Sprague-Dawley rats (22–33 days old) were supplied by Monash University, Central Animal House, Melbourne. All animals were killed by decapitation and their pituitary glands collected into Dulbecco phosphate-buffered saline (DPBS; Commonwealth Serum Laboratories (C.S.L.), Melbourne) containing 7·5 mM glucose.

Pituitary Cell Isolation

Adult male rats

Dispersed pituitary cells from anterior pituitary glands were prepared as described previously (Scott *et al.* 1980; Scott and Burger 1981) using a combination of trypsin and DNase treatment. The isolated pituitary cells were suspended in Dulbecco's Modified Eagles' Medium (DMEM; C.S.L., Melbourne) containing 5% (v/v) horse serum, 2·5% (v/v) fetal calf serum, 1% (w/v) glutamine, 1% (w/v) non-essential amino acids and penicillin (400 U/ml). Volumes of 500 μl containing 125 000 viable cells were dispensed at random into 24 multi-well plates (Costar 3524, Cambridge, Mass., U.S.A.) and incubated at 37°C in a water-saturated atmosphere of 95% air : 5% CO_2, which maintained the medium pH at 7·2–7·3. After allowing for cell attachment (12–18 h), test substances, in a volume of 100 μl, were added to the medium and incubation continued for a further 72 h. The culture medium was removed by gentle suction and the cells lysed with 0·1% (w/v) Triton X-100 for the measurement of pituitary FSH cell content (Scott and Burger 1981).

Immature female rats

Dispersed pituitary cells from whole pituitary glands were prepared using procedures described above for adult male rats except that a single enzymic treatment with trypsin was used for cell dispersion. The enzymic reaction containing pituitary tissue (from 40 to 45 rats) in 10 ml DPBS with 0·1% (w/v) bovine serum albumin (BSA), 7·5 mM glucose and 0·5% (w/v) trypsin was gently stirred and terminated after incubation for 15 min at 37°C. The trypsin solution was replaced with 15 ml DMEM containing 10% (v/v) fetal calf serum, 1% (w/v) glutamine, non-essential amino acids and penicillin (100 U/ml). After gentle stirring at 37°C for 15 min the DMEM solution was replaced with 15 ml DPBS (Ca-, Mg-free) containing 2 mM EDTA and 0·1% (w/v) BSA and stirred for a further 5 min. The tissue fragments were then transferred to a 10-ml siliconized glass centrifuge tube and washed three times in 10 ml DPBS (Ca-, Mg-free) at room temperature. The tissue fragments were dispersed into single cells by gentle suction and extrusion through a siliconized Pasteur pipette in 1·5 ml DPBS (Ca-, Mg-free). The dispersed

cells were transferred to another siliconized centrifuge tube and made up to 10 ml with DMEM. Any cell debris at the bottom of the tube was removed with a pipette and the cell suspension centrifuged at 900 *g* for 2 min at room temperature. The cells were resuspended in DMEM, centrifuged again and resuspended in DMEM. A fraction was taken for counting of cells by haemocytometer and cell viability was estimated by trypan blue (0·2%, w/v) exclusion test. Cell yield per pituitary was $1 \cdot 5–1 \cdot 9 \times 10^6$ and cell viability was greater than 92%. Aliquots of 50 μl volume containing 180 000 viable cells were then dispensed into multi-well plates (Costar 3524 or 3548) containing 550 μl of DMEM and inhibin standard (rete testis fluid standard, RTFS; Baker *et al.* 1985) or test substance. Incubation was performed at 37°C in water-saturated atmosphere (as described above for pituitary cells from adult male rats) for 48 h and the media removed for the measurement of FSH and LH by radioimmunoassays (Gibson *et al.* 1979; Lee *et al.* 1981). All samples within a single experiment were measured in the <10% coefficient of variation region of the standard curves (Burger *et al.* 1972). Reference standards for FSH and LH were NIADDK-rat FSH-RP1 and NIADDK-rat LH-I6, respectively. For experiments which involved determination of cell content of FSH and LH the cells were lysed with 500 μl of 0·1% Triton X-100.

Statistical Analysis

All samples were assayed in quadruplicate at each dose level and the FSH dose–response curve was expressed as the percentage inhibition from control (without exposure to inhibin). Regression analysis was performed using a computer program, and the slope (*b*), index of precision (λ), and the significance of regression (Finney's G), were calculated (Finney 1964). For the determination of assay sensitivity the dose of inhibin causing 40% suppression (ED_{40}) from control wells was calculated. Where multiple preparations were compared with the reference preparation (RTFS) the relative potencies were calculated by using the method of Borth (1960) for multiple bioassays and expressed in terms of an original inhibin standard (ovine testicular lymph protein, OTLP) with assigned potency of 1 unit (U) of activity per milligram protein (Eddie *et al.* 1979).

Results

Time Course of Inhibin Action

The effects of different incubation times (24, 48, 72 h) by various inhibin preparations on pituitary FSH release and content in cells prepared from immature female rats were determined and the data are presented in Table 1 and Fig. 1. Pituitary cell cultures were prepared as described above and each culture well contained 150 000–180 000 viable cells. Initially, the inhibin standard (RTFS) was examined for suppression of pituitary FSH release and content with time (Table 1). There was no suppression of FSH cell content within the first 24 or 48 h of incubation but by 72 h, the cell content of FSH was significantly decreased ($F_{5,18} = 23 \cdot 2$, $P < 0 \cdot 01$) with about 30% suppression at the highest dose (2000 mU per well) of RTFS. In contrast, media content of FSH was significantly depressed ($F_{5,18} = 53 \cdot 4$, $P < 0 \cdot 01$) by all doses of RTFS within 24 h of incubation. The degree of suppression was similar at 48 and 72 h (Fig. 1*a*). Using inhibin preparation from various animal species the magnitude of inhibition of FSH secretion was also comparable at 48 and 72 h of incubation. The individual and overall statistical (regression) analysis of the dose–response curves is summarized in Table 2. As can be seen from the results (F statistic of 0·99, 1·22 and 2·09) the combined slopes did not depart from parallelism at any time of incubation, indicating that the different preparations of inhibin suppressed the secretion of FSH in a similar manner. Although significant inhibition of FSH was observed at 24 h of incubation the indices of precision (λ) for the different dose–responses exhibited by the various inhibin preparations were better at 48 and 72 h of incubation. Similar observations were also obtained for Finney's G values. On the basis of the above observations,

Table 1. Time course of effects of inhibin standard (RTFS) on pituitary FSH release and cell content

Pituitary cells from immature female rats were incubated with different doses of RTFS and the culture media removed at 24, 48 and 72 h after incubation. The cells remaining in the culture plates were lysed with 500 μl of 0·1% (v/v) Triton X-100 and the concentrations of FSH in the cell culture media and lysates were measured by RIA. Values represent mean (s.d.) μg rat FSH-RP1/ml; $n = 4$ per point. Within each time after incubation, mean values with different letter superscripts are significantly different ($P < 0.05$) by Duncan's new multiple range test (Duncan 1957)

Dose RTFS (mU/well)		FSH release after incubation for: 24 h	48 h	72 h
0	Cell	4·95(0·37)	4·74(0·30)	3·50(0·16)
	Media	1·31(0·10)^{a}	3·40(0·43)^{a}	4·73(0·20)^{a}
125	Cell	4·40(0·41)	4·02(0·67)	3·32(0·15)
	Media	0·91(0·10)^{b}	2·49(0·19)^{b}	3·60(0·12)^{b}
250	Cell	4·35(0·40)	4·47(0·45)	3·01(0·13)
	Media	0·66(0·08)^{c}	1·56(0·05)^{c}	2·45(0·32)^{c}
500	Cell	4·19(0·20)	4·01(0·68)	2·69(0·23)
	Media	0·41(0·08)^{d}	0·94(0·10)^{d}	1·24(0·09)^{d}
1000	Cell	4·55(0·35)	4·04(0·51)	2·50(0·28)
	Media	0·36(0·11)^{e}	0·57(0·07)^{e}	0·72(0·10)^{e}
2000	Cell	4·24(0·49)	3·89(0·17)	2·41(0·12)
	Media	0·38(0·14)^{de}	0·50(0·08)^{e}	0·66(0·05)^{e}

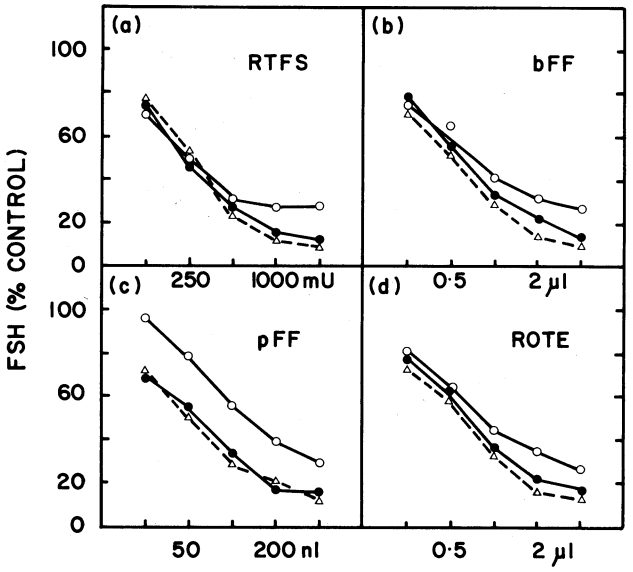

Fig. 1. Suppression of basal secretion of FSH from pituitary cells by varying doses and volumes of different inhibin preparations after 24 (○), 48 (●) and 72 (△) h of incubation. (*a*) RTFS; (*b*) bFF; (*c*) pFF; (*d*) ROTE. Results shown are means of quadruplicate wells expressed as percentage of control (no inhibin) for each of the three periods.

the bioactivities of various preparations of inhibin were determined using conditions of 48 h incubation and basal release of pituitary FSH. The data for the inhibition of FSH secretion after 48 h of incubation with RTFS, bovine follicular fluid (bFF), porcine follicular fluid (pFF) and rat ovarian tissue extract (ROTE) preparations are depicted in Fig. 2.

Table 2. Time course of effects of inhibin on basal secretion of FSH from pituitary cells

Results (data from Table 1) were expressed initially as percentage of control (no inhibin) for each of the time periods and analysed by regression analysis by the method of Borth (1960). The values in the linear region of the dose–response curves were used in the analyses and the slopes (b), indices of precision (λ = s.d./b) and Finney's G values for each dose–response curve and the combined analysis for each time period are summarized in this table. The combined analysis of variance for each time period indicates no significant departures from parallelism ($P > 0.05$)

Time of incubation	Statistic[A]	Preparation[B] RTFS	bFF	pFF	ROTE	Combined analysis
24 h	b	−27·9	−21·8	−23·3	−21·1	−22·9
	λ	−0·10	−0·13	−0·15	−0·12	−0·12
	g	0·073	0·046	0·028	0·038	$F_{3,48} = 0·99$
48 h	b	−27·8	−28·1	−24·8	−28·4	−27·3
	λ	−0·05	−0·07	−0·05	−0·11	−0·07
	g	0·007	0·013	0·007	0·029	$F_{3,48} = 1·22$
72 h	b	−35·9	−30·5	−29·9	−29·2	−30·7
	λ	−0·06	−0·08	−0·08	−0·04	−0·06
	g	0·008	0·016	0·048	0·005	$F_{3,42} = 2·09$

[A] g = Finney's G = F statistic for significance/F statistic for regression.
[B] bFF, bovine follicular fluid; pFF, porcine follicular fluid; ROTE, rat ovarian tissue extract.

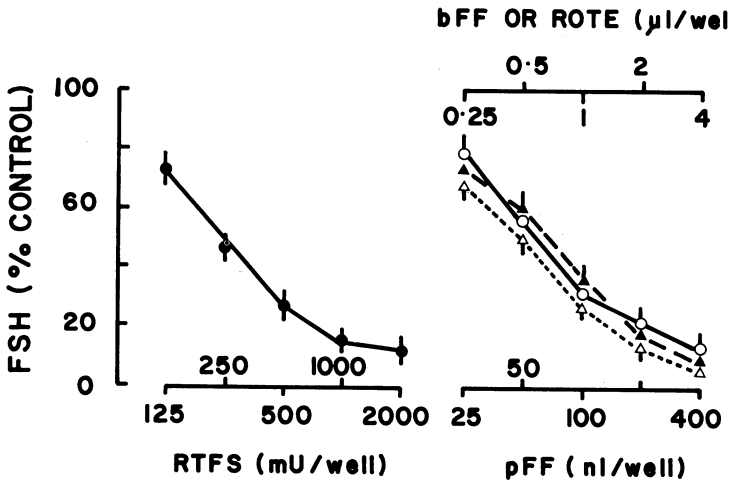

Fig. 2. Dose–response inhibition of FSH secretion from pituitary cells by various preparations of inhibin after 48 h of incubation. Statistical analysis of the data are presented in Table 2. Results shown are means of quadruplicate wells expressed as percentage of control (no inhibin) and the vertical bars indicate standard deviations. ● RTFS. ○ bFF. ▲ ROTE. △ pFF.

Table 3. Effect of inhibin standard (RTFS) on basal secretion of FSH and LH from pituitary cells of immature female rats

The pooled results of three experiments are shown. In each experiment, inhibin standard (RTFS) was added to quadruplicate wells and the percentage inhibition from control (no inhibin) of FSH and LH concentrations in the culture media after 48 h of incubation was calculated. Results represent mean (s.e.m.). As can be seen there was no significant reduction of LH secretion by inhibin standard

RTFS (mU/well)	62·5	125	250	500	1000
Inhibition of FSH (as % of control):	80·8(1·5)	65·8(3·2)	43·8(3·3)	27·8(3·1)	19·6(3·7)
Inhibition of LH (as % of control):	87·6(5·2)	89·7(3·3)	79·0(4·4)	85·9(5·3)	84·9(6·9)

Table 4. Characteristics of inhibin bioassays performed using pituitary cells from immature female and adult male rats

See also Fig. 3. The various parameters of the bioassays were compared by *t*-tests and the significance of the differences are presented. Values represent mean (s.d.) [range]

Parameter	Immature female	Adult male	*P*
No. of assays	25	13	—
Slope ($-b$)	27·4(2·8) [22·0–31·9]	19·8(4·3) [13·0–26·2]	<0·001
Index of precision (λ)	0·08(0·03) [0·04–0·17]	0·13(0·06) [0·07–0·25]	<0·005
Finney's G	0·017(0·014) [0·003–0·060]	0·045(0·039) [0·011–0·159]	<0·005
ED_{40} (mU)	167(27) [113–240]	677(182) [407–948]	<0·001

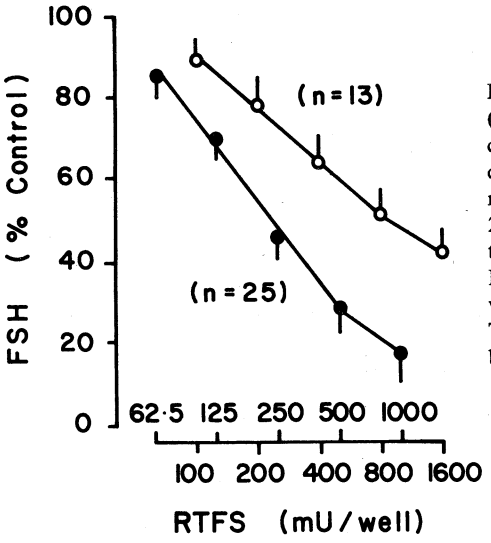

Fig. 3. Effect of inhibin standard (RTFS) on the release (●) and cell content (○) of FSH from pituitary cells of immature female and adult male rats respectively. The data from 25 and 13 assays performed at about the same period of time are shown. Results shown represent means and the vertical bars indicate standard errors. The characteristics of the two inhibin bioassays are summarized in Table 4.

Specificity of Bioassay

The specificity of the inhibin bioassay, using pituitary cells from immature female rats, was examined by investigating (*a*) the effects of inhibin on pituitary LH secretion, and (*b*) the effects of non-inhibin containing protein preparations on basal FSH secretion.

In contrast to FSH concentrations which were suppressed to <20% of controls with 1 U of inhibin per well, there was no dose-related suppression of pituitary LH secretion in the presence of increasing doses of inhibin RTFS standard (Table 3). Furthermore, non-inhibin containing preparations added to pituitary cells in culture did not affect the secretion of FSH. The preparations tested were PMSG (0·3–5 U per well), rat FSH-RP1 (25–400 ng per well), rat LH-I6 (0·25–4 ng per well), and rat prolactin-B6 (5–80 ng per well). These results, therefore, indicate the specificity of the bioassay for inhibin substances.

Comparison of Bioassays Performed using Pituitary Cells from Immature Female and Adult Male Rats

A comparison of the characteristics of inhibin bioassays using cells from immature female rats (as described above) and cells from adult male rats (as described previously by Scott *et al.* 1980) was made. These assays were performed at about the same period of time by the same technicians and the results are summarized in Table 4 and in Fig. 3. As can be seen the slopes of the dose–response curves using cells from immature rats were significantly ($P < 0·001$) steeper than those using cells from mature male rats (mean of $-27·4$ versus $-19·8$ respectively). Similarly, the indices of precision and the Finney's G values were superior in the bioassay using female pituitary cells. The sensitivity of the bioassay (as indicated by the ED_{40} values) was at least four times greater than for the cell content assay method.

Discussion

We have described a rapid (2-day), simple, precise and specific *in vitro* bioassay for the measurement of inhibin activity based on inhibition of pituitary FSH secretion from cells of immature female rats. The assay was simple in that firstly, it required a single enzymic treatment with trypsin for the dispersion of pituitary cells and secondly, it involved no preincubation of cells before addition of test substances and no changes of culture media during the course of the bioassay. Furthermore, whole pituitary glands were used instead of separation of anterior lobes from other parts of the pituitary gland prior to cell dispersion (Eddie *et al.* 1979; Scott *et al.* 1980), inevitably saving time in assay procedures. Our observations in obtaining >90% cell viability could be explained partly by the shorter cell dispersion times in comparison with other previously published procedures. We have found that assays performed with cells containing <90% cell viability invariably led to poor dose–response inhibition curves and were, in general, invalid bioassays with poor assay precision (personal observations, Lee *et al.*). This latter observation should be an important consideration in maintaining good quality inhibin bioassays with high precision and reproducibility and is currently adopted in our laboratory. The above comments are likely reasons for the superior assay precision and reproducibility of the basal FSH release method reported here.

The observations of steeper slopes and greater sensitivities of the basal release assay in comparison to the cell content method are in accord with previous observations

(Eddie *et al.* 1979; Scott *et al.* 1980; Channing *et al.* 1985). In this report the greater slope (−27·4) of the release assay compared with −19·8 for the cell content method suggests that a greater degree of inhibition of FSH release by inhibin may occur in contrast to a lesser effect on inhibition of pituitary FSH biosynthesis (Table 4). Alternatively the levels of FSH in the culture media represents a sum of both inhibition of release and biosynthesis of pituitary FSH. We have determined the biopotency of a bovine follicular fluid inhibin standard (Robertson *et al.* 1986) in both assay systems and a significantly lower ($P < 0{\cdot}01$) potency was obtained in the release assay (371[88] U/ml, mean[s.d.], $n = 7$ assays) compared with the cell content assay (545[154] U/ml, $n = 14$ assays). The reasons for the discrepancy are unclear in spite of using the same inhibin RTFS as reference standard in both assay systems. One likely explanation could be the source and species of inhibin used, each exerting different degrees of inhibition of release and biosynthesis of FSH in the different pituitary cell types. Further studies are needed to explore this possibility. In Fig. 1*c* it is clear that porcine follicular fluid inhibin was less suppressive than other species of inhibin after 24 h of pituitary cell incubation.

The sensitivity of the bioassay, as judged by the ED_{40} values, is remarkably similar to values obtained by others using basal release assays (Ling *et al.* 1985; Robertson *et al.* 1986). In the latter case, the same inhibin reference standard was used and the ED_{50} of 0·2 U per well was comparable to the values reported here (ED_{40} of 0·17 U per well). When expressed in terms of purified inhibin (200 000 U/mg protein; Robertson *et al.* 1985) the sensitivity (ED_{40}) of the bioassay is equivalent to 1 ng of bovine follicular fluid inhibin protein. Ling *et al.* (1985) reported ED_{50} values of 0·5–0·9 ng/ml for a purified porcine follicular fluid inhibin preparation. Furthermore, these authors reported no suppression of pituitary LH secretion by their purified inhibin preparations, in concordance with the observations made here (Table 3). The specificity of the bioassay is further substantiated by the lack of FSH suppression by non-inhibin-containing preparations. We have now applied this inhibin bioassay to the measurement of inhibin in ovarian and peripheral blood samples of rats during sexual development (Lee *et al.* 1984), in granulosa cell culture samples (Lee 1984), and in screening inhibin monoclonal antibodies (Lee *et al.* 1986). It is proposed that the above rapid, sensitive, and precise inhibin bioassay be applied to the measurement of inhibin samples with low inhibin activity and to the screening of large numbers of fractions during inhibin purification.

Acknowledgments

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